Essential Roles of EphB Receptors and EphrinB Ligands in Endothelial Cell Function and Angiogenesis

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Abstract

Eph receptor tyrosine kinases and their Ephrin ligands represent an important signaling system with widespread roles in cell physiology and disease. Receptors and ligands in this family are anchored to the cell surface; thus Eph/Ephrin interactions mainly occur at sites of cell-to-cell contact. EphB4 and EphrinB2 are the Eph/Ephrin molecules that play essential roles in vascular development and postnatal angiogenesis. Analysis of expression patterns and function has linked EphB4/EphrinB2 to endothelial cell growth, survival, migration, assembly, and angiogenesis. Signaling from these molecules is complex, with the potential for being bidirectional, emanating both from the Eph receptors (forward signaling) and from the Ephrin ligands (reverse signaling). In this review, we describe recent advances on the roles of EphB/EphrinB protein family in endothelial cell function and outline potential approaches to inhibit pathological angiogenesis based on this understanding.

I. INTRODUCTION

The Eph receptor family, which includes 14 members, constitutes the largest family of tyrosine kinase receptors in mammals. Eph receptors and their Ephrin (Eph receptor interacting) ligands form a system of cell communication with widespread roles in physiology and disease. In mammals, there are nine EphA (EphA1–8, and EphA10) receptors, which promiscuously bind five glycosylphosphatidylinositol (GPI)-linked EphrinA ligands and five EphB (EphB1–4 and EphB6) receptors, which promiscuously bind three transmembrane EphrinB ligands (Chrencik *et al.*, 2006; Egea and Klein, 2007; Kullander and Klein, 2002; Pasquale, 2010). There are some exceptions: EphA4 can bind both A-type and most B-type Ephrins, EphB2 can bind EphrinA5, and EphB4 essentially binds only EphrinB2 (Table I). Typically, Eph receptor–Ephrin ligand interactions occur at the cell's surface at sites of cell-to-cell contact and result in bidirectional signaling from the Eph receptor (forward signaling) and from the Ephrin ligand (reverse signaling) (Chrencik *et al.*, 2006; Egea and Klein, 2007; Pasquale, 2010).

EphrinB2 and its receptor EphB4 play critical roles in the development of the vascular system and contribute to the function of the adult vasculature (Pitulescu and Adams, 2010). Knockout mice lacking EphrinB2 or EphB4 expression and mice with deletion of EphrinB2 targeted to the endothelial cells display a severely compromised vascular system and die at midgestation (Adams *et al.*, 1999; Gerety and Anderson, 2002; Gerety *et al.*, 1999). The targeted deletion ofEphrinB2 inmural cells (pericytes and smoothmuscle cells) leads to diffuse tissue edema, hemorrhaging, and perinatal death of the mice (Foo *et al.*, 2006). EphrinB is phosphorylated in angiogenic blood vessels, and inhibition of phosphorylation-dependent or PDZ-dependent signaling down-stream of EphrinB ligands prevents endothelial cell sprouting and the proper assembly of endothelial cells with other endothelial cells and with pericytes (Salvucci *et al.*, 2006, 2009; Sawamiphak *et al.*, 2010b;Wang *et al.*, 2010b).

In this review, we will present current information on structural features, signaling mechanisms, and expression and function of the EphB/EphrinB proteins in the context of blood vessel physiology and pathology.

II. STRUCTURAL FEATURES AND INTERNALIZATION

The extracellular portion of Eph receptors (both A and B families) includes a globular, ligand-binding domain; a cysteine-rich EGF-like region; and two fibronectin-type III repeats (Himanen and Nikolov, 2003; Himanen *et al.*, 2001; Toth *et al.*, 2001). The intracytoplasmic portion consists of a short membrane-proximal region, a tyrosine kinase domain, a sterile alpha motif (SAM) protein–protein interaction domain, and a PDZ-binding C-terminal motif (Fig. 1A; Chrencik *et al.*, 2006). The B Ephrins display an extracellular Eph receptor-binding domain, a single-pass transmembrane region, a short intracellular domain with several sites for tyrosine and serine phosphorylation, and a C-terminal PDZ-binding motif (Fig. 1A; Chrencik *et al.*, 2006).

Initial high-affinity interaction between the Eph receptor globular domain and a protruding loop of EphrinB leads to conformational changes of receptor and ligand, and the formation of heterodimers of an Eph receptor with an Ephrin molecule (Himanen *et al.*, 2001; Toth *et al.*, 2001). Other residues in the Eph receptor contribute to a lower-affinity binding interface for much of the extracellular region of EphrinB2, leading to the association of two homodimers to form a tetrameric complex comprising two Eph receptors and two Ephrin molecules (Chrencik *et al.*, 2006; Himanen and Nikolov, 2003). Eph/Ephrin tetramers can aggregate into larger clusters through several low-affinity Eph–Eph and Ephrin–Ephrin interactions identified in the extracellular domains (Fig. 1B; Smith *et al.*, 2004).

One of the reported consequences of high-affinity interactions involving EphB and EphrinB expressed on adjacent cells is internalization of both molecules into Eph or Ephrin-expressing cells (Lauterbach and Klein, 2006; Mann *et al.*, 2003; Marston *et al.*, 2003; Zimmer *et al.*, 2003). This process provides a mechanism for removal of Eph receptors and ligands from the cell surface and termination of receptor/ligand adhesive interactions, which may explain the occurrence of cell repulsion (Egea and Klein, 2007). EphB–EphrinB complexes, detected in intracellular vesicles as full-length proteins, are believed to be the result of *trans*-endocytosis from one cell to the adjacent cell (Fig. 2). The direction of endocytosis seems to be dependent on the direction of signaling. For example, if a cell expressing EphB2 contacts a cell expressing EphrinB1 in which the C-terminal domain is truncated, endocytosis of the complex occurs preferentially into the EphB2-expressing cell (Zimmer *et al.*, 2003). By contrast, if EphB2 is signaling-deficient, the internalization occurs in the EphrinB1-expressing cell. If, however, both EphB and EphrinB are signaling-impaired, internalization of the Eph/Ephrin complex is not observed (Zimmer *et al.*, 2003). Although the biochemical basis for internalization of Eph/Ephrin is currently poorly defined, EphB1 was found to be associated with caveolin-1, suggesting the potential involvement of caveolae (Vihanto *et al.*, 2006), and EphrinB1 was found to be associated with clathrin-coated vesicles, suggesting a clathrin-dependent mechanism (Vihanto *et al.*, 2006). Once internalized, signaling (reverse or forward) has been shown to persist within the recipient cell (Marston *et al.*, 2003).

In addition to undergoing internalization of themselves, EphB and EphrinB can promote the internalization of the surrounding membrane and other proteins. For example, EphB signaling has been shown to modulate the clathrin-mediated endocytosis of amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid-type glutamate receptors in neurons (Irie *et al.*, 2005), and EphrinB signaling has been reported to promote the internalization of VEGFR2 and VEGFR3 in endothelial cells (Sawamiphak *et al.*, 2010b; Wang *et al.*, 2010b).

III. BIDIRECTIONAL SIGNALING

Eph receptor signaling induced by Ephrin binding (forward signaling) is initiated by autophosphorylation and Src family kinases-mediated phosphorylation of the intracellular tyrosine residues, resulting in the activation of the tyrosine kinase catalytic domain (Kalo and Pasquale, 1999; Knoll and Drescher, 2004; Kullander and Klein, 2002; Murai and Pasquale, 2003). Once the Eph receptors are phosphorylated, adaptor proteins containing Src-homology 2 (SH2) domains can bind and initiate phosphorylation of downstream substrates (Brantley-Sieders *et al.*, 2004; Kalo and Pasquale, 1999; Murai and Pasquale, 2003; Wybenga-Groot *et al.*, 2001; Zisch *et al.*, 2000). Activated Eph receptors can also mediate other protein–protein interactions via the SAM and PDZ-binding motifs, which contribute to signaling (Chrencik *et al.*, 2006; Kullander and Klein, 2002; Noren and Pasquale, 2004). Key components of Eph signaling are the Rho family of GTPases, including RhoA, Cdc42, and Rac, which are involved in the regulation of the actin cytoskeleton and cell shape, movement, and adhesion (Klein, 2009; Ogita *et al.*, 2003; Shamah *et al.*, 2001). Rho GTPases shuttle between an inactive (GDP-bound) and a signaling-active (GTP-bound) state, and this transition is regulated by guanidine nucleotide exchange factors (GEFs), which activate the Rho GTPases (Holland *et al.*, 1997). Phosphorylated Eph receptors have been shown to associate and activate a number of Rho GEFs, including Vav2, Tiam, Kalirin, and Intersectin (Cowan *et al.*, 2005; Klein, 2009; Murai and Pasquale, 2005; Sahin *et al.*, 2005). In addition, phosphorylated EphB has been shown to induce the ubiquitination and degradation of the GEF Ephexin 5, which binds to EphB and inhibits RhoA activity (Margolis *et al.*, 2010).

Unlike Eph receptors, B Ephrins do not possess intrinsic catalytic activity, and thus rely on the recruitment of signaling molecules to signal. EphrinB signaling upon receptor engagement and clustering (reverse signaling) is initiated by the recruitment and activation of Src family kinases, which phosphorylate specific tyrosine residues in the intracytoplasmic domain of B Ephrins (Palmer *et al.*, 2002; Fig. 3A). The activation of Src kinases is dependent upon metalloproteinase and presenilin-1-γ-secretase-mediated enzymatic cleavage of an intracellular peptide of EphrinB that binds to Src inhibiting its association with the inhibitory protein Csk (Georgakopoulos *et al.*, 2006). Phosphorylated EphrinB provides a docking site for the SH2 domain-containing adaptor protein Grb4 and the signal transducer and activator of transcription STAT3 (Bong *et al.*, 2007; Cowan and Henkemeyer, 2001; Segura *et al.*, 2007; Tanaka *et al.*, 2003; Xu and Henkemeyer, 2009). The EphrinB–Grb4 complex results in the activation of focal adhesion kinase (FAK) catalytic activity and recruitment of the G-protein-coupled receptor kinase interacting protein (GIT) 1 (Cowan and Henkemeyer, 2001; Segura *et al.*, 2007). Grab4 can also associate with other proteins involved in cytoskeleton regulation, including dynamin, Cbl-associated protein (CAP/ponsin), the Abl-interacting protein (Abi-1), and p21-activated kinase (PAK1; Cowan and Henkemeyer, 2001). Upon binding to EphrinB, STAT3 undergoes Jak-2-dependent phosphorylation and migrates to the nucleus, where it regulates a variety of target genes, thus linking membrane EphrinB to the nucleus (Bong *et al.*, 2007).

EphrinB can also become phosphorylated in the absence of receptor engagement. In the chicken retina, FGF can induce tyrosine phosphorylation of endogenous EphrinB, presumably through the coexpressed FGF receptor (Chong *et al.*, 2000; Lee *et al.*, 2009). In fibroblasts, activation of the PDGF receptor induces EphrinB1 phosphorylation (Bruckner *et al.*, 1997), as does activation of Tie2 by angiopoietin-1 in endothelial cells (Adams *et al.*, 1999).

A number of studies have highlighted the importance of tyrosine phosphorylation-independent signaling by EphrinB originating from protein–protein interactions involving

the C-terminal PDZ-binding motif (Egea and Klein, 2007; Lin *et al.*, 1999; Noren and Pasquale, 2004). Genetic studies have shown that knock-in mice expressing a mutant EphrinB2, in which the five tyrosines have been substituted to prevent phosphorylation, are only modestly affected (Makinen *et al.*, 2005). In contrast, knock-in mice expressing a mutant EphrinB2, in which the PDZ-interacting domain was disrupted, present major defects in lymphatic vascular (Makinen *et al.*, 2005) and retinal vessel development (Sawamiphak *et al.*, 2010b).

Biochemical studies have provided examples of phosphorylation-independent EphrinB signaling. In one such example, both the WTand phosphorylation-deficient EphrinB2 proteins were reported to similarly regulate the internalization of VEGFR2, whereas the PDZ-mutant EphrinB2 did not. This observation suggested that the binding of activated EphrinB2 to a PDZ-containing protein (yet to be identified) mediates the internalization of VEGFR2 or VEGFR3 (Sawamiphak *et al.*, 2010b; Fig. 3B). In another example, the EphrinB cytoplasmic domain was reported to bind to the PDZ-containing RGS3 (regulator of G-protein signaling) protein, which can regulate G-protein-coupled receptor signaling (Lu *et al.*, 2001; Fig. 3C). Another PDZ-dependent signaling pathway involves the interaction between EphrinB and PAR-3, a scaffold protein member of the Par (partitioning defective) complex (Lin *et al.*, 1999), which has been shown to stimulate Cdc42-induced Rac activation (Lin *et al.*, 1999; Nishimura *et al.*, 2005). Dishevelled, a scaffold protein that plays important roles in the Wnt signaling pathway, has been shown to bind to unphosphorylated EphrinB as well as to tyrosine-phosphorylated EphrinB, a binding which is mediated by Grb4 (Boutros and Mlodzik, 1999; Tanaka *et al.*, 2003). The phosphorylation-independent EphrinB1 binding of Dishevelled was found to require the presence of the DEP (Dishevelled, Egl–10, Pleckstrin), but not the PDZ domain, of Dishevelled (Lee *et al.*, 2006). Functionally, the binding of Dishevelled to EphrinB1 has been shown to mediate signals through the Rho small GTPase pathway (Lee *et al.*, 2006; Tanaka *et al.*, 2003; Fig. 3D). More recently, Par-6, another member of the Par protein family, was found to bind to EphrinB1 at the C-terminus through a non-PDZ-binding motif (Lee *et al.*, 2009). This Par-6/EphrinB1 complex was found to compete with the small GTPase Cdc42 for binding to Par-6, providing evidence that Par-6 is a mediator of EphrinB1 signaling (Lee *et al.*, 2009; Fig. 3E). Recent genetic experiments have further suggested the existence of phosphorylation and PDZ-independent EphrinB signaling, potentially mediated by Grb4 docking via the EphrinB SH3 domain or the association with Dishevelled (Dravis and Henkemeyer, 2011).

There is evidence of cross-regulation between phosphorylation and PDZ-dependent EphrinB signaling. For example, the tyrosine phosphatase PTB-BL, which contains a PDZ motif, is recruited to the active EphrinB and can negatively regulate EphrinB phosphorylation and Src activity (Palmer *et al.*, 2002; Fig. 3F).

Many of the signaling pathways initiated by EphB/EphrinB signaling described above have been identified in endothelial cells and have been linked to various endothelial cell functions (Chrencik *et al.*, 2006). EphB4 signaling has been shown to activate the Akt, PI3K (phosphatidylinositol 3-kinase), and the MAPK (mitogen activated protein kinases) pathways promoting endothelial cell proliferation and migration (Steinle *et al.*, 2002). EphB2 and EphB4 signaling was found to enhance SDF1/CXCL12-induced Akt phosphorylation (Salvucci *et al.*, 2006). EphrinB phosphorylation was reported to transiently activate Src family kinases, which are positive regulators of EphrinB phosphorylation, inducing endothelial cell sprouting (Palmer *et al.*, 2002; Salvucci *et al.*, 2009). Also, EphrinB phosphorylation was reported to induce Jak2-dependent STAT3 phosphorylation, contributing to endothelial cell assembly onto extracellular matrix (Salvucci *et al.*, 2009). EphrinB2 stimulation resulted in the activation of the PI3K and MAPK pathways in vascular

endothelial cells promoting their proliferation (Steinle *et al.*, 2003) and migration (Maekawa *et al.*, 2003). EphrinB1 activation promoted JNK phosphorylation through interaction of the C-terminal domain with PDZ-containing proteins enhancing endothelial cell attachment and migration (Huynh-Do *et al.*, 2002).

Studies *in vivo* have revealed the importance of EphrinB2–PDZ interaction for reverse signaling underlying the development of lymphatic vessels (Makinen *et al.*, 2005), and the regulation of VEGFR2 and VEGFR3 internalization and signaling in blood vessels thereby modulating endothelial cell growth, survival, and migration (Sawamiphak *et al.*, 2010b). Although the biochemical basis for signaling from EphrinB/PDZ interactions has not been clarified in endothelial cells, the contribution of Rho family GTPases might be important in this context, analogous to the situation in neuronal cells and other cells (Egea and Klein, 2007; Lee *et al.*, 2006; Noren and Pasquale, 2004; Tanaka *et al.*, 2003, 2004).

IV. ROLE OF B-TYPE EPHS AND EPHRINS IN VASCULAR DEVELOPMENT

The formation of blood vessels starts early during development of the mouse embryo, and the presence of functional vessels becomes necessary around midgestation. Two processes contribute to the development of vascular structures: vasculogenesis, the process by which endothelial cell precursors (angioblasts) form endothelial tubules, which contributes to the formation of the primary vascular plexus in the yolk sac, and angiogenesis, the process by which an existing vasculature forms sprouts and generates new vessels, thus expanding and remodeling an existing vascular bed, which contributes to the formation of most vessels during development and after birth.

Genetic experiments in mice have demonstrated that the global deletion of EphB4 or EphrinB2 leads to embryonic death with marked defects in the "remodeling" of the primary vascular plexus, suggesting that these molecules are critical to early vascular development (Adams *et al.*, 1999; Gerety *et al.*, 1999;Wang *et al.*, 1998). Consistent with this interpretation, deletion of EphrinB2 targeted to the developing vasculature produced a similar phenotype (Gerety and Anderson, 2002). The phenotype of EphB4-null or EphrinB2-null mice was indistinguishable, suggesting that forward signaling, reverse signaling, or a combination of forward and reverse signaling is necessary for normal vascular development. Mutant mice, in which the carboxy-terminal cytoplasmic tail of EphrinB2 was truncated (residues 264–336), displayed a very similar vascular phenotype to that of EphrinB2-null mice (Adams *et al.*, 2001), suggesting that EphrinB2 reverse signaling is critical to early vascular development. A subsequent study using the same EphrinB2 C-terminal mutation in a different construct concluded that the cytoplasmic domain of EphrinB2 is not essential for vascular development as the mice were born live (Cowan *et al.*, 2004). This study suggested that the discordant results were attributable to differences in trafficking of the mutant EphrinB2 to the cell surface and that the defective vascular development was attributable to defective stimulation of Eph signaling (Cowan *et al.*, 2004). Nonetheless, cardiovascular development was compromised also in these mice, suggesting that EphrinB2 reverse signaling is critical at later stages of cardiovascular development (Cowan *et al.*, 2004). Other genetic experiments examined the relative signaling contribution of phosphorylation and the PDZ-binding domain of EphrinB2 to vascular development (Makinen *et al.*, 2005). Knock-in mice expressing a mutant EphrinB2, in which the conserved tyrosine residues were mutated to prevent phosphorylation, had no appreciable blood vascular defects (Makinen *et al.*, 2005). Similarly, knock-in mice expressing a PDZ-mutant EphrinB2 were born normally without apparent blood vascular defects (Makinen *et al.*, 2005). However, these EphrinB2 PDZ-mutant knock-in mice exhibited marked defects in lymphatic vessel development, whereas the phosphorylation-deficient knock-in mice had only minor defects in the lymphatic vessels (Makinen *et al.*, 2005). Additional experiments using EphrinB2 PDZ-

mutant mice concluded that EphrinB2 signaling is required for the normal development of retinal vessels (Sawamiphak *et al.*, 2010b). Together, these results suggested that EphrinB2 reverse signaling mediated by the PDZ-binding domain is a critical contributor to lymphatic and retinal blood vessel development (Makinen *et al.*, 2005; Sawamiphak *et al.*, 2010b). EphrinB2-targeted deletion in pericytes and smooth muscle cells caused perinatal lethality associated with developmental defects in small-diameter blood vessels, which were not properly covered with smooth muscle cells/pericytes (Foo *et al.*, 2006). Similar to the observations in mutant mice with a targeted deletion of EphrinB2 in the endothelium, overexpression of EphrinB2 in the endothelium during early development caused marked vascular defects and premature death at midgestation, providing evidence for a dosage-dependent function of EphrinB2 during vascular development (Wang *et al.*, 2010b).

Several studies have revealed the importance of EphB4 and EphrinB2 in the determination of arterial–venous fate of endothelial cells (Adams *et al.*, 1999; Gerety *et al.*, 1999; Wang *et al.*, 1998; Zhong *et al.*, 2001; Fig. 4). Studies in zebrafish demonstrated that angioblasts migrating to the trunk (where they form the dorsal aorta and the cardinal vein) are predetermined to either an arterial or a venous fate based on their selective expression of the arterial marker EphrinB2 or venous marker EphB4. Initially, angioblasts assemble into a single precursor vessel from which the venous-fated, EphB4-expressing endothelial cells migrate and segregate to form the cardinal vein, whereas the EphrinB2-expressing cells do not move ventrally (Herbert *et al.*, 2009). Various approaches to limit EphrinB2 or EphB4 function have established that this process of cell segregation is regulated by Eph/Ephrin interactions (Herbert *et al.*, 2009). For example, if EphrinB2 expression is silenced by morpholinos or if EphrinB2 signaling is prevented by a C-terminal EphrinB2 deletion, the dorsal aorta contains very few cells. Conversely, if EphB4 is targeted with morpholinos, the cardinal vein is depleted of cells.

V. ANGIOGENESIS AND SPROUTING ANGIOGENESIS

Several mechanisms have been proposed to underlie the process of angiogenesis, that is, the formation of new vessels from existing vessels, including sprouting, elongation/widening, incorporation of circulating endothelial precursors, and formation of a lumen. There is evidence for a critical contribution of EphB4 and EphrinB2 function in several steps in angiogenesis.

EphrinB2 is expressed in the endothelium and mural cells of adult arteries, arterioles, and capillaries in many tissues, and levels of EphrinB2 expression increase in angiogenic vessels under physiological and pathological conditions (Gale *et al.*, 2001; Korff *et al.*, 2008; Shin *et al.*, 2001). EphrinB is broadly phosphorylated in angiogenic vessels of the retina, skin wounds, and tumor vessels, but not in the resting endothelium (Salvucci *et al.*, 2009). EphB4 is expressed most prominently in endothelial cells of venous derivation (Wang *et al.*, 1998). This pattern of segregated expression of EphrinB2 and EphB4 has raised questions about the sites and extent of receptor/ligand interactions, since they would be limited to arterial/vein boundaries. However, expression of EphrinB2 and EphB4 partially overlaps in retinal vessels (Wang *et al.*, 2010b), and endothelial cells derived from many sources, including the umbilical vein, human aorta, and dermal microvasculature, were found to express EphrinB2 and EphB4. This suggested that EphrinB2 and EphB4 have much broader avenues for interaction than previously appreciated (Gale *et al.*, 2001; Salvucci *et al.*, 2006; Shin *et al.*, 2001). In addition, certain tumor cells express EphB4 and other members of the B- and A-type receptors, providing an opportunity for functional interactions with angiogenic tumor vessels that express EphrinB2 (Fukai *et al.*, 2008; Noren *et al.*, 2006; Zhuang *et al.*, 2010).

Angiogenic sprouting is characterized by the appearance of a pioneering endothelial cell, identified as a "tip" cell, which forms filopodial extensions and is locally invasive and motile in response to surrounding VEGF-A gradients (Gerhardt *et al.*, 2003; Ferrara *et al.*, 2003; Ruhrberg *et al.*, 2002; Fig. 5). Other endothelial cells, named "stalk" cells, follow the tip cell and form the extending sprout at the base of the stalk (Gerhardt *et al.*, 2003). The Notch and VEGF signaling pathways play critical roles in determining the distinctive roles of tip and stalk cells. Tip and stalk cells have both been shown to be responsive to local VEGF-A gradients (Ferrara *et al.*, 2003), but this responsiveness appears to be modulated by opposing functions of the Notch ligands Dll4 and Jag1 (Benedito *et al.*, 2009). VEGF-A induces the expression of the Notch ligand Dll4 in tip cells, which interacts with Notch1/4 expressed on the adjacent stalk cells reducing their expression of VEGFR2 and thus limiting stalk cell responsiveness to VEGF-A (Lobov *et al.*, 2007; Suchting *et al.*, 2007). A second Notch ligand, Jag1, is expressed by stalk cells and serves to stimulate Notch1/4 on tip cells contributing to their responsiveness to VEGF-A (Benedito *et al.*, 2009). Another potential regulator of tip and stalk cell function is soluble VEGFR1. Stalk cells produce soluble VEGFR1, which neutralizes VEGF-A by competing with receptor binding, and may thus further limit or modulate responses to VEGF-A in the growing sprout (Chappell *et al.*, 2009).

Recent studies have outlined an important role of EphrinB2 signaling in sprouting angiogenesis, particularly in the regulation of tip cell function. Developing retinal vessels grow by sprouting (Gariano and Gardner, 2005) and widely express EphrinB2, which is concentrated on the tip cells and their protrusions (Sawamiphak *et al.*, 2010b; Wang *et al.*, 2010b). EphrinB2 is diffusely phosphorylated in the developing retinal vessels, but phosphorylation subsides once the retinal vessels have fully developed (Salvucci *et al.*, 2009; Fig. 6). Expression of a PDZ-mutant EphrinB2 impaired the development of retinal vessels, providing evidence that EphrinB2 signaling is critical to retinal vessel development (Sawamiphak *et al.*, 2010b). In particular, the PDZ-mutant tip cells displayed a significantly reduced ability to form filopodia. This ability of EphrinB2 to promote filopodia extension was confirmed *in vitro* using primary endothelial cells stimulated with EphB4-Fc (Sawamiphak *et al.*, 2010b) and retinal explants *ex vivo* (Sawamiphak *et al.*, 2010a).

In the mouse retina, filopodia from adjacent tip cells have been shown to bridge with each other to form connections between developing sprouts, which is an initial step leading to the development of a lumen that permits blood flow in the new vessel (Bentley *et al.*, 2009). The formation of such connecting bridges requires endothelial cell expression of EphrinB and signaling (Sawamiphak *et al.*, 2010b). A similar process of bridging between endothelial cells can be observed *in vitro* when primary endothelial cells are incubated onto extracellular matrix to form a characteristic network (Salvucci *et al.*, 2006, 2009). Endothelial-to-endothelial cell assembly onto extracellular matrix *in vitro* is characterized first by the appearance of needle-like extensions and protrusions from endothelial cells resulting in the joining or endothelial cells with each other (Fig. 7A). The appearance of these needle-like protrusions is followed by extensive changes in the cytoskeletal structure and shape of the joined cells, movement of the cell nuclei along the cytoplasm, and the thickening of connecting bridges (Salvucci *et al.*, 2006, 2009). The initial steps in this process of endothelial-to-endothelial cell assembly are associated with time-dependent phosphorylation of EphrinB2 (Salvucci *et al.*, 2009; Fig. 7B). This phosphorylation is likely induced by EphB/EphrinB interactions as specific peptide inhibitors of EphB prevent the formation of cord-like structures on Matrigel (Fig. 7C). A number of experiments provide evidence that such phosphorylation is critical to EphrinB2 function as a regulator of endothelial-to-endothelial cell assembly: if EphrinB2 is silenced in endothelial cells or if the endothelial cells express a signaling-deficient EphrinB2, the assembly of endothelial cells into networks is markedly reduced. Other studies have also shown that if EphrinB2 is overexpressed,

appropriate endothelial cell assembly is prevented, providing evidence that an optimal EphrinB2 expression level is required (Salvucci *et al.*, 2009; Sawamiphak *et al.*, 2010b; Wang *et al.*, 2010b).

An important question relates to the mechanism by which EphrinB2 is activated in sprouting endothelium, particularly in tip cells where it seems to play an essential role in the path-finding, probing, and extension function of tip cells. One of the possibilities is that, in the retina, astrocytes provide a source of cell-associated EphB4 that stimulates the developing retinal vasculature (Sawamiphak *et al.*, 2010b). Another possibility is that retinal endothelial cells express EphB4, which can stimulate EphrinB2 in interacting endothelial cells (Wang *et al.*, 2010b). Experiments *in vitro* using primary endothelial cells provide evidence that both EphB4 and EphrinB2 can be simultaneously expressed and that the assembly of vascular networks is dependent upon cell-to-cell interactions resulting in EphrinB signaling induced by endothelial cell-derived EphB4 (Salvucci *et al.*, 2006, 2009). Blocking such EphB/EphrinB cell-to-cell interactions prevents endothelial cell assembly into vascular networks (Salvucci *et al.*, 2006, 2009).

What is the mechanism by which EphrinB2 signaling contributes to sprouting angiogenesis, including "tip" cell function and endothelial cell assembly? Two recent studies in mouse and zebrafish have unveiled a role of EphrinB2 signaling in the regulation of VEGFR2 and VEGFR3 function. If EphrinB2 is silenced or if a signaling-deficient (PDZ-mutant) EphrinB2 is expressed in endothelial cells, VEGFR2 and VEGFR3 signaling is defective, as evidenced by reduced phosphorylation of the receptor following stimulation by the cognate ligand. In addition, activation of the downstream VEGFR signaling components Rac1, Erk1/2, and Akt is reduced (Sawamiphak *et al.*, 2010b; Wang *et al.*, 2010b). Recent studies have concluded that VEGFR trafficking away from the plasma membrane into the endocytic compartment contributes or is necessary for VEGFR function in response to appropriate VEGF ligands (Lampugnani *et al.*, 2006; Lanahan *et al.*, 2010; Sawamiphak *et al.*, 2010b; Wang *et al.*, 2010b). Thus, by promoting the internalization of VEGFR2 and VEGFR3, EphrinB2 signaling may critically regulate VEGFR function in response to the VEGF ligand. It was also observed that EphrinB2 signaling induces some degree of VEGFR2 internalization in the absence of exogenous VEGF (Sawamiphak *et al.*, 2010b). There is evidence for the existence of an endogenous pool of VEGF, which is not secreted outside the cells but contributes to endothelial cell function; it is possible that VEGFR internalized by EphrinB2 signaling is activated by autocrine endogenous VEGF (Lampugnani *et al.*, 2006; Lee *et al.*, 2007).

Besides serving as inducers of EphrinB2 reverse signaling, EphB receptors have also been reported to play a signaling role of their own in endothelial cells. Two studies have concluded that EphB4 forward signaling in endothelial cells represses endothelial cell migration, adhesion, and proliferation *in vitro*, and may serve to repel endothelial cells from each other and maintain arterial/venous boundaries in capillary beds (Fuller *et al.*, 2003; Kim *et al.*, 2002). Thus, in these studies, EphB4 forward signaling appeared to have opposite effects to those of EphrinB2 reverse signaling in the context of endothelial cell-sprouting angiogenesis. However, other studies have suggested that EphB4 forward signaling in endothelial cells promotes some degree of endothelial cell proliferation and angiogenesis (Stein *et al.*, 1997, 2002). A detailed analysis of EphB2/4 expression and signaling in the context of endothelial cell assembly into cord-like structures *in vitro* showed characteristic kinetic changes suggestive of a role of EphB forward signaling (Salvucci *et al.*, 2006). When first dispersed on extracellular matrix, primary endothelial cells broadly express surface EphB2/4, but as the cells contact each other to form cord structures, EphB2/4 are internalized and no longer detected on the cell surface (Salvucci *et al.*, 2006). In addition, EphB2/4 become phosphorylated with kinetics suggestive of their activation in association

with the occurrence of cell-to-cell contact and receptor internalization (Salvucci *et al.*, 2006). EphB receptor signaling has been linked to the activation of secondary molecules that regulate cytoskeleton structure and cell movement, particularly small Rho family GTPases (Egea and Klein, 2007; Noren and Pasquale, 2004). Consistent with this ability of Eph signaling to regulate cell structure in response to cell-to-cell contact, a typical feature of endothelial cell assembly on extracellular matrix is the time-dependent activation of F (filamentous)-actin filaments in conjunction with cell-to-cell contact and changes in cell shape (Salvucci *et al.*, 2006). The *in vivo* relevance of these observations remains to be determined, and progress on the role of EphB4 forward signaling in endothelial cell-sprouting angiogenesis will likely benefit from use of mutant mice-expressing signaling-deficient forms of EphB targeted to the endothelial cells.

VI. VESSEL REMODELING AND STABILIZATION

Once formed through sprouting angiogenesis, newly formed vessels undergo a number of changes as a result of the establishment of blood flow, changes in tissue metabolism, endothelial growth factor availability, and other factors. Some of the newly formed vessels regress, whereas others mature through the establishment of a basal membrane composed of extracellular matrix proteins produced by endothelial cells and surrounding stromal cells. A critical step in this process of maturation is the recruitment of pericytes/smooth muscle cells to the nascent vessel, which stabilize the vessel wall and regulate endothelial cell survival, growth, and permeability (Armulik *et al.*, 2005; Hellstrom *et al.*, 2001). Electron microscopy studies have shown that pericytes are inserted into the basal membrane of vessels and come into direct contact with the endothelium through cytoplasmic extensions that penetrate the basement membrane and push deeply onto the endothelial cell surface membrane (Cuevas *et al.*, 1984). Pericytes are found around some blood capillaries, precapillary arterioles, postcapillary venules, and collecting venules, but pericyte coverage of vessels is partial and variable (ranges between 10% and 50%), depending on the tissue. The highest pericyte coverage is found in the central nervous system, where pericytes may regulate the blood–brain barrier functions (Armulik *et al.*, 2010); in the retina, the frequency of pericytes is similar to that of the endothelial cells. Although the derivation of pericytes/mural cells is still somewhat controversial, recent studies suggest that such cells are similar to mesenchymal stem cells, derive from the bone marrow, and display multilineage potential, which explains in part their morphological and phenotypic diversity (Crisan *et al.*, 2008).

Genetic mouse models have implicated several genes in the generation and proper assembly of pericytes, including the PDGF/PDGFR-1, Ang-1/Tie2, and the spingosine 1-phosphate (S1P/S1P1) genes. PDGFB and PDGF-Rβ-null mice die during development with microvascular aneurisms and lack of pericyte coverage on some vessels (Hellstrom *et al.*, 2001). Ang1- or Tie2-null mice die at midgestation with poorly organized basal membranes and reduced coverage/detachment of pericytes (Dumont *et al.*, 1994; Sato *et al.*, 1995; Suri *et al.*, 1996). Conversely, the overexpression of Ang1 promotes pericyte coverage and vessel leakage resistance (Suri *et al.*, 1998; Thurston *et al.*, 1999). Disruption of the s1p1 gene in mice causes prenatal death with vascular abnormalities characterized by defective pericyte coverage (Liu *et al.*, 2000).

EphrinB2 also plays an important role in pericyte function. The mural-cell-specific inactivation of EphrinB2, using PDGF-cre mice, caused the embryos to have edema and extensive hemorrhaging in a variety of tissues and was associated with perinatal death of the mutant mice (Foo *et al.*, 2006). Interestingly, the EphrinB2-null pericytes appeared morphologically normal in many of these mice, but they associated poorly with the vessels, showing a scattered distribution resulting in incomplete vessel coverage (Foo *et al.*, 2006).

During mouse development, EphrinB2 is expressed in the mural cells that cover arteries and veins (Foo *et al.*, 2006). In the adult mouse, EphrinB2 is expressed in a proportion of smooth muscle cells/pericytes surrounding arteries (Shin *et al.*, 2001). Importantly, levels of EphrinB2 expression in smooth muscle cells are comparable to those detected in the arterial endothelium (Shin *et al.*, 2001). Human smooth muscle cells located in the media of adult coronary arteries are partially positive for EphrinB2 (Korff *et al.*, 2008). Human bone marrow-derived mesenchymal stem cells, which either represent pericytes or are related to pericytes, express a functional EphrinB, which can be phosphorylated by EphB4-Fc (Salvucci *et al.*, 2009).

The silencing of EphrinB2 in pericytes/smooth muscle cells derived from the mouse aorta has remarkable effects on cell morphology: the cells become elongated, do not properly spread, and show numerous active lamellipodia protrusions, without evidence of polarization and without cell-to-cell contact (Foo *et al.*, 2006). Some of these defects appeared to be cell-contact-independent, suggesting that EphrinB2 may have cell-autonomous roles (Foo *et al.*, 2006). Functionally, EphrinB2-deficient smooth muscle cells displayed increased, but random, migration, which was associated with defective formation of focal adhesions. Such phenotype was reversed by reexpression of EphrinB2 or activation of Rho-like GTPases (Foo *et al.*, 2006). Consistent with EphrinB2 playing a role in mural cell function, EphB4 (expressed by endothelial cells) induced activation of EphrinB2 on mural cells and enhanced mural/endothelial cell association within tumor blood vessels (Erber *et al.*, 2006).

In vitro, mesenchymal cells/pericytes, which express EphrinB2, characteristically make contact with endothelial cells on extracellular matrix, and when this contact occurs, EphrinB2 phosphorylation is clearly and transiently detected in the mesenchymal stem cells/pericytes (Salvucci *et al.*, 2009). The silencing of EphrinB2 in bone marrow-derived mesenchymal cells/pericytes prevented their proper assembly with endothelial cells (Salvucci *et al.*, 2009; Fig. 8), suggesting that phosphorylation-dependent EphrinB2 signaling is critical for pericytes to make proper contact with endothelial cells (Salvucci *et al.*, 2009).

Little is known about the role of EphB receptors and their signaling in pericytes. Human mesenchymal stem cells express EphB2 and EphB4 (Salvucci *et al.*, 2009), and smooth muscle cells derived from human thymus also express EphB4 (Korff *et al.*, 2008). Activation of EphB4 in smooth muscle cells reduced their ability to migrate, suggesting that EphB4 signaling in pericytes may secure their attachment to the endothelial cells stabilizing the vessel wall (Korff *et al.*, 2008). Vascular smooth muscle cells also express EphA4 receptors, which can be activated by EphrinA1 and by many EphrinB ligands (Ogita *et al.*, 2003). When EphA4 is activated in smooth muscle cells, their ability to form lamellipodia and to spread is inhibited, a process that was linked to inhibition of the small GTPase Rac1 and the kinase Pak1 (Deroanne *et al.*, 2003).

VII. PHYSIOLOGICAL ANGIOGENESIS IN THE ADULT

Physiological angiogenesis in the adult is limited: it only occurs in conjunction with the menstrual cycle and pregnancy, in the skeletal bone during growth, and during wound repair.

The critical role of VEGF as a promoter of angiogenesis during the menstrual cycle, embryo implantation, and pregnancy are well recognized, but little is known about the potential roles of Eph/Ephrins in this context. Studies on corpora lutea have shown that ovulation was followed by a rapid increase in the expression of EphrinB1 and EphrinB2 in the luteinizing granulose (Egawa *et al.*, 2003; Xu *et al.*, 2006) and suggested the occurrence of EphB/

EphrinB interactions between the granulosa cells of the corpus luteum and proximal angiogenic capillaries (Egawa *et al.*, 2003; Xu *et al.*, 2006).

Placental implantation of the embryo involves the aggressive invasion of maternal tissues by the embryonic/fetal cytotrophoblasts. This process ultimately results in the cytotrophoblast invasion of the maternal spiral arterioles that supply blood to the placenta, creating a chimeric vasculature composed of maternal and fetal cells (Fisher *et al.*, 1989; Librach *et al.*, 1991). The invasive behavior of the embryonic/fetal cytotrophoblasts is accompanied by remarkable changes in gene expression and phenotype; in particular, they acquire vascular-type markers (Damsky *et al.*, 1992; Zhou *et al.*, 1997) and produce proangiogenic factors (Zhou *et al.*, 2003). Important changes occurring in the cytotrophoblasts as they penetrate the uterine wall are the loss of EphB4 expression and an increase in EphrinB2 expression.Notch signaling is a critical regulator of the divergent expression of EphrinB2 and EphB4 in these cells (Hunkapiller *et al.*, 2011; Red-Horse *et al.*, 2005). This phenotypic change in embryonic/fetal cytotrophoblasts has been proposed to underlie the selective tropism of the cytotrophoblasts toward the maternal arterioles, thus limiting interactions with the maternal venules (Red-Horse *et al.*, 2005).

Angiogenesis in the context of skin wound healing occurs through endothelial cell sprouting from capillaries at the edge of the wound when the dermis is injured. The process is driven by VEGF-A (Bao *et al.*, 2009) that is first produced by platelets, which contribute to the formation of the blood clot that initially seals the wound. Inflammatory monocytes and neutrophils are additional sources of VEGF in the wound and represent a local defense system against pathogens. They are recruited by a variety of signals, including complement components, platelet degranulation products, and potentially bacterial particles (Banks *et al.*, 1998; Mohle *et al.*, 1997). Sprouting wound vessels are components of the "granulation tissue," which is a hallmark of wounds undergoing repair. Besides sprouting vessels, the granulation tissue contains a fibrin clot, fibroblasts, collagen, and inflammatory cells (Bao *et al.*, 2009; Murohara *et al.*, 1998). Granulation tissue undergoes extensive remodeling in repairing wounds, and the process is orchestrated by a series of enzymes including MMP1, MMP2, urokinase-type, and tissue-type plasminogen activators, and plasminogen activator inhibitor (Schultz and Wysocki, 2009).

A study reported that circulating mouse neutrophils express EphrinA2 and B2 (Schruefer *et al.*, 2006). Other studies have demonstrated that inflammatory macrophages express EphrinB2 and suggested a role for monocytederived EphrinB2 in the stimulation of venous endothelium that expresses EphB4 (Yuan *et al.*, 2000, 2004). Recent studies have confirmed the expression of EphrinB2 in the developing dermal vasculature of mice (Wang *et al.*, 2010b). In a murine model of wound healing in the skin, the sprouting endothelial cells from the margins of the wound expressed EphrinB2, which was prominently and time-dependently phosphorylated in these cells, indicative of active signaling (Salvucci *et al.*, 2009; Fig. 9A). In contrast, EphrinB2 was not phosphorylated in the surrounding normal vessels or in the new capillaries once sprouting had subsided and the granulation tissue was well developed (Salvucci *et al.*, 2009). Additionally, EphrinB2 expression and phosphorylation were transiently detected in wound-infiltrating inflammatory cells (Salvucci *et al.*, 2009).

VIII. PATHOLOGICAL ANGIOGENESIS

A. Neovascular Disorders of the Eye

Intraocular neovascularization is a pathological complication of many eye diseases, including diabetic retinopathy, retinopathy of prematurity, and age-related (neovascular, wet-type) macular degeneration (AMD). Since these conditions represent the major cause

for blindness, control of pathological angiogenesis is an important therapeutic goal. Retinal ischemia and increased VEGF expression are often the principal causes of pathological retinal angiogenesis (Alon *et al.*, 1995; Fraisl *et al.*, 2009; Miller *et al.*, 1994). Oxygen levels and the levels of VEGF are tightly regulated in the normal retina (Stenzel *et al.*, 2011). Hypoxia regulates expression of VEGF largely through the activities of the transcription factors HIFs (hypoxia inducing factors) (Semenza, 2003; Tang *et al.*, 2011). The HIF-1α subunit is maintained at low levels during normoxia through an oxygen-dependent degradation process, which is mediated by the von Hippel Lindau (VHL) protein, a component of the E3 ubiquitin ligase complex (Semenza, 2011). Once the oxygen pressure drops below 5%, HIF-1α is not sufficiently degraded, resulting in the activation of genes involved in the maintenance of oxygen homeostatic levels. One of these target genes is VEGF, which promotes the growth of blood vessels toward hypoxic zones (Ferrara *et al.*, 2003; Gerhardt *et al.*, 2003). Consistent with its role in the degradation of HIF-1α, loss-of-function mutations of VHL result in increased tumor angiogenesis (Pugh and Ratcliffe, 2003).

Abnormally high levels of VEGF and pathological angiogenesis also characterize the wet-type AMD (Jager *et al.*, 2008). In this condition, choroidal neovascularization causes exudation and hemorrhaging, which impair the central portion of the retina that is responsible for high-resolution vision (Jager *et al.*, 2008). VEGF is detected at high levels in the choroidal membranes from patients with wet AMD (Kvanta *et al.*, 1996; Lopez *et al.*, 1996); various approaches to limit VEGF activity in the eye have demonstrated a marked clinical benefit to the patients.

A number of mouse models of ischemia-induced intraocular neovascularization have been developed, including the retinopathy of immaturity (ROP) model, which has been useful to study ischemia-induced retinal angiogenesis (Smith *et al.*, 1994). In this model, 7-day-old mice are exposed to high (75%) oxygen tension for a period of 5 days and then are exposed to room air. High oxygen tension causes physiological postnatal retinal angiogenesis to come to a stop and promotes degeneration of the retinal vessels that had recently sprouted. Once the retinas are reexposed to air oxygen, retinal neovascularization resumes with vigorous retinal vessel sprouting after 3 days (Smith *et al.*, 1994). These sprouting retinal vessels express EphrinB2, which is phosphorylated (Salvucci *et al.*, 2009), indicative of activity (Fig. 9B). Once the sprouting subsides in the retinal vessels, EphrinB2 phosphorylation also subsides, without change in the levels of EphrinB2 expression (Salvucci *et al.*, 2009). These results show that hyperoxia-induced retinal neovascularization is associated with EphrinB2 activation and thus is similar in this respect to the occurrence of EphrinB2 phosphorylation during physiological postnatal angiogenesis of the retina.

B. Tumor Angiogenesis

Angiogenesis, a hallmark of a growing malignancy, often starts when the tumors are quite small in size (1–2mm) and is principally driven by VEGF-A produced by the tumor cells and by cells in the tumor microenvironment. Tumor hypoxia is believed to represent an important inducer of tumor-associated VEGF (Dayan *et al.*, 2008; Ferrara and Kerbel, 2005; Mantovani *et al.*, 2002; Murdoch *et al.*, 2008), which is an indispensable driver of tumor angiogenesis in preclinical mouse models (Ferrara and Kerbel, 2005; Kim *et al.*, 1993). Tumor angiogenesis resembles physiological angiogenesis but presents notable differences (Chung *et al.*, 2011). A principal difference is that tumor angiogenesis is not self-limited but appears to be a self-perpetuating and persistent process that is not turned off once vessels are formed. Tumor vessels are disorganized compared to normal vessels and display a number of abnormalities, including fragility, reduced number of pericytes, and leakiness with a propensity to produce exudates and to bleed. These abnormalities have been linked to excess VEGF-A, and thus VEGF neutralization has been reported to somewhat "normalize" the

tumor vasculature (Jain, 2005). The tumor cells, particularly melanomas and glioblastomas, may undergo a process of transformation, named "vasculogenic mimicry," such that the tumor cells or the differentiated tumor stem cells acquire phenotypic characteristics of endothelial cells and become components of the vessel wall (Hendrix *et al.*, 2003a,b; Ricci-Vitiani *et al.*, 2010; Wang *et al.*, 2010a).

A number of preclinical and clinical studies using inhibitors of VEGF have identified VEGF-independent pathways of tumor angiogenesis, which may account for the poor responses and/or acquired resistance to VEGF neutralization (Shojaei *et al.*, 2007). One such pathway involves the neutrophilderived proangiogenic factor Bv8 (also known as prokineticin 2), which drives endothelial cell proliferation and migration in the absence of VEGF-A (Shojaei *et al.*, 2007, 2009). Other pathways markedly modulate VEGF expression and/or VEGF-A-induced responses, both physiologically and in cancer, including the Dll4/Notch (Hellstrom *et al.*, 2007; Noguera-Troise *et al.*, 2006; Ridgway *et al.*, 2006), Angiopoietin-1/Tie2 and Angiopoietin-2/Tie2 (Augustin *et al.*, 2009), platelet-derived growth factors (PDGFs)/PDGF-receptor β (Bergers *et al.*, 2003), and TGFβ1/TGFβ receptor II (Chung *et al.*, 2011; Fridlender *et al.*, 2009). The Eph/Ephrin signaling pathways can also regulate VEGF/VEGFR signaling in the context of cancer, but the system is complex (Bruckner *et al.*, 1997; Pasquale, 2010; Sawamiphak *et al.*, 2010a,b).

The functions of the Eph/Ephrin system in cancer are complex due to the fact that many tumor cells express Eph receptors and ligands, albeit to varying degrees (Davalos *et al.*, 2006; Huang *et al.*, 2007; Noren and Pasquale, 2007; Zhuang *et al.*, 2010), which can stimulate or be stimulated by Eph/Ephrin molecules in the tumor endothelium and in other cells of the tumor microenvironment. Thus, the outcome of Eph receptor and ligand signaling in the tumor cells differs in different tumors (no effect, as opposed to inhibition or stimulation of tumor growth) (Batlle *et al.*, 2002; Dohn *et al.*, 2001; Dopeso *et al.*, 2009; Jin *et al.*, 2006; Kumar *et al.*, 2009; Miao *et al.*, 2009; Noren and Pasquale, 2007; Noren *et al.*, 2006; Ruhe *et al.*, 2007; Smith *et al.*, 2004; Yu *et al.*, 1999).

There are a few situations that best illustrate the complex roles of Eph/Ephrin in tumor growth. For example, a number of studies have provided evidence that EphB2/EphB4 signaling in certain tumor cells suppresses tumor growth or promotes tumor dormancy. In particular, it was reported that soluble EphrinB-Fc reduced colon and breast cancer cell growth *in vitro* and tumor progression in mice by pharmacologically activating tumor cell-associated EphB4 (Batlle *et al.*, 2002; Noren and Pasquale, 2007; Noren *et al.*, 2006). Consistent with a tumor-suppressor role of EphB4 signaling, a dominant-negative form of EphB4 promoted colorectal tumor growth (Dopeso *et al.*, 2009). Also, consistent with the notion that EphB2 and EphB4 act as inhibitors of tumor growth, EphB2 and EphB4 are mostly inactive in tumors as judged by low levels of phosphorylation in many malignant cells and are expressed at higher levels in benign tumors of the same lineage (Kumar *et al.*, 2009; Miao *et al.*, 2005; Noren and Pasquale, 2007; Noren *et al.*, 2006). Thus, it was proposed that EphB signaling in the tumor cells may inhibit oncogenic signaling pathways operating in the tumor cells thereby reducing tumor growth, may promote repulsive interactions between the EphB-expressing tumor cells and the EphrinB1-expressing normal tissue that would restrict tumor cell invasion, or may promote tumor cell adherence to each other preventing local tissue infiltration (Cortina *et al.*, 2007; Kemp *et al.*, 2009; Pasquale, 2010).

In contrast with these results, other studies have indicated that EphB4 signaling promotes tumor growth, perhaps due to a loss of tumor-inhibitory function or to acquisition of ligand-independent activities (Pasquale, 2010). In some studies, activation of tumor-associated EphB4 enhanced tumor growth, motility, and metastasis of prostate, bladder, colorectal, and

melanoma cancer cells (Xia *et al.*, 2005, 2006; Yang *et al.*, 2006). Also, knockdown of EphB4 in colorectal and ovarian cancer cells inhibited tumor growth and metastasis (Kumar *et al.*, 2007, 2009). Tumor-associated expression of EphB4 stimulated tumor angiogenesis and tumor growth by activating EphrinB2 on the tumor endothelium (Noren *et al.*, 2006), and overexpression of EphrinB2 in ovarian and melanoma tumor cells correlated with increased tumor invasion and poor prognosis, perhaps by promoting EphB4 activation in the tumor cells (Castellvi *et al.*, 2006; Meyer *et al.*, 2005). Deregulated expression of EphB4 and EphrinB2 in KSHV (Kaposi's sarcoma-associated herpes virus)-infected KS cells is also believed to contribute to tumor growth (Masood *et al.*, 2005).

The complexities of Eph/Ephrin interactions in growing tumors and the difficulties in generalizing their function in cancer suggest that careful experimentation with controlled systems may be useful. In line with this, a simplified tumor model unequivocally showed that EphrinB2 signaling in tumor-associated vessels promotes tumor angiogenesis, much alike the situation in physiological angiogenesis (Sawamiphak *et al.*, 2010b). Using mouse tumor models of intracranial gliomas and subcutaneous astrocytoma, the authors showed that tumor growth and tumor angiogenesis were significantly reduced in EphrinB2-deficient tumor-bearing mice in comparison to wild-type control mice (Sawamiphak *et al.*, 2010b). The interpretation of these results is that sprouting angiogenesis in tumors resembles physiological angiogenesis and as such is similarly regulated by EphrinB2 signaling via modulation of VEGF-A/VEGFR2 function (Sawamiphak *et al.*, 2010b; Wang *et al.*, 2010b). Consistent with EphrinB signaling playing an important role in tumor angiogenesis, the angiogenic tumor vessels in experimental MOPC315 tumors displayed an intensely phosphorylated EphrinB, particularly at the tumor margins where angiogenesis was most prominent (Salvucci *et al.*, 2009; Fig. 9C). In addition, tumors that express EphB4 promote angiogenesis through interaction with EphrinB2 expressed by the tumor endothelium (Noren *et al.*, 2006).

IX. THERAPEUTIC IMPLICATIONS

Pathological angiogenesis in the adult is mostly associated with neovascular diseases of the eye, including wet macular degeneration and diabetic retinopathy, and with tumor angiogenesis, which contributes to tumor growth in many types of cancer. It is now clear that EphrinB2 signaling plays a critical role in promoting VEGF-induced endothelial cell sprouting and orchestrating endothelial-to-endothelial and endothelial-to-pericyte assembly. For these reasons, EphrinB2 and its ligands are desirable therapeutic targets for antiangiogenic therapy. However, the complexities of cancer, where Eph/Ephrin interactions involve not only the tumor vasculature but also the tumor cells and other cells resulting in strikingly different tumor outcomes, are a challenge for the development of Eph/Ephrin-based cancer drugs. Thus, potential application of Eph/Ephrin targeting to cancer treatment will need additional study and validation for individual cancer types, stage, and other variables.

A number of approaches have demonstrated to effectively inhibit Eph/Ephrin interactions. Monomeric soluble EphB4 ectodomain, which inhibits EphB4 forward signaling and EphrinB2 reverse signaling, has been shown to reduce angiogenesis *in vivo* and experimental tumor growth in mice (Kertesz *et al.*, 2006; Scehnet *et al.*, 2009). EphB4 agonist antibodies could be useful in the context of selected EphB4-expressing tumors as they could promote EphB4 tumor-suppressor activity and at the same time inhibit EphrinB2-induced tumor angiogenesis (Krasnoperov *et al.*, 2010; Noren and Pasquale, 2004; Noren *et al.*, 2006). Specific antibodies to EphB2 have been generated that can effectively block interaction with Ephrins and kill EphB2-expressing tumor cells when conjugated with the cytotoxic drug auristatin (Mao *et al.*, 2004; Xu *et al.*, 2009). Phage display allowed the

identification of specific peptides that bind EphB2 and EphB4 at nanomolar concentrations and can readily inhibit endothelial-to-endothelial cell assembly *in vitro* and experimental angiogenesis *in vivo* (Chrencik *et al.*, 2006, 2007; Koolpe *et al.*, 2005; Salvucci *et al.*, 2006, 2009). A neutralizing antibody to EphrinB2 was reported to reduce vessel number, but not size, in an experimental model of human glioblastoma (Li *et al.*, 2011).

X. CONCLUSIONS

In this review, we have discussed evidence for a role of the B family of Ephs and Ephrins in angiogenesis. We have described how B Ephs and Ephrins signaling play critical roles in developmental and postnatal angiogenesis in physiology and disease. Thus, B Ephs and Ephrins are promising targets to modulate angiogenesis. Several approaches to block EphB/EphrinB function seem to be very effective at reducing angiogenesis in experimental models. However, many complexities of EphB and EphrinB signaling are not understood, particularly how they integrate with other signaling pathways. The context-dependent functions of B Ephs and Ephrins in cancer are poorly understood and may require a better understanding of the role of Eph/Ephrin in cell interactions between tumor cells and the tumor microenvironment as well as understanding how EphB/EphrinB signaling integrates with oncogenic signaling pathways. Research in the coming years will likely decipher the diverse functions of Ephs and Ephrins in the context of cancer. This will be an important advance that may open a wide range of therapeutic opportunities.

Acknowledgments

REFERENCES

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R. Roles of ephrinB ligands EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, sprouting angiogenesis. Genes Dev. 1999; 13:295–306. [PubMed: 9990854]

Adams RH, Diella F, Hennig S, Helmbacher F, Deutsch U, Klein R. The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. Cell. 2001; 104:57–69. [PubMed: 11163240]

Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels has implications for retinopathy of prematurity. Nat. Med. 1995; 1:1024–1028. [PubMed: 7489357]

Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Circ. Res. 2005; 97:512–523. [PubMed: 16166562]

Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, He L, Norlin J, Lindblom P, Strittmatter K, Johansson BR, Betsholtz C. Pericytes regulate the blood–brain barrier. Nature. 2010; 468:557–561. [PubMed: 20944627]

Augustin HG, Koh GY, Thurston G, Alitalo K. Control of vascular morphogenesis homeostasis through the angiopoietin–tie system. Nat. Rev. Mol. Cell Biol. 2009; 10:165–177. [PubMed: 19234476]

Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C, Selby PJ. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements cancer biology. Br. J. Cancer. 1998; 77:956–964. [PubMed: 9528841]

Bao P, Kodra A, Tomic-Canic M, Golinko MS, Ehrlich HP, Brem H. The role of vascular endothelial growth factor in wound healing. J. Surg. Res. 2009; 153:347–358. [PubMed: 19027922]

Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H. Beta-catenin TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell. 2002; 111:251–263. [PubMed: 12408869]

Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, Adams RH. The Notch ligands Dll4 Jagged1 have opposing effects on angiogenesis. Cell. 2009; 137:1124–1135. [PubMed: 19524514]

Bentley K, Mariggi G, Gerhardt H, Bates PA. Tipping the balance: robustness of tip cell selection, migration fusion in angiogenesis. PLoS Comput. Biol. 2009; 5:e1000549. [PubMed: 19876379]

Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes endothelial cells in the tumor vasculature with kinase inhibitors. J. Clin. Invest. 2003; 111:1287–1295. [PubMed: 12727920]

Bong YS, Lee HS, Carim-Todd L, Mood K, Nishanian TG, Tessarollo L, Daar IO. ephrinB1 signals from the cell surface to the nucleus by recruitment of STAT3. Proc. Natl. Acad. Sci. U.S.A. 2007; 104:17305–17310. [PubMed: 17954917]

Boutros M, Mlodzik M. Dishevelled: at the crossroads of divergent intracellular signaling pathways. Mech. Dev. 1999; 83:27–37. [PubMed: 10507837]

Brantley-Sieders D, Schmidt S, Parker M, Chen J. Eph receptor tyrosine kinases in tumor tumor microenvironment. Curr. Pharm. Des. 2004; 10:3431–3442. [PubMed: 15544526]

Bruckner K, Pasquale EB, Klein R. Tyrosine phosphorylation of transmembrane ligands for Eph receptors. Science. 1997; 275:1640–1643. [PubMed: 9054357]

Castellvi J, Garcia A, de la Torre J, Hernandez J, Gil A, Xercavins J, Ramon y Cajal S. Ephrin B expression in epithelial ovarian neoplasms correlates with tumor differentiation angiogenesis. Hum. Pathol. 2006; 37:883–889. [PubMed: 16784989]

Chappell JC, Taylor SM, Ferrara N, Bautch VL. Local guidance of emerging vessel sprouts requires soluble Flt-1. Dev. Cell. 2009; 17:377–386. [PubMed: 19758562]

Chong LD, Park EK, Latimer E, Friesel R, Daar IO. Fibroblast growth factor receptor-mediated rescue of x-ephrin B1-induced cell dissociation in Xenopus embryos. Mol. Cell. Biol. 2000; 20:724–734. [PubMed: 10611251]

Chrencik JE, Brooun A, Recht MI, Kraus ML, Koolpe M, Kolatkar AR, Bruce RH, Martiny-Baron G, Widmer H, Pasquale EB, Kuhn P. Structure thermodynamic characterization of the EphB4/Ephrin-B2 antagonist peptide complex reveals the determinants for receptor specificity. Structure. 2006; 14:321–330. [PubMed: 16472751]

Chrencik JE, Brooun A, Recht MI, Nicola G, Davis LK, Abagyan R, Widmer H, Pasquale EB, Kuhn P. Three-dimensional structure of the EphB2 receptor in complex with an antagonistic peptide reveals a novel mode of inhibition. J. Biol. Chem. 2007; 282:36505–36513. [PubMed: 17897949]

Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. Nat. Rev. Cancer. 2011; 10:505–514. [PubMed: 20574450]

Cortina C, Palomo-Ponce S, Iglesias M, Fernandez-Masip JL, Vivancos A, Whissell G, Huma M, Peiro N, Gallego L, Jonkheer S, Davy A, Lloreta J, et al. EphB– ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells. Nat. Genet. 2007; 39:1376–1383. [PubMed: 17906625]

Cowan CA, Henkemeyer M. The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals. Nature. 2001; 413:174–179. [PubMed: 11557983]

Cowan CA, Yokoyama N, Saxena A, Chumley MJ, Silvany RE, Baker LA, Srivastava D, Henkemeyer M. Ephrin-B2 reverse signaling is required for axon pathfinding cardiac valve formation but not early vascular development. Dev. Biol. 2004; 271:263–271. [PubMed: 15223333]

Cowan CW, Shao YR, Sahin M, Shamah SM, Lin MZ, Greer PL, Gao S, Griffith EC, Brugge JS, Greenberg ME. Vav family GEFs link activated Ephs to endocytosis axon guidance. Neuron. 2005; 46:205–217. [PubMed: 15848800]

Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell. 2008; 3:301–313. [PubMed: 18786417]

Cuevas P, Gutierrez-Diaz JA, Reimers D, Dujovny M, Diaz FG, Ausman JI. Pericyte endothelial gap junctions in human cerebral capillaries. Anat. Embryol. (Berl). 1984; 170:155–159. [PubMed: 6517350]

Damsky CH, Fitzgerald ML, Fisher SJ. Distribution patterns of extracellular matrix components adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. J. Clin. Invest. 1992; 89:210–222. [PubMed: 1370295]

Davalos V, Dopeso H, Castano J, Wilson AJ, Vilardell F, Romero-Gimenez J, Espin E, Armengol M, Capella G, Mariadason JM, Aaltonen LA, Schwartz S Jr, et al. EPHB4 survival of colorectal cancer patients. Cancer Res. 2006; 66:8943–8948. [PubMed: 16982731]

Dayan F, Mazure NM, Brahimi-Horn MC, Pouyssegur J. A dialogue between the hypoxia-inducible factor the tumor microenvironment. Cancer Microenviron. 2008; 1:53–68. [PubMed: 19308685]

Deroanne C, Vouret-Craviari V, Wang B, Pouyssegur J. EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway. J. Cell Sci. 2003; 116:1367–1376. [PubMed: 12615978]

Dohn M, Jiang J, Chen X. Receptor tyrosine kinase EphA2 is regulated by p53- family proteins induces apoptosis. Oncogene. 2001; 20:6503–6515. [PubMed: 11641774]

Dopeso H, Mateo-Lozano S, Mazzolini R, Rodrigues P, Lagares-Tena L, Ceron J, Romero J, Esteves M, Landolfi S, Hernandez-Losa J, Castano J, Wilson AJ, et al. The receptor tyrosine kinase EPHB4 has tumor suppressor activities in intestinal tumorigenesis. Cancer Res. 2009; 69:7430–7438. [PubMed: 19738063]

Dravis C, Henkemeyer M. Ephrin-B reverse signaling controls septation events at the embryonic midline through separate tyrosine phosphorylation-independent signaling avenues. Dev. Biol. 2011; 355:138–151. [PubMed: 21539827]

Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A, Breitman ML. Dominant-negative targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev. 1994; 8:1897–1909. [PubMed: 7958865]

Egawa M, Yoshioka S, Higuchi T, Sato Y, Tatsumi K, Fujiwara H, Fujii S. Ephrin B1 is expressed on human luteinizing granulosa cells in corpora lutea of the early luteal phase: the possible involvement of the B class Eph–ephrin system during corpus luteum formation. J. Clin. Endocrinol. Metab. 2003; 88:4384–4392. [PubMed: 12970314]

Egea J, Klein R. Bidirectional Eph–ephrin signaling during axon guidance. Trends Cell Biol. 2007; 17:230–238. [PubMed: 17420126]

Erber R, Eichelsbacher U, Powajbo V, Korn T, Djonov V, Lin J, Hammes HP, Grobholz R, Ullrich A, Vajkoczy P. EphB4 controls blood vascular morphogenesis during postnatal angiogenesis. EMBO J. 2006; 25:628–641. [PubMed: 16424904]

Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature. 2005; 438:967–974. [PubMed: 16355214]

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF its receptors. Nat. Med. 2003; 9:669–676. [PubMed: 12778165]

Fisher SJ, Cui TY, Zhang L, Hartman L, Grahl K, Zhang GY, Tarpey J, Damsky CH. Adhesive degradative properties of human placental cytotrophoblast cells in vitro. J. Cell Biol. 1989; 109:891–902. [PubMed: 2474556]

Foo SS, Turner CJ, Adams S, Compagni A, Aubyn D, Kogata N, Lindblom P, Shani M, Zicha D, Adams RH. Ephrin-B2 controls cell motility adhesion during blood-vessel-wall assembly. Cell. 2006; 124:161–173. [PubMed: 16413489]

Fraisl P, Mazzone M, Schmidt T, Carmeliet P. Regulation of angiogenesis by oxygen metabolism. Dev. Cell. 2009; 16:167–179. [PubMed: 19217420]

Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell. 2009; 16:183–194. [PubMed: 19732719]

Fukai J, Yokote H, Yamanaka R, Arao T, Nishio K, Itakura T. EphA4 promotes cell proliferation migration through a novel EphA4-FGFR1 signaling pathway in the human glioma U251 cell line. Mol. Cancer Ther. 2008; 7:2768–2778. [PubMed: 18790757]

Fuller T, Korff T, Kilian A, Dandekar G, Augustin HG. Forward EphB4 signaling in endothelial cells controls cellular repulsion segregation from ephrinB2 positive cells. J. Cell Sci. 2003; 116:2461–2470. [PubMed: 12734395]

Gale NW, Baluk P, Pan L, Kwan M, Holash J, DeChiara TM, McDonald DM, Yancopoulos GD. Ephrin-B2 selectively marks arterial vessels neovascularization sites in the adult, with expression in both endothelial smooth-muscle cells. Dev. Biol. 2001; 230:151–160. [PubMed: 11161569]

Gariano RF, Gardner TW. Retinal angiogenesis in development disease. Nature. 2005; 438:960–966. [PubMed: 16355161]

Georgakopoulos A, Litterst C, Ghersi E, Baki L, Xu C, Serban G, Robakis NK. Metalloproteinase/ Presenilin1 processing of ephrinB regulates EphB-induced Src phosphorylation signaling. EMBO J. 2006; 25:1242–1252. [PubMed: 16511561]

Gerety SS, Anderson DJ. Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. Development. 2002; 129:1397–1410. [PubMed: 11880349]

Gerety SS, Wang HU, Chen ZF, Anderson DJ. Symmetrical mutant phenotypes of the receptor EphB4 its specific transmembrane ligand ephrin-B2 in cardiovascular development. Mol. Cell. 1999; 4:403–414. [PubMed: 10518221]

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J. Cell Biol. 2003; 161:1163–1177. [PubMed: 12810700]

Hellstrom M, Gerhardt H, Kalen M, Li X, Eriksson U, Wolburg H, Betsholtz C. Lack of pericytes leads to endothelial hyperplasia abnormal vascular morphogenesis. J. Cell Biol. 2001; 153:543–553. [PubMed: 11331305]

Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, Yoon K, Rossant J, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature. 2007; 445:776–780. [PubMed: 17259973]

Hendrix MJ, Seftor EA, Hess AR, Seftor RE. Molecular plasticity of human melanoma cells. Oncogene. 2003a; 22:3070–3075. [PubMed: 12789282]

Hendrix MJ, Seftor EA, Hess AR, Seftor RE. Vasculogenic mimicry tumour-cell plasticity: lessons from melanoma. Nat. Rev. Cancer. 2003b; 3:411–421. [PubMed: 12778131]

Herbert SP, Huisken J, Kim TN, Feldman ME, Houseman BT, Wang RA, Shokat KM, Stainier DY. Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. Science. 2009; 326:294–298. [PubMed: 19815777]

Himanen JP, Nikolov DB. Eph signaling: a structural view. Trends Neurosci. 2003; 26:46–51. [PubMed: 12495863]

Himanen JP, Rajashankar KR, Lackmann M, Cowan CA, Henkemeyer M, Nikolov DB. Crystal structure of an Eph receptor–ephrin complex. Nature. 2001; 414:933–938. [PubMed: 11780069]

Holland SJ, Gale NW, Gish GD, Roth RA, Songyang Z, Cantley LC, Henkemeyer M, Yancopoulos GD, Pawson T. Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. EMBO J. 1997; 16:3877–3888. [PubMed: 9233798]

Huang F, Reeves K, Han X, Fairchild C, Platero S, Wong TW, Lee F, Shaw P, Clark E. Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection. Cancer Res. 2007; 67:2226–2238. [PubMed: 17332353]

Hunkapiller NM, Gasperowicz M, Kapidzic M, Plaks V, Maltepe E, Kitajewski J, Cross JC, Fisher SJ. A role for Notch signaling in trophoblast endovascular invasion in the pathogenesis of pre-eclampsia. Development. 2011; 138:2987–2998. [PubMed: 21693515]

Huynh-Do U, Vindis C, Liu H, Cerretti DP, McGrew JT, Enriquez M, Chen J, Daniel TO. Ephrin-B1 transduces signals to activate integrin-mediated migration, attachment angiogenesis. J. Cell Sci. 2002; 115:3073–3081. [PubMed: 12118063]

Irie F, Okuno M, Pasquale EB, Yamaguchi Y. EphrinB-EphB signalling regulates clathrin-mediated endocytosis through tyrosine phosphorylation of synaptojanin 1. Nat. Cell Biol. 2005; 7:501–509. [PubMed: 15821731]

Jager RD, Mieler WF, Miller JW. Age-related macular degeneration. N. Engl. J. Med. 2008; 358:2606–2617. [PubMed: 18550876]

Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 2005; 307:58–62. [PubMed: 15637262]

Jin YJ, Wang J, Qiao C, Hei TK, Brandt-Rauf PW, Yin Y. Anovel mechanism for p53 to regulate its target gene ECK in signaling apoptosis. Mol. Cancer Res. 2006; 4:769–778. [PubMed: 17050670]

Kalo MS, Pasquale EB. Signal transfer by Eph receptors. Cell Tissue Res. 1999; 298:1–9.

Kemp HA, Cooke JE, Moens CB. EphA4 EfnB2a maintain rhombomere coherence by independently regulating intercalation of progenitor cells in the zebrafish neural keel. Dev. Biol. 2009; 327:313–326. [PubMed: 19135438]

Kertesz N, Krasnoperov V, Reddy R, Leshanski L, Kumar SR, Zozulya S, Gill PS. The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4- EphrinB2 interaction, modulates angiogenesis, inhibits tumor growth. Blood. 2006; 107:2330–2338. [PubMed: 16322467]

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature. 1993; 362:841–844. [PubMed: 7683111]

Kim I, Ryu YS, Kwak HJ, Ahn SY, Oh JL, Yancopoulos GD, Gale NW, Koh GY. EphB ligand, ephrinB2, suppresses the VEGF- angiopoietin 1-induced Ras/mitogen-activated protein kinase pathway in venous endothelial cells. FASEB J. 2002; 16:1126–1128. [PubMed: 12039842]

Klein R. Bidirectional modulation of synaptic functions by Eph/ephrin signaling. Nat. Neurosci. 2009; 12:15–20. [PubMed: 19029886]

Knoll B, Drescher U. Src family kinases are involved in EphA receptor-mediated retinal axon guidance. J. Neurosci. 2004; 24:6248–6257. [PubMed: 15254079]

Koolpe M, Burgess R, Dail M, Pasquale EB. EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity. J. Biol. Chem. 2005; 280:17301–17311. [PubMed: 15722342]

Korff T, Braun J, Pfaff D, Augustin HG, Hecker M. Role of ephrinB2 expression in endothelial cells during arteriogenesis: impact on smooth muscle cell migration monocyte recruitment. Blood. 2008; 112:73–81. [PubMed: 18445690]

Krasnoperov V, Kumar SR, Ley E, Li X, Scehnet J, Liu R, Zozulya S, Gill PS. Novel EphB4 monoclonal antibodies modulate angiogenesis inhibit tumor growth. Am. J. Pathol. 2010; 176:2029–2038. [PubMed: 20133814]

Kullander K, Klein R. Mechanisms functions of Eph ephrin signalling. Nat. Rev. Mol. Cell Biol. 2002; 3:475–486. [PubMed: 12094214]

Kumar SR, Masood R, Spannuth WA, Singh J, Scehnet J, Kleiber G, Jennings N, Deavers M, Krasnoperov V, Dubeau L, Weaver FA, Sood AK, et al. The receptor tyrosine kinase EphB4 is overexpressed in ovarian cancer, provides survival signals predicts poor outcome. Br. J. Cancer. 2007; 96:1083–1091. [PubMed: 17353927]

Kumar SR, Scehnet JS, Ley EJ, Singh J, Krasnoperov V, Liu R, Manchanda PK, Ladner RD, Hawes D, Weaver FA, Beart RW, Singh G, et al. Preferential induction of EphB4 over EphB2 its implication in colorectal cancer progression. Cancer Res. 2009; 69:3736–3745. [PubMed: 19366806]

Kvanta A, Algvere PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. Invest. Ophthalmol. Vis. Sci. 1996; 37:1929–1934. [PubMed: 8759365]

Lampugnani MG, Orsenigo F, Gagliani MC, Tacchetti C, Dejana E. Vascular endothelial cadherin controls VEGFR-2 internalization signaling from intracellular compartments. J. Cell Biol. 2006; 174:593–604. [PubMed: 16893970]

Lanahan AA, Hermans K, Claes F, Kerley-Hamilton JS, Zhuang ZW, Giordano FJ, Carmeliet P, Simons M. VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. Dev. Cell. 2010; 18:713–724. [PubMed: 20434959]

Lauterbach J, Klein R. Release of full-length EphB2 receptors from hippocampal neurons to cocultured glial cells. J. Neurosci. 2006; 26:11575–11581. [PubMed: 17093078]

Lee HS, Bong YS, Moore KB, Soria K, Moody SA, Daar IO. Dishevelled mediates ephrinB1 signalling in the eye field through the planar cell polarity pathway. Nat. Cell Biol. 2006; 8:55–63. [PubMed: 16362052]

Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, Ferrara N, Nagy A, Roos KP, Iruela-Arispe ML. Autocrine VEGF signaling is required for vascular homeostasis. Cell. 2007; 130:691–703. [PubMed: 17719546]

Lee HS, Mood K, Battu G, Ji YJ, Singh A, Daar IO. Fibroblast growth factor receptor-induced phosphorylation of ephrinB1 modulates its interaction with Dishevelled. Mol. Biol. Cell. 2009; 20:124–133. [PubMed: 19005214]

Li JL, Sainson RC, Oon CE, Turley H, Leek R, Sheldon H, Bridges E, Shi W, Snell C, Bowden ET, Wu H, Chowdhury PS, et al. DLL4-Notch signaling mediates tumor resistance to anti-VEGF therapy in vivo. Cancer Res. 2011; 71:6073–6083. [PubMed: 21803743]

Librach CL, Werb Z, Fitzgerald ML, Chiu K, Corwin NM, Esteves RA, Grobelny D, Galardy R, Damsky CH, Fisher SJ. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J. Cell Biol. 1991; 113:437–449. [PubMed: 1849141]

Lin D, Gish GD, Songyang Z, Pawson T. The carboxyl terminus of B class ephrins constitutes a PDZ domain binding motif. J. Biol. Chem. 1999; 274:3726–3733. [PubMed: 9920925]

Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, et al. Edg-1, the G proteincoupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J. Clin. Invest. 2000; 106:951–961. [PubMed: 11032855]

Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, Wiegand SJ. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc. Natl. Acad. Sci. U.S.A. 2007; 104:3219–3224. [PubMed: 17296940]

Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest. Ophthalmol. Vis. Sci. 1996; 37:855–868. [PubMed: 8603870]

Lu Q, Sun EE, Klein RS, Flanagan JG. Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein selectively inhibits G protein-coupled chemoattraction. Cell. 2001; 105:69–79. [PubMed: 11301003]

Maekawa H, Oike Y, Kanda S, Ito Y, Yamada Y, Kurihara H, Nagai R, Suda T. Ephrin-B2 induces migration of endothelial cells through the phosphatidylinositol- kinase pathway promotes angiogenesis in adult vasculature. Arterioscler. Thromb. Vasc. Biol. 2003; 23:2008–2014. [PubMed: 14500293]

Makinen T, Adams RH, Bailey J, Lu Q, Ziemiecki A, Alitalo K, Klein R, Wilkinson GA. PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. Genes Dev. 2005; 19:397–410. [PubMed: 15687262]

Mann F, Miranda E, Weinl C, Harmer E, Holt CE. B-type Eph receptors ephrins induce growth cone collapse through distinct intracellular pathways. J. Neurobiol. 2003; 57:323–336. [PubMed: 14608666]

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002; 23:549–555. [PubMed: 12401408]

Mao W, Luis E, Ross S, Silva J, Tan C, Crowley C, Chui C, Franz G, Senter P, Koeppen H, Polakis P. EphB2 as a therapeutic antibody drug target for the treatment of colorectal cancer. Cancer Res. 2004; 64:781–788. [PubMed: 14871799]

Margolis SS, Salogiannis J, Lipton DM, Mandel-Brehm C, Wills ZP, Mardinly AR, Hu L, Greer PL, Bikoff JB, Ho HY, Soskis MJ, Sahin M, et al. EphBmediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. Cell. 2010; 143:442–455. [PubMed: 21029865]

Marston DJ, Dickinson S, Nobes CD. Rac-dependent trans-endocytosis of ephrinBs regulates Eph–ephrin contact repulsion. Nat. Cell Biol. 2003; 5:879–888. [PubMed: 12973357]

Masood R, Xia G, Smith DL, Scalia P, Still JG, Tulpule A, Gill PS. Ephrin B2 expression in Kaposi sarcoma is induced by human herpesvirus type 8: phenotype switch from venous to arterial endothelium. Blood. 2005; 105:1310–1318. [PubMed: 15471957]

Meyer S, Hafner C, Guba M, Flegel S, Geissler EK, Becker B, Koehl GE, Orso E, Landthaler M, Vogt T. Ephrin-B2 overexpression enhances integrin-mediated ECM-attachment migration of B16 melanoma cells. Int. J. Oncol. 2005; 27:1197–1206. [PubMed: 16211213]

Miao H, Strebhardt K, Pasquale EB, Shen TL, Guan JL, Wang B. Inhibition of integrin-mediated cell adhesion but not directional cell migration requires catalytic activity of EphB3 receptor tyrosine kinase. Role of Rho family small GTPases. J. Biol. Chem. 2005; 280:923–932. [PubMed: 15536074]

Miao H, Li DQ, Mukherjee A, Guo H, Petty A, Cutter J, Basilion JP, Sedor J, Wu J, Danielpour D, Sloan AE, Cohen ML, et al. EphA2 mediates ligand-dependent inhibition ligand-independent promotion of cell migration invasion via a reciprocal regulatory loop with Akt. Cancer Cell. 2009; 16:9–20. [PubMed: 19573808]

Miller JW, Adamis AP, Shima DT, D'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B, et al. Vascular endothelial growth factor/vascular permeability factor is temporally spatially correlated with ocular angiogenesis in a primate model. Am. J. Pathol. 1994; 145:574–584. [PubMed: 7521577]

Mohle R, Green D, Moore MA, Nachman RL, Rafii S. Constitutive production thrombin-induced release of vascular endothelial growth factor by human megakaryocytes platelets. Proc. Natl. Acad. Sci. U.S.A. 1997; 94:663–668. [PubMed: 9012841]

Murai KK, Pasquale EB. 'Eph'ective signaling: forward, reverse crosstalk. J. Cell Sci. 2003; 116:2823–2832. [PubMed: 12808016]

Murai KK, Pasquale EB. New exchanges in eph-dependent growth cone dynamics. Neuron. 2005; 46:161–163. [PubMed: 15848793]

Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat. Rev. Cancer. 2008; 8:618–631. [PubMed: 18633355]

Murohara T, Horowitz JR, Silver M, Tsurumi Y, Chen D, Sullivan A, Isner JM. Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide prostacyclin. Circulation. 1998; 97:99–107. [PubMed: 9443437]

Nishimura T, Yamaguchi T, Kato K, Yoshizawa M, Nabeshima Y, Ohno S, Hoshino M, Kaibuchi K. PAR-6-PAR-3 mediates Cdc42-induced Rac activation through the Rac GEFs STEF/Tiam1. Nat. Cell Biol. 2005; 7:270–277. [PubMed: 15723051]

Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, Lin HC, Yancopoulos GD, Thurston G. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature. 2006; 444:1032–1037. [PubMed: 17183313]

Noren NK, Pasquale EB. Eph receptor–ephrin bidirectional signals that target Ras Rho proteins. Cell. Signal. 2004; 16:655–666. [PubMed: 15093606]

Noren NK, Pasquale EB. Paradoxes of the EphB4 receptor in cancer. Cancer Res. 2007; 67:3994–3997. [PubMed: 17483308]

Noren NK, Foos G, Hauser CA, Pasquale EB. The EphB4 receptor suppresses breast cancer cell tumorigenicity through an Abl–Crk pathway. Nat. Cell Biol. 2006; 8:815–825. [PubMed: 16862147]

Ogita H, Kunimoto S, Kamioka Y, Sawa H, Masuda M, Mochizuki N. EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells. Circ. Res. 2003; 93:23–31. [PubMed: 12775584]

Palmer A, Zimmer M, Erdmann KS, Eulenburg V, Porthin A, Heumann R, Deutsch U, Klein R. EphrinB phosphorylation reverse signaling: regulation by Src kinases PTP-BL phosphatase. Mol. Cell. 2002; 9:725–737. [PubMed: 11983165]

Pasquale EB. Eph receptors ephrins in cancer: bidirectional signalling beyond. Nat. Rev. Cancer. 2010; 10:165–180. [PubMed: 20179713]

Pitulescu ME, Adams RH. Eph/ephrin molecules—a hub for signaling endocytosis. Genes Dev. 2010; 24:2480–2492. [PubMed: 21078817]

Pugh CW, Ratcliffe PJ. The von Hippel-Lindau tumor suppressor, hypoxiainducible factor-1 (HIF-1) degradation, cancer pathogenesis. Semin. Cancer Biol. 2003; 13:83–89. [PubMed: 12507560]

Red-Horse K, Kapidzic M, Zhou Y, Feng KT, Singh H, Fisher SJ. EPHB4 regulates chemokine-evoked trophoblast responses: a mechanism for incorporating the human placenta into the maternal circulation. Development. 2005; 132:4097–4106. [PubMed: 16107476]

Ricci-Vitiani L, Pallini R, Biffoni M, Todaro M, Invernici G, Cenci T, Maira G, Parati EA, Stassi G, Larocca LM, De Maria R. Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. Nature. 2010; 468:824–828. [PubMed: 21102434]

Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, Kowalski J, Watts RJ, Callahan C, Kasman I, Singh M, Chien M, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature. 2006; 444:1083–1087. [PubMed: 17183323]

Ruhe JE, Streit S, Hart S, Wong CH, Specht K, Knyazev P, Knyazeva T, Tay LS, Loo HL, Foo P, Wong W, Pok S, et al. Genetic alterations in the tyrosine kinase transcriptome of human cancer cell lines. Cancer Res. 2007; 67:11368–11376. [PubMed: 18056464]

Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev. 2002; 16:2684–2698. [PubMed: 12381667]

Sahin M, Greer PL, Lin MZ, Poucher H, Eberhart J, Schmidt S, Wright TM, Shamah SM, O'Connell S, Cowan CW, Hu L, Goldberg JL, et al. Ephdependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse. Neuron. 2005; 46:191–204. [PubMed: 15848799]

Salvucci O, de la Luz Sierra M, Martina JA, McCormick PJ, Tosato G. EphB2 EphB4 receptors forward signaling promotes SDF-1-induced endothelial cell chemotaxis branching remodeling. Blood. 2006; 108:2914–2922. [PubMed: 16840724]

Salvucci O, Maric D, Economopoulou M, Sakakibara S, Merlin S, Follenzi A, Tosato G. EphrinB reverse signaling contributes to endothelial mural cell assembly into vascular structures. Blood. 2009; 114:1707–1716. [PubMed: 19411631]

Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron- Maguire M, Gridley T, Wolburg H, Risau W, Qin Y. Distinct roles of the receptor tyrosine kinases Tie-1 Tie-2 in blood vessel formation. Nature. 1995; 376:70–74. [PubMed: 7596437]

Sawamiphak S, Ritter M, Acker-Palmer A. Preparation of retinal explant cultures to study ex vivo tip endothelial cell responses. Nat. Protoc. 2010a; 5:1659–1665. [PubMed: 20885378]

Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, Acker-Palmer A. Ephrin-B2 regulates VEGFR2 function in developmental tumour angiogenesis. Nature. 2010b; 465:487–491. [PubMed: 20445540]

Scehnet JS, Ley EJ, Krasnoperov V, Liu R, Manchanda PK, Sjoberg E, Kostecke AP, Gupta S, Kumar SR, Gill PS. The role of Ephs, Ephrins, growth factors in Kaposi sarcoma implications of EphrinB2 blockade. Blood. 2009; 113:254–263. [PubMed: 18836096]

Schruefer R, Sulyok S, Schymeinsky J, Peters T, Scharffetter-Kochanek K, Walzog B. The proangiogenic capacity of polymorphonuclear neutrophils delineated by microarray technique by measurement of neovascularization in wounded skin of CD18- deficient mice. J. Vasc. Res. 2006; 43:1–11. [PubMed: 16227701]

Schultz GS, Wysocki A. Interactions between extracellular matrix growth factors in wound healing. Wound Repair Regen. 2009; 17:153–162. [PubMed: 19320882]

Segura I, Essmann CL, Weinges S, Acker-Palmer A. Grb4 GIT1 transduce ephrinB reverse signals modulating spine morphogenesis synapse formation. Nat. Neurosci. 2007; 10:301–310. [PubMed: 17310244]

Semenza GL. Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer. 2003; 3:721–732. [PubMed: 13130303]

Semenza GL. Regulation of metabolism by hypoxia-inducible factor 1. Cold Spring Harb. Symp. Quant. Biol. 2011 Jul 22. [Epub ahead of print].

Shamah SM, Lin MZ, Goldberg JL, Estrach S, Sahin M, Hu L, Bazalakova M, Neve RL, Corfas G, Debant A, Greenberg ME. EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. Cell. 2001; 105:233–244. [PubMed: 11336673]

Shin D, Garcia-Cardena G, Hayashi S, Gerety S, Asahara T, Stavrakis G, Isner J, Folkman J, Gimbrone MA Jr, Anderson DJ. Expression of ephrinB2 identifies a stable genetic difference between arterial venous vascular smooth muscle as well as endothelial cells, marks subsets of

microvessels at sites of adult neovascularization. Dev. Biol. 2001; 230:139–150. [PubMed: 11161568]

Shojaei F, Wu X, Malik AK, Zhong C, Baldwin ME, Schanz S, Fuh G, Gerber HP, Ferrara N. Tumor refractoriness to anti-VEGF treatment is mediated by CD11b +Gr1+ myeloid cells. Nat. Biotechnol. 2007; 25:911–920. [PubMed: 17664940]

Shojaei F, Wu X, Qu X, Kowanetz M, Yu L, Tan M, Meng YG, Ferrara N. G-CSF-initiated myeloid cell mobilization angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. Proc. Natl. Acad. Sci. U.S.A. 2009; 106:6742–6747. [PubMed: 19346489]

Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R, D'Amore PA. Oxygen-induced retinopathy in the mouse. Invest. Ophthalmol. Vis. Sci. 1994; 35:101–111. [PubMed: 7507904]

Smith FM, Vearing C, Lackmann M, Treutlein H, Himanen J, Chen K, Saul A, Nikolov D, Boyd AW. Dissecting the EphA3/Ephrin-A5 interactions using a novel functional mutagenesis screen. J. Biol. Chem. 2004; 279:9522–9531. [PubMed: 14660665]

Stein E, Schoecklmann H, Daniel TO. Eph family receptors ligands in vascular cell targeting assembly. Trends Cardiovasc. Med. 1997; 7:329–334. [PubMed: 21235905]

Steinle JJ, Meininger CJ, Forough R, Wu G, Wu MH, Granger HJ. Eph B4 receptor signaling mediates endothelial cell migration proliferation via the phosphatidylinositol 3-kinase pathway. J. Biol. Chem. 2002; 277:43830–43835. [PubMed: 12235151]

Steinle JJ, Meininger CJ, Chowdhury U, Wu G, Granger HJ. Role of ephrin B2 in human retinal endothelial cell proliferation migration. Cell. Signal. 2003; 15:1011–1017. [PubMed: 14499344]

Stenzel D, Lundkvist A, Sauvaget D, Busse M, Graupera M, van der Flier A, Wijelath ES, Murray J, Sobel M, Costell M, Takahashi S, Fassler R, et al. Integrin-dependent -independent functions of astrocytic fibronectin in retinal angiogenesis. Development. 2011; 138:4451–4463. [PubMed: 21880786]

Suchting S, Freitas C, le Noble F, Benedito R, Breant C, Duarte A, Eichmann A. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation vessel branching. Proc. Natl. Acad. Sci. U.S.A. 2007; 104:3225–3230. [PubMed: 17296941]

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell. 1996; 87:1171–1180. [PubMed: 8980224]

Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, Sato TN, Yancopoulos GD. Increased vascularization in mice overexpressing angiopoietin-1. Science. 1998; 282:468–471. [PubMed: 9774272]

Tanaka M, Kamo T, Ota S, Sugimura H. Association of Dishevelled with Eph tyrosine kinase receptor ephrin mediates cell repulsion. EMBO J. 2003; 22:847–858. [PubMed: 12574121]

Tanaka M, Ohashi R, Nakamura R, Shinmura K, Kamo T, Sakai R, Sugimura H. Tiam1 mediates neurite outgrowth induced by ephrin-B1 EphA2. EMBO J. 2004; 23:1075–1088. [PubMed: 14988728]

Tang M, Chen B, Lin T, Li Z, Pardo C, Pampo C, Chen J, Lien CL, Wu L, Ai L, Wang H, Yao K, et al. Restraint of angiogenesis by zinc finger transcription factor CTCF-dependent chromatin insulation. Proc. Natl. Acad. Sci. U.S.A. 2011; 108:15231–15236. [PubMed: 21896759]

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science. 1999; 286:2511–2514. [PubMed: 10617467]

Toth J, Cutforth T, Gelinas AD, Bethoney KA, Bard J, Harrison CJ. Crystal structure of an ephrin ectodomain. Dev. Cell. 2001; 1:83–92. [PubMed: 11703926]

Vihanto MM, Vindis C, Djonov V, Cerretti DP, Huynh-Do U. Caveolin-1 is required for signaling membrane targeting of EphB1 receptor tyrosine kinase. J. Cell Sci. 2006; 119:2299–2309. [PubMed: 16723736]

Wang HU, Chen ZF, Anderson DJ. Molecular distinction angiogenic interaction between embryonic arteries veins revealed by ephrin-B2 its receptor Eph- B4. Cell. 1998; 93:741–753. [PubMed: 9630219]

Wang R, Chadalavada K, Wilshire J, Kowalik U, Hovinga KE, Geber A, Fligelman B, Leversha M, Brennan C, Tabar V. Glioblastoma stem-like cells give rise to tumour endothelium. Nature. 2010a; 468:829–833. [PubMed: 21102433]

Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Luthi U, Barberis A, Benjamin LE, et al. Ephrin- B2 controls VEGF-induced angiogenesis lymphangiogenesis. Nature. 2010b; 465:483–486. [PubMed: 20445537]

Wybenga-Groot LE, Baskin B, Ong SH, Tong J, Pawson T, Sicheri F. Structural basis for autoinhibition of the Ephb2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region. Cell. 2001; 106:745–757. [PubMed: 11572780]

Xia G, Kumar SR, Masood R, Zhu S, Reddy R, Krasnoperov V, Quinn DI, Henshall SM, Sutherland RL, Pinski JK, Daneshmand S, Buscarini M, et al. EphB4 expression biological significance in prostate cancer. Cancer Res. 2005; 65:4623–4632. [PubMed: 15930280]

Xia G, Kumar SR, Stein JP, Singh J, Krasnoperov V, Zhu S, Hassanieh L, Smith DL, Buscarini M, Broek D, Quinn DI, Weaver FA, et al. EphB4 receptor tyrosine kinase is expressed in bladder cancer provides signals for cell survival. Oncogene. 2006; 25:769–780. [PubMed: 16205642]

Xu NJ, Henkemeyer M. Ephrin-B3 reverse signaling through Grb4 cytoskeletal regulators mediates axon pruning. Nat. Neurosci. 2009; 12:268–276. [PubMed: 19182796]

Xu Y, Zagoura D, Keck C, Pietrowski D. Expression of Eph receptor tyrosine kinases their ligands in human Granulosa lutein cells human umbilical vein endothelial cells. Exp. Clin. Endocrinol. Diabetes. 2006; 114:590–595. [PubMed: 17177142]

Xu Z, Jin H, Qian Q. Humanized anti-EphB4 antibodies for the treatment of carcinomas vasculogenesis-related diseases. Expert Opin. Ther. Pat. 2009; 19:1035–1037. [PubMed: 19552516]

Yang NY, Pasquale EB, Owen LB, Ethell IM. The EphB4 receptor-tyrosine kinase promotes the migration of melanoma cells through Rho-mediated actin cytoskeleton reorganization. J. Biol. Chem. 2006; 281:32574–32586. [PubMed: 16950769]

Yu J, Zhang L, Hwang PM, Rago C, Kinzler KW, Vogelstein B. Identification classification of p53-regulated genes. Proc. Natl. Acad. Sci. U.S.A. 1999; 96:14517–14522. [PubMed: 10588737]

Yuan K, Jin YT, Lin MT. Expression of Tie-2, angiopoietin-1, angiopoietin-2, ephrinB2 EphB4 in pyogenic granuloma of human gingiva implicates their roles in inflammatory angiogenesis. J. Periodontal Res. 2000; 35:165–171. [PubMed: 10929871]

Yuan K, Hong TM, Chen JJ, Tsai WH, Lin MT. Syndecan-1 up-regulated by ephrinB2/EphB4 plays dual roles in inflammatory angiogenesis. Blood. 2004; 104:1025–1033. [PubMed: 15126321]

Zhong TP, Childs S, Leu JP, Fishman MC. Gridlock signalling pathway fashions the first embryonic artery. Nature. 2001; 414:216–220. [PubMed: 11700560]

Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, Damsky CH. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? J. Clin. Invest. 1997; 99:2139–2151. [PubMed: 9151786]

Zhou Y, Bellingard V, Feng KT, McMaster M, Fisher SJ. Human cytotrophoblasts promote endothelial survival vascular remodeling through secretion of Ang2, PlGF, VEGF-C. Dev. Biol. 2003; 263:114–125. [PubMed: 14568550]

Zhuang G, Brantley-Sieders DM, Vaught D, Yu J, Xie L, Wells S, Jackson D, Muraoka-Cook R, Arteaga C, Chen J. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. Cancer Res. 2010; 70:299–308. [PubMed: 20028874]

Zimmer M, Palmer A, Kohler J, Klein R. EphB–ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion. Nat. Cell Biol. 2003; 5:869–878. [PubMed: 12973358]

Zisch AH, Pazzagli C, Freeman AL, Schneller M, Hadman M, Smith JW, Ruoslahti E, Pasquale EB. Replacing two conserved tyrosines of the EphB2 receptor with glutamic acid prevents binding of SH2 domains without abrogating kinase activity biological responses. Oncogene. 2000; 19:177–187. [PubMed: 10644995]
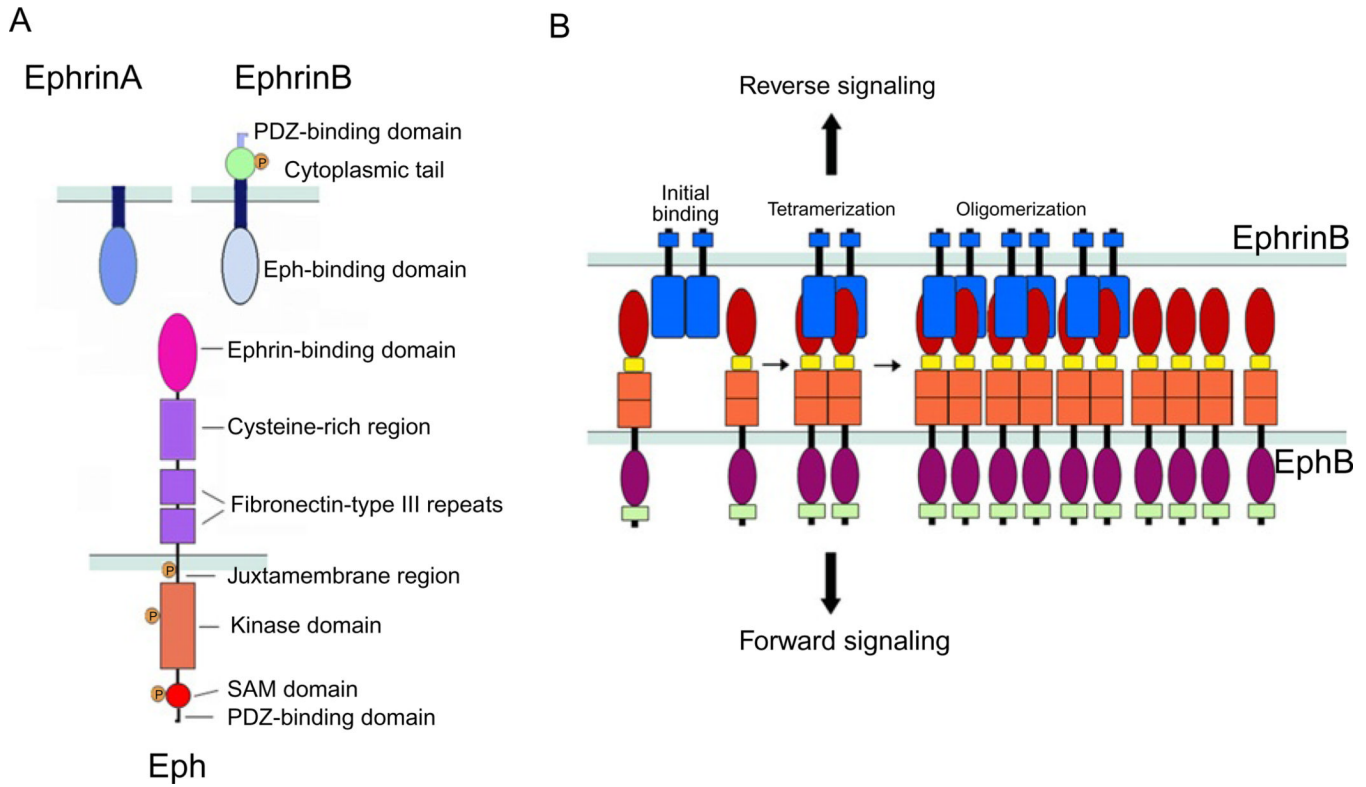
Fig. 1.
(A) Schematic representation of the domain structure and binding interfaces of Ephrins and Eph receptors. EphrinA ligands are attached to the cell surface through a glycosylphosphatidylinositol (GPI)-anchor; the extracellular domain contains an Eph receptor-binding domain that is connected to the transmembrane segment. EphrinB ligands are transmembrane proteins with an extracellular Eph receptor-binding domain connected to a transmembrane segment, which is followed by a short intracellular domain. The Eph receptors include an extracellular domain composed of an Ephrin-binding domain, a cysteine-rich segment that contains an epidermal growth factor (EGF)-like motif, and two fibronectin-type III domains; and a cytoplasmic region that contains a juxtamembrane region, the kinase domain, a sterile a-motif (SAM), and a binding site for PDZ-containing proteins. (B) Representation of initial binding of cell surface Eph and Ephrin molecules to form heterotetramers, which initiate signaling, and subsequent oligomerization to form large receptor/ligand clusters that expand laterally through hemophilic interactions between Eph receptors.
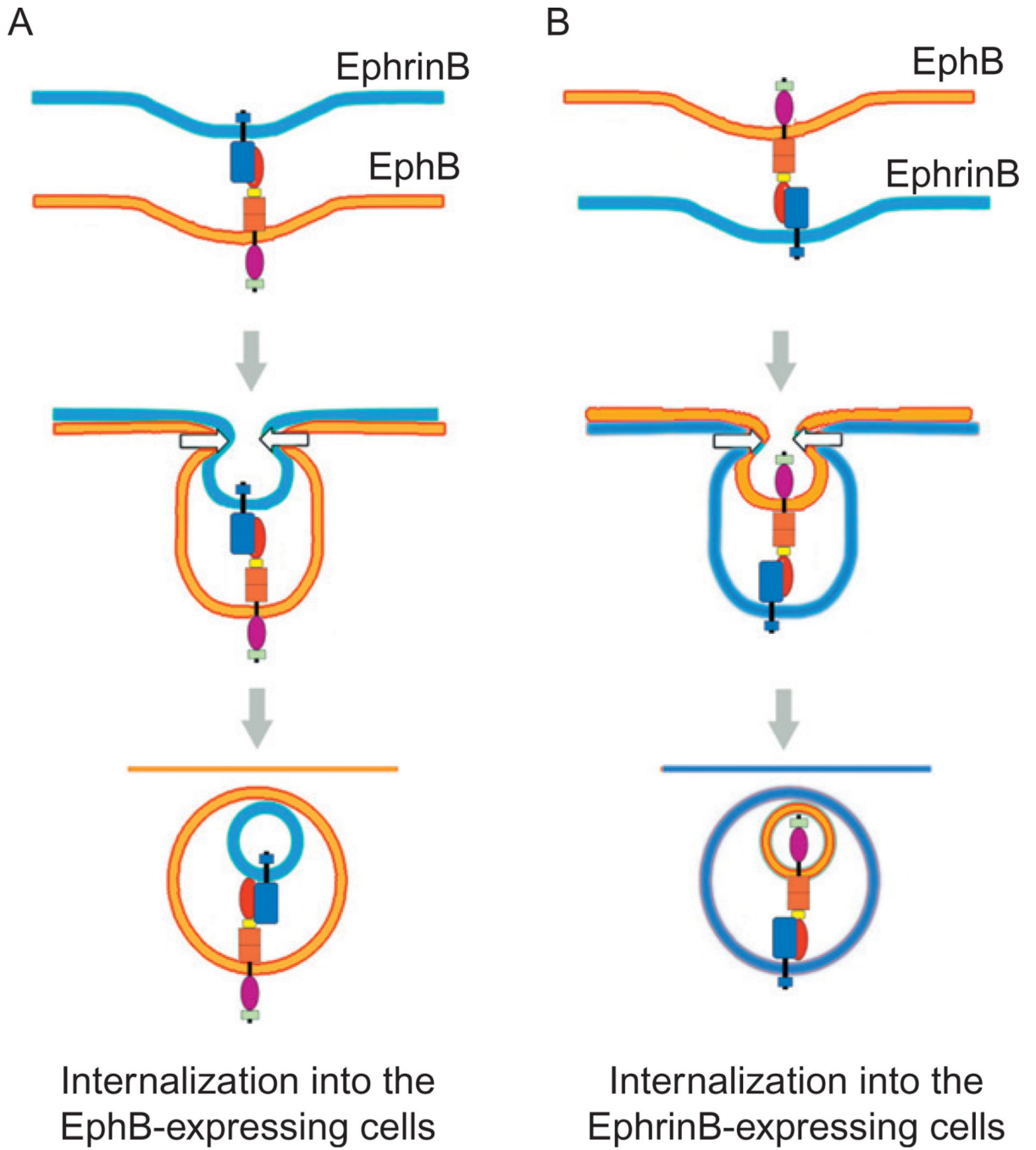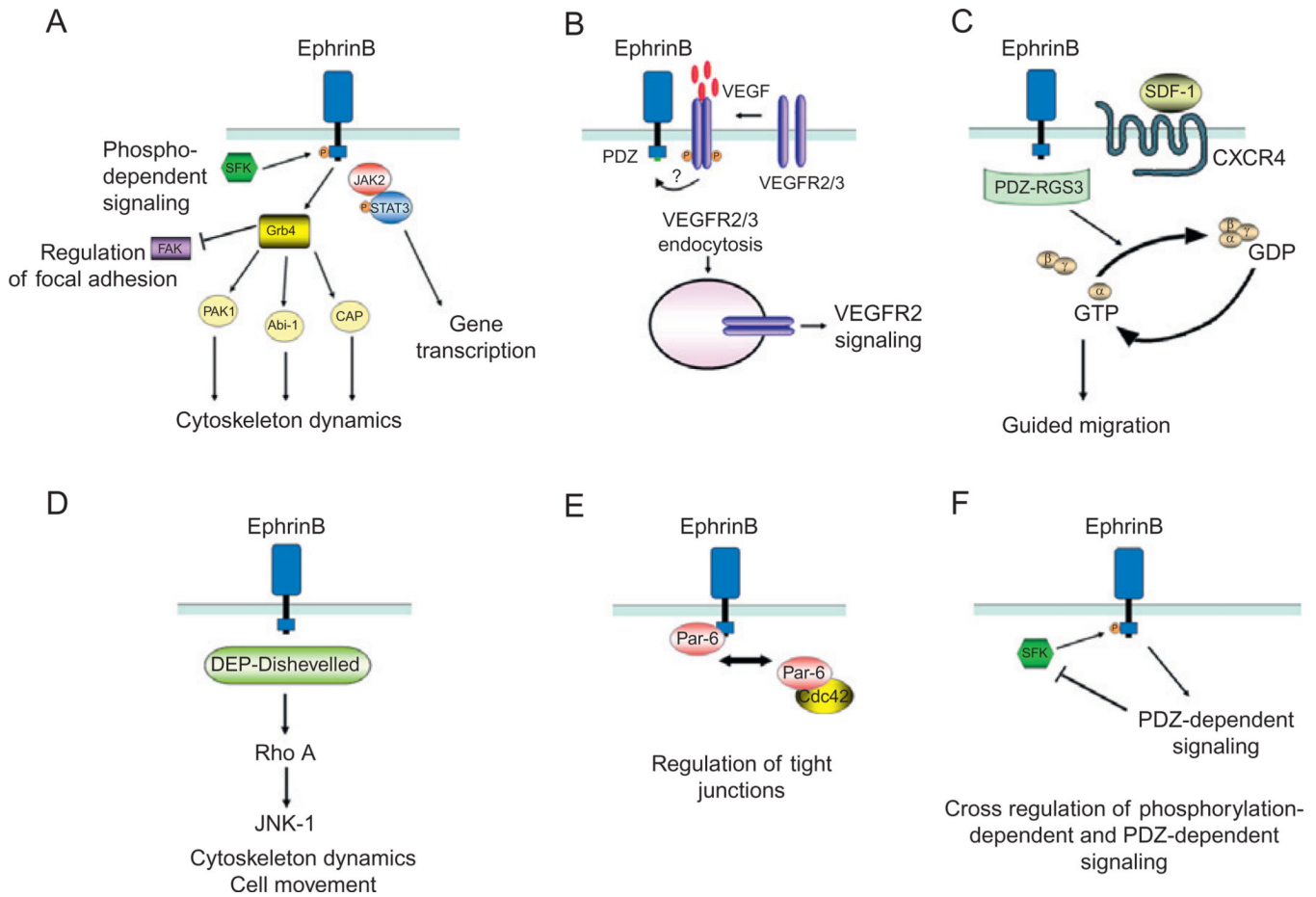
Fig. 2.
Interaction of EphrinB with EphB can lead to (A) *trans*-endocytosis in the direction of the receptor (forward endocytosis) or (B) *trans*-endocytosis in the direction of the ligand (reverse endocytosis). Both processes lead to the internalization of full-length ligand and receptor.

Fig. 3.
Mechanisms of EphrinB reverse signaling. (A) EphrinB activation by EphB receptor leads to the recruitment of Src family kinases (SFKs) that phosphorylate EphrinB intracellular domain at tyrosine residues. The adaptor molecule Grb4, which contains a Src-homology-2 (SH2) domain, is recruited to the phosphorylated EphrinB and initiates a number of signaling events that regulate cytoskeleton dynamics and focal adhesions. The phosphorylated EphrinB can also recruit the Jak2/STAT3 complex; the phosphorylated STAT3 translocates to the nucleus regulating expression of target genes. (B) Activated EphrinB regulates the internalization of VEGFR2 and VEGFR3 through PDZ-mediated binding of yet unknown protein(s). After VEGF binding, VEGFR is phosphorylated; signaling from activated VEGFR requires internalization, which is positively regulated by EphrinB signaling. (C) RGS3 is a PDZ-containing protein that constitutively binds to EphrinB and links signaling from the G-protein-coupled receptor CXCR4 to EphrinB. CXCR4 signaling in response to the ligand SDF1 is induced by the dissociation of Gβγ and GTP-Gα subunits. PDZ-RGS3 can inhibit CXCR4 signaling by enhancing the GTPase activity of the Gα subunit, resulting in the reformation of the inactive heterodimeric CXCR4 receptor. (D) The scaffold protein Dishevelled binds EphrinB through its DEP domain and mediates signaling via the Rho small GTPase pathway. (E) The scaffold protein Par6 associates with EphrinB resulting in the loss of tight junctions. PAR6 forms a complex with activated apical protein kinase C (aPKC) and Cdc42-GTP; the complex localizes to the apical cell junctions where it regulates tight junctions. EphrinB1 can compete with Cdc42 for binding to Par6 and thus reduce tight junctions. (F) Cross-regulation between EphrinB phosphorylation- and PDZ-dependent signaling pathways. The phosphatase PTB-BL,

recruited through its PDZ domain to EphrinB cytoplasmic domain, inactivates Src and dephosphorylates EphrinB.
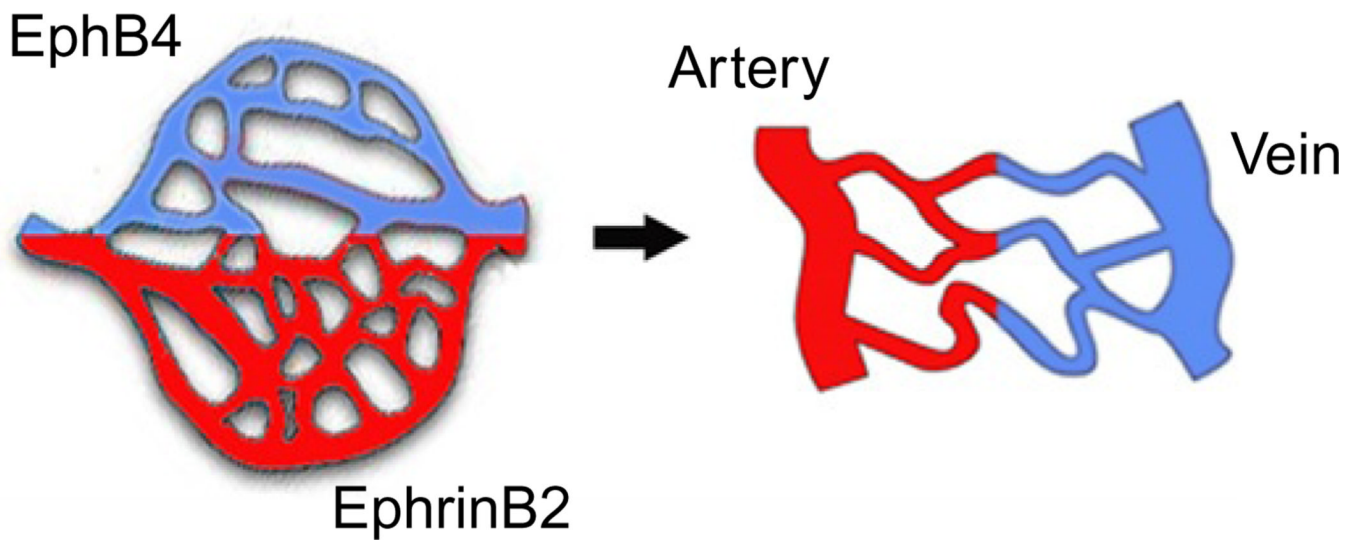
Fig. 4.
A role for EphB4 and EphrinB2 in arterial and venous fate determination. In the primitive vascular plexus, presumptive arterial and venous territories are marked by the distinctive expression of EphrinB2 or EphB4, prior to the formation of EphrinB2[+] arteries and EphB4^{+} veins.
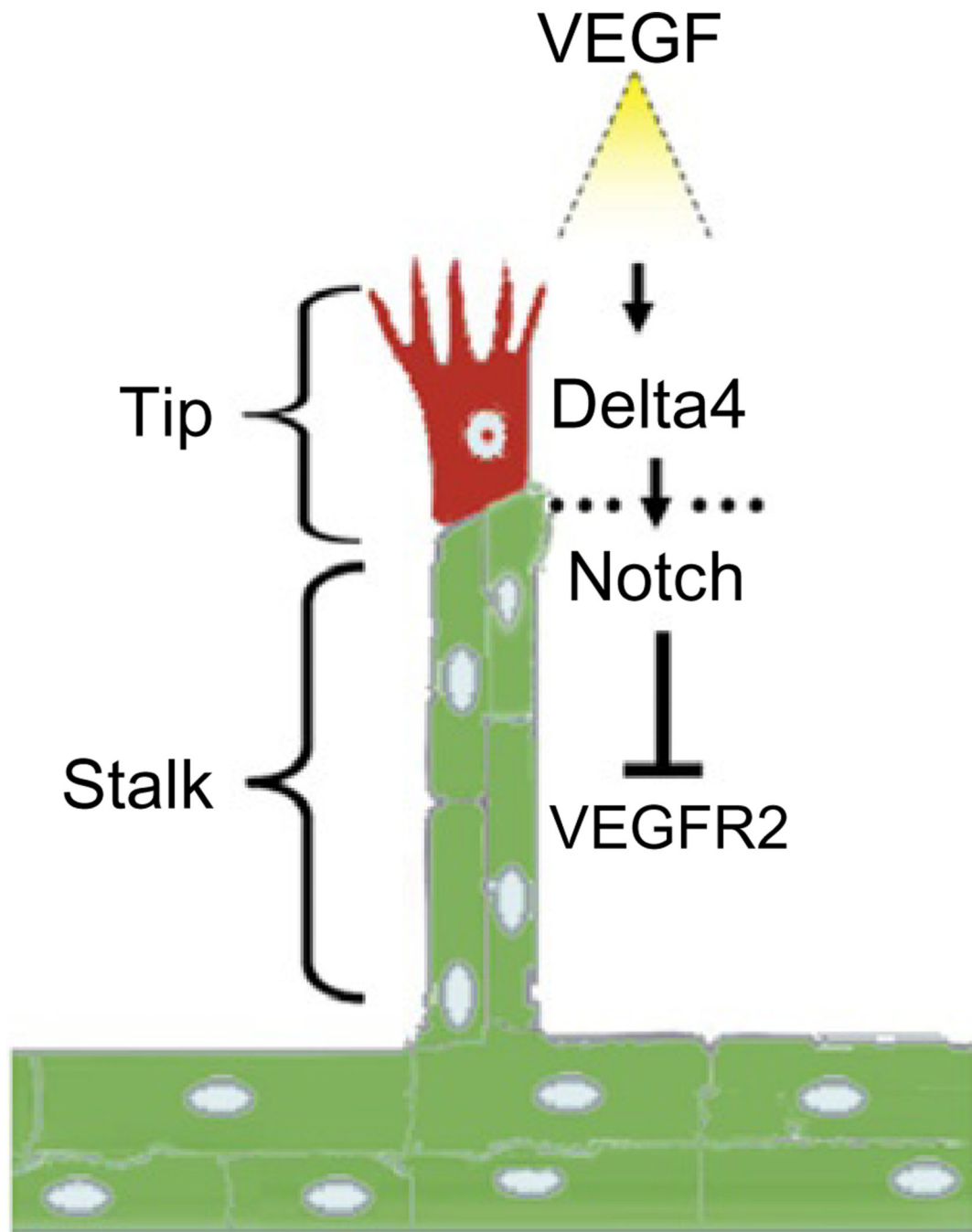
VEGF

Tip

Delta4

Notch

Stalk

VEGFR2

Fig. 5.
Angiogenesis is often initiated by the sprouting of endothelial cells from existing vessels. The leading cell in the sprout, the so-called tip cell, is characterized by filopodia extensions; the cells at the base of the sprout, the so-called stalk cells, are proliferating. VEGF/ VEGFR2, the Notch ligands Delta4 and Notch signaling participate in orchestrating sprouting angiogenesis.
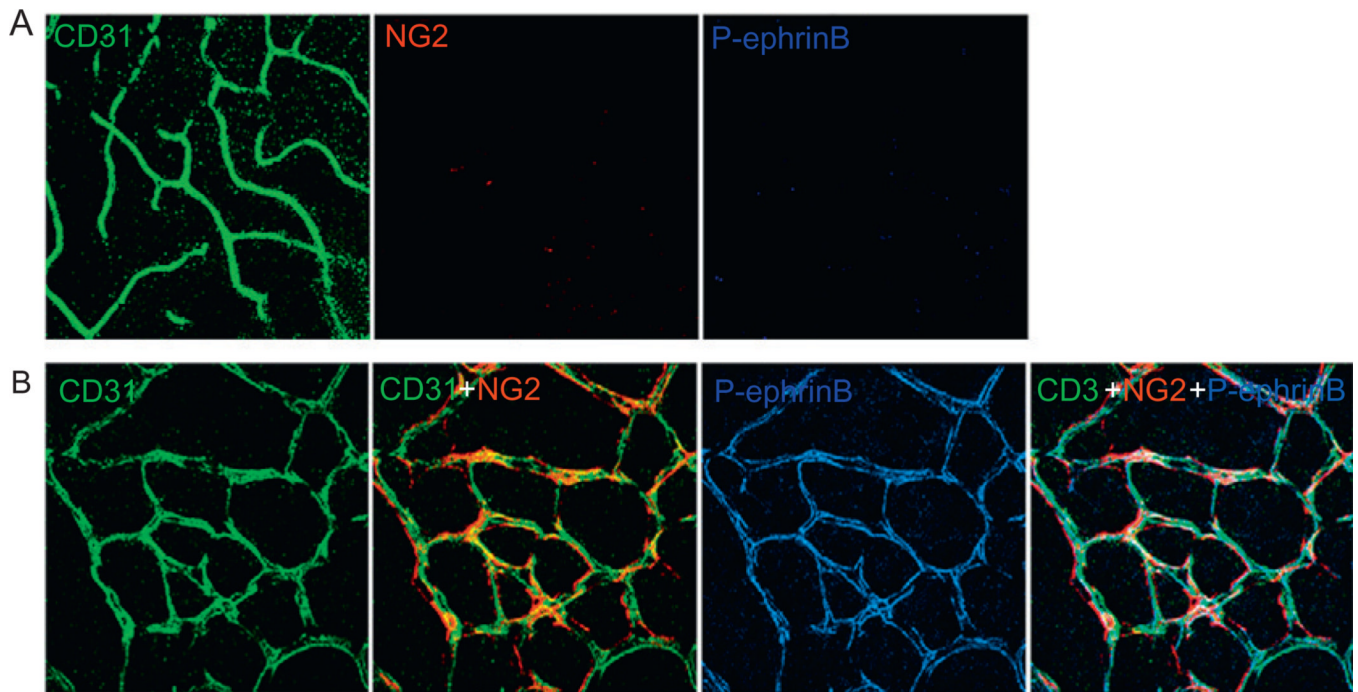
Fig. 6.
EphrinB is phosphorylated in remodeling but not in fully developed retinal vessels. (A) EphrinB phosphorylation is not detected in fully developed mouse retinal vessels, including the CD31+ endothelial cells and theNG2+ pericytes. (B) EphrinB2 is widely phosphorylated in the remodeling retinal vessels from 6-day-old mice, both in the endothelial cells and in the pericytes.
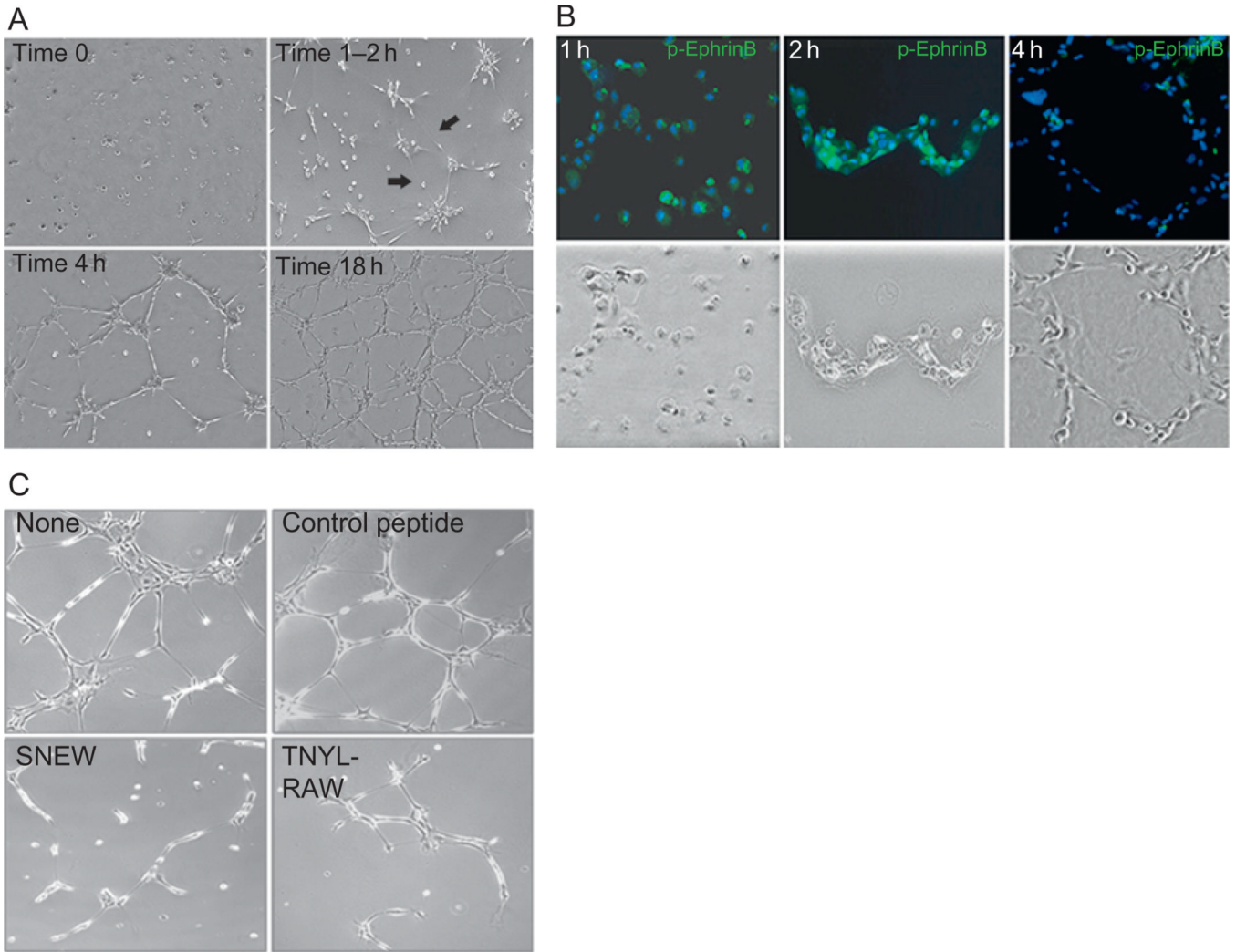
Fig. 7.
Primary endothelial cells assemble into cord-like structures on extracellular matrix, a
process that requires EphB/EphrinB interaction. (A) Time-dependent assembly of primary
endothelial cells on Matrigel. Note the appearance after 1–2h of needle-like endothelial cell
extensions that eventually form the initial bridging of endothelial cells with each other. (B)
EphrinB2 is broadly and time-dependently phosphorylated in endothelial cells as they
assemble into cord-like structures. (C) The EphB receptor inhibitors SNEW and TNYL-
RAW peptides prevent the assembly of endothelial cells into cord-like structures.
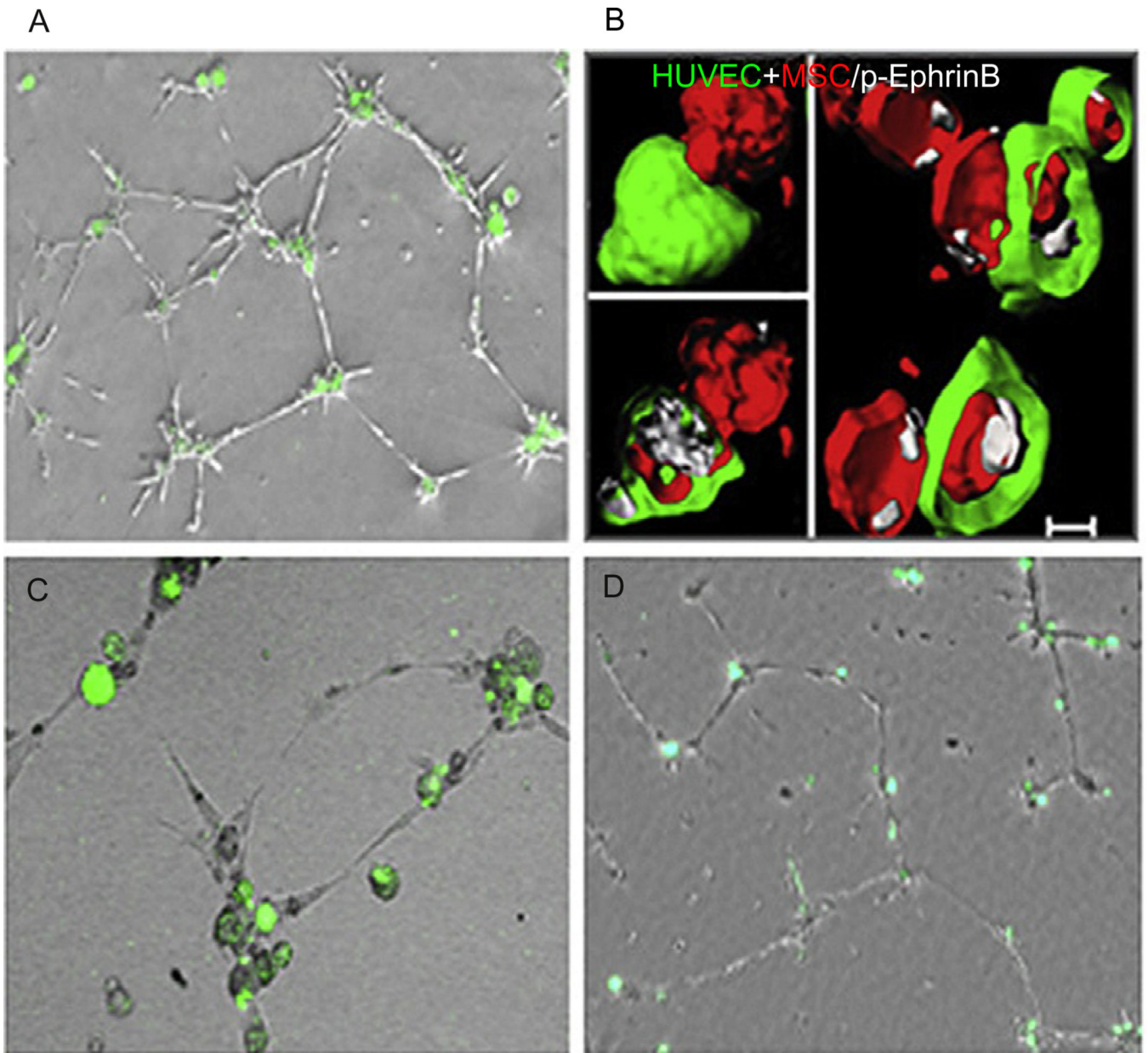
A

B

Fig. 8.
Endothelial cells and mesenchymal stem cells/pericytes assemble in cord-like structures on
Matrigel, with mesenchymal stem cells (green) in the nodes anchoring the cord structures.
(B) EphrinB is phosphorylated (white) at points of contact between endothelial cells
(HUVEC green) and mesenchymal stem cells (MSC red) forming cord-like structures. (C)
The silencing of EphrinB2 in mesenchymal stem cells (green) prevents their assembly with
endothelial cells (unstained). (D) The silencing of EphrinB2 in endothelial cells (unstained)
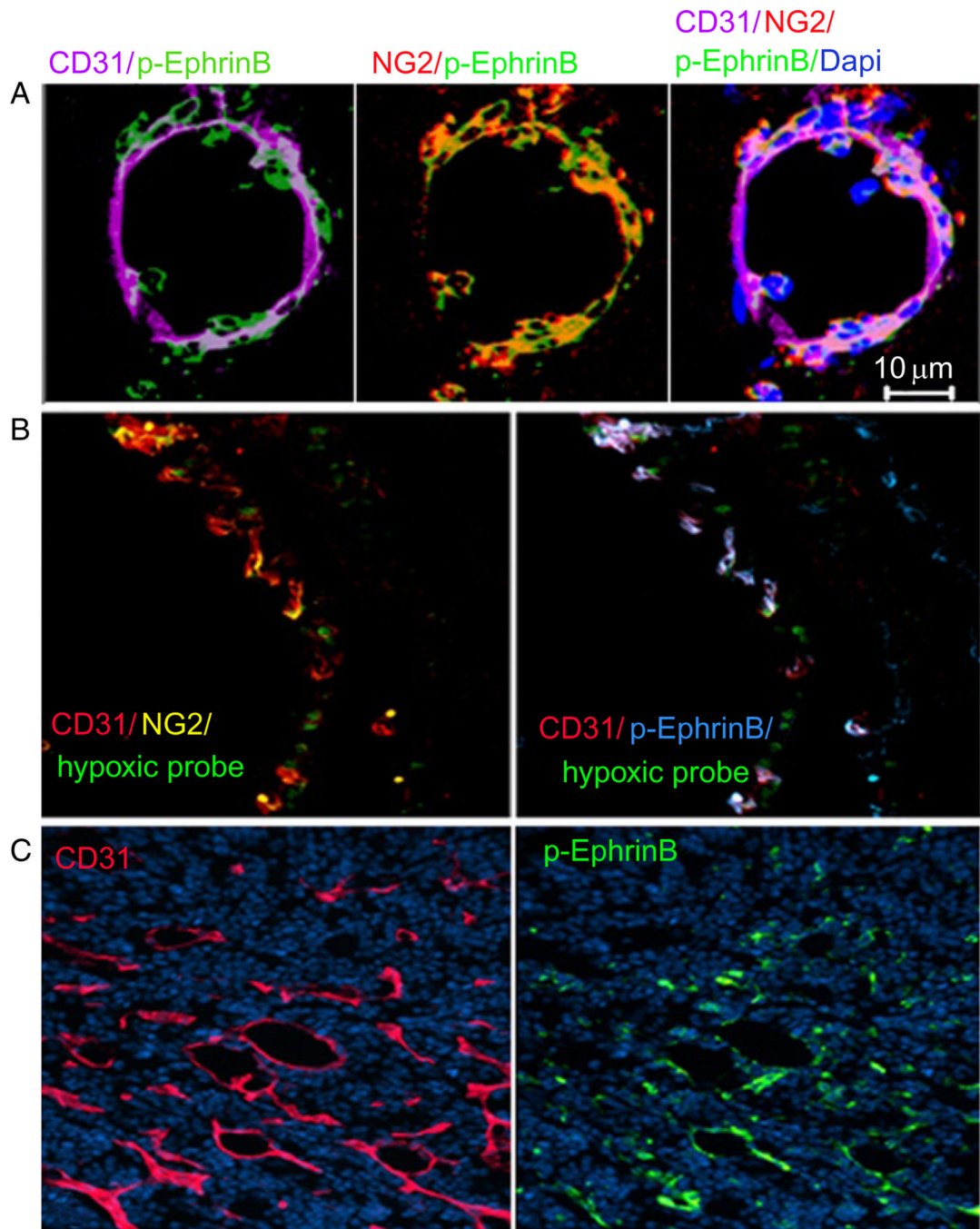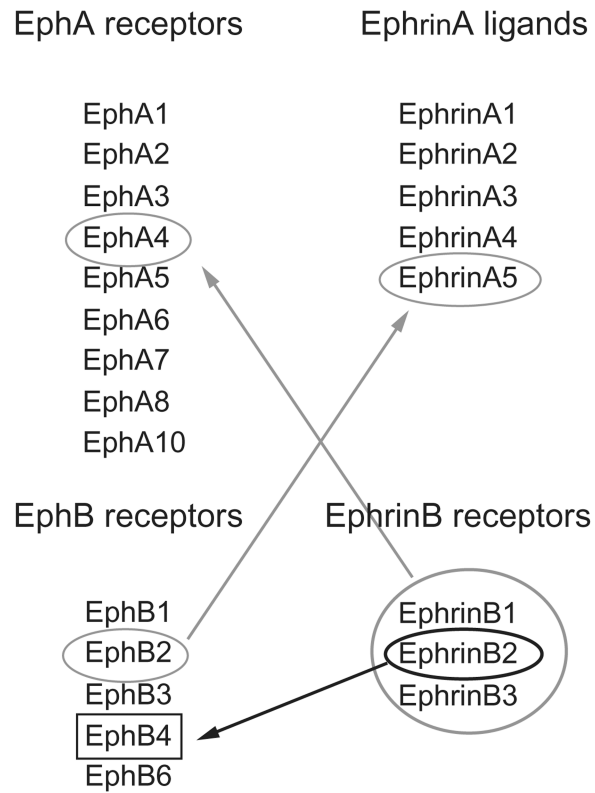prevents their assembly with MSC (green).

Fig. 9.
(A) EphrinB is phosphorylated in angiogenic vessels associated with skin wound healing.
(B) EphrinB is phosphorylated in angiogenic retinal vessels induced by hypoxia (areas of hypoxia visualized in green; ROP model). (C) EphrinB is phosphorylated in angiogenic tumor vessels. CD31 identifies endothelial cells; NG2 pericytes/mural cells.

Table I

Listing of EphA, EphrinA, EphB, and EphrinB Proteins

EphA receptors   EphrinA ligands

EphA1   EphrinA1
EphA2   EphrinA2
EphA3   EphrinA3
EphA4   EphrinA4
EphA5   EphrinA5
EphA6
EphA7
EphA8
EphA10

EphB receptors   EphrinB receptors

EphB1   EphrinB1
EphB2   EphrinB2
EphB3   EphrinB3
EphB4
EphB6

Although EphrinA ligands generally bind to EphA receptors, there are exceptions noted by the red arrows. Although EphrinB ligands generally bind all EphB receptors, EphrinB2 essentially binds only to EphB4.