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Leading Edge
Review

Eph-Ephrin Bidirectional Signaling in Physiology and Disease

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Receptor tyrosine kinases of the Eph family bind to cell surface-associated ephrin ligands on neighboring cells. The ensuing bidirectional signals have emerged as a major form of contact-dependent communication between cells. New findings reveal that Eph receptors and ephrins coordinate not only developmental processes but also the normal physiology and homeostasis of many adult organs. Imbalance of Eph/ephrin function may therefore contribute to a variety of diseases. The challenge now is to better understand the complex and seemingly paradoxical signaling mechanisms of Eph receptors and ephrins, which will enable effective strategies to target these proteins in the treatment of diseases such as diabetes and cancer.

Eph-Ephrin Bidirectional Signaling

Since its discovery two decades ago, the Eph family of receptor tyrosine kinases has been implicated in an increasing number of physiological and pathological processes in many cell types and different organs. Therefore, elucidating the mechanism of action of the Eph receptors and their signaling networks is important for understanding developmental processes, the physiology of adult organs and, as is becoming increasingly evident, the pathogenesis of many diseases. Eph receptors have diverse activities, including widespread effects on the actin cytoskeleton, cell-substrate adhesion, intercellular junctions, cell shape, and cell movement (Egea and Klein, 2007; Himanen et al., 2007; Pasquale, 2005). In addition, effects on cell proliferation, survival, differentiation, and secretion have also been described. These activities depend on the interaction of the Eph receptors with the ephrins (*Eph* r*eceptor in*teracting proteins). In the human genome, there are nine EphA receptors that bind to five GPI-linked ephrin-A ligands and five EphB receptors that bind to three transmembrane ephrin-B ligands. Interactions are promiscuous within each class, and some Eph receptors can also bind to ephrins of the other class.

Several of the domains in the Eph receptor extracellular region can bind to the ephrins. The amino-terminal "ephrin-binding" domain contains a high-affinity binding site that mediates receptor-ephrin interaction between cells (Figure 1) (Himanen et al., 2007; Wimmer-Kleikamp and Lackmann, 2005). Two additional lower-affinity ephrin-binding sites have also been identified in the ephrin-binding domain and the cysteine-rich region, which are thought to facilitate clustering of multiple Eph-ephrin complexes. The Eph fibronectin type III domain closer to the membrane can also bind to ephrins, if they are located on the same cell surface.

Downstream Signaling

A distinctive feature of Eph-ephrin complexes is their ability to generate bidirectional signals that affect both the receptor-expressing and ephrin-expressing cells (Pasquale, 2005). Eph receptor "forward" signaling depends on the tyrosine kinase domain, which mediates autophosphorylation as well as phosphorylation of other proteins, and on the associations of the receptor with various effector proteins. Ephrin-B "reverse" signaling also depends in part on tyrosine phosphorylation of the ephrin cytoplasmic region (mediated by Src family kinases and some receptor tyrosine kinases) and on associated proteins. Most Eph receptors and the B-type ephrins also have a carboxy-terminal PDZ domain-binding site, which is particularly important for the physiological functions of ephrin-B (Egea and Klein, 2007). The mechanisms of reverse signaling for ephrin-A are less understood, but these GPI-linked ephrins probably use associated transmembrane proteins to fulfill their signaling function. Several candidates have been reported at meetings, including the p75 low-affinity nerve growth factor receptor (T.R. McLaughlin et al., 2007, Soc. Neurosci., abstract).

Eph receptors and ephrins use some common signaling effectors, such as Src family kinases and Ras/Rho family GTPases, which are particularly important for the organization of the actin cytoskeleton and cell adhesion (Figure 1). Some signaling connections may apply only to a particular Eph class, including those between EphA receptors and the Rho exchange factor Ephexin or between EphB receptors and the exchange factors Intersectin and Kalirin. Others are more selective. For example, the lipid phosphatase Ship2 was found to interact only with EphA2, and the GTPase-activating proteins SPAR/E6TP1 interacted only with EphA4 and EphA6 among several EphA and EphB receptors examined (Richter et al., 2007; Zhuang et al., 2007).

An emerging theme is that Eph receptors and ephrins activate complex bidirectional signaling networks that often include signaling pathways with opposite effects (Figure 1). This may explain why differences in cellular context can dramatically alter the outcome of Eph/ephrin stimulation. Furthermore, the degree of Eph/ephrin clustering may not only affect signal strength but may also differentially regulate

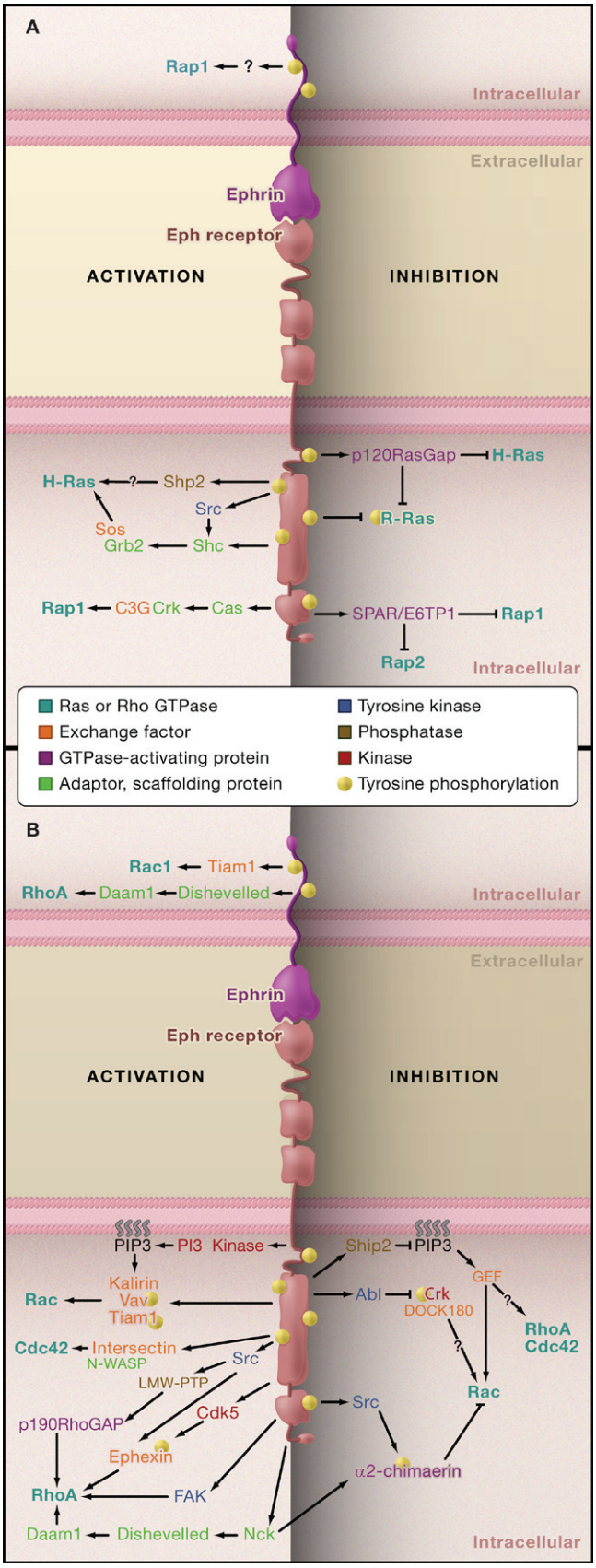

Figure 1. Eph Receptor-Ephrin Bidirectional Pathways Regulate GTPases

(A) Regulation of Ras GTPases. (B) Regulation of Rho GTPases. The domain structure of an Eph receptor is shown schematically, including from the N terminus: ephrin-binding domain, cystein-rich region, two fibronectin type III domains, transmembrane segment, juxtamembrane domain, kinase domain, SAM domain, and PDZ domain-binding site. The domain structure of an ephrin-B ligand is also shown, including the Eph-binding domain, linker region, transmembrane segment, cytoplasmic region, and PDZ domain-binding site. The pathways shown have been characterized with one or several Eph receptors/ephrins. For example, in (A) Shp2 has been linked to EphA2; Shc-Grb2 to EphA2 and EphB1; Cas-Rap1 to EphB1; and SPAR/E6TP1 to EphA4 and EphA6. In (B), α2-chimaerin has been linked to EphA4; FAK to EphA2 and EphB2; Ship2 to EphA2; Abl-Crk to EphB4; Ephexin family members to EphA receptors; and Kalirin, Tiam1, and Intersectin to EphB receptors. Tyrosine phosphorylation is shown only for some effectors where it has a demonstrated role in Eph-ephrin bidirectional signaling. The location of the arrows does not imply the involvement of a particular Eph or ephrin domain. The relative activation of different pathways and their effects on cell behavior may depend on the ephrin levels, degree of receptor clustering, and cellular context. The question marks indicate signaling connections that have not been conclusively demonstrated downstream of Eph receptors or ephrins. PIP3, phosphatidylinositol (3,4,5) phosphate; GEF, guanine nucleotide exchange factor; LMW-PTP, low-molecular-weight phosphotyrosine phosphatase.

downstream pathways thus leading to variable outcomes (Pasquale, 2005; Poliakov et al., 2004). Further increasing versatility, forward and reverse signaling can also be independently regulated, for example through Eph receptor dephosphorylation (Konstantinova et al., 2007). In addition, interactions between Eph receptors and ephrins located on the same cell surface appear to represent a mechanism for silencing bidirectional signaling, although it is unclear under what circumstances Eph receptors and ephrins intermingle rather than segregate in different microdomains of the plasma membrane (Egea and Klein, 2007).

Processing of Eph-Ephrin Complexes

A well-characterized effect of Eph forward signaling is retraction of the cell periphery following contact with ephrin-expressing cells (Pasquale, 2005). This repulsive response is particularly important for axon guidance and sorting of Eph-expressing cells from ephrin-expressing cells during development. Several mechanisms can explain how the initial adhesive contact evolves into cell separation. One is removal of the adhesive Eph-ephrin complexes from the cell surface by endocytosis of vesicles containing plasma membrane fragments derived from both cells (Egea and Klein, 2007). An implication of this unusual mechanism is that the two cells exchange Eph receptors or ephrins and possibly their associated proteins, which may continue to signal from intracellular compartments. Another way to convert cell adhesion into repulsion is proteolytic cleavage (Egea and Klein, 2007; Himanen et al., 2007). Studies have shown that metalloproteases and other proteases can cleave the extracellular portions of EphB receptors and ephrins. The remaining membrane-anchored fragments are further cleaved by γ-secretase, followed by proteasomal degradation.

Proteolytic cleavage not only terminates the adhesive Eph-ephrin interaction and causes downregulation of the proteins, but it can also generate Eph/ephrin fragments with new activities. For example, the ephrin-B cytoplasmic pep-

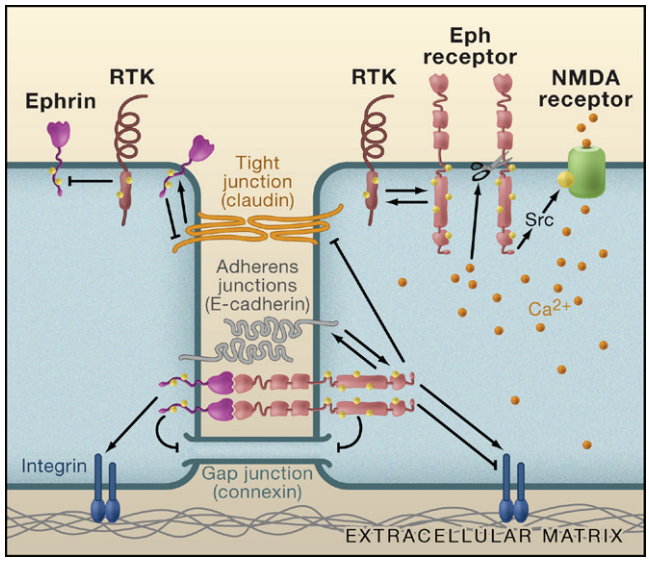

Figure 2. Crosstalk between Eph-Ephrins and Other Receptors
Some forms of crosstalk occur at epithelial cell junctions, others have been reported in neurons and other cell types. RTK, receptor tyrosine kinase; yellow circles, tyrosine phosphorylation; the scissors indicate proteolytic cleavage.

tide released by γ-secretase activates the tyrosine kinase Src, which in turn phosphorylates the cytoplasmic domain of intact B-type ephrins and perhaps other substrates (Egea and Klein, 2007). Furthermore, the soluble Eph and ephrin extracellular portions released by metalloproteases might reach distant cells and trigger effects that are independent of cell-cell contact. They could, for example, function as monomeric inhibitors of bidirectional signaling. Alternatively, soluble A-type ephrins oligomerized by transglutamination may serve to activate EphA receptors at a distance (Alford et al., 2007).

Crosstalk with Other Signaling Systems

Although bidirectional signaling is their best characterized modus operandi, Eph receptor and ephrins may also function independently of each other and/or in concert with other cell-surface communication systems (Figure 2). For example, recent studies have proposed that members of the epidermal growth factor (EGF) receptor family can coopt EphA2 as an effector to promote cell motility and proliferation, perhaps independently of ephrin stimulation (Brantley-Sieders et al., 2008; Larsen et al., 2007). Other studies have shown association and synergistic responses of fibroblast growth factor (FGF) receptors and EphA4, and that phosphorylation by FGF receptors inhibits ephrin-B1 activities (Arvanitis and Davy, 2008). Intricate links between EphB/ephrin-B and Wnt signaling have also been revealed in different model systems. EphB receptors and Ryk, a Wnt receptor containing an inactive tyrosine kinase domain, can physically associate and likely function together in craniofacial development and axon guidance (Arvanitis and Davy, 2008; Schmitt et al., 2006). Furthermore, both EphB receptors and B-type ephrins can signal through components of the noncanonical Wnt pathway (Figure 1B) (Kida et al., 2007; Lee et al., 2006). This pathway in turn causes endocytic removal of EphB receptors from the cell surface, whereas canonical Wnt signaling upregulates EphB transcripts and downregulates ephrin-B transcripts (Clevers and Batlle, 2006; Kida et al., 2007).

E-cadherin-dependent intercellular adhesion can also regulate Eph receptor expression, cell-surface localization, and ephrin-dependent activation (Arvanitis and Davy, 2008; Ireton and Chen, 2005). The regulation is reciprocal, and EphB signaling drives E-cadherin to the cell surface thus promoting the formation of epithelial adherens junctions and enabling EphB/ephrin-B-dependent cell sorting. Conversely, inhibiting EphB-ephrin-B binding was found to disturb adherens junctions (Cortina et al., 2007; Noren and Pasquale, 2007). EphA2 overexpression, on the other hand, has been shown to destabilize adherens junctions through a pathway involving Src, the low-molecular-weight phosphotyrosine phosphatase, and p190RhoGAP, resulting in increased RhoA activity (Figure 1B) (Fang et al., 2008). The Eph system also affects integrin-mediated cell communication with the extracellular environment (Bourgin et al., 2007; Pasquale, 2005; Wimmer-Kleikamp and Lackmann, 2005).

Crosstalk of EphA2 or ephrin-B1 with claudins, which are components of epithelial tight junctions, has been implicated in the regulation of cell adhesion and intercellular permeability (Arvanitis and Davy, 2008). Some claudins can also cause ephrin-B1 tyrosine phosphorylation independently of EphB receptors. Gap junction proteins are also critical for Eph/ephrin function in cell sorting, insulin secretion, and osteogenic differentiation (Davy et al., 2006; Konstantinova et al., 2007; Poliakov et al., 2004).

Reciprocal communication also occurs between EphB receptors and calcium channels (Figure 2). Following ephrin binding, EphB2 associates with the NMDA receptors, which are calcium channels, and promotes clustering of these neurotransmitter receptors at synapses (Yamaguchi and Pasquale, 2004). Moreover, activation of Src family kinases downstream of EphB2 leads to NMDA receptor phosphorylation, which increases NMDA-dependent calcium influx. Interestingly, increased intracellular calcium in turn promotes proteolytic degradation of EphB2, demonstrating that Eph levels can be regulated by intracellular calcium independently of ephrin binding (Litterst et al., 2007).

More information on Eph signaling mechanisms and crosstalk with other signaling systems can be found in recent reviews (Arvanitis and Davy, 2008; Egea and Klein, 2007; Himanen et al., 2007; Noren and Pasquale, 2004; Pasquale, 2005; Poliakov et al., 2004).

Neural Development, Plasticity, and Regeneration

The activities of Eph receptors and ephrins in the nervous system have been extensively studied. Neurons form complex networks where electrical signals travel from axonal to dendritic processes through specialized junctions called synapses. Here, neurotransmitters released from the presynaptic terminal in response to electrical signals activate postsynaptic ion channel receptors that initiate new electrical and chemical signals in the postsynaptic neuron. The network of neuronal processes is embedded among surrounding glial cells, which regulate many properties of the neurons including their

ability to form synapses. Eph-ephrin bidirectional signaling is important not only for the communication between neurons but also for that between neurons and glial cells (Yamaguchi and Pasquale, 2004).

Development of Neuronal Connections

Eph receptors and ephrins are highly expressed in the developing nervous system, where they have well-known roles in the establishment of neuronal connectivity by guiding axons to the appropriate targets and regulating the formation of synaptic connections. The trajectories of many axonal projections depend on Eph receptors and ephrins distributed in gradients or forming boundaries (Luo and Flanagan, 2007; Pasquale, 2005; Poliakov et al., 2004). A number of Ras/Rho regulatory proteins have been implicated over the years in axon guidance by the Eph receptors, including several guanine nucleotide exchange factors for Rho GTPases (Figure 1B). Only recently four simultaneous studies have also implicated a GTPase-activating protein for Rac1, α2-chimaerin, as a critical EphA4 effector (Beg et al., 2007; Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007). Remarkably, α2-chimaerin mutant mice have defects in the formation of cortical and spinal motor circuits that phenocopy those in the EphA4 knockout mice, indicating that α2-chimaerin is essential for certain axon guidance decisions that depend on EphA4. Mice lacking the adaptor proteins Nck1 and Nck2 in the nervous system also exhibited similar defects, suggesting that Nck adaptors, which can bind both EphA4 and α2-chimaerin, may also play a role in the pathway (Fawcett et al., 2007; Wegmeyer et al., 2007).

In vitro and in vivo analyses of hippocampal and cortical neurons have revealed that the EphB receptors and B-type ephrins regulate multiple steps in the assembly and maturation of the pre- and postsynaptic sides of excitatory synapses. Interestingly, different Eph receptor domains can control different aspects of synaptogenesis. The EphB2 extracellular region, for example, is sufficient to promote the assembly of presynaptic structures even when expressed in non-neuronal cells (Kayser et al., 2006). This activity requires the ephrin-binding domain, suggesting a *trans*-synaptic interaction with axonal ephrins. This ability of EphB2 to promote presynaptic specializations, however, may vary in different brain regions because it was detected in cortical but not hippocampal neurons. Activation of ephrin-B reverse signaling by postsynaptic EphB2 has also been recently implicated in the morphological and functional maturation of developing retinotectal synapses in the *Xenopus* optic tectum (Lim et al., 2008). The EphB2 extracellular portion also associates with NMDA neurotransmitter receptors and promotes their clustering at synapses following ephrin-B stimulation (Dalva et al., 2007). Furthermore, EphB2 promotes AMPA neurotransmitter receptor clustering and endocytosis, and these activities respectively depend on the PDZ domain-binding site of EphB2 and its kinase activity.

Most excitatory synapses are located on small dendritic protrusions called dendritic spines, which compartmentalize the postsynaptic space from the dendritic shaft, but some are also located on the dendritic shaft (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). EphB receptors selectively promote the formation of the synapses located on spines and also play a critical role in spine maturation, which results in the characteristic mushroom shape determined by the actin cytoskeleton. Studies with cultured neurons have implicated several nucleotide exchange factors for Rho GTPases in EphB-dependent spine elaboration, including Kalirin, Intersectin, and Tiam1 (Figure 1B) (Tolias et al., 2007; Yamaguchi and Pasquale, 2004). It is not known whether these exchange factors function in different subsets of dendritic spines and whether there are differences in their effects on the spines.

Ephrin-B ligands are also found postsynaptically, and ephrin-B3 expressed in non-neuronal cells can drive the formation of presynaptic structures in cocultured neurons, presumably by interacting with axonal Eph receptors (Aoto et al., 2007). Interestingly, ephrin-B3 overexpression and knockdown using short-interfering RNAs (siRNAs) in cultured hippocampal neurons have shown that the excitatory synapses induced by ephrin-B3 are located on the dendritic shaft. Consistent with this, the ephrin-B3 knockout mice have fewer shaft synapses in hippocampal area CA1 than wild-type mice. The synaptogenic activity of ephrin-B3 depends on the scaffolding protein GRIP1, which may help ephrin-B3 clustering by interacting with its PDZ domain-binding site. Treatment of cultured hippocampal neurons with EphB2 Fc (a soluble form of the EphB2 extracellular region dimerized by fusion with the Fc portion of an antibody) has also been shown to promote synapse formation and dendritic spine maturation, presumably through ephrin-B1 and/or ephrin-B2 and a reverse signaling mechanism involving recruitment of the adaptors Grb4 and GIT1 (Segura et al., 2007).

It will be interesting to further investigate the involvement of the Eph system in process extension and synaptogenesis of the new neurons that continue to be generated in the hippocampus and the olfactory system throughout life (Chumley et al., 2007). In particular, the integration of newly generated neurons in the hippocampal circuitry seems to be important for the behavioral effects of antidepressants, an area where the involvement of Eph receptors has not yet been explored (Sahay and Hen, 2007).

Plasticity of Neuronal Circuits

Eph receptors and ephrins persist in the adult brain, particularly in regions where neuronal circuits continue to be remodeled in response to environmental changes (Yamaguchi and Pasquale, 2004). Indeed, studies with mutant mice have shown that the Eph system regulates the plasticity of neuronal connections in structures such as the hippocampus, where changes in synapse number and size are important for learning and memory. Although the synaptic localization of Eph receptors and ephrins has not been fully characterized, it is becoming apparent that it may differ depending on the brain region and even in different synapses from the same neuron (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). For example, as discussed above, in cortical neurons EphB2 is in spine synapses and ephrin-B3 seems to be in shaft synapses. B-type ephrins are presynaptic in area CA3 of the mouse hippocampus and the *Xenopus* optic tectum but postsynaptic in area CA1 of the hippocampus.

EphB receptors are also postsynaptic in area CA1, and it is unclear whether they are in the same dendritic spines as B-type ephrins or in mutually exclusive subpopulations of spines. To complicate matters further, EphA4, which is the Eph receptor most highly expressed in the adult hippocampus and can interact with all ephrins, has been detected by electron microscopy not only in spines but also in presynaptic terminals (Tremblay et al., 2007).

Electrophysiological measurements using hippocampal slices have demonstrated that the Eph system plays a role in paradigms of activity-dependent synaptic plasticity that model learning and memory (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). These include long-term potentiation (LTP), where high-frequency electrical stimulation increases synaptic strength; long-term depression (LTD), where low-frequency stimulation reduces synaptic strength; and depotentiation, where low-frequency stimulation reverses the effects of LTP. In an initial study, ephrin-A5 Fc treatment caused an LTP-like effect whereas EphA Fc inhibited LTP (Yamaguchi and Pasquale, 2004). The mechanisms underlying these effects, which likely depend on EphA4 and possibly other less abundant EphA receptors, remain unclear. EphA4 in the dendritic spines of hippocampal neurons has been implicated in communication with astrocytes, which express ephrin-A3 on their perisynaptic processes. EphA4 activation by ephrin has been recently shown to inhibit the Rap1 and Rap2 GTPases and integrin activity and to promote RhoA and PLCγ activity (Figure 1), causing spine retraction and synapse loss as well as changes in spine shape (Bourgin et al., 2007; Fu et al., 2007; Richter et al., 2007; Zhou et al., 2007). These effects of EphA4 forward signaling would be predicted to affect synaptic plasticity, perhaps enabling an influence of astrocytes on synaptic function.

Electrophysiological measurements have also shown reduced LTP and LTD at hippocampal synapses of area CA1 in EphB2 and EphA4 knockout mice, although basal synaptic transmission was normal (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). For both receptors, however, knockin mutants lacking the kinase domain rescued the defects, suggesting that EphB2 and EphA4 forward signaling is not required for these forms of synaptic plasticity. Because synaptic plasticity in area CA1 depends on postsynaptic mechanisms, EphB2 may regulate plasticity by associating with NMDA ion channel receptors and by promoting their synaptic localization. Alternatively, EphB2 and/or EphA4 may stimulate reverse signaling through postsynaptic ephrins.

Studies with mutant mice have also shown that reverse signaling by postsynaptic ephrin-B2 plays an essential role in synaptic plasticity in area CA1 of the hippocampus (Bouzioukh et al., 2007; Yamaguchi and Pasquale, 2004). The PDZ domain-binding site of ephrin-B2 is required for LTP, LTD, and depotentiation, whereas the tyrosine phosphorylation sites are only important for LTP. The involvement of ephrin-B3 in synaptic plasticity in area CA1 remains to be clarified because different groups have reported either defective or normal LTP in ephrin-B3 knockout mice (Dalva et al., 2007). Reverse signaling by presynaptic B-type ephrins has been implicated in the regulation of LTP in area CA3, which depends on presynaptic mechanisms. This effect is due to *trans*-synaptic bidirectional communication with postsynaptic EphB2, possibly regulating presynaptic vesicle release. Similarly, presynaptic ephrin-B signaling has been recently shown to enhance presynaptic glutamate release and postsynaptic glutamate responsiveness in developing *Xenopus* retinotectal synapses, where EphB2 is also localized postsynaptically (Lim et al., 2008).

Given the involvement of the Eph system in the regulation of dendritic spine morphology and synaptic plasticity, its dysfunction would be predicted to cause learning and memory deficits. Indeed, some Eph/ephrin mutations and hippocampal infusion of Eph/ephrin Fc fusion proteins have been shown to affect learning and memory performance in mice (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). It will be interesting to investigate whether Eph/ephrin dysfunction may cause some forms of mental retardation and the accompanying dendritic spine abnormalities, and whether downregulation of EphB2 cell-surface clusters by soluble amyloid β protein has a role in the synapse/spine degeneration and memory loss characteristic of Alzheimer's disease (Lacor et al., 2007). Repeated exposure to drugs of abuse also causes long-lasting changes in the neuronal circuits of certain brain regions, including hippocampus and cortex, and alterations in Eph receptor/ephrin expression might contribute to some of these effects (Bahi and Dreyer, 2005). Better understanding of how Eph bidirectional signaling regulates synaptic plasticity may suggest new strategies to help counteract the cognitive and behavioral problems associated with mental retardation, aging, or drug addiction.

Repair after Injury

Upregulation of multiple Eph receptors and ephrins has been detected at sites of nervous system injury (Du et al., 2007). In some cases, developmental expression patterns are recapitulated. In others, new patterns develop under the regulation of cytokines, hypoxia, and other factors present at sites of injury. Some of the Eph receptors/ephrins expressed in neural cells may provide guidance cues enabling the re-establishment of appropriate connections, but they may also hinder proper axon regrowth through their repulsive signaling (Wu et al., 2007). Eph receptors and ephrins present in inflammatory cells and meningeal fibroblasts that infiltrate the injury site can also engage in bidirectional signaling with Eph proteins upregulated in neural cells, with consequences for regeneration. For example, EphB3 expressed in the macrophages recruited to the injured mouse optic nerve promotes sprouting of damaged retinal axons, which express ephrin-B3 (Liu et al., 2006). Furthermore, the interplay between EphB2 expressed in invading meningeal fibroblasts and ephrin-B2 expressed in reactive astrocytes after rat spinal cord transection appears to promote the segregation of the two cell types and the formation of the glial scar and surrounding basal lamina.

The EphA4 receptor is emerging as an inhibitor of nerve regeneration. After lesions to the spinal cord, this receptor accumulates in both damaged corticospinal axons and reactive astrocytes (Du et al., 2007; Fabes et al., 2007). Analysis of EphA4 knockout mice and infusion of an EphA4

antagonistic peptide in the intrathecal space surrounding the rat spinal cord suggest that EphA4 forward signaling plays a role in the axon retraction that occurs after lesion and also hinders subsequent axon sprouting/regeneration and behavioral recovery. This could be due to interaction of axonal EphA4 with both ephrin-B2 expressed in reactive astrocytes and ephrin-B3 expressed in myelin. EphA4 in reactive astrocytes may also play a role in the formation of the glial scar, which forms a barrier impeding axon regeneration. According to these still preliminary but intriguing studies, strategies to inhibit EphA4 function promise to be beneficial for the treatment of spinal cord injury. More extensive studies on the involvement of the Eph system in different regions of the central nervous system after various types of injury will help identify possible Eph-based strategies to improve recovery.

Despite the progress over many years in elucidating the activities of Eph bidirectional signaling in neural development, plasticity, and repair, new exciting roles continue to be discovered for these molecules. That a single Eph receptor, or ephrin, can affect multiple processes through different signaling mechanisms underscores how effectively the complexity and versatility of the Eph system have been exploited in the nervous system.

Immune Function

Many Eph receptors and ephrins are expressed in lymphoid organs and lymphocytes, suggesting that they have immunoregulatory properties (Wu and Luo, 2005). For example, the Eph system seems to play a role in immune processes where cell contact-dependent communication is critical, such as the development of thymocytes into mature T cells within the thymus and the subsequent differentiation of activated T cells into effector cells in the periphery.

Several studies have shown that perturbing Eph-ephrin interactions in thymic organ culture with Eph or ephrin Fc fusion proteins interferes with thymocyte survival and maturation (Alfaro et al., 2007; Munoz et al., 2006; Wu and Luo, 2005). Defects in thymocyte maturation have also been observed in EphA4 knockout mice, which have greatly decreased numbers of peripheral T cells. These defects appear to result from abnormal development of the stromal cells of the thymic cortex, which express EphA4 and support thymocyte survival and maturation. Preliminary observations suggest that EphB2 and EphB3 knockout mice also have a disorganized thymic architecture and decreased numbers of thymocytes. These findings suggest that the Eph system is important for the structural organization of the thymus and for guiding the movement of thymocytes through the different thymic compartments that support their gradual maturation into T cells.

Other studies have shown that the Eph receptors modulate responses mediated by the T cell receptor (TCR) and may represent a class of costimulatory receptors. EphB6 is the Eph receptor whose function in immune regulation has been best characterized (Wu and Luo, 2005). This receptor is highly expressed in the thymus, where it is present in a substantial fraction of thymocytes, particularly those double positive for CD4 and CD8. EphB6 has also been detected in a fraction of peripheral $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, where its levels appear to be dynamically regulated by rapid synthesis and removal. Although EphB6 lacks kinase activity, stimulation of T cells with anti-EphB6 antibodies or ephrin-B ligands leads to increased tyrosine phosphorylation and intracellular signaling. EphB6 phosphorylation may occur through association with coexpressed EphB receptors, such as EphB1 and possibly EphB4. Several cytoplasmic signaling molecules known to participate in TCR signaling, such as the adaptor and ubiquitin ligase Cbl, associate with EphB6 and have been implicated in its effects.

There is substantial evidence that EphB receptors modulate T cell responses (Alfaro et al., 2007; Wu and Luo, 2005; Yu et al., 2006). First, these receptors cluster with activated T cell receptors in aggregated lipid rafts. Second, clustering of EphB receptors with immobilized anti-EphB6 antibodies or ephrin-B Fc ligands lowers the activation threshold of T cells responding to suboptimal TCR ligation. EphB activation also promotes T cell proliferation, production of interferon γ (but not interleukins 2 and 4), and cytotoxic T cell activity. These effects involve upregulation of the p38 and p42/44 MAP kinases. Third, EphB6-negative T cells purified from human peripheral blood or from the spleen of EphB6 knockout mice show impaired TCR signaling, proliferation, and cytokine secretion in vitro. Fourth, the EphB6 knockout mice show impaired cellular immune responses despite having normal T cell numbers. Thus, EphB receptor ligation enhances the effects of weak TCR signaling, suggesting that EphB receptors promote positive thymocyte selection and T cell responses to antigen-presenting cells. On the other hand, in thymocytes and Jurkat T cells EphB receptor signaling has also been reported to blunt the effects of high TCR signaling, such as interleukin-2 secretion and induction of apoptosis. Hence, EphB receptor ligation might also inhibit the effects of strong TCR signaling, such as the negative selection of self-reactive thymocytes.

Physiologically, EphB receptors in T cells are likely activated through interactions with ephrin-B ligands expressed by other T cells as well as other cell types, such as thymic epithelial cells and antigen-presenting cells (Wu and Luo, 2005). Interestingly, these Eph interactions may facilitate T cell responses in lymphoid organs, where T cells and antigen-presenting cells have sustained contact to promote differentiation of naive T cells into effectors.

EphA receptors and A-type ephrins are also expressed in thymocytes and T cells (Freywald et al., 2006; Wu and Luo, 2005) and have also been reported to modulate TCR signaling. For example, stimulation of $CD4^+$ $CD8^+$ double-positive thymocytes with ephrin-A1 Fc inhibits interleukin-2 secretion and apoptosis induced by strong TCR activation. This suggests that EphA receptors modulate negative selection of self-reactive thymocytes, which depends on apoptosis triggered by strong TCR stimulation. Ephrin-A1 is also expressed in $CD4^+$ helper T cells, where it may have a functional effect through reverse signaling because its ligation with antibodies has been reported to suppress TCR responses. Furthermore, the EphA system has been proposed to modulate

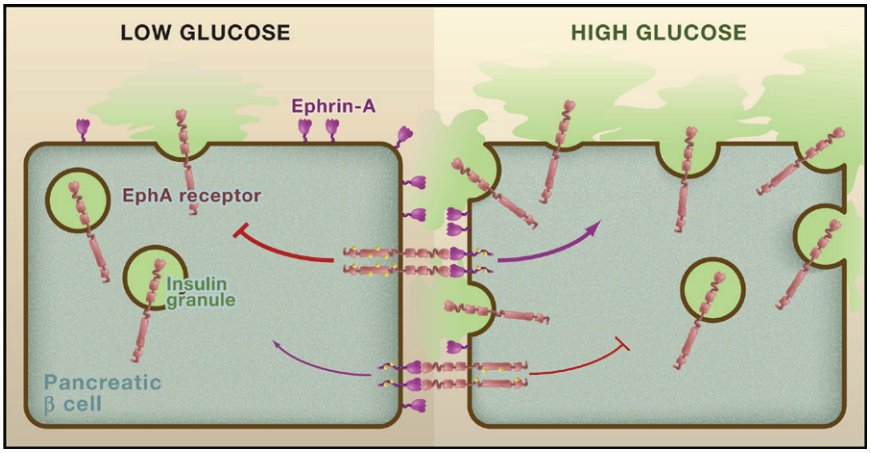

Figure 3. EphA-Ephrin-A Bidirectional Signaling and Insulin Secretion

When glucose levels are low, forward signaling predominates inhibiting insulin secretion; when glucose levels are high, reverse signaling predominates promoting insulin secretion. Ephrin-A molecules are mainly on the cell surface whereas Eph receptor molecules are also in the secretory granules. Thicker lines indicate stronger signals; yellow circles, tyrosine phosphorylation.

thymocyte and T cell migratory responses to chemokines (such as SDF1) and integrin-dependent adhesion, which guide thymocyte movements within the thymus and T cell trafficking between the blood, lymphoid tissues, and sites of extravasation (Hjorthaug and Aasheim, 2007; Sharfe et al., 2008; Wu and Luo, 2005). Signaling molecules that have been implicated in EphA-dependent regulation of T cell migration include the cytoplasmic tyrosine kinases Lck and Pyk2, the exchange factor Vav1, and Rho family GTPases. However, more work is needed to establish the physiological significance of the EphA-dependent chemotactic and adhesive responses observed in vitro.

Eph receptors and ephrins are also expressed in B lymphocytes, but their effects in these cells have not been characterized (Aasheim et al., 2000; Nakanishi et al., 2007). Clearly, more work is needed to refine our knowledge of Eph bidirectional signaling in the immune system. As in other organs, the role of these molecules is likely to be complex and involve the coordinated activities of different Eph receptors and ephrins that have intertwined and partially overlapping functions. Careful expression studies and evaluation of immunological defects in compound Eph and ephrin conditional knockout mice will be particularly useful for dissecting these roles. It will also be important to determine whether defects in Eph function contribute to immunological disorders and hematopoietic malignancies where Eph proteins are highly expressed (Nakanishi et al., 2007).

Glucose Homeostasis and Diabetes

The β cells in the pancreas adjust their secretion of insulin in response to glucose levels in the blood in order to maintain glucose homeostasis in the body. Communication between β cells clustered in pancreatic islets has long been known to modulate insulin secretion, but the underlying molecular mechanisms were unknown. A recent study using cultured cells and mouse models shows that β cells communicate via EphA receptors and ephrin-A ligands (Konstantinova et al., 2007). Remarkably, EphA forward signaling (which inhibits insulin secretion) and ephrin-A reverse signaling (which enhances insulin secretion) can be differentially regulated in pancreatic cells (Figure 3). When glucose is low, EphA forward signaling predominates, decreasing basal insulin secretion. Glucose causes EphA receptor dephosphorylation, leading to downregulation of EphA forward signaling without inhibition of ephrin-A reverse signaling. Thus, reverse signaling predominates when glucose is high, increasing insulin secretion. A further twist is that although ephrin-A ligands are mainly localized on the plasma membrane, EphA receptors are also in the intracellular insulin secretory granules. This suggests that EphA levels on the plasma membrane, and therefore EphA-ephrin-A complexes, increase upon insulin release. This causes a negative feedback loop that limits insulin secretion through increased EphA signaling when glucose levels are low and a positive feedback loop that potentiates secretion through increased ephrin-A signaling when glucose levels are high (Figure 3).

Although further studies will be required to fully elucidate the signaling pathways underlying these effects, some evidence suggests that the opposite effects of EphA and ephrin-A signaling depend on differential regulation of Rac1 GTPase activity and actin filament assembly as well as gap junction communication. A number of intriguing questions also remain. First, do EphB receptors and ephrin-B ligands—which are also expressed in pancreatic β cells—contribute to the regulation of glucose homeostasis or have other functions? Second, do these results in the pancreas reveal a general mechanism by which Eph receptors and ephrins regulate exocytosis in other secretory systems? Third, do the Eph-dependent defects in insulin secretion play a role in type 2 diabetes and might the ability of the EphA/ephrin-A system to affect insulin release be exploited in the treatment of diabetes?

Bone Maintenance and Bone Remodeling Diseases

Developmental deficiencies in EphB/ephrin-B signaling can cause skeletal malformations. These include cleft palate, defective development of the skull vault, craniosynostosis, and other bone abnormalities observed in EphB2/EphB3 and ephrin-B1 mutant mice and in individuals harboring ephrin-B1 mutations that cause the X-linked developmental disorder craniofrontonasal syndrome (Davy et al., 2006; Pasquale, 2005). Interestingly, mosaic ephrin-B1 expression in calvarial osteoblast precursors—due to random X chromosome inactivation in ephrin-B1 heterozygous females—causes abnormal cell sorting leading to defects in bone development. Genetic and other evidence supports a model

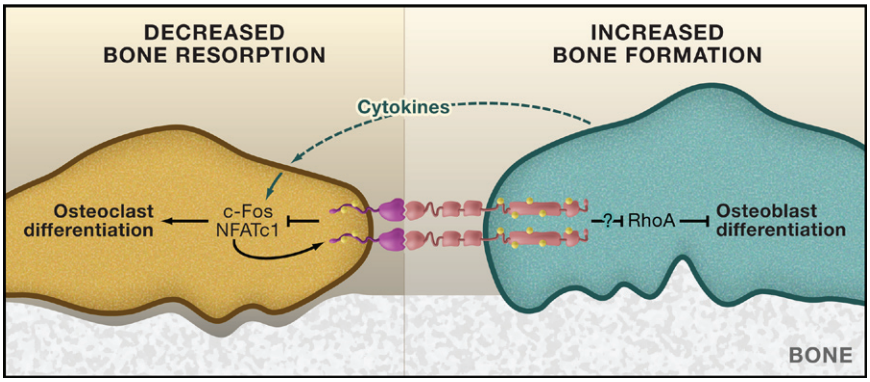

Figure 4. EphB-Ephrin-B Bidirectional Signaling in Bone Formation

Osteoblasts secrete cytokines that upregulate ephrin-B2 in osteoclast precursors. Ephrin-B ligands in osteoclasts interact with EphB receptors in osteoblasts generating bidirectional signals that inhibit osteoclast differentiation and promote osteoblast differentiation.

in which EphB-ephrin-B1 bidirectional signaling at the ectopic boundaries that form between ephrin-B1 positive and negative osteoblast precursors leads to impaired gap junction communication, which inhibits osteoblast differentiation and delays ossification of developing calvarial bones.

Besides these developmental roles, EphB-ephrin-B bidirectional signaling between osteoblasts and osteoclasts has been implicated in the regulation of bone homeostasis in the adult (Zhao et al., 2006). Bones continue to be remodeled throughout life, a process controlled by dynamic reciprocal communication between osteoclasts, which degrade bone, and osteoblasts, which form bone. Gain- and loss-of-function experiments in culture have shown that cytokines produced by osteoblasts activate the transcription factors c-Fos and NFATc1 in osteoclast precursors. This promotes osteoclast differentiation and also increases ephrin-B2 expression (Figure 4). Several Eph receptors present in osteoblasts can stimulate ephrin-B reverse signaling in osteoclasts, which represses osteoclast differentiation through a negative feedback loop that inhibits c-Fos and NFATc1 and requires the ephrin PDZ domain-binding site.

The communication between osteoclasts and osteoblasts is bidirectional and forward signaling by EphB4—and possibly other coexpressed Eph receptors—promotes the differentiation of osteoblasts, which deposit new bone at sites of resorption by osteoclasts (Figure 4). The Eph forward signaling pathway responsible for osteoblast differentiation may involve RhoA inactivation. Hence, cell contact-dependent communication between Eph receptors and ephrins limits osteoclast differentiation and enhances osteoblast differentiation, inducing a shift from bone resorption to bone formation. Indeed, transgenic overexpression of EphB4 in osteoblasts has been shown to increase bone mass in mice.

These findings suggest that interventions targeting the EphB system may be helpful in the prevention and treatment of bone remodeling diseases, such as osteoporosis and osteopetrosis. It will be important, however, to elucidate the role of bidirectional signaling between osteoblasts, which in addition to EphB receptors also express B-type ephrins. Another unresolved issue with possible therapeutic implications is whether Eph-ephrin interactions between cancer cells and osteoblasts or osteoclasts may play a role in bone metastatic disease.

Intestinal Homeostasis

The intestine is lined by a monolayer of epithelial cells that control the absorbance of nutrients and the secretion of protective mucus and antimicrobial agents. The intestinal epithelium undergoes continuous self-renewal throughout life, and homeostasis is maintained by the balance of cell proliferation, differentiation, and apoptosis. A recent study has shown that a few cycling cells located at the bottom of invaginations called crypts can generate all intestinal epithelial lineages and therefore likely represent the long sought-after intestinal stem cells (Barker et al., 2007). The stem cells give rise to rapidly proliferating transit-amplifying cells, which differentiate while migrating toward the top of the crypts. In the small intestine, epithelial cells continue to migrate toward the tips of protrusions called villi, where they die and are shed into the intestinal lumen.

The canonical Wnt/β-catenin/Tcf signaling pathway is a critical regulator of homeostasis in the intestinal epithelium, in part through its ability to promote the transcription of EphB receptors and inhibit that of ephrin-B ligands (Clevers and Batlle, 2006). As the newly generated epithelial cells migrate, they gradually lose EphB expression and acquire ephrin-B expression as they move away from the source of Wnt secreted by surrounding mesenchymal cells at the bottom of the crypts. This creates countergradients of EphB and ephrin-B expression along the crypt axis, with high EphB expression at the bottom of the crypts and high ephrin-B expression at the top and in the villi. A population of secretory cells in the small intestine, called Paneth cells, also undergoes renewal but remains interspersed with the stem cells at the bottom of the crypts. Unlike other intestinal epithelial cells, Paneth cells can differentiate when Wnt levels are high. They also maintain high EphB3 expression after differentiation, which is important for their localization.

Analysis of EphB2/EphB3 and ephrin-B1 knockout mice, and knockin mice expressing a dominant-negative form of EphB2 replacing the wild-type receptor, has shown that EphB-dependent repulsive signaling restricts intermingling of the proliferating and differentiated cells (Clevers and Batlle, 2006; Cortina et al., 2007). Interestingly, crosstalk with E-cadherin appears to play a crucial role (Figure 2). EphB forward signaling promotes E-cadherin-mediated cell adhesion in colorectal cancer cells, and E-cadherin is required for the in vitro sorting of EphB- and ephrin-B-expressing cells into separate cell clusters.

Perturbation of EphB forward signaling in the mouse through genetic manipulations or administration of soluble forms of the ephrin-B2 or EphB2 extracellular domains has also implicated the EphB system in intestinal epithelial cell

proliferation (Holmberg et al., 2006). Cell proliferation was decreased on the sides of the crypts and not at the bottom, suggesting that the EphB system promotes the proliferation of transit-amplifying cells.

It will be important to also examine the role of the EphA/ephrin-A system in intestinal homeostasis because uneven mRNA expression along the crypts of the colon has also been reported for several EphA receptors and ephrin-A1 (Kosinski et al., 2007). EphA2 and ephrin-A1 have also been suggested to regulate epithelial barrier function in the intestine (Rosenberg et al., 1997). Future studies to explore whether Eph receptors and ephrins may play a role in intestinal diseases, such as inflammatory bowel disease, or in the restoration of the injured intestinal epithelium (Hafner et al., 2005; Rosenberg et al., 1997) will provide a more complete understanding of the Eph system in intestinal homeostasis and disease. The EphB system has also been implicated in colorectal cancer (see next section). The Eph bidirectional signaling pathways in normal and transformed intestinal epithelial cells also await a comprehensive investigation.

Cancer

Besides their expression in normal tissues, Eph receptors and/or ephrins are present, and often upregulated, in essentially all types of cancer cells (Ireton and Chen, 2005; Noren and Pasquale, 2007). In many cases this may be due to oncogenic signaling pathways, hypoxia, or inflammatory cytokines. For example, the Wnt/β-catenin/Tcf pathway promotes EphB expression in colorectal cancer cells and the Ras-MAP kinase pathway promotes EphA2 expression in breast cancer cells. Interestingly, activation of these two pathways also results in ephrin downregulation and, as a consequence, low Eph receptor activation. Indeed, Eph receptor forward signaling does not necessarily aid the tumorigenic process. Tumor suppressor activities have been reported for Eph signaling in colorectal, breast, prostate, and skin cancer cells both in vitro and in vivo. However, the decreased tumorigenicity of cancer cells in which Eph receptor expression was experimentally decreased suggests that these receptors can also have tumor-promoting effects. The role of ephrin reverse signaling in cancer cells is poorly characterized, although several ephrins have been reported to promote cell transformation and cancer cell migration/invasion (Campbell et al., 2006; Meyer et al., 2005; Tanaka et al., 2007). To complicate matters further, the Eph system is also operational in the tumor microenvironment. The effects of Eph-ephrin bidirectional signaling have been mostly studied in tumor endothelial cells, whereas information on other types of tumor stromal cells is very limited. In order to design rational strategies to target the Eph system for cancer therapy, we need to further elucidate how Eph receptors and ephrins influence the behavior of cancer cells, cancer stromal cells, and also cancer stem cells. Below we discuss work on several cancers, which exemplifies our current understanding of the Eph system in oncogenic transformation.

Colorectal Cancer

The same signaling proteins that control physiological self-renewal in the intestine can also initiate malignant transformation when mutations subvert their activity. Thus, constitutive activation of the Wnt/β-catenin/Tcf pathway leads to the formation of adenomas and colorectal cancer (Clevers and Batlle, 2006). As in the normal intestine, the pathway also upregulates EphB expression in the early stages of tumorigenesis. Despite their reported ability to promote proliferation in the intestinal epithelium, the EphB receptors appear to have a tumor suppressor role in colorectal cancer. Indeed, in advanced human colorectal cancers expression of different EphB receptors is lost in a large fraction of the tumor cells, and there is strong association of tumor histological grade and patient survival with EphB silencing (Batlle et al., 2005). Intriguingly, hypoxia may explain the coordinated downregulation of multiple EphB receptors in advanced cancers because hypoxia-inducible factor-1 can compete with Tcf-4 for binding to nuclear β-catenin, leading to silencing of Tcf-4 target genes (Kaidi et al., 2007).

Reduced EphB activity accelerates the progression of colorectal cancer. This is supported by studies with the $Apc^{Min/+}$ mouse model, where poorly differentiated and aggressive colorectal adenocarcinomas develop in mice lacking EphB3 or ephrin-B1 and in mice expressing dominant-negative EphB2 but not in control mice (Batlle et al., 2005; Cortina et al., 2007). A possible mechanism inhibiting the expansion of EphB-positive tumor cells involves E-cadherin-dependent spatial restriction by surrounding epithelial cells that express ephrin-B ligands. The involvement of the EphA/ephrin-A system in colorectal cancer remains to be investigated using mouse models, to follow up on cell culture studies suggesting oncogenic effects of coexpressed EphA2 and ephrin-A1 (Wimmer-Kleikamp and Lackmann, 2005).

Breast Cancer

EphA2 and EphB4 are the Eph receptors most extensively studied in breast cancer, although our understanding of their activities is far from complete (Ireton and Chen, 2005; Macrae et al., 2005; Noren and Pasquale, 2007). Both receptors are widely expressed but poorly tyrosine phosphorylated in human breast cancer cell lines, suggesting a low level of ephrin-dependent activation. Indeed, the levels of ephrin-B2—the preferred ligand for EphB4—are low in these cell lines, and high EphA2 expression also correlates with low ephrin-A expression. Intriguingly, even when ephrin-A1 is present, its ability to activate EphA2 may be impaired in breast cancer cells that lack E-cadherin. These data suggest that if EphA2 and EphB4 have oncogenic activity in human breast cancer cell lines, this activity must be either independent of ephrin stimulation or manifest itself when ephrin stimulation is low.

Overexpression of EphA2 in a human mammary epithelial cell line has been shown to cause oncogenic transformation (Ireton and Chen, 2005; Noren and Pasquale, 2007). Despite the fact that EphA2 was poorly tyrosine phosphorylated, the overexpressing cells acquired the ability to grow in soft agar and form tumors in mice. Furthermore, they had decreased estrogen dependence and sensitivity to the drug tamoxifen. On the other hand, EphA2 knockdown by RNA interference or with antisense oligonucleotides has been shown to inhibit the tumorigenicity of several types of cancer cells, including a breast cancer cell line. Similarly, EphB4 knockdown inhibited breast cancer cell survival, migration, and invasion, and also tumor growth in a mouse xenograft model.

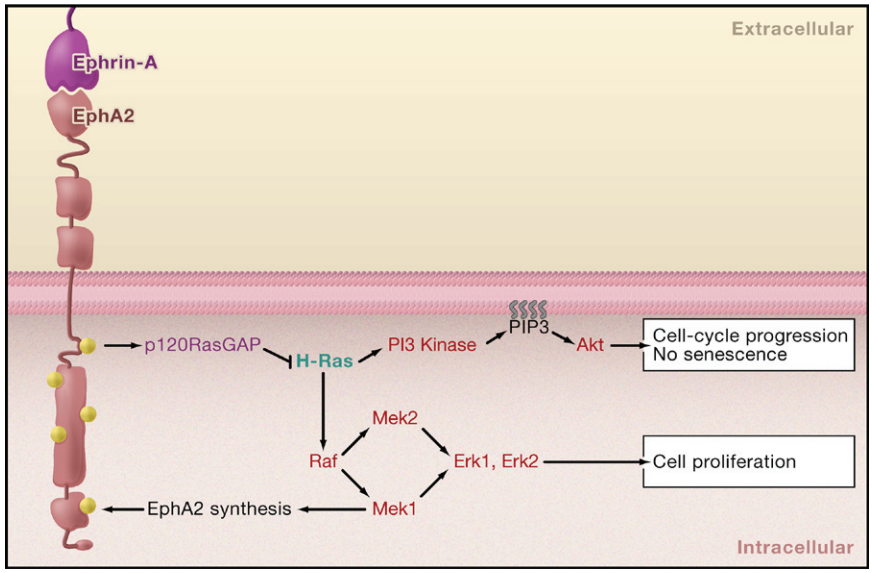

Figure 5. EphA2, Cell-Cycle Arrest, and Cellular Senescence

Raf-activating mutations upregulate the levels of EphA2, which may contribute to cell-cycle arrest and senescence through inhibition of H-Ras-PI3 kinase-Akt. In cells without activated Raf, EphA2 also inhibits the MAP kinase pathway.

The mechanisms underlying these oncogenic effects of Eph receptors that appear to be poorly activated are unclear. Some evidence suggests that ephrin-independent crosstalk with oncogenic signaling pathways may be involved. For example, EphA2 has been found to enhance tumor cell proliferation and motility in cells overexpressing EGF receptor family members, an activity that likely contributes to tumorigenesis and metastatic progression in a mouse ErbB2 mammary adenocarcinoma model (Brantley-Sieders et al., 2008; Larsen et al., 2007). The Eph receptors might also serve as scaffolds for constitutively associated signaling proteins, somehow affecting their localization and signaling ability to promote cell transformation. One study has shown that when transformed by EphA2 overexpression, mammary epithelial cells deposit more fibronectin, which plays a role in their survival (Hu et al., 2004). Oncogenic signaling pathways that may be activated by low ephrin levels could also be responsible for the tumorigenic effects of EphA2 and EphB4 in breast cancer cells.

Low versus high Eph forward signaling might have opposite effects on tumorigenicity, as has been shown for other cellular properties (Pasquale, 2005; Poliakov et al., 2004). EphA2 dephosphorylation by the low-molecular-weight phosphotyrosine phosphatase has been shown to promote mammary epithelial cell transformation, presumably by inhibiting EphA2 forward signaling (Noren and Pasquale, 2007; Wimmer-Kleikamp and Lackmann, 2005). Furthermore, EphA2 and EphB4 activation with soluble ephrin ligands or activating antibodies decreases the malignant properties of human breast cancer cell lines. Activation of EphA2 inhibited growth in soft agar, fibronectin deposition, cell survival, and tumor growth in a breast cancer xenograft model (Ireton and Chen, 2005). Inhibition of Ras activity downstream of EphA2 likely plays an important role in these tumor suppressor effects by inhibiting downstream MAP kinases and possibly also the PI3 kinase-Akt pathway (Figure 5) (Menges and McCance, 2007). EphB4 activation also inhibits breast cancer cell growth and migration (Noren and Pasquale, 2007). These effects involve activation of Abl family tyrosine kinases and tyrosine phosphorylation of the adaptor protein Crk, likely inhibiting Rac activity (Figure 1B). Curiously, high levels of ephrin stimulation produce effects similar to EphA2 or EphB4 knockdown in cultured breast cancer cells. Further studies are needed to elucidate the mechanisms underlying the antioncogenic effects of ephrin stimulation versus downregulation of Eph receptor expression.

A possible working hypothesis is that high levels of ephrin-dependent EphA2 and EphB4 forward signaling suppress tumorigenesis whereas low levels of forward signaling or crosstalk with oncogenic signaling pathways promote tumorigenicity. However, in contrast to its tumor suppressor effects in human breast cancer cells, EphA2 kinase activity appears to promote tumorigenesis in mouse 4T1 mammary tumor cells, which express ephrin-A1 (Brantley-Sieders et al., 2006). In these cells, EphA2 kinase activity promotes VEGF secretion, RhoA activation, and cell motility in vitro as well as tumor growth and metastasis in mouse models. EphA2 is also tyrosine phosphorylated and coexpressed with ephrin-A1 in other types of cancer cells, including malignant melanoma cells, suggesting divergent roles for EphA2 in cell transformation depending on the cellular context (Ireton and Chen, 2005). Perhaps, cancer cells that endogenously express highly activated Eph receptors have evolved mechanisms to neutralize their tumor suppressor signals. For example, Ras- and Raf-activating mutations could counteract some of the antioncogenic effects of activated EphA2 (Figure 5) (Menges and McCance, 2007).

Skin Cancer and Melanoma

The most common types of skin cancer are derived from either melanocytes or keratinocytes, and EphA2 appears to have different effects in the two types of cancer cells. In melanoma, ephrin-A1-mediated activation of EphA2 and possibly other EphA receptors promotes proliferation (Easty and Bennett, 2000; Hess et al., 2007). Intriguingly, EphA2 has also been found to associate with vascular endothelial cadherin and promote the formation of blood vessel-like structures by malignant melanoma cells, a role similar to that of EphA2 in tumor endothelial cells (see below). In contrast, a recent study has shown that susceptibility to chemically induced keratinocyte transformation is enhanced in EphA2 knockout mice (Guo et al., 2006). Furthermore, despite the observed upregulation of EphA2 in mouse as well as human keratinocyte-derived skin carcinomas, the tumors lacking EphA2 grow faster and are more invasive.

Similar to the EphB/ephrin-B interplay in colorectal cancer, ephrin-A1 expression in the surrounding skin appears to restrict expansion of the EphA2-positive tumor cells. Inhibition of Ras-dependent pathways may explain these tumor suppressor effects of EphA2.

Bidirectional signaling through other Eph receptors and ephrins can also have diverse effects on melanoma malignancy. For example, EphB4 activation by coexpressed ephrin-B2 in the aggressive SW1 mouse melanoma cell line promotes RhoA activation, leading to increased ameboid migration (Noren and Pasquale, 2007). In contrast, EphB4 activation with ephrin-B2 Fc in the human MDA-MB-435 cell line (which has low endogenous ephrin-B2 expression) inhibits proliferation, survival, migration, and invasion in vitro as well as tumor growth in a mouse xenograft model through a pathway involving Abl and Crk. It should be noted that a recent study provides strong evidence that the currently available stocks of MDA-MB-435 cells, which were previously believed to be of breast cancer origin, are instead derived from a melanoma line (Rae et al., 2007).

In addition to promoting EphB signaling, endogenous ephrin-B2 expressed in melanoma cells has also been found to associate with β1-integrins and promote cell adhesion and migration, suggesting a role in tumor progression through reverse signaling and crosstalk with integrins (Figure 2) (Meyer et al., 2005). The EphA4 receptor is expressed in melanocytes but downregulated in aggressive melanoma cells, suggesting that EphA4 has a role as a melanoma tumor suppressor (Easty and Bennett, 2000). EphB6 is also downregulated during melanoma progression, but this receptor lacks kinase activity and thus may function differently from other Eph receptors (Hafner et al., 2003).

Tumor Angiogenesis

Besides being expressed in cancer cells, Eph receptors and ephrins are also present in the tumor vasculature, where they promote angiogenesis (Brantley-Sieders and Chen, 2004; Heroult et al., 2006; Noren and Pasquale, 2007). Because blood vessels are critical for tumor growth and metastasis, this is an important aspect of the oncogenic effects of Eph-ephrin bidirectional signaling. The main roles in tumor angiogenesis have so far been attributed to EphA2 forward signaling and ephrin-B2 reverse signaling based on a series of in vitro and in vivo experiments with mouse tumor models, including analysis of angiogenesis in EphA2 knockout mice. Interestingly, EphA2 is not expressed in the embryonic vasculature or the adult quiescent vasculature. Interaction with ephrin-A1 present in tumor endothelial cells as well as tumor cells is responsible for activating endothelial EphA2. Signaling effectors that have been implicated in the angiogenic activity of EphA2 include PI3 kinase, Vav guanine nucleotide exchange factors, and Rac1 (Figure 1B). Activation of these effectors presumably impacts the actin cytoskeleton, thus regulating endothelial cell shape and migration. Interestingly, EphA2 appears to be required for VEGF-induced endothelial cell migration and assembly into capillary-like structures (Chen et al., 2006).

Ephrin-B2 is also widely expressed in the vasculature of many tumors, which is not surprising given that this ephrin is found in the embryonic arterial vasculature and its expression in endothelial cells is upregulated by hypoxia and VEGF (Brantley-Sieders and Chen, 2004; Heroult et al., 2006; Noren and Pasquale, 2007). Ephrin-B2 reverse signaling can be stimulated by interaction with EphB4 expressed in the tumor vasculature and in tumor cells. Indeed, increased levels of the EphB4 extracellular portion on the surface of a cancer cell line have been shown to increase tumor growth through effects on the vasculature. EphB4 activation by ephrin-B2 in circulating endothelial progenitor cells also increases their recruitment to sites of neovascularization through selectin-mediated adhesion (Foubert et al., 2007). It will be interesting to investigate whether this also contributes to tumor neovascularization.

Given the divergent effects of Eph receptors and ephrins in cancer cells, Eph-based anticancer therapies involving vascular targeting seem the most straightforward. Indeed, various approaches to interfere with EphA2-ephrin-A or EphB-ephrin-B2 binding using soluble Eph extracellular domains have consistently resulted in inhibition of tumor growth in various mouse models (Heroult et al., 2006; Ireton and Chen, 2005; Noren and Pasquale, 2007; Wimmer-Kleikamp and Lackmann, 2005). However, targeting the Eph system will also affect the tumor cells, likely with variable outcomes depending on the tumor type. Ultimately, the efficacy of each Eph-based targeting strategy will have to be evaluated empirically in appropriate in vivo tumor models.

Cancer Stem Cells

An emerging theme in cancer therapy is the possible importance of targeting the "cancer stem cells," which are the cells that can repopulate the tumor and cause recurrence even when most of the tumor mass has been eliminated. Because Eph receptors/ephrins are expressed in various other types of stem cells, they are also likely to be present in cancer stem cells (Pasquale, 2005). However, characterization of the Eph system in stem cells is still at an early stage. Positive as well as negative effects on proliferation, apoptosis, and differentiation have been reported depending on the Eph/ephrin involved and the type of stem cell. An area of particular interest is the role of Eph-ephrin bidirectional signaling in the communication between stem cells and their supporting niche cells. Intriguingly, a recent study has implicated Eph receptor-dependent inhibition of the Ras-MAP kinase pathway in the asymmetric division of at least two different precursor cells in the ascidian embryo (Picco et al., 2007; Shi and Levine, 2008). It was shown that contact with asymmetrically localized ephrin-expressing neighboring cells triggers polarized Eph receptor activity, driving specification of one of the two daughter cells to a neural rather than notochord fate or to a mesodermal rather than an endodermal fate. It will be interesting to investigate whether Eph-ephrin interactions with niche cells might have a similar role in the self-renewal versus differentiation choice during asymmetric stem cell division. Knowing the effects of Eph-ephrin signaling in cancer stem cells will likely be important in deciding how to target these molecules for anticancer therapy.

Henipavirus Infection

It was recently discovered that ephrin-B2 and ephrin-B3 serve as the cell entry receptors for Nipah and Hendra viruses, two emerging paramyxoviruses comprising the newly defined

Henipavirus genus (Bonaparte et al., 2005; Negrete et al., 2005, 2006). Although the natural host for henipaviruses is the fruit bat, outbreaks in farm animals and transmission to humans have repeatedly occurred in recent years. The high evolutionary conservation of the ephrins explains the ability of Nipah and Hendra viruses to infect a wide range of animal species. In humans, these viruses are highly lethal and are classified as category 4 containment pathogens. The tissue distribution of ephrin-B2 in the vascular system and both ephrin-B2 and ephrin-B3 in the nervous system are consistent with the tissue tropism of the viruses. Both Nipah and Hendra viruses bind to the same region of ephrin-B2 and ephrin-B3 that also mediates high-affinity binding to EphB receptors. It will therefore be interesting to determine whether disruption of EphB/ephrin-B function, or activation of reverse signals following ephrin-B clustering by the tetrameric viral attachment glycoprotein, play a role in disease pathogenesis. From a therapeutic perspective, it will also be important to determine if soluble forms of the ephrin-B2 and EphB4 extracellular domains, which inhibit henipavirus infection in cell culture, may also be useful as prophylactic agents. Furthermore, various soluble forms of the henipavirus G protein, which binds ephrin-B2 with subnanomolar affinity, may have therapeutic applications to stimulate or inhibit angiogenesis, depending on their ability to activate or block reverse signaling.

Concluding Remarks

Additional roles of Eph receptors and ephrins in adult physiology beyond those discussed in the previous sections have been discovered, and the list continues to grow. For example, hypoxia reportedly stimulates upregulation of ephrin-B2 in bone marrow stromal cells, which in turn activates EphB4 signaling in hematopoietic progenitor cells (Pasquale, 2005). This causes the detachment of the progenitor cells from the stroma and their differentiation into red blood cells, suggesting an Eph-dependent mechanism to maintain oxygen homeostasis in the blood. An involvement of the Eph system in blood clotting has also been demonstrated, where EphA4 and ephrin-B1 expressed in human platelets contribute to the stabilization of the blood clot through an integrin-dependent mechanism (Arvanitis and Davy, 2008). Eph/ephrin-dependent regulation of the permeability of intercellular junctions likely plays a role in glomerular filtration in the kidney. In particular, ephrin-B1 has been recently identified as a potentially important component of the slit diaphragm of podocytes (Hashimoto et al., 2007). Analysis of mutant mice has revealed that EphB2-ephrin-B2 bidirectional signaling controls the ionic homeostasis of the vestibular endolymph fluid in the inner ear and, therefore, has a potential role in vertigo and positional nystagmus (Dravis et al., 2007). Furthermore, given that several Eph receptors and ephrins are expressed in inflammatory cells and upregulated by inflammatory cytokines, the Eph system likely has multiple roles in inflammation (Ivanov and Romanovsky, 2006). EphB-ephrin-B interactions have also been implicated in the development of chronic neuropathic pain following tissue damage (Du et al., 2007). It can be expected that new discoveries clarifying the mechanisms of the known and yet to be discovered Eph physiological activities will keep the spotlight on the Eph field for years to come.

However, several factors could accelerate progress. It is becoming apparent that expression of Eph receptors and ephrins undergoes dynamic spatial and temporal regulation at the transcriptional and posttranscriptional levels, not only during development but also in the adult and probably in diseased tissues. Knowing the relative abundance and cellular localization of Eph receptors and ephrins, and their subcellular localization, is critical for understanding biological function. Therefore, to determine precisely which Eph receptors or ephrins are involved in a particular physiological process, or should be targeted in a particular disease, there is an urgent need for validated and specific antibodies that will enable detailed expression studies. It is also becoming clear that Eph receptors and ephrins can use multiple signaling mechanisms to achieve different effects and that their downstream pathways are often intertwined with other signaling networks. The availability of conditional knockout mice where gene inactivation can be spatially and temporally regulated, and of knockin mice in which a mutated Eph/ephrin replaces the wild-type protein, will be critical for understanding physiological functions and elucidating the in vivo importance of particular downstream signaling pathways. Functional antibodies and chemical genetics approaches also hold great promise for moving the field forward, particularly as more antibodies, peptides, and chemical compounds that can selectively modulate the function of individual Eph receptors and ephrins become available (Himanen et al., 2007; Noren and Pasquale, 2007; Pasquale, 2005). These tools also have the potential to be used for the selective targeting of only a particular Eph/ephrin domain, thus enabling a detailed mechanistic characterization of the multiple activities of these proteins. Systems biology approaches to integrate Eph signaling pathways with other signaling networks will also be helpful. A thorough understanding of Eph-ephrin bidirectional activities will provide new perspectives on physiology, disease pathogenesis, and potential therapies.

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