

Survey

The ephrins and Eph receptors in angiogenesis

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Abstract

Eph receptors are a unique family of receptor tyrosine kinases that play critical roles in embryonic patterning, neuronal targeting, vascular development and adult neovascularization. Engagement of Eph receptors by ephrin ligands mediates critical steps of angiogenesis, including juxtacrine cell–cell contacts, cell adhesion to extracellular matrix, and cell migration. Recent evidence from *in vitro* angiogenesis assays and analysis of mice deficient for one or more members of the Eph family establishes the role of Eph signaling in sprouting angiogenesis and blood vessel remodeling during vascular development. Furthermore, elevated expression of Eph receptors and ephrin ligands is associated with tumors and associated tumor vasculature, suggesting that Eph receptors and their ephrin ligands also play critical roles in tumor angiogenesis and tumor growth. This review will focus on the relevance of Eph receptor signaling in embryonic and adult neovascularization, and possible contributions to tumor growth and metastasis. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Ephrins; Eph receptors; Receptor tyrosine kinases; Vascular development; Angiogenesis

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1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a multi-step process involving a diverse array of molecular signals. These include factors that stimulate endothelial cell proliferation, migration, and assembly, as well as recruitment of perivascular cells and extracellular matrix remodeling. Endothelial cell receptor tyrosine kinases (RTK) have been recognized as critical mediators of angiogenesis. These are the vascular endothelial growth factor (VEGF) receptor, Tie, and Eph RTKs [27,63,71]. The functions of both VEGF/VEGF receptor and angiopoietins/Tie-2 receptor families in vascular development and angiogenesis are well studied, and reviewed elsewhere [10,18,22,23,27,38,52,63,71]. The Eph receptor tyrosine kinase family, however, represents a new class of RTKs, and its role in angiogenesis is just beginning to emerge.

First discovered in a human cDNA library screen for homologous sequences to the viral oncogene *vfps* [31], Eph receptors are a unique class of RTK. First, unlike other families of RTKs, which bind to soluble ligands, Eph receptors interact with cell surface-bound ephrin ligands. Ephrins attach to the cell membrane either through a glycosylphosphatidyl inositol (GPI) anchor or a transmembrane domain. Second, distinct from other RTK ligands, ephrins do not promote cell proliferation; rather, they mediate cellular repulsion, adhesion, cell attachment to extracellular matrices, and cell migration in various cell types. Finally, these receptor–ligand interactions activate signaling pathways in a bi-directional fashion, through both the Eph receptors and ephrin ligands (also referred to as counter receptors).

Eph receptor tyrosine kinases and their ephrin ligands mediate many important biological processes in both invertebrates and vertebrates [33]. Their functions are best studied in the nervous system, where Eph and ephrin molecules are involved in patterning the developing hind-brain rhombomeres, axon pathfinding, and guiding neural crest cell migration [24]. Recently, a series of publications has demonstrated the essential role of Eph/ephrin in vascular development during embryogenesis and in adult angiogenesis. This review will focus mainly on the contributions of Eph and ephrin signaling to the process of developmental and pathological blood vessel formation.

2. Structure of Eph RTKs and ephrin ligands

The Eph family of receptor tyrosine kinases is the largest known family of RTKs identified, consisting of at least 14 receptors and 8 ligands [13,26]. Homologues of Eph receptors and ephrin ligands have been identified in vertebrate and invertebrate species, such as mice, *Xenopus laevis*, zebrafish and *Caenorabhditis elegans* [13]. For clarity, the Eph receptors have been divided in two subclasses, classes

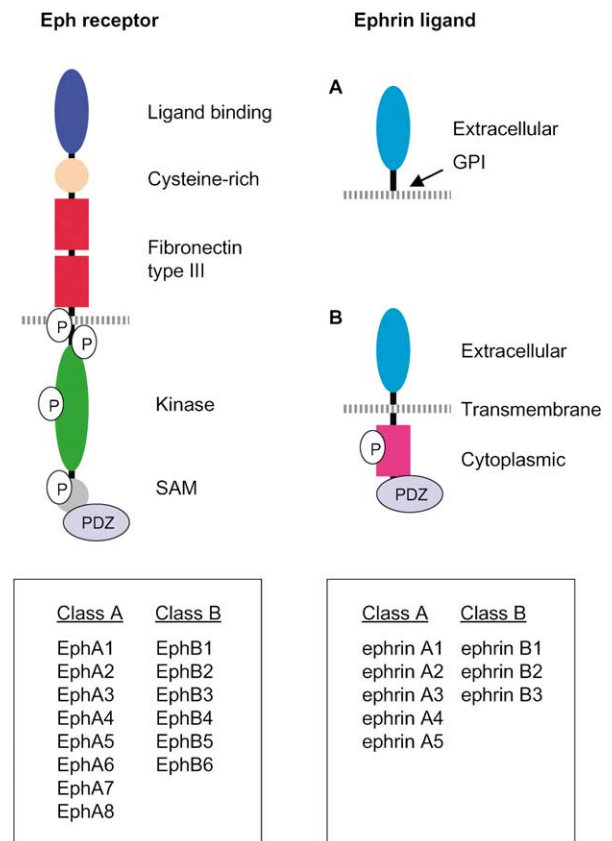


Fig. 1. Domain structure and signaling of Eph receptors and ephrin ligands. Both GPI-anchored ephrinA and transmembrane ephrinB ligands interact with the N-terminal globular domain of Eph receptors for ligand binding. The globular domain is followed by a cysteine-rich region and two fibronectin type III repeats, which contain a dimerization motif. Phosphorylated tyrosine residues provide docking sites for SH2 domain-containing signaling proteins. SAM domains form homodimers and may regulate receptor dimerization. Signaling proteins containing PDZ domains dock to the carboxy terminus of Eph receptors and ephrinB ligand.

A and B, depending on the type of interaction with the ephrin ligands. In general, Eph class A (EphA) receptors bind to GPI anchored ephrin ligands (ephrinA), while Eph class B receptors (EphB) bind to ephrin ligands containing transmembrane domains (ephrinB) (Fig. 1). The only receptor with significant binding affinity for both classes A and B ephrins is EphA4 [26].

Consistent with other types of RTKs, both A and B Eph class receptors contain a single transmembrane spanning domain. The extracellular region of the Eph receptor is glycosylated, and consists of a ligand binding domain containing immunoglobulin-like motifs, followed by a cysteine-rich domain and two fibronectin III-like repeats. The intracellular region consists of a juxtamembrane region, a single tyrosine kinase domain, and PDZ binding motif (PSD-95 post-synaptic density protein, Discs large, Zona occludens tight junction protein) within the non-catalytic region of the COOH-terminus [7,65]. The kinase domain and juxtamembrane region contain tyrosine residues, and

phosphorylation of these tyrosine residues creates docking sites for interactions with signaling proteins containing SH2/SH3 (Src-Homology-2/3) domains. The PDZ binding motif binds to PDZ domain-containing proteins, which are thought to serve as scaffolds for the assembly of multi-protein signaling complexes at the membrane.

Of the eight ligands known, five are attached to cell surface by GPI linkage and three by transmembrane domains. Analyses of amino acid sequences of ephrin ligands indicate that each ligand consists of a signal peptide at the amino terminus, followed by a conserved receptor binding region containing conserved cysteine residues and a spacer region. At the carboxy terminus, the class A ligands contain a hydrophobic region comprising the GPI linkage. In contrast, ephrinB ligands contain a transmembrane domain and a cytoplasmic domain containing PDZ-binding motif and conserved tyrosine residues that may be phosphorylated and serve as docking sites for proteins containing SH2/SH3 domains. These structural motifs control ligand binding, receptor and ligand binding, and regulate the binding of ephrins to specific Eph receptors to elicit distinct biological responses.

3. Bi-directional signaling between Eph receptors and ephrin ligands

Eph/ephrin signaling is complex in several respects. First, within each subclass, receptor–ligand binding is promiscuous. With few exceptions, one ligand is able to bind to multiple receptors; and conversely, one receptor can bind to multiple ligands within the same subclass [26]. Thus, the specificity of ligand–receptor interaction apparently comes from the cell-specific localization of these molecules in vivo. Another unique aspect of the ligand–receptor interaction in the Eph family is that the extent of receptor activation is dependent on the oligomerization state of the ligands. The effects on receptor phosphorylation and activation of downstream signaling pathways differ when soluble recombinant ephrin ligands are presented to receptor either as ephrin–Fc dimers, or as clustered multimers. For example, in human renal microvascular endothelial cells, presentation of ephrinB1–Fc to endogenous EphB1 in a non-clustered form results in receptor phosphorylation and a decrease in cell attachment to fibronectin, while clustered ephrinB1 can activate the EphB1 and induce cell attachment and capillary tubule formation [62]. Moreover, as both A and B class ephrins are capable of inducing signal transduction cascades upon binding Eph receptors, the initiation of bi-directional signaling through both receptor- and ligand-bearing cells also represents complexity in signaling. Thus, reciprocal and bi-directional signaling between Eph receptors and ephrin ligands, as discussed below, adds yet another level of complexity in Eph signaling. Taken together, these findings suggest that Eph signaling requires a combinatorial complexity in order to achieve precise in vivo regulation of

the diverse array of functions, from embryonic patterning, neuronal targeting, to angiogenesis.

3.1. Eph receptor downstream signaling

Binding of Eph receptors to their ligands induces receptor dimerization and subsequent *trans*-phosphorylation by the cytoplasmic kinase domains of the two receptors. Multiple tyrosine residues important for autophosphorylation and recruitment of adaptor proteins have been mapped to the juxtamembrane and kinase domains of the receptor. These phosphorylated tyrosine residues serve as docking sites for a number of cytoplasmic signaling proteins, including Ras-GTPase activating protein (RasGAP), Src and Abl family of non-receptor tyrosine kinases, low molecular weight phosphotyrosine phosphatase (LMW-PTP), phospholipase C γ , phosphatidylinositol 3-kinase, and adaptor proteins SLAP, Grb2, Grb10, and Nck [9,24,40]. Furthermore, PDZ binding motifs located at the C terminus of the Eph receptors bind to PDZ-domain-containing proteins such as AF6, a Ryk receptor tyrosine kinase interacting protein, Pick 1, a protein kinase C-interacting protein, Syntenin, a syndecan-interacting protein, and Grip1 and Grip2, two glutamate receptor interacting proteins [7,32,65].

Many of the proteins identified in the Eph signaling pathways have been implicated in regulating cell morphology, attachment and motility. For example, Eph receptors are found to regulate integrin-dependent cell adhesion through activation of c-Jun kinase via Nck-interacting Ste20 kinase in endothelial and neuronal cells [4,37]. In addition, focal adhesion kinase (FAK) was implicated in the regulation of cell adhesion in prostate carcinoma PC-3 cells [50]. As long suspected, Eph receptor signaling also regulates actin dynamics via small GTPases of the Rho family. Treatment of EphA-receptor-expressing cells with ephrinA5 increased the levels of GTP-bound Rho while reducing the levels of GTP-bound Rac, and ephrinA5-induced growth-cone collapse was suppressed by inhibition of Rho GTPase activity and the Rho-associated kinase ROCK [66]. A recent report showed that activation of EphA4 receptor recruits Ephexin (Eph-interacting exchange factor), a guanine nucleotide exchange factor, providing a direct link between Eph receptors and Rho family GTPases [59]. Taken together, these studies demonstrated the role of Eph receptor signaling in changing of cellular morphology and architecture, cell attachment and cell motility.

3.2. Signal transduction through ephrins

The cytoplasmic and transmembrane domains of ephrinB ligands are analogous to those of conventional receptor molecules. Similar to the receptor, ephrinB ligands share a single transmembrane domain, a cytoplasmic region, and a C-terminal PDZ binding motif. The cytoplasmic domains of ephrinB ligands become phosphorylated on tyrosine

residues following receptor binding [8,34]. It is currently unknown what downstream signaling molecules bind to the phosphorylated tyrosine residues. A search for proteins interacting with ephrinB ligands have yielded clones encoding PDZ domain-containing proteins, including Syn-tenin, Grip, Pick 1, Phip, the phosphotyrosine phosphatase FAP-1 [43,65], and more recently PDZ-RGS, a PDZ containing protein with GTPase stimulating activity, which regulates SDF-1 induced chemoattraction [44].

Despite lacking a cytoplasmic domain, ephrinA ligands also appear to be capable of signal transduction. There are precedents for GPI anchored proteins to signal, and a number of such proteins are involved in immune responses [57]. Recent data have shown that signaling through ephrinA ligands may be mediated by the recruitment of adapter proteins. The ephrinA ligands have been localized to lipid microdomains or rafts, which contain other signaling molecules such as G proteins and caveolin proteins, indicating ephrinA ligands may activate a number of signaling pathways [54]. Stimulation of ephrinA8 by EphA5-Fc has been shown to recruit and activate Fyn, a member of the Src kinase family. Fyn, in turn, activates p80 kinase to induce cytoskeletal changes in fibroblasts and increase cell adhesion to fibronectin through increasing the number of focal adhesions [17]. EphrinA2 and ephrinA5 have also been localized to lipid microdomains in HEK293 transfected cells. Stimulation by EphA3 results in integrin-dependent cell adhesion to laminin as well as tyrosine phosphorylation of an unknown 120 KD protein [36]. These observations indicate that ephrinA ligands are capable of transducing signals to influence actin cytoskeletal changes, attachment, and migration.

Functional evidence of bi-directional signaling came from studies in zebrafish and homozygous null mice. Mellitzer et al., developed a zebrafish animal cap assay to study cell intermingling between two populations of cell expressing either Eph receptor or ephrin ligand. Juxtaposition of these two populations of cell leads to restriction of cell intermingling and establishment of boundary. However, expression of truncated forms of either EphB2 or ephrinB2 lacking cytoplasmic domains in the animal cap results in cell intermingling across the border, indicating that signaling pathways directing cellular repulsion are activated through the ligand as well as the receptor [48]. More recently, the functional role of ephrin cytoplasmic domain is demonstrated in *ephrinB2^{ΔC/ΔC}* mice expressing a mutant ephrinB2 with cytoplasmic domain deletion [1]. *ephrinB2^{ΔC/ΔC}* mice exhibit vascular remodeling defects that are reminiscent of one subset of the phenotypes in *ephrinB2^{-/-}* null mice, indicating that the signaling through ephrinB2 is required for vascular development. Interestingly, unlike *ephrinB2^{-/-}* null mice, no defects in neural crest cell migration are present in *ephrinB2^{ΔC/ΔC}* mice. These results indicate that ephrinB2 signaling is required for vascular development, but is dispensable for neural crest cell migration during embryogenesis.

4. Regulation of Eph receptor/ligand expression and activities

Regulation of Eph ligand and receptor expression and activity occurs at many levels. Such regulation includes induction of Eph/ephrin transcription, re-distribution of Eph/ephrin molecules within the cell membrane upon cell stimulation, and internalization of receptor–ligand complex. Interestingly, many of the known modulators of Eph/ephrin signaling are known regulators of angiogenesis.

4.1. TNF- α and VEGF

Expression of ephrin ligands may be induced by growth factors and cytokines in various cell types. EphrinA1, the first ephrin ligand identified, was shown to be an immediate early gene product induced by tumor necrosis factor alpha (TNF- α) in cultured human umbilical venous endothelial cells (HUVEC) [35]. Unlike other angiogenic factors induced by TNF- α [6,60], we have shown that induction of ephrinA1 does not require NF- κ B or p42/44 MAPK signaling. Instead, TNF-induction of ephrinA1 requires the activation of both JNK and p38MAPK signaling pathways [12], each of which has been shown to regulate actin reorganization and cell migration in endothelial cells [14,61]. Thus, regulation of ephrinA1 expression through p38MAPK and JNK is consistent with the proposed roles of ephrinA1 in endothelial cell migration and blood vessel assembly.

In addition to TNF- α , ephrinA1 is also induced by lipopolysaccharides (LPS), interleukin-1 beta (IL-1 β) [35], and more recently discovered, by vascular endothelial growth factor (VEGF) (Cheng et al., submitted for publication) in HUVEC and microvascular endothelial cells. Our study revealed that similar to TNF- α , VEGF induces ephrinA1 as an immediate early gene product. Blocking class A Eph receptor signaling inhibits VEGF-induced endothelial cell survival, migration, sprouting in vitro and corneal angiogenesis in vivo, suggesting EphA receptor activation is required for VEGF-induced angiogenesis (Cheng et al., submitted for publication).

4.2. Transcriptional regulators of Eph receptors and ephrins

Homeobox-containing transcription factors have emerged as one of the major regulators of Eph expression. In the developing hindbrain, we have identified an enhancer element in the gene encoding EphA2 that is sufficient for rhombomere 4-specific expression in the developing hindbrain during embryogenesis. This element contains multiple Hox–Pbx consensus binding sites that bind to both Hoxa1/Pbx1 and Hoxb1/Pbx1 protein in vitro. Co-expression of either Hoxa1 or Hoxb1 with Pbx1 trans-activated EphA2 enhancer-dependent reporter gene expression. These data, together with observations of reduced EphA2 expression in *hoxa1* and *hoxb1* double mutant mice,

suggest that expression of *epha2* gene in rhombomere 4 is directly regulated by Hoxa1 and Hoxb1 homeobox transcription factors [11]. EphrinA1 expression also appears to be regulated by *hox* genes. Inhibition of expression of *hoxb3* by anti-sense oligonucleotides results in reduced expression of ephrinA1 in human microvascular endothelial cells, suggesting that *hoxb3* is involved in regulating ephrinA1 expression in endothelial cells [51]. Furthermore, Sonic Hedgehog and GH6 transcription factor are shown to regulate EphA3 expression in the retina [58]. In addition to homeobox-containing transcription factors, Krox-20 was found to directly activate the transcription of EphA4 (*sek-1*) in rhombomere 3 and 5 [64]. These data suggest that homeobox transcription factors may be common mediators of Eph/ephrin expression induced by many diverse signals.

4.3. Regulation of Eph receptor activities

Most receptor tyrosine kinase signals are terminated by removal of the soluble ligand–transmembrane receptor complex from the cell surface through internalization and subsequent degradation or dissociation. However, ephrins are membrane-anchored rather than soluble ligands, suggesting the existence of alternative strategies. Using ephrinB1–GFP fusion proteins, we have recently followed the movement of ephrinB1 molecules with time after its binding to EphB1 receptor (Roberts et al., unpublished results). When ephrinB1–GFP expressing cells are juxtapositioned next to EphB1-expressing cells, EphB1–ephrinB1 ligand–receptor complexes are endocytosed bi-directionally between adjacent cells upon ligand/receptor activation. Thus, receptor/ligand-mediated reciprocal endocytosis represents a novel mechanism for protein transfer between juxtacrine signaling cells. In support of this observation, Kinch and co-workers also show that upon binding to ligands, EphA2 receptor is rapidly internalized and degraded through a ubiquitin-mediated pathway (Kinch, personal communication).

An alternative mechanism for termination of ephrin signals in neurons is demonstrated by Hattori et al. [29]. After ligand–receptor engagement, ephrinA ligand is cleaved from the plasma membrane by a metalloprotease known as Kuzbanian. As receptor signaling is down-regulated upon ephrin cleavage, this solubilization has been hypothesized as a possible regulatory mechanism for termination of Eph/ephrin signaling. Taken together, these recent data revealed novel mechanisms of modulating Eph receptor and ephrin ligand activity.

5. Roles in cellular proliferation, migration, cell–cell and cell–matrix interactions

5.1. Regulation of cellular proliferation

Unlike many angiogenic or growth factors such as VEGF, EGF and bFGF, ephrins are not known to directly promote cellular proliferation or transformation. However,

over-expression of EphA1 in fibroblasts and EphA2 in normal breast epithelial cells promotes colony growth in soft agar and tumor formation in nude mice, indicating the ability of EphA2 receptor to transform normal cells [45,73]. Further evidence of Eph/ephrin involvement in cell proliferation came from the ability of Eph/ephrin signaling to regulate cell growth induced by other growth factors. Stimulation of EphA2 receptor by ephrinA1 in bovine aortic endothelial cells and mouse embryonic fibroblasts attenuates cell growth induced by PDGF, VEGF and EGF, apparently via inhibition of ras-mediated Erk pathways [49]. Thus, while it appears that mis-regulated expression of Eph receptors promotes transformation, normal expression of Eph receptors may function to negatively regulate cellular proliferation.

5.2. Regulation of cell–cell attachment

As cell surface bound molecules, Eph and ephrin signaling mediates cell-to-cell contacts. During neural development, Eph receptors and ligands often display reciprocal expression patterns on adjacent tissue compartments such as between neighboring rhombomere segments or between projecting axons and their targets. Bi-directional signaling of Eph receptors and ephrin ligands mediates cell–cell repulsion to establish rhombomere boundaries and to guide axons to their targets [24,42]. In addition to mediating cellular repulsive signals, interaction of two cell populations expressing either EphB receptors or ephrinB ligand reduces the number of gap junctions between these two populations of cells, preventing the communication of these two groups of cells [48].

Another mechanism of Eph receptor-regulated cell–cell contacts involves the cadherin family of adhesion molecules. EphA2 is expressed on the cell membrane at sites of cell-to-cell contact in normal breast epithelial cells. However, the localization of EphA2 is drastically changed in malignant breast adenocarcinoma cells lacking E-cadherin expression [72]. Further evidence of a functional relationship between Eph receptors and cadherins came from studies in embryonic stem (ES) cells. Loss of E-cadherin in ES cells results in cellular dissociation and reduction of endogenous EphA2 expression, while over-expression of E-cadherin rescues EphA2 expression [56]. Furthermore, injection of *Xenopus* EphA4 mRNA into *Xenopus* blastomeres causes blastomere dissociation, and injection of C-cadherin mRNA can rescue cell adhesion [70]. Taken together, these studies indicate that EphA receptors may function downstream of the cadherin family of adhesion molecules to mediate cadherin-induced cell attachment.

5.3. Regulation of cell–matrix contacts and cellular migration

Cell migration, a critical biological process for growth-cone pathfinding as well as angiogenesis, requires the adhesion to and dissociation of cells from extracellular matrices.

Activation of Eph/ephrin signaling has been shown to affect cell attachment via integrin-dependent mechanisms. Understanding of this mechanism, however, appears to be complicated, and might depend on specific cell types and experimental systems. In PC-3 prostate cancer cells transfected with EphA2, stimulation with ephrinA1–Fc results in an inhibition of cell adhesion and spreading on laminin and fibronectin, but not on poly-L-lysine, a non-integrin-dependent attachment factor. Further examination of EphA2 receptor-mediated attachment indicates that EphA2 directly inhibits integrin receptor function through dephosphorylation of FAK kinase [50]. In endothelial cells and P19 teratocarcinoma cells, EphB1 activation exerts the opposite effect, that is, to promote integrin-mediated cell attachment [37]. Integrin-mediated mechanisms also play an important role in ephrin-mediated cell–matrix adhesion. Stimulation of ephrinA8 in transfected NIH3T3 cells with EphA5–Fc results in activation of Fyn kinase and Erk pathways, enhanced cell attachment and spreading to laminin and fibronectin substrates, and an increase in focal adhesions [16]. Furthermore, stimulation of endothelial cells expressing endogenous ephrinB1 with EphB1 induces integrin-dependent cell attachment, migration and angiogenesis (Huynh-Do et al., submitted for publication). Taken together, these data suggest that signaling through either ephrin ligands or Eph receptors affect cell–matrix attachment through an integrin-dependent manner.

6. Function in vascular development during embryogenesis

The assembly of endothelial cells and supporting mesenchyme into mature and functional blood vessels is a multi-step process involving a diverse array of molecular signals. Ligands for receptor tyrosine kinases (RTK) such as VEGFs and angiopoietins are well known as critical mediators of blood vessel formation during embryogenesis. Recent studies from *in vitro* angiogenesis assays and *in vivo* homozygous null mice now firmly establish an essential role of Eph receptors and their ephrin ligands in vascular development.

6.1. Expression pattern in vascular development

Expression of ephrin ligands and Eph receptors during embryogenesis is highly dynamic, with expressions in various sites during different stages of development [33]. These sites include the organizer, or node, during gastrulation, different rhombomere segments during hindbrain patterning, neural crest cells that migrate to branchial arches during cranial morphogenesis, somites, and developing vasculature. Consistent with a role in vascular development, *in situ* hybridization of ephrinA1 showed expression in the mesoderm and pre-endocardial cells at E7.25 day, the dorsal aorta at E8.5 day, in the primary head vein, intersomitic vessels and

limb bud vasculature at E9.5 day [47]. Other class A Eph molecules have also been found to be expressed in the blood vessel lining of the aorta, branchial arch arteries, umbilical vein, primary head vein, and the endocardium of the heart [25].

Recently, class B members of the Eph/ephrin family have also been described in the developing cardiovascular system. Most interestingly, expression of *LacZ* reporter gene under the control of endogenous ephrinB2 or EphB4 promoter/enhancer in mice lacking one allele of ephrinB2 or EphB4 revealed complementary expression of ephrinB2 in arterial endothelial cells and its cognate receptor, EphB4 in venous endothelial cells [68]. Although later studies also found low levels of EphB4 on arterial endothelial cells and ephrinB2 on paravascular supporting cells [2], ephrinB2 and EphB4 are the best markers available for arterial and venous endothelial cells at very early stage of development. Other EphB molecules were also expressed at the sites of neovascularization. EphrinB1 is co-expressed with EphB1 in endothelium in the developing kidney [15]. EphB3 is expressed predominantly in veins, as is EphB4, but is also detected in some arteries such as those found in the yolk sac [2]. In addition to expression in endothelial cells, ephrinB2 and EphB2 are also expressed in mesenchymal supporting cells, suggesting a role of Eph receptors and ephrin ligands in vessel wall development through interactions between endothelium and mesenchyme [2].

6.2. Phenotypes in homozygous null mice

Recent studies from targeted disruption of Eph receptors and ligands in mice, together with studies *in vitro*, now firmly establish the role of ephrins/Eph in vascular development (Table 1). Disruption of the *ephrinB2* gene results in retardation in overall growth at E10, and embryonic lethality at E10.5 in homozygous null mice with a 100% penetrance. Vasculogenesis is halted at the primary plexus stage, endothelial cells are disorganized, and features of angiogenic remodeling such as branching and sprouting are absent. Defects in differentiation of mesenchyme-derived cells are also detected in the yolk sac, suggesting disrupted interactions between endothelial and mesenchymal-derived support cells [68]. Overall, these defects are detected in both arterial and venous blood vessels, such as those in the yolk sac and head. Since ephrinB2 is expressed in arterial endothelial cells, the defects in venous blood vessels indicate that absence of ephrinB2 not only affects signaling transduced through the receptor, but also through the ligand [68]. In support of this notion, expression of truncated ephrinB2 lacking cytoplasmic domain in ‘knock-in’ mice reveals angiogenic remodeling defects reminiscent to those in ephrinB2 knockout mice, indicating that the cytoplasmic tail is required for ligand reverse signaling [1]. Targeted deletion of EphB4 phenocopies the loss of ephrinB2, suggesting that this receptor/ligand pair cooperates in the regulation of vascular development in embryo [28]. Disruption of other individual member of B class

Table 1
Defects resulting from homozygous mutations of Eph/ephrin genes

Gene knock out/knock in	Phenotype	Reference
<i>ephrinB2</i> ^{-/-}	Embryonic lethal, die at E10.5 Defective vessel remodeling and sprouting Heart trabeculation defects Defects in guidance of migrating cranial neural crest cells	[1,68]
<i>ephrinB2</i> ^{ΔC/ΔC}	Defects in angiogenic remodeling similar to those observed in <i>ephrinB2</i> ^{-/-} No guidance defects of neural crest cell migration	[1]
<i>ephB3</i> ^{-/-}	No vascular defects Defects in the formation of corpus callosum	[2,55]
<i>ephB2</i> ^{-/-}	No vascular defect Defects in pathfinding of commissural axons	[2] [30]
<i>ephB2</i> ^{-/-} <i>ephB3</i> ^{-/-}	Embryonic lethal, die at E10.5 (~30%) Defects in neural development Defective vessel remodeling, similar to those observed in <i>ephrinB2</i> ^{-/-}	[55] [5] [2]
<i>ephB4</i> ^{-/-}	Embryonic lethal, die at E10.5 Defective vessel remodeling, similar to those observed in <i>ephrinB2</i> ^{-/-}	[28]

Eph receptors, EphB2 or EphB3, does not lead to overt defects in developing vasculature. However, mice deficient in both EphB2 and EphB3 display a similar vascular defect to that of ephrinB2 or EphB4 knock out mice. This vascular phenotype in *ephB2*^{-/-}, *ephB3*^{-/-} mice exhibits 30% penetrance, indicating functional compensation by other EphB receptors.

Taken together, expression data and functional studies in mutant mice suggest that Eph/ephrin molecules play critical roles in the developing vasculature at multiple levels. First, interaction between ephrinB2 and EphB4 at the arterial–venous interface might restrict arterial and venous endothelial cell intermingling, thereby stimulating formation of new capillary sprouts. Furthermore, juxtacrine expression of ligand and receptor between adjacent endothelial cells might be required for establishing contact-dependent communication and promote vascular assembly. Finally, reciprocal expression of Eph/ephrin molecules between endothelial cells and adjacent mesenchymal cells might be important for the differentiation of mesenchymal cells into perivascular supporting cells, which is critical for the maintenance of a stable and mature vessel.

7. Functions in tumorigenesis and angiogenesis

7.1. Effects on tumor cells

Elevated expression and activity of Eph receptors have been correlated with the growth of solid tumors. EphA1, the first Eph receptor identified, was found to be expressed in erythropoietin producing hepatoma cells [31], and in breast, liver, lung, and colon carcinoma [46]. Over-expression of EphA1 in NIH3T3 cells led to the foci formation in soft agar and promoted tumor formation in nude mice [45]. EphA2 has also been found to be upregulated in a number of human

tumors including melanoma, breast, prostate, and colon carcinoma, and the higher expression levels were found to correlate with the more malignant and metastatic tumors [19,20,53,67,73]. Consistent with this observation, EphA2 was expressed in the undifferentiated and invasive mammary tumors of mice expressing the H-Ras transgene, but not in the well-differentiated and non-metastatic mammary tumors of c-Myc expressing mice [3]. Furthermore, our study revealed that EphA2 is expressed in other tumor types in mice, including pancreatic islet carcinoma in RIP-Tag transgenic mice, and in 4T1 and EMT6 malignant breast carcinoma cell-derived tumors in BalB/c mice (Brantley et al., submitted for publication). Other Eph receptors of both A and B classes are also over-expressed in a variety of cancers and tumor cell lines, including melanoma, gastric, esophageal, and colon cancer, and hematopoietic tumors [20,41,69].

The upregulated expression of Eph receptors or ephrins may have several roles in promoting tumorigenesis. First, over-expression of ephrinA1 in melanoma cells correlates with an increase in tumor cell growth, indicating that ephrins may act as a cell survival factor or a promoter of abnormal cell growth in tumor cells [21]. Second, elevated Eph receptor levels in tumor could affect cell–cell attachment through interaction with cadherins. Loss of E-cadherin has been shown to affect the expression and subcellular localization of several Eph receptors and ephrins [56,72]. Conversely, cadherin function may also be regulated by Eph receptors and ephrin ligands, since ectopic expression of ephrinB1 or activated EphA4 in early *Xenopus* embryos disrupted cadherin-dependent cell adhesion [39,70]. Although the precise nature of this reciprocal regulation remains to be determined, the loss of cellular adhesion is a hallmark of metastatic cancer. Third, inappropriate expression and regulation of Eph receptors could also affect cell–matrix interaction by modulating integrin activity. Miao et al. reported that activation of EphA2 receptor in PC-3 prostate carcinoma

cells induced transient inhibition of integrin-mediated cell adhesion through rapid recruitment of the protein tyrosine phosphatase SHP2 and subsequent dephosphorylation and inactivation of focal adhesion kinase (FAK) [50]. Zhou et al. described that activation of EphB2 resulted in reduced integrin-mediated cell adhesion in a heterologous cell system, apparently through phosphorylation of R-Ras and interfering with the ability of small GTPase to support integrin activity [74]. In a proof-of-principle experiment, over-expression of EphA2 in normal breast epithelial cells results in malignant tumors in athymic mice [73]. Taken together, dysregulation of Eph receptor activity may enhance tumor cell motility, invasion and metastasis through affecting cell survival, cell–cell and cell–matrix attachment.

7.2. Roles in tumor angiogenesis

The growth of solid tumors is highly dependent on the ability to recruit blood vessels, which supply the tumor with growth factors and oxygen necessary for tumor survival, growth and malignancy. Although it is now clear that Eph receptors and ephrin ligands play a critical role in vascular development during embryogenesis, the function of these molecules in pathological angiogenesis has not been well characterized. A survey of expression patterns of Eph molecules in tumor vasculature revealed that the ephrinA1 and EphA2 ligand–receptor pair is consistently expressed in endothelial cells of tumor associated vessels in a variety of tumors, including tumor xenographs (MDA-MB-435 human breast cancer and KS1767 human Kaposi's sarcoma) and human tumor specimens (lung anaplastic adenocarcinoma and squamous carcinoma, gastric cancer, colon carcinoma, and kidney clear cell carcinoma) [53]. We have determined the expression patterns of ephrinA1 and EphA2 in two murine tumor models that are angiogenesis-dependent, the RIP-Tag islet carcinoma transgenic model and 4T1 transplantable metastatic mammary carcinoma model (Brantley et al., submitted for publication). EphrinA1 ligand was expressed predominantly in tumor tissue, and EphA2 receptor expression was mainly associated with tumor vasculature. In addition, a soluble EphA2 receptor inhibited tumor neovascularization in a dorsal vascular window assay (Brantley et al., submitted for publication). These data suggest a role of ephrin/Eph molecules in promoting angiogenesis in tumor.

The precise mechanism of how Eph/ephrin regulates tumor angiogenesis is not known. However, from the available data it is conceivable that Eph/ephrin-dependent tumor neovascularization is mediated by the interplay of Eph receptors/ephrin ligands expressed by tumor cells and endothelial cells (Fig. 2). Ephrins expressed on the tumor cells may function as contact-dependent organizing molecules to guide incoming vessel that express EphA2 receptor (Fig. 2A). Alternatively, angiogenic factors such as VEGF or TNF- α in the tumor microenvironment may induce the expression and/or activation of ephrins in endothelial cells,

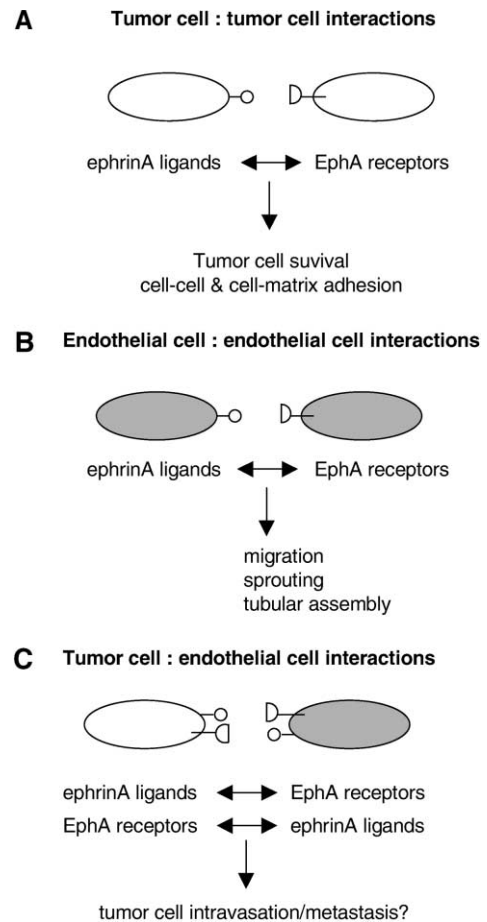


Fig. 2. Possible functions of Eph/ephrin signaling in tumor growth, angiogenesis, and metastasis. (A) EphA/ephrinA signaling in tumor cells may affect tumor cell survival, cell–cell and cell–matrix adhesion. (B) EphA/ephrinA signaling in endothelial cells promotes endothelial cell migration, sprouting, and vessel assembly. (C) EphA/ephrinA signaling between tumor cells and endothelial cells might affect tumor cell intravasation and metastasis.

as suggested in studies from cultured endothelial cells (Daniel, personal communication; Cheng et al., submitted for publication). The ephrins may then interact with Eph receptors on adjacent endothelial cells to promote endothelial cell sprouting, migration, and capillary tube formation through bi-directional signaling (Fig. 2B). Furthermore, the interactions between tumor cells and host blood vessel may provide a mechanism for tumor cell to intravasate into blood stream, facilitating tumor metastasis (Fig. 2C). Regardless of mechanisms, Eph receptors and ephrin ligands would be attractive candidates as tumor prognostic markers and potential targets for therapeutic intervention in cancer.

8. Perspectives

Eph receptors and ephrin ligands mediate cell–cell interaction in many important biological processes. While

their functions in the nervous system are well studied, the work on ephrin/Eph functions in the vasculature is only just beginning. Many important questions remain to be addressed. What are the signaling mechanisms downstream of Eph/ephrins that govern interactions between endothelial cells, endothelial and perivascular supporting cells, and endothelial and tumor cells? Do they regulate cell adhesion or de-adhesion? Are they attractive signals to induce angiogenic sprouting or repulsive signals to restrict cell intermingling? Do they have a role in tumor metastasis by facilitating tumor cell intravasation? How are the expression and activities of Eph/ephrin molecules regulated? Do Eph receptors/ephrins cooperate with other angiogenic factors, such as VEGF or angiopoietins, in regulating the growth of new blood vessels? Just as molecular inhibitors for VEGF have been developed to combat tumorigenesis, further dissection of the molecular mechanisms of Eph/ephrin-dependent angiogenesis will contribute to a better understanding of the process of blood vessel formation, and may provide potential targets for therapeutic intervention in cancer.

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