Regulation of signaling interactions and receptor endocytosis in growing blood vessels

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Abstract
Blood vessels and the lymphatic vasculature are extensive tubular networks formed by endothelial cells that have several indispensable functions in the developing and adult organism. During growth and tissue regeneration but also in many pathological settings, these vascular networks expand, which is critically controlled by the receptor EphB4 and the ligand ephrin-B2. An increasing body of evidence links Eph/ephrin molecules to the function of other receptor tyrosine kinases and cell surface receptors. In the endothelium, ephrin-B2 is required for clathrin-dependent internalization and full signaling activity of VEGFR2, the main receptor for vascular endothelial growth factor. In vascular smooth muscle cells, ephrin-B2 antagonizes clathrin-dependent endocytosis of PDGFRβ and controls the balanced activation of different signal transduction processes after stimulation with platelet-derived growth factor. This review summarizes the important roles of Eph/ephrin molecules in vascular morphogenesis and explains the function of ephrin-B2 as a molecular hub for receptor endocytosis in the vasculature.

Keywords: endocytosis, endothelial cells, Eph, ephrin, mural cells, receptor

Introduction

Blood vessels are essential during development and throughout adult life. They are among the first structures that form in the early vertebrate embryo and extend rapidly to match the increasing metabolic needs of the growing organism. Before the onset of heart beat, mesodermal precursor cells give rise to hemangioblast precursors, which will migrate, differentiate into endothelial cells and assemble the first vessels in a process called vasculogenesis.1 This early step in vascular development generates a single aorta at the embryonic midline.2 The CV arises later than the DA and is followed by the formation of highly branched interconnected capillary networks.3-6 The latter is achieved through angiogenesis, which is the expansion of an existing vessel network through endothelial cell (EC) proliferation, migration and vessel sprouting. During sprouting, specialized tip cells migrate and lead the sprout towards the avascular space by emitting filopodia and lamellipodia that sense the growth factor gradient (Fig. 1B).7,8 More proliferative stalk cells follow the invasive tip cells and form the base of endothelial sprouts, but ECs can also actively compete for the leading position.8,9 Such cell-shuffling events during sprouting angiogenesis are promoted by heterogeneous adhesion interactions between endothelial cells.10 When stable EC-EC connections are formed, newly assembled vessel branches get lumenized and stabilized by mural cells.11

There is also a second endothelial network, the lymphatic vasculature, which unidirectionally transports interstitial liquid, proteins, lipids and immune cells from distal tissues through collecting ducts and lymph nodes into the venous branch of the circulation.12 In the embryo, the first lymphatic endothelial cells (LEC) differentiate from the roof of the CV and from additional venous sources, bud off and migrate as streams of connected cells between the CV and superficial venous plexus before they form the first lumenized lymphatic vessels.13-15 Further rounds of LEC sprouting and mitosis lead to the formation of peripheral lymphatic vasculature in a process termed lymphangiogenesis.13,16,17 After the completion of developmental growth, blood vessels and the lymphatic vasculature in most tissues acquire a mature and quiescent phenotype that is characterized by the absence of endothelial sprouting and proliferation.18,19 However, vascular networks retain plasticity that permits growth in response to tissue damage and in a range of disease processes.20,21

Apart from endothelial cells, mural cells of the mesenchymal lineage, namely pericytes and vascular smooth muscle cells

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(VSMC), are another essential component of vascular networks. Pericytes, which associate with the abluminal (basal) surface of endothelial tubes in capillaries and post-capillary venules, are thought to stabilize blood vessels, prevent vascular leakage, and contribute to vascular maturation and quiescence. VSMCs, which cover arteries and veins, are highly plastic and help the vessel wall to resist against the laterally acting forces in the circulatory system. In mature blood vessels, VSMCs differentiate and express markers important for the regulation of contraction, and, upon injury, can dedifferentiate by losing the expression of these contractile markers. Loss or dedifferentiation of VSMCs is associated with aneurysms, i.e., the local ballooning of vessels.

Many studies have focused on the signaling processes controlling angiogenesis and lymphangiogenesis. This has led to the identification of numerous key signals with conserved roles in physiological and pathological vessel growth. Among those signals, many secreted growth factors and the corresponding receptors of the tyrosine kinase superfamily have particularly important functions.

**Receptor Tyrosine Kinases in the Vascular System**

Growth, migration and differentiation processes in the developing vascular system are critically controlled by receptor tyrosine kinases (RTKs). Vascular endothelial growth factors (VEGFs) activate their receptors on endothelial cells and thereby promote the expansion of the blood vessel and lymphatic vessel networks. Within the VEGF family, VEGF-A (also known as vascular permeability factor) is the most important regulator of angiogenesis and levels of this growth factor need to be tightly regulated. Accordingly, loss of only one copy of the *Vega* gene led to early embryonic lethality of the heterozygous mice. The related growth factor VEGF-C is essential for many aspects of lymphangiogenesis. Three other family members, VEGF-B, VEGF-D and placental growth factor (PIGF), have distinct biological activities. Additional VEGF homologues, VEGF-E and VEGF-F, were identified in viruses and snake venom, respectively.

Alternative splice variants and processed forms of VEGFs increase the complexity of this pathway. VEGFs, in particular VEGF-A, are produced by many cell types and act mainly as paracrine factors on endothelial cells, but autocrine expression of VEGF-A has been also shown to be essential for endothelial cell survival.

Different VEGF family growth factors can bind with high affinities to one or several of the cognate receptor tyrosine kinases, namely VEGFR1/Flt1, VEGFR2/Flik1/KDR and VEGFR3/
Flt4. VEGF-A binds VEGFR1 and VEGFR2. While the latter mediates the important pro-angiogenic functions of VEGF-A, VEGFR1 in ECs predominately as a high affinity trap that prevents VEGF-A binding to and signaling through VEGFR2. VEGFR3 is the main receptor for VEGF-C and, accordingly, is indispensable for lymphangiogenesis. Expression of VEGFR3 during angiogenesis and the phenotypes of knockout mice and zebrafish mutants indicate that this receptor is also important for blood vessel growth. In addition to VEGF receptors, VEGFs can bind to heparan sulfate proteoglycans (HSPGs), neuropilins (NRPs) and integrins, which are important for VEGF presentation or complex formation with VEGFRs. All VEGF receptors are structurally similar and consist of extracellular Ig-like domains, a single transmembrane region, an intracellular kinase domain, and a less conserved C-terminal tail. VEGF ligand binding to VEGFRs can occur in cis or trans (via HSPGs expressed on adjacent cells) and thereby induces receptor homo- or heterodimerization, activation of the kinase domain and signaling through various downstream pathways.

The angiopoietins (Ang) ligands and Tie receptors, which are expressed by vascular and haematopoietic cells, are important for morphogenesis and homeostasis of blood vessels and lymphatic vessels. While inactivation of the Tek gene encoding Tie-2 in mice led to midgestation lethality due to defects in capillary plexus remodeling and maturation, hematopoiesis and heart development, loss of Tie-1, an orphan receptor without known ligands, resulted in later embryonic lethality due to impaired vascular integrity without defects in hematopoiesis. Four angiopoietins (Ang-1-4) can bind to the receptor Tie-2. Although Ang-1 and Ang-2 have similar structures and bind with similar affinities to Tie-2, they have distinct functions. Ang-1 promotes vascular growth, maintenance and maturation regulating endothelial cell survival, prevents apoptosis and inhibits inflammation, whereas Ang-2 can promote EC death and vascular regression. While Ang-1 deficient mice die in utero with similar phenotype to Tie-2 deficient embryos, Ang-2 KO mice are born normally and die postnatally due to lymphatic defects. Although it was thought that Ang-2 has an antagonistic role by binding Tie-2 and preventing Ang-1-induced receptor activation, some evidence indicates that Ang-2 can bind and signal through Tie-2 and integrins in certain settings.

The receptors for platelet-derived growth factors (PDGFs), PDGFRα and PDGFRβ, are evolutionary and structurally related to VEGFRs. They are bound by one or more isoforms of 5 different disulphide-linked dimers PDGF growth factors (PDGF-AA, -BB, -AB, -CC, and -DD). Ligand-receptor interaction leads to receptor homo- or heterodimerization, conformational change, and kinase activation, which triggers auto- and transphosphorylation processes. This is followed by binding of various signaling and docking molecules leading to the activation of several signaling pathways, which are required for cellular proliferation, migration and survival (for a review see ref. ). In the vasculature, PDGF-B is expressed by endothelial cells and enriched in the tip cell area. PDGF-B signals in a paracrine fashion to PDGFRβ-expressing mural cells and thereby promotes their proliferation, directional migration and vessel wall assembly. Both Pdgfrβ and Pdgfrb KO mice are lethal during late embryonic gestation due to capillary dilation and rupture of microaneurisms resulting in widespread hemorrhaging. Mutants showed increased pericyte/VSMC detachment and reduced mural cell coverage of the endothelium, which led to renal, vascular, cardiac and placental defects. In the adult, activation of PDGFRβ expressed by fibroblasts and myofibroblasts regulates interstitial fluid pressure of tissues and prevents edema formation by inducing contraction of these cell types.

**Eph/ephrin** interactions play important roles in numerous morphogenetic processes including angiogenesis and lymphangiogenesis. While there is little evidence for a major role of A-class receptors and their GPI-anchored ephrin ligands in the developing vasculature, several important functions have been identified for the transmembrane protein ephrin-B2 and the receptor EphB4. In classical forward signaling, ephrin ligands activate Eph receptors on neighboring cells (in trans), which frequently translates into cell-cell repulsion that leads to cell sorting, the segregation of ligand-expressing and receptor-expressing cells into distinct tissue domains, and boundary formation (Fig. 2A and B). In reverse signaling, Eph receptors induce signaling processes in ephrin-presenting cell, which often promotes cell-cell adhesion and cell-substrate adhesion. Ephrin-B reverse signaling relies on its scaffolding activity and involves the phosphorylation of several conserved tyrosine residues in the cytoplasmic domain, the recruitment of cytoplasmic adapters and binding of PDZ domain-containing proteins to the C-terminal PDZ motif. It is currently unknown whether the outcome of Eph-ephrin interactions in vascular cells can be explained through presentation of ligands and receptors in distinct membrane microdomains of the same cell, as has been proposed for EphA and ephrin-A molecules. Distinct signaling outputs downstream of the B-class ligand ephrin-B3 are mediated by the SH2/SH3 adaptor Grb4 and the PDZ domain proteins PICK1 and syntenin, respectively.

Eph/ephrin interactions require direct cell-cell contact because Eph receptors are only efficiently activated by membrane-bound ligands and not by the soluble forms. These interactions and signaling outputs can also depend on differential receptor ectodomain binding affinity for a specific ligand. The analysis of EphA4 ectodomain structure showed that interactions with ephrin can induce conformational changes in the ligand-binding domain of the receptor besides its ability to increase the local concentration of the receptor above the critical value required for Eph/ephrin clustering. This clustering is necessary for the initiation of further signaling processes at cell-cell contacts (Fig. 2C). Gradually, through a multistep process, higher-order clusters will assemble. The extent of receptor clustering was shown to determine the strength of the signaling in living cells. While trimeric or tetrameric small-sized EphB2 receptor clusters can induce cellular signaling, cellular responses got stronger the more active, multimeric (trimeric or greater) EphB2 clusters
Interestingly, even ligand-unbound receptors have the ability to interact with Eph clusters that thereby expand beyond the interface of ephrin ligand interactions (Fig. 2C). Receptor clustering is also modulated by other Eph interacting proteins, which bind to the EphB cytoplasmic part. Accordingly, deletion of SAM and PDZ binding motifs in EphB2 increased clustering and signaling of this receptor.
Regulation of Blood Vessel Morphogenesis by Eph/ephrin Signaling

As mentioned above, EphB4 and its ligand ephrin-B2 constitute the only receptor/ligand pair within the Eph/ephrin family with essential and well-defined roles in angiogenesis (Fig. 3A). EphB4 and its ligand ephrin-B2 were embryonically lethal at midgestation and showed no defects in vasculogenesis but compromised angiogenic blood vessel growth and remodeling. From zebrafish to human and from early embryos to adults, ephrin-B2 expression dominates in arterial domains, while EphB4 is more prominent in the venous compartment. Another receptor, EphB1, shows venous restricted expression in the skin after the capillary plexus has formed, which is maintained into adulthood. However, venous expression of EphB1 is lost in other adult organs.

In contrast, ephrin-B2 and EphB4 are widely perceived as markers for arterial and venous territories, respectively, throughout development and in adult life. In mouse, their expression starts as early as embryonic day 8.25 and marks large regions of the primary vascular plexus prior to the formation of arteries and veins. It is well possible that a cell-cell repulsion mechanism helps at this stage to establish clear borders between the developing arterial and venous vascular compartments.

VEGF and Notch signaling have been shown to positively control ephrin-B2 expression. In arterial angioblast precursors, VEGF-A/VEGFR2 act together with the neuropilin-1 (NRP1) co-receptor to activate Notch signaling. Notch promotes the arterial and suppresses the venous fate of ECs, and thereby orchestrates the arterial-venous differentiation program. VEGF regulates also the early arterial expression of Dll4, a key ligand of Notch receptors in the endothelium, via ETS family transcription factors. SOXF transcription factors interact in a combinatorial fashion with Rbpj, a key transcriptional regulator downstream of active Notch, which upregulates Dll4 expression and promotes arterial differentiation. Following the initial VEGF-dependent induction of Dll4, Notch activity is required to maintain expression of the Delta ligand and also promotes the expression of target genes such as ephrin-B2. In venous endothelium, COUP-TFII, a nuclear orphan receptor transcription factor, and PI3K-Akt signaling suppress Notch activity, NRP1 expression and MAP kinase signaling, which results in the repression of arterial differentiation. Consequently, Notch down-regulation led to reduced expression of ephrin-B2 and upregulation of EphB4.

Ephrin-B2 and EphB4 expression is detected in the 2 first axial vessels of the embryonic body, the dorsal aorta and the cardinal vein (Fig. 1A). Although the balanced formation of these big vessels was compromised after the inactivation of these genes, arterial-venous differentiation itself was not affected. In zebrafish, the DA assembles first and more dorsal, which is followed by the assembly of the CV more ventral to the midline. Accordingly, arterial and venous angioblasts precursors are localized in separate stripes along the midline and migrate in separate waves, in temporal relation to expression of the Erv2 transcription factor. Interestingly, in zebrafish, the DA and the CV are thought to be derived from a common precursor vessel by ventral sprouting, which is controlled by repulsive EphB4/ephrin-B2 signaling (Fig. 1A). Although cells of venous origin were not formally shown to populate this precursor vessel, it was proposed that venous-fated angioblasts segregate, sprout and migrate ventrally to assemble the CV, which is thereby segregated from the DA. While expression of ephrin-B2 in endothelial cells or angioblasts limited their ability to move ventrally into the CV, expression of EphB4a, one of the 2 paralogous gene products in zebrafish, promoted this migration process. Cells injected with ephrin-B2a-targeting morpholinos or overexpressing a C-terminally truncated (reverse signaling-incompetent) version of the ligand were more abundant in the CV and reduced in the DA. The opposite effect was obtained with morpholinos targeting EphB4a.

Similar to zebrafish, longitudinal connections between the DA and the CV that could correspond to migrating endothelial cells have been reported in chick and mouse embryos (Fig. 1A). Recently, the existence of an arterial-venous segregation process through endothelial cell migration was demonstrated in mouse via time-lapse imaging of early embryos. These data indicate that the mouse DA contains a mixed population of precursor cells with venous or arterial signature as well as a small number of cells expressing both markers, which might reflect that arterio-venous commitment is not yet specified. Similar to the processes in zebrafish, EphB4/ephrin-B2 signaling regulates CV formation from the DA by promoting ventral sprouting and by repelling venous ECs away from those with an arterial fate. Venous endothelial cells were enriched in the DA and strongly reduced in the CV of EphB4-deficient mice, while the total number of cells was not changed.

The sorting of lymphatic endothelial cells from the venous ECs during the venous-lymphatic segregation process and formation of the first lymphatic vessel structures resembles aspects of arterial-venous segregation events. Although it is clear that ephrin-B2 and EphB4 are expressed by lymphatic vessels and play important roles in lymphangiogenesis, further studies are needed to address whether they promote cell segregation similar to the processes in the DA/CV. It has been shown that ephrin-B2 reverse signaling is necessary for the lymphatic sprouting from the primitive plexus, a process that gives rise to the dermal lymphatic vasculature. The remodeling of the primary lymphatic vessels into a more organized network was also affected by loss of the C-terminal ephrin-B2 PDZ motif but not by the impairment of ephrin-B2 tyrosine phosphorylation. EphB4/ephrin-B2 signaling also controls valve formation and maintenance in the lymphatic vasculature. Somewhat unexpectedly, ephrin-B2 was found to be highly enriched in the venous valves where it is also required for valve formation and maintenance.

Besides its expression in endothelial cells, ephrin-B2 was also found in mural cells (Fig. 3B). Whereas endothelial specific inactivation of the Ephb2 gene led to lethality during early embryo development due to impaired angiogenesis and heart morphogenesis, mural cell-specific Ephb2 mutants developed to term and died perinatally. This finding most likely reflects the essential roles of endothelial cells in the growth of the embryonic vasculature, whereas mural cell function in mice only becomes indispensable after birth.
Ephrin-B2 modulates VEGF- and PDGF receptors endocytosis in vascular cells. (A) VEGFR endocytosis and turnover is higher in sprouting cells compared to established vessels. Ephrin-B2 and its interacting partners, the clathrin-associated sorting protein Dab2 and the cell polarity regulator PAR-3, control VEGFR activity, clathrin-dependent VEGFR internalization and downstream intracellular signal transduction. These interactions and activities dynamically regulate vascular endothelial cell sprouting, motility and proliferation. It is currently unknown whether VEGF signaling is also modulated by processes downstream of EphB4. (B) PDGF-B-induced PDGFRβ activation leads to clathrin-dependent PDGFRβ internalization followed by ERK1/2 and JNK activation in VSMCs. This process is limited by ephrin-B2, which sequesters PDGFRβ into a caveolin-dependent, passive pool and positively regulates Tiam1 expression and Rac1 activation. Eph-dependent ephrin-B2 internalization in interacting VSMCs induces the endocytosis of inactive (unphosphorylated) PDGFRβ. PDGF-induced ERK1/2 activation counteracts Tiam1 expression, which reduces VSMC spreading, migration and proliferation.
**Regulation of VEGF Receptor Endocytosis by Eph/ephrin Signaling**

Eph/ephrin signaling regulates endothelial sprouting angiogenesis, which has been studied best in the postnatal murine retina (Fig. 3A). Ephrin-B2 and EphB4 show partially overlapping expression in the angiogenic growth front and are presumably also active in the newovascular sprouts and blind-ended capillaries during hypoxia-induced angiogenesis in oxygen-induced retinopathy, a model for pathological vessel growth in the eye. Ephrin-B2 overexpression stimulated endothelial motility and invasiveness in cultured cells and transgenic mice. Conversely, ephrin-B2 conditional deletion in endothelium or deletion of the C-terminal PDZ binding motif (in ephrin-B2ΔV mutants) impaired sprouting angiogenesis in physiological and pathological conditions. When the ephrin-B2 cytoplasmic domain was replaced by β-galactosidase, retina vascularization and filopodia formation were reduced. Likewise, revascularization and the formation of vascular malformations, so-called neovascular tufts, were attenuated when these mutants were analyzed under conditions of oxygen-induced retinopathy.

The 2 main signaling pathways known to reciprocally regulate and cooperate in the specification of tip-stalk cell phenotypes during angiogenesis are VEGF and Notch. VEGF promotes Dll4 ligand expression in tip cells. Dll4 activates its Notch receptor in the neighboring stalk cells, which is thought to further repress VEGF receptor expression in these cells and inhibit sprouting. Surprisingly, Notch is a weak regulator of VEGFR2 expression, while being a potent inhibitor of VEGFR3 protein expression. Accordingly, when Notch was inhibited in postnatal mice, VEGFR3 was found to promote unproductive, excessive angiogenesis in a VEGF-independent fashion.

One of the critical molecular events involved in VEGF signaling regulation is endocytosis. While the traditional view was that endocytosis leads to receptor degradation and the termination of signaling, there is increasing evidence for RTK signaling from endocytic vesicles after internalization. Within the plasma membrane, VEGFR2 co-localizes with caveolin-1 or is found at the endothelial cell-cell contacts in adherens junctions. During angiogenesis, these junctions get less stable and VEGFR2 is no longer blocked by interaction with Vescadherin or several phosphatases. In response to VEGF binding, VEGFR2 is rapidly internalized, which occurs mainly in a clathrin and dynamin-dependent fashion, and is trafficked to Rab5+ vesicles. VEGFR2 in endosomes was found in a complex with Nrps1, dynamin-binding protein synectin/GIPC and myosin-VI, a reverse transport motor. VEGFR2 next moves from early antigen 1 (EEA1)/Rab5+ endosomes either into recycling (Rab11+ or Rab4+) endosomes or into multivesicular bodies (MVBs) and late endosomes (Rab7+) prior to recycling or degradation respectively.

High rates of VEGF uptake, VEGF receptor endocytosis and turnover have been detected in sprouting endothelial cells of the early postnatal retina (Fig. 3A). Here, ephrin-B2 regulates VEGF signaling by promoting clathrin-mediated endocytosis of VEGFR2 and VEGFR3. Ephrin-B2 recruits Dab2, a clathrin-associated-sorting protein (CLASP), which associates with the cell polarity factor Par3 in clathrin-coated vesicles. Accordingly, endothelial specific deletion of Dab2 or Par3 impaired VEGF receptor internalization and phenocopied sprouting defects seen in EC-specific Ephb2 mutants.

Ligand-dependent VEGFR2 and VEGFR3 endocytosis is spatially regulated and predominantly takes place at the leading angiogenic front, which is also a site of high VEGF2 transcription. Accordingly, while VEGFR2/R3 protein steady state levels were surprisingly low in sprouts at the leading edge of the growing retinal vasculature, levels of the 2 receptors were profoundly increased in sprouts after blockade of protein degradation or inhibition of clathrin-mediated endocytosis. These experiments also indicated that VEGF receptor internalization and turnover in the more mature vessels of the central retina is much lower, which was attributed to negative modulation of Dab2-dependent internalization by atypical protein kinase C (aPKC). aPKC-λ and aPKC-ζ can phosphorylate Dab2 in proximity of its cargo-binding PTB domain, which inhibited the interaction with VEGFR2 and VEGFR3. Immunostaining of phosphorylated (active) aPKC was weak in sprouts at the angiogenic growth front (i.e. the area with high VEGF receptor turnover and activity), while strong phospho-aPKC staining marked more mature vessels with low VEGFR internalization. Accordingly, postnatal inactivation of the gene encoding aPKC-λ in ECs increased the uptake of fluorescently labeled VEGF through VEGFR2 in the central retina, and the abundance of filopodial protrusions was greatly increased in mutant vessels.

Interestingly, not only endocytosis but also VEGF signaling activity is regulated by ephrin-B2, Dab2, Par3, and aPKC. In the absence of ephrin-B2 signaling, stimulated VEGF receptors were retained on the cell surface, which was accompanied by reduced tyrosine phosphorylation of these molecules and impaired downstream activation of Rac1, Akt and MAP kinase Erk1/2 (Fig. 3A). Inhibition of aPKC in cultured cells led to accelerated VEGFR2 and VEGFR3 internalization and strongly increased VEGF-A and VEGF-C-dependent MAP kinase activation. While the stimulation of EphB4 forward or ephrin-B2 reverse signaling triggered some VEGF receptor internalization even in the absence of VEGF stimulation, this process failed to induce significant activation of the VEGF pathway.

**Regulation of PDGF Receptor B Endocytosis by Eph/ephrin Signaling**

The recruitment of mural cells strongly depends on PDGF-B/ PDGFRB signaling. Activation of this pathway suppresses the differentiation and promotes the proliferation of VSMCs, pericytes and other mesenchymal cell types. Upon ligand binding, PDGF receptors accumulate in coated pits at the cell membrane and internalize mainly in a clathrin- and dynamin-dependent
brane compartments, which preferentially contained the active, led to redistribution of PDGFR in membrane fractions from control VSMCs, the loss of ephrin-B2-deficient VSMCs showed increased activation of the kinase activity showed perinatal lethality and thereby phenotype of the EphB receptors controlling mural cell function requires further investigation, but expression of EphB2, EphB3 and EphB4 was observed in cultured murine VSMCs. Cell contact-dependent interactions between pericytes and endothelial Eph/ephrin molecules are also possible.

In cultured ephrin-B2-deficient VSMCs, signaling of the SH2-containing adaptor proteins Crk and Crk-associated substrate, p130(CAS), which control cell migration and cell-matrix adhesion, were altered. Accordingly, the cells showed defects in focal adhesion formation, cell adhesion, spreading, and directional migration, whereas cell motility was increased. Interestingly, many of these defects did not require cell-cell contact arguing that ephrin-B2 can control mural cell function in a cell-autonomous fashion and without interacting with receptors in trans. The molecular function of ephrin-B2 in mural cells is linked to PDGFRB plasma membrane distribution, endocytosis and signaling. Ephb2 mutant aorta lysates or PDGF-B-stimulated ephrin-B2-deficient VSMCs showed increased activation of PDGFRB, MAP kinase Erk1/2 and JNK, which was linked to more rapid, clathrin-dependent internalization of PDGFRB. While ephrin-B2 co-localized with caveolin and PDGFRB in the membrane fractions from control VSMCs, the loss of ephrin-B2 led to redistribution of PDGFRB into clathrin-containing membrane compartments, which preferentially contained the active, phosphorylated form of the receptor. These findings indicated that ephrin-B2 is a negative regulator of clathrin-mediated PDGFRB endocytosis and thereby MAP kinase and JNK signaling. At the same time, aortas from mice lacking ephrin-B2 in VSMCs or cultured Ephb2 KO smooth muscle cells also showed impaired activation of small GTPase Rac1 and strongly reduced expression of the Rac GTP Exchange Factor Tiam1 (Fig. 3B). Re-expression of full-length Tiam1 in Ephb2 KO cells partially rescued VSMC spreading, migration, focal adhesion formation, and proliferation. Thus, ephrin-B2 modulates not only the internalization of PDGFRB but is required for the balanced activation of different signaling transduction cascades in the PDGF pathway.

Stimulation of ephrin-B2 internalization with soluble, recombinant EphB4 fusion protein also triggered the clustering and endocytosis of surface PDGFRB, but only from the inactive, unphosphorylated pool (Fig. 3B). Under these conditions, residual PDGFRB at the cell surface was more readily activated by PDGF-B leading to enhanced autophosphorylation and MAP kinase signaling similar to ephrin-B2-deficient VSMCs. These results suggest that the presence/absence of ephrin-B2 or its interactions with EphB-expressing cells can strongly modulate PDGFRB activity and downstream signaling in mural cells. Future work should integrate these findings with the role of other known co-receptors and interaction partners of PDGFRB. Low density lipoprotein receptor-related protein 1 (LRP1), a transmembrane protein that can bind lipoprotein, proteases, growth factors, cytokines and matrix proteins, has been shown to associate with PDGFRB and modulates its expression, internalization and signaling. LRP6, another member of the LDL receptor-related protein family, can also bind PDGFRB and enhance the degradation of the RTK, which led to reduced PDGF-dependent VSMC proliferation. As a final example, the expression of Neuropilin-1 in VSMCs was shown to promote PDGF-stimulated smooth muscle cell migration.

Role of Ephrin-B2 in Kidney Damage and Fibrosis

Imbalance of receptor endocytosis due to the absence or increased activation of ephrin-B2 can lead to disease-like conditions in different tissues. In kidney, microvascular disease causes peritubular capillary (PTC) rarefaction. Here, pericytes migrate away from the capillary wall and differentiate into myofibroblasts, which results in kidney fibrosis. As a consequence, peritubular capillaries become less stable and regress. After inactivation of the Ephb2 gene in pericytes, mutant capillaries in embryonic skin were less stable due to impaired pericyte association, a phenomenon that mimics processes during the onset of fibrosis in kidney. During kidney injury, ephrin-B2 reverse signaling protected against PTC rarefaction by promoting angiogenesis and vascular stability. Pericytes were protected against myofibroblasts conversion and activation, which limited fibrogenesis. Cultured primary kidney microvascular endothelial cells from ephrin-B2ΔV mice showed impaired migration and proliferation after VEGF-A stimulation, which was linked to reduced VEGFR2 activation and internalization. Interestingly, in these mice, inhibition of VEGFR2 or PDGFRB with blocking antibodies or soluble receptor ectodomains was shown to ameliorate microvascular rarefaction, pericyte detachment and differentiation into myofibroblasts, which led to reduced fibrotic scar.
formation. However, future work will have to address whether this role of ephrin-B2 in pericytes is directly linked to the function of PDGFRβ or other receptors. These findings point at important disease-promoting roles of deregulated VEGFR2 or PDGFR signaling, which might be linked to Eph/ephrin molecules and/or other regulators of RTK endocytosis and signaling.

The examples above highlight how ephrin-B2 controls the function of RTKs in different cell types, which has fundamental impact on processes such as cell migration, proliferation, adhesion, cell-cell communication, or the assembly of functional tissue compartments. Important roles of Eph/ephrin molecules in trafficking, surface presentation and internalization have been also reported outside the vascular system and for receptors without tyrosine kinase activity. Thus, the regulation of a trafficking, surface presentation and internalization have been unraveled. What is the exact mechanistic role of ephrin-B2 and other Eph/ephrin molecules in receptor trafficking and endocytosis? What other molecules, adapters and interaction partners are involved? What is the precise nature of the endosomal compartments enabling receptor tyrosine kinase signaling after internalization? What are the exact contributions of ephrin reverse and Eph forward signaling in these processes and how do they affect the clathrin machinery and other endocytic pathways as well as the trafficking, recycling or degradation of interacting RTKs and surface receptors? Understanding these key questions will be undoubtedly challenging but, given the many important roles of Eph/ephrin regulation in almost every cell type and organ system, also worthwhile. As there is more and more evidence for roles of Eph receptors and ephrin ligands in disease processes, better insight into the underlying mechanism might also provide important leads for the development of novel therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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