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Eph receptors and ephrin ligands: embryogenesis to tumorigenesis

Vincent C Dodelet[1] and Elena B Pasquale*,[1]

[1]*The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, California, CA 92037, USA*

Protein tyrosine kinase genes are the largest family of oncogenes. This is not surprising since tyrosine kinases are important components of signal transduction pathways that control cell shape, proliferation, differentiation, and migration. At 14 distinct members, the Eph kinases constitute the largest family of receptor tyrosine kinases. Although they have been most intensively studied for their roles in embryonic development, increasing evidence also implicates Eph family proteins in cancer. This review will address the recent progress in understanding the function of Eph receptors in normal development and how disregulation of these functions could promote tumorigenesis. *Oncogene* (2000) **19,** 5614–5619.

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Introduction

The Eph receptors have been divided into two groups based on sequence homology: EphA and EphB receptors (Eph-Nomenclature-Committee, 1997; Pasquale, 1997). The ligands for the Eph receptors are the ephrins, which share the distinctive property of being membrane-bound. The five ephrins of the A subclass are linked to the membrane through a GPI linkage, while the three ephrins of the B subclass are transmembrane proteins. Although the ephrins bind promiscuously to Eph receptors, some specificity has been demonstrated (Gale *et al*., 1996). The ligands of the ephrin-A subclass bind preferentially to the EphA receptors whereas the ligands of the B subclass bind preferentially to the B receptors.

The Eph receptors have a ligand-binding domain at the amino terminus (Labrador *et al*., 1997). This globular domain is followed by a central cysteine-rich region, and two fibronectin type III repeats near the membrane-spanning segment. The cytoplasmic region of the Eph receptors contains a conserved kinase domain flanked by less conserved juxtamembrane region and C-terminal tail (Pasquale, 1997) (Figure 1).

Bidirectional signaling

An intriguing feature of the Eph receptors is their involvement in bidirectional signaling (Bruckner *et al*., 1997; Holland *et al*., 1996). Recent evidence has shown that signaling pathways can be propagated not only downstream of the Eph receptors but also downstream of the ephrin ligands.

*Correspondence: EB Pasquale

The Eph receptors become phosphorylated at specific tyrosine residues in the cytoplasmic domain following ligand binding (Figure 1). Phosphorylated motifs serve as sites of interaction with certain cytoplasmic signaling proteins such as non-receptor tyrosine kinases of the Src and Abl families and the non-receptor phosphotyrosine phosphatase LMW–PTP (low molecular weight phosphotyrosine phosphatase); the enzymes phospholipase C γ, phosphatidylinositol 3-kinase, and Ras GTPase activating protein; the adaptors SLAP, Grb2, Grb10, and Nck; and SHEP1, a R-Ras- and Rap-1A-binding protein (reviewed by Bruckner and Klein, 1998; Dodelet *et al*., 1999; Flanagan and Vanderhaeghen, 1998; Kalo and Pasquale, 1999). Furthermore, through their C terminus the Eph receptors associate with PDZ (*p*ostsynaptic density protein, *d*isc large, *z*ona occludens) domain-containing proteins such as AF6, a candidate effector for Rap1A (Linnemann *et al*., 1999), Pick1, a protein kinase C-interacting protein, Syntenin, a syndecan-interacting protein, and Grip1 and Grip2, two glutamate receptor interacting proteins (Buchert *et al*., 1999; Hock *et al*., 1998; Torres *et al*., 1998). Many of these proteins, which are candidates for mediating the activities of the Eph receptors, have been implicated in regulating cell morphology, attachment and motility.

The transmembrane ephrin-B ligands also become phosphorylated on tyrosine residues following receptor binding (Bruckner *et al*., 1997; Holland *et al*., 1996). The identity of the ephrin-B phosphorylation sites and their functional significance are unknown. However, such phosphorylation suggests ephrin-B association with a tyrosine kinase. This association may explain how the ephrin-B ligands, which lack enzymatic activity, are able to transduce signals. Ephrin-B ligands have also been shown to interact through their C terminus with several PDZ domain-containing proteins such as Syntenin, Grip, Pick1, Phip and the phosphotyrosine phosphatase FAP-1 (Lin *et al*., 1999; Torres *et al*., 1998) (Figure 1). These proteins may also contribute to ephrin-B signaling pathways. Finally, recent evidence suggests that the ephrin-A ligands can also signal, even though they lack a cytoplasmic region (Davy *et al*., 1999).

Although some of the proteins involved in Eph receptor and ephrin signal transduction have been identified, the exact mechanisms at play are still not well understood.

Functions of Eph receptors and ephrin ligands

Activation of Eph receptors does not appear to have a major effect on cell proliferation. Rather, Eph receptors in conjunction with their ephrin ligands have been shown to control the directional movement of

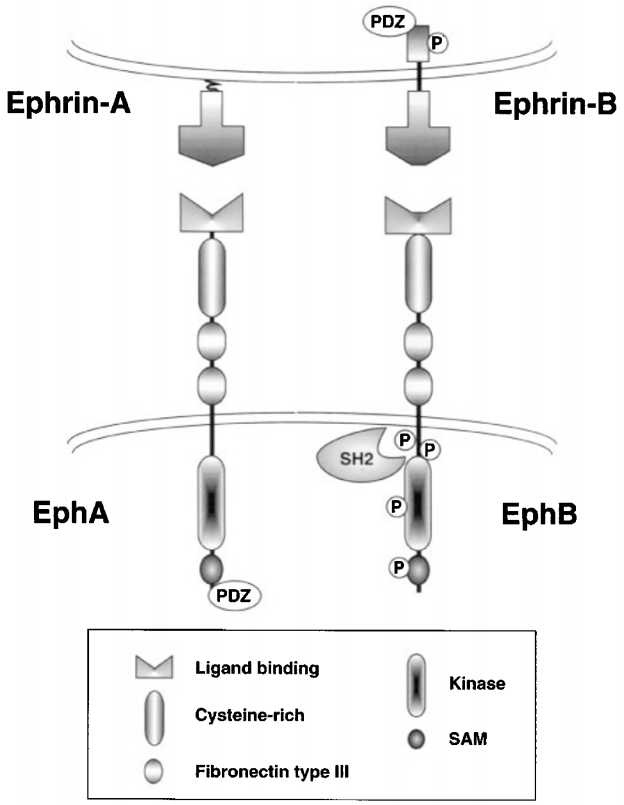

Figure 1 Interactions and signal transduction of Eph receptors and ephrins. Upon cell-cell contact Eph receptors and ephrins engage in a class specific manner, with GPI-linked ephrin-A ligands binding to EphA receptors and transmembrane ephrin-B ligands binding to EphB receptors. Binding of ephrins leads to receptor clustering, which in turn leads to receptor activation and subsequent autophosphorylation of multiple tyrosine residues (shown for EphB). These phosphorylated tyrosine residues provide docking sites for SH2 domain-containing signaling proteins. Upon receptor binding, ephrin-B ligands also become phosphorylated on tyrosine, presumably via an unidentified associated tyrosine kinase. It is unknown at present whether SH2 domain-containing proteins also dock to phosphorylated ephrin-B ligands. Signaling proteins containing PDZ domains dock to the carboxy terminus of the Eph receptors (shown for EphA) and the ephrin-B ligands

cells and neuronal growth cones and the establishment of the embryonic body plan, likely by regulating cytoskeletal organization and cell adhesion (Bruckner *et al*., 1997; Drescher, 1997; Flanagan and Vanderhaeghen, 1998; Pasquale, 1997). Eph activities are important in neurons and other types of cells, such as neural crest cells and endothelial cells. The functions and expression patterns of Eph family proteins suggest that they also play a role in cancer progression and metastasis.

Cell sorting

In many developing tissues, areas where Eph receptors are expressed confine with areas where ephrin ligands are expressed (Flenniken *et al*., 1996; Gale *et al*., 1996). Consistent with these expression patterns, the Eph receptor-ligand system restricts intermingling between adjacent populations of cells (Mellitzer *et al*., 1999). Eph family proteins contribute to segmental patterning of the hindbrain, because they prevent the intermixing of cells from adjacent segments (rhombomeres) and from adjacent streams of neural crest cells (Smith *et al*., 1997; Xu *et al*., 1995, 1999). Furthermore, the lamina-specific and area-specific patterns of expression of EphA proteins in the embryonic primate cerebral cortex suggest a role in early specification of presumptive functional domains (Donoghue and Rakic, 1999). A notable example of cell segregation mediated by Eph proteins outside the nervous system is that of EphB4, a receptor expressed in endothelial cells of embryonic veins, and ephrin-B2, a ligand for EphB4 that is expressed in endothelial cells of arteries (Adams *et al*., 1999; Gerety *et al*., 1999; Wang *et al*., 1998).

Axon guidance

A classical example of Eph receptor-mediated axon guidance is the specification of retinotectal topography. Temporal retinal axons, which have high EphA3 expression, grow to the anterior tectum, where expression of its ligands, ephrin-A2 and ephrin-A5, is low (Cheng and Flanagan, 1994; Drescher *et al*., 1995). *In vitro* experiments have confirmed that both of these ligands behave as repulsive guidance molecules for axons that express their receptors (Cheng and Flanagan, 1994; Drescher *et al*., 1995; Monschau *et al*., 1997). Furthermore, changing the distribution of ephrin-A2 *in vivo* in the chicken optic tectum by retroviral expression caused temporal axons to follow aberrant trajectories that avoided ectopic areas of high ligand expression (Nakamoto *et al*., 1996). Gene knock out experiments confirmed that the repulsive activities of the ephrin-A2 and ephrin-A5 ligands are required *in vivo* for the proper guidance and mapping of retinal axons in the mammalian midbrain (Feldheim *et al*., 1998; Frisen *et al*., 1998). Examples of Eph receptor-mediated axon guidance, however, are not confined to the retinotectal/retinocollicular projection. Disruption of the ephrin-A5 gene, for example, caused a distortion of the body map in the neocortical somatosensory area (Vanderhaeghen *et al*., 2000) and loss of topographic precision in a sensory projection to the forebrain (Feldheim *et al*., 1998). Furthermore, inactivation of the EphA4 gene caused major pathfinding defects in the corticospinal tract (Dottori *et al*., 1998), inactivation of the EphB3 receptor gene affected the pathfinding of the axons in the corpus callosum (Orioli *et al*., 1996), and inactivation of the EphB2 gene caused inappropriate pathway selection at the midline in commissural axons that innervate the ear (Cowan *et al*., 2000). EphB receptors may also play a role in pathfinding of longitudinally projecting spinal cord commissural axons (Imondi *et al*., 2000).

Gene knock out experiments suggest that ephrin-B ligands can also transduce signals in the axons in which they are expressed (Birgbauer *et al*., 2000; Henkemeyer *et al*., 1996). For example, signals transduced by ephrin-B ligands upon interaction with EphB receptor ectodomains appear to guide the growth of axons forming the brain anterior commissure and, possibly, retinal ganglion cell axons. In fact, these axons project abnormally in mice lacking certain EphB genes, but the guidance errors are ameliorated by expression of a truncated form of EphB2 lacking the kinase domain and containing the ectodomain. Thus, the intrinsic signaling function of EphB2 appears to be dispensable

for guidance of these axons while the ectodomain plays an essential role, presumably by activating ephrin-B ligand signaling pathways.

Functions in synapses

Eph family proteins may be involved not only in guiding axons to their targets, but also in the development of initial neuronal contacts into synapses and in synapse maintenance. The recently described localization of Eph receptors and ephrins in neuronal synapses (Buchert *et al*., 1999; Torres *et al*., 1998) suggests previously unexpected roles for these proteins. They may activate bidirectional signaling pathways in synapses, and contribute to synaptic plasticity by destabilizing synapses. Alternatively, the interaction between the EphB2 and ephrin-B1 ectodomains may play adhesive roles. Synaptic localization of EphB2 and ephrin-B1 likely depends on their binding to proteins containing PDZ domains that are concentrated at neuronal synapses (Buchert *et al*., 1999; Craven and Bredt, 1998; Torres *et al*., 1998).

Angiogenesis

Recent evidence suggests that the Eph receptors are one of the families of receptor tyrosine kinases that, in conjunction with their ligands, control blood vessel formation. Ephrin-A1, the first ligand for an Eph receptor to be identified, was cloned from human umbilical vein endothelial cells (HUVECs) as a gene induced by tumor necrosis factor α (TNFα) (Holzman *et al*., 1990). Ephrin-A1 transcripts were also detected in embryonic endothelial cells during early organogenesis but not in adult tissues (Flenniken *et al*., 1996; McBride and Ruiz, 1998; Takahashi and Ikeda, 1995), suggesting a physiological role for this ligand in vascular morphogenesis. An angiogenic activity for ephrin-A1 was demonstrated *in vivo*, in a rat corneal angiogenesis assay (Pandey *et al*., 1995b). In this assay, ephrin-A1 specifically mediated the angiogenic effects of TNFα, but not FGF, suggesting that induction of ephrin-A1 and subsequent activation of its receptor, EphA2, could be responsible for the angiogenic effects of TNFα. Ephrin-A1 does not cause endothelial cell proliferation, but acts as a chemoattractant for endothelial cells.

Three other Eph receptors (EphB2, EphB3 and EphB4) and their ligands (ephrin-B1 and ephrin-B2) have been recently implicated in the formation of the circulatory system of the mouse embryo (Adams *et al*., 1999; Gerety *et al*., 1999; Wang *et al*., 1998). Ephrin-B2 is expressed in arterial endothelial cells, whereas EphB4 has complementary expression and is confined to venous endothelial cells. EphB4 and ephrin-B2 may, therefore, define boundaries between arterial and venous endothelial cells. However, cell-cell interactions between Eph receptors and ephrins are not restricted to the borders between arteries and veins: ephrin-B1 is widely expressed in both arterial and venous endothelial cells and EphB3 is expressed in veins and some arteries. Finally, expression of ephrin-B2 and EphB2 in mesenchyme adjacent to vessels suggest that Eph family proteins also mediate interactions between mesenchymal cells and endothelial cells. Indeed, mice lacking ephrin-B2, and a proportion of double mutants deficient in EphB2 and EphB3, exhibit severe defects in the remodeling of the embryonic vascular system.

Eph receptor and ephrin ligand expression in cancer

The first receptor of this gene family, EphA1, was originally isolated from an **e**rythropoietin-**p**roducing **h**epatoma cell line (Hirai *et al*., 1987). Carcinomas of breast, liver, lung, and colon showed elevated expression of this receptor, despite the absence of gene amplification or rearrangement (Hirai *et al*., 1987; Maru *et al*., 1988). Additionally, overexpression of EphA1 in NIH3T3 cells resulted in the formation of foci in soft agar and tumors in nude mice (Maru *et al*., 1990).

The expression levels of several other receptors of both the A and B class were also observed to be elevated in various tumor types. EphA2 was found to be overexpressed in nearly all melanoma cell lines tested, whereas it was not detected in normal melanocytes (Easty *et al*., 1995). Interestingly, EphA2 expression levels were significantly higher in cell lines from distant metastases than in primary melanomas. EphA3 was identified as an antigen present on the surface of a pre-B cell leukemic cell line and was also found to be overexpressed in the absence of gene amplification or rearrangements in some hemopoietic tumors and several lymphoid tumor cell lines (Wicks *et al*., 1992). EphB2 was found to be overexpressed in about one third of 31 human tumor cell lines examined, in 75% of the gastric tumors examined, and in some esophageal and colon cancers (Kiyokawa *et al*., 1994). EphB3 was detected in tumor cell lines of breast and epidermoid origins (Bohme *et al*., 1993).

Analysis of human breast carcinoma tissue by *in situ* hybridization showed that expression of EphB4 is elevated in primary infiltrating ductal breast carcinomas with a high grade of malignancy (Berclaz *et al*., 1996). Particularly strong expression was observed in grade III carcinomas, in the outer layer of the tumor cell mass, which are the layers most likely to release tumor cells that may give rise to metastases. In transgenic mouse models of mammary carcinogenesis, EphB4 and EphA2 were detected in the undifferentiated and invasive mammary tumors of mice expressing the H-Ras oncogene, but not in the well-differentiated and non-metastatic mammary tumors of c-Myc expressing mice (Andres *et al*., 1994). Progression of the malignant process in the H-Ras tumors correlated with the transition of EphB4 expression from myoepithelial cells to the anaplastic tumor cells of epithelial origin (Nikolova *et al*., 1998). Expression of EphB4 mRNA was also detected in all human breast carcinoma cell lines examined, including MCF7, MDA-MB-231, SKBR, T47D, BT20 and BT474 (Bennett *et al*., 1994; Berclaz *et al*., 1996).

It therefore seems that a positive correlation is emerging between the level of expression of Eph receptors in tumor tissue and the observed degree of malignancy. As for the ephrin ligands, too little data exists to establish if such a correlation exists. Indeed, the expression patterns of ephrins in tumors have only begun to be characterized, and it is clear that additional studies are necessary. Upregulation of ephrin-A1 and ephrin-B2 has been reported in

melanomas (Easty *et al*., 1999; Vogt *et al*., 1998), but in a mouse mammary tumor model, ephrin-B2 expression, which is normally restricted to the luminal epithelial cells, was lost at the onset of tumorigenesis (Nikolova *et al*., 1998). This last observation may be of great interest since transmembrane ephrin B ligands have been shown to inhibit the transforming activity of oncogenic tyrosine kinases (Bruckner *et al*., 1997).

Mechanisms of tumorigenesis

Molecular control of integrin activity – enhanced cell motility, invasion and metastasis

In spite of this wealth of information correlating cell transformation with increased expression of the Eph receptors, the molecular mechanisms underlying the specific roles of the Eph family in tumor formation, progression, and metastasis have only started to be understood. Recent work has highlighted the ability of Eph receptors to affect cell-matrix attachment by modulating integrin activity. Activation of endogenous EphA2 in PC-3 prostate carcinoma cells with ephrin-A1 ligand was shown to induce transient inhibition of integrin-mediated cell adhesion (Miao *et al*., 2000). Rapid recruitment of the protein tyrosine phosphatase SHP2 to activated EphA2 lead to dephosphorylation and thus inactivation of focal adhesion kinase (FAK). Zou et al. demonstrated that the small GTPase R-Ras, which regulates integrin binding (Zhang *et al*., 1996), can be tyrosine phosphorylated by EphB2 (Zou *et al*., 1999). This phosphorylation most probably leads to an inability of R-Ras to interact with downstream effectors, and ultimately to decreased integrin activation and cell adhesion.

However, the density of ephrin ligand may ultimately determine the effects of Eph signaling on integrin-mediated adhesion. In an attempt to understand the mechanism whereby Eph receptors guide cell migration in response to ligand gradients, Huynh-Do *et al*. (1999) plated cells expressing EphB1 on surfaces coated with defined extracellular matrix components and varying densities of ephrin-B1 ligand. Lower densities of ligand stimulated integrin-mediated attachment in an Eph receptor dependent fashion, whereas higher densities did not, despite similar levels of activation of the receptor, as evidenced by the level of tyrosine phosphorylation (Huynh-Do *et al*., 1999). Interestingly, the oligomeric state of activating ligand seems to determine the nature of the signaling complex that is recruited to the receptor (Stein *et al*., 1998). Thus it is probable that, *in vivo*, cell contact induced Eph receptor activation may influence directional cell motility by modulating integrin function either positively or negatively, dependent on the ratio of receptor to ligand densities. Overexpression of Eph receptors may therefore impart tumors with increased sensitivity to ligand-induced stimulation, favoring decreased cell adhesion, increased cell motility, and a concomitantly higher degree of tissue invasiveness. Alternatively, the presence of ligand may be unnecessary since overexpression of Eph receptors can in and of itself promote receptor activation (Zisch *et al*., 1997), and may thus be sufficient to promote these effects. In fact, given the anti-oncogenic effects observed for the ephrin-B ligands and the ligand-independent activation potential of the Eph receptors, conditions in tumor tissue where ligand expression is low and receptor expression is high may be the most favorable for tumor growth and metastasis.

Eph receptors may also influence cell-cell attachment by interacting with certain cell adhesion molecules. Loss of E-cadherin, the main cell adhesion molecule of early embryonic and adult epithelial cells, has been shown to affect the expression and subcellular localization of several Eph receptors and ephrins (Orsulic and Kemler, 2000; Zantek *et al*., 1999). Reciprocally, cadherin function may be regulated by Eph receptors and ephrins, since ectopic expression of ephrin-B1 or activated EphA4 in early Xenopus embryos disrupted cadherin dependent cell adhesion (Jones *et al*., 1998; Winning *et al*., 1996). The molecular mechanisms involved in this cross-regulation are still undetermined. However, because tissue disorganization and abnormal cell adhesion are hallmarks of the more advanced stages of cancer, the inappropriate expression and regulation of an Eph receptor would be expected to increase tumor metastatic potential.

Modulation of cell adhesion is only one aspect, albeit a crucial one, of cell motility. The general process of cellular guidance in which the Eph receptors have been implicated also involves reorganization of cytoskeletal elements to allow for changes in cell morphology during movement. The small GTPases of the Rac/Rho family are prime candidates for mediating these effects, and a link between the Eph receptors and these GTPases has recently been established. Stimulation of retinal ganglion cells by ephrin-A5 was shown to induce growth cone collapse in a Rho dependent manner (Wahl *et al*., 2000). Inhibition of Rho or of the downstream Rho effector, Rho kinase (ROCK), dramatically reduced ephrin-A5 induced collapse and even allowed for the continuing advance of growth cones in the presence of ligand. Since Rho proteins are known regulators of the actin cytoskeleton (reviewed in Mackay and Hall, 1998; Zohn *et al*., 1998) and have been shown, *in vitro*, to be essential for cellular invasion (Banyard *et al*., 2000), their activation by Eph receptors may very well contribute to increased tumor invasion and metastasis.

Promotion of angiogenesis – enhanced tumor growth

Although it is now clear that the Eph receptors and ephrins play a significant role in the formation of the embryonic vasculature, their precise contribution to pathogenic neovascularization is not well understood. However, the existing data does allow us to put forth a few interesting hypotheses. Since ephrins have been shown, in *in vitro* experiments, to promote the organization and assembly of endothelial cells into capillary-like structures and to stimulate sprouting from existing vessels (Daniel *et al*., 1996; Pandey *et al*., 1995a), it would seem likely that these molecules may be involved in similar processes during tumor growth. Proliferation of the tumor mass is dependent on an adequate blood supply, and many tumors secrete angiopoietic factors for new vessel recruitment (Folkman, 1995; Hanahan *et al*., 1996; Yancopoulos *et al*., 1998). A complex interplay could occur between Eph receptors/ephrins expressed by tumor cells and en-

dothelial cells. For example, secreted ephrins may function as diffusible chemoattractants for endothelial cells (as has been reported for ephrin-A1 (Pandey *et al*., 1995a)), and tumor expressed Eph receptors may act as contact-dependent organizing molecules to appropriately guide the incoming vessels.

Many other possibilities exist but, even if function remains elusive, the fact that blood vessels and tumor cells express Eph receptors and ephrins makes these molecules stand out as attractive candidates as prognostic tumor markers and targets for therapeutic intervention in cancer.

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