Review

Differential Regulation of EphA2 in Normal and Malignant Cells

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Eph receptor tyrosine kinases (RTK) comprise the largest family of tyrosine kinases encoded in the human genome.^{1,2} Fourteen Eph receptors and eight ephrin ligands have been identified to date and these molecules are increasingly understood to play important roles in disease and development.^{3–5}

Eph receptor family members share structural and functional similarities. Their extracellular regions include an N-terminal ligand binding domain,^{6,7} a cysteine-rich motif, and two fibronectin-like repeats. Also, Eph receptors can be distinguished from other RTKs in that they all recognize ligands, known as ephrins, which are anchored to the membrane of apposing cells.^{1,2,8,9} Ephrins do not necessarily share extensive homology and are so grouped based on their abilities to bind Eph receptors. The ephrins have been separated into two classes based on the means by which they are anchored to the cell membrane.^{3,9} EphrinA ligands are linked to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage, whereas EphrinB ligands encode for a transmembrane domain.

Based on the identity of their ligands, the Eph receptors themselves have been classified into either EphA or EphB subfamilies.⁹ These families share a degree of specificity, which is determined by a four-amino-acid loop on the extracellular surface.¹⁰ Moreover, Eph receptors and ephrin ligands each have overlapping specificity.^{1,2,8} Several ligands can bind to one receptor and, in turn, several receptors can bind to one ligand. In general, however, EphA receptors bind EphrinA ligands and EphB receptors bind EphrinB ligands.^{1,2,8,11–13}

Ligand binding typically triggers tyrosine phosphorylation of Eph receptors.² In particular, two tyrosines near the transmembrane domain are highly conserved and phosphorylated in response to ligand binding.^{14,15} These residues appear to be critical for function, as mutations of these tyrosines abolish the enzymatic activity of certain Eph kinases.¹⁵ In addition, tyrosine phosphor-

ylation creates binding sites for signaling or adapter proteins (Figure 1).¹⁶ Other sites of protein-protein interaction are also mediated by sterile α motifs (SAM)^{17,18} and PDZ (postsynaptic density protein, disc large, zona occludens) binding motifs¹⁹ located near the C-terminal end of some Eph receptors.

Eph receptors have been studied extensively in the developing nervous system, where they regulate patterning during neural development.^{2,11–13,20,21} At the cellular level, ephrin binding causes Eph receptors to initiate signals that promote cell-cell repulsion and these events appear to assist axon guidance and neural organization.¹

Overexpression and Functional Alterations of EphA2 in Cancer

Unlike most Eph kinases, which are primarily expressed during development, EphA2 is primarily found in adult human epithelial cells.²² The cellular functions of EphA2 in normal epithelia are not well understood, but work with tumor-based models suggests potential roles for EphA2 in the regulation of cell growth, survival, migration, and angiogenesis.^{23–27}

Unlike other receptor tyrosine kinases, ligand binding is not necessary for EphA2 tyrosine kinase activity.^{28,29} Rather, the ligand appears to regulate EphA2 subcellular localization and its interactions with downstream adapter and signaling proteins.^{26,52,54,61,64} It is presently unclear why EphA2, unlike other Eph kinases, does not require tyrosine phosphorylation of the receptor for its enzymatic activity. One explanation may be revealed by phosphopeptide mapping studies, which failed to identify phosphorylation of the putative “activation loop” tyrosine

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Figure 1. Biological and biochemical pathways linked with EphA2. Shown is a synthesis of the reported protein interactions and cellular consequences of EphA2 signaling. The **solid lines** denote areas of known positive regulation, whereas **dotted lines** represent a negative regulatory event. **Dashed lines** indicate situations where it is presently unresolved whether this pathway represents a positive or negative regulatory event.

at residue 772, even under conditions where EphA2 is phosphorylated on other residues (M.S. Kinch et al, unpublished). Future structure-function studies will be necessary to determine the relative importance of an “activation loop” motif in governing EphA2 enzymatic activity.

EphA2 expression is frequently elevated in cancer. High levels of EphA2 have been reported using multiple and diverse cell models and clinical specimens, including breast cancer,^{28–30} colon cancer,²⁴ prostate cancer,³¹ non-small cell lung cancers,³² and aggressive melanomas (Table 1.^{33,34} However, EphA2 does not appear to simply function as a marker but as an active participant in malignant progression. For example, ectopic overexpression of EphA2 in non-transformed mammary epithelial cells is sufficient to promote a malignant phenotype as defined using *in vitro* and *in vivo* standards.²⁹

Despite its overexpression in cancer, the EphA2 on malignant cells is not tyrosine-phosphorylated and functions differently than the EphA2 in non-transformed epithelial cells (Figure 2.^{28–30} This deficiency arises, in part, because malignant cells generally have unstable cell-cell contacts,^{35–37} which prevent EphA2 from productively interacting with its membrane-anchored ligands.²⁸ In particular, the ability of EphA2 to bind its ligands is highly sensitive to the proper expression and function of the E-cadherin adhesion protein, which is also a powerful

Figure 2. Consequences of EphA2 stimulation in malignant cells. Shown is a model of differential behavior of EphA2 on tumor cells in the presence or absence of ligand (EphrinA1-Fc) or monoclonal antibody binding. “Activated” EphA2 negatively regulates extracellular matrix (ECM) attachments, which impacts a variety of different cellular behaviors (**solid lines** indicate positive regulation and **dotted lines** indicate negative regulation). Stimulation also promotes EphA2 degradation. Similarly, decreased ECM contacts appear to increase in cell-cell attachments, which causes EphA2 to be degraded by endogenous ligands. In the converse situation (as occurs in malignant cells), high levels of unphosphorylated EphA2 increase ECM attachment, which in turn destabilizes cell-cell contacts and further favors EphA2 accumulation. Similarly, overexpression of LMW-PTP suppresses the phosphorylation of EphA2 and thereby promotes malignant character.

suppressor of metastasis.³⁸ Consequently, the EphA2 localizes from sites of cell-cell contact between non-transformed epithelial cells to membrane-ruffling in aggressive tumor cells.²⁸ E-cadherin expression is restricted to epithelial cells and it is presently unclear whether other cadherin family members similarly regulate EphA2-ligand binding. Indeed, emerging evidence from our laboratories implicates a role for VE-cadherin in the regulation of EphA2 function in endothelial cells but future investigation will be necessary to determine whether and how different cadherins regulate EphA2.

Tyrosine phosphorylation of EphA2 in normal cells is also regulated by an associated phosphatase.³⁹ Initial evidence for linked phosphatase activity arose from the finding that EphA2 was rapidly dephosphorylated under conditions that prevent ligand binding. We subsequently identified LMW-PTP (also known as HCPTP) as a phosphatase that interacts with, and dephosphorylates EphA2.³⁹ Moreover, LMW-PTP is overexpressed in many malignant cells, where it functions as a powerful oncoprotein.³⁹ Notably, the highest levels of LMW-PTP are consistently found in those tumor cells that have high levels of unphosphorylated EphA2.³⁹ Based on these findings, we asked if LMW-PTP might regulate EphA2 expression or function and indeed, ectopic overexpression of LMW-PTP causes the dephosphorylation and up-regulation of EphA2.³⁹ Moreover, the oncogenic activities of LMW-PTP require EphA2 since antisense or antibody-based inhibition of EphA2 expression prevents LMW-PTP-mediated malignant transformation.³⁹

Genetic Regulation of EphA2

In the human genome, the EphA2 locus is located on chromosome 1p36.1, which has been identified as a genetic “hot spot” in cancer.⁴⁰ The 1p36 chromosome

Table 1. EphA2 Overexpression in Cell Models and Clinical Specimens of Human Cancer

Specimens and models	Reference no.
Clinical specimens	
Metastatic melanoma	33
Breast cancer	29, 51
Prostate cancer	31
Non-small cell lung cancer	32, 51
Colon cancer	51
Cell models	
Metastatic melanoma	33, 34
Breast cancer	29, 30, 51
Prostate cancer	31
Colon cancer	24

segment is deleted in some cancers but amplified in others.^{40–44} Thus, future investigation could determine whether EphA2 might contribute to the malignant character associated with these genomic changes.

EphA2 is also regulated by transcriptional and post-translational mechanisms. A recent report showed that EphA2 transcription is regulated by p53,⁴⁵ which is frequently mutated in cancer.^{46–48} P53 appears to regulate EphA2 through a response element in the EphA2 promoter and this appears to be involved in the regulation of cell survival.⁴⁵ The regulation of EphA2 by p53 is notable, given that p53 is generally understood to function as a tumor suppressor in many cancers. At first glance, the positive regulation of EphA2 by p53 appears to be inconsistent with the high levels of EphA2 in many cancers. However, mutations in p53 function have also been described, which contribute to an aggressive phenotype and future studies could determine whether these activating mutations function, in part, by upregulating EphA2.^{46–48} Such information could be important for determining whether EphA2 is regulated early or late during tumor progression.

Other mechanisms also contribute to EphA2 overexpression in cancer. For example, EphA2 mRNA levels are up-regulated in the Ras-transformed mammary epithelial cells.³⁰ Similarly, high levels of EphA2 gene expression have been detected in the mammary tumors of transgenic mice that overexpress oncogenic H-Ras.⁴⁹ Since the downstream consequences of Ras signaling have been the subject of much investigation, this knowledge could ultimately help to identify mechanisms that cause EphA2 overexpression in cancer.

In mammary epithelial cells, EphA2 expression is also negatively regulated by a pathway involving estrogen and *c-myc*.⁵⁰ For example, exposure of non-transformed breast epithelial cells to physiological levels of estradiol was sufficient to down-regulate EphA2 levels. This mechanism is notable in light of evidence that the highest levels of EphA2 are found in the most aggressive breast cancer cell models, which lack estrogen receptor (ER). Thus, we postulate that loss of hormone sensitivity may override this repressive mechanism and thereby contribute to widespread overexpression of EphA2 that has been observed in aggressive breast cancers.^{28–30,50,51}

Regulation of EphA2 Protein Stability

Emerging evidence suggests that high levels of EphA2 can arise in tumor cells as a result of increased protein stability.⁵² We initially formulated this hypothesis after comparing non-transformed and malignant variants of MCF-10A cells.³⁰ EphA2 protein levels were disproportionately higher than EphA2 mRNA levels as cells progressed toward a more aggressive phenotype. Similar comparisons of tumor-derived cells revealed that EphA2 protein levels are 50- to 500-fold higher in some malignant cell models despite comparable levels of EphA2 mRNA.^{29,30}

With other RTKs, ligand stimulation promotes receptor internalization and degradation.⁵³ Since differential ligand stimulation distinguishes the EphA2 on non-trans-

formed *versus* malignant cells,²⁸ we postulated that decreased ligand-mediated degradation might have contributed to high levels of EphA2 on tumor cells.^{30,52} To test this, we used monoclonal antibodies or an engineered ligand, EphrinA1-Fc. Both treatments restore EphA2 activation on tumor cells independent of their need for stable cell-cell contacts.^{28,29,50,54,55} Each stimulus restored EphA2 activation and caused subsequent degradation of EphA2 protein.^{52,55} The activated EphA2 was degraded by both lysosomal and proteosomal mechanisms, and chemical inhibitors of those pathways prevented EphA2 degradation.⁵²

Further support for the concept that EphA2 accumulates on the surface of malignant cells was provided by studies of EphA2-associated phosphatases. For example, overexpression of LMW-PTP decreased the phosphotyrosine content of EphA2, which caused EphA2 to accumulate on the surface of malignant cells.³⁹ Despite the fact that this EphA2 was not itself tyrosine-phosphorylated, it retained enzymatic activity, which was consistent with the fact that EphA2 expression was necessary for LMW-PTP-mediated malignant transformation.

To decipher the mechanisms governing EphA2 protein stability, we asked if ligand stimulation would cause EphA2 to interact with downstream adapter proteins that might promote EphA2 degradation.⁵² We focused on the Cbl adapter protein based on evidence linking c-Cbl with the degradation of other receptor tyrosine kinases.^{56–58} Antibody-cross-linking or EphrinA1-Fc increased the association of EphA2 with c-Cbl in both malignant and non-transformed cells.⁵² Moreover, overexpression of c-Cbl decreased EphA2 protein levels, presumably by increasing the efficiency of protein degradation. In the converse experiment, dominant negative forms of Cbl (v-Cbl and 70Z-Cbl) inhibited EphA2 protein degradation.

The molecular basis of EphA2-Cbl binding is presently unclear. EphA2 contains a consensus binding site for c-Cbl at tyrosine 813 (YXXXP).²² However, conservative mutations of this site do not prevent ligand-mediated degradation (J. Walker-Daniels, unpublished). Rather, c-Cbl may interact with EphA2 through a different site or via an intermediate. Consistent with this, EphA2 was recently shown to interact with GRB2, which can interact with c-Cbl to mediate ubiquitination and degradation of other receptor tyrosine kinases.^{59,60}

Our studies have used a soluble ligand, whereas EphA2 normally is stimulated by a membrane-anchored ligand.⁸ Thus, it is formally possible that differences in cellular organization or signaling might distinguish artificial EphA2 stimulation in malignant cells from its normal function(s). Future analysis will be necessary to investigate these unanswered questions.

Biochemical Consequences of EphA2 Signaling

Recent investigation has greatly expanded the understanding of EphA2 protein interactions and the downstream biochemical and biological consequences of EphA2 signaling. Ligand stimulation has several conse-

quences on the biochemical properties of EphA2; notable among these are increased phosphotyrosine content and protein-protein interactions.²⁶

Ligand stimulation increases the phosphotyrosine content of EphA2 on tumor cells.^{28–30,52,54,55,61} This outcome appears to result largely from receptor autophosphorylation, since ligand stimulation of enzymatically-deficient mutants of EphA2 fails to cause receptor autophosphorylation (M.S. Kinch, unpublished information). We cannot entirely exclude that other tyrosine kinases might also contribute to EphA2 phosphotyrosine content. For example, EphA2 autophosphorylation of certain sites could create docking proteins for other kinases that subsequently phosphorylate additional sites, such as has been reported with the Src-mediated phosphorylation of the EGF receptor.^{62,63} In this context, it is intriguing that phosphorylated EphA2 interacts with the Src-like adapter protein (SLAP), whose SH2 domain is highly homologous to the SH2 domain of c-Src.⁶⁴

Tyrosine-phosphorylated EphA2 interacts with several proteins via interactions with SH2 or PTB domains (Figure 1). These interactions include binding to Shc, Grb2, SHP-2, SLAP, and PI 3-kinase.^{26,52,54,61,64} The downstream consequences of these interactions include direct induction of the enzymatic activities of PI 3-kinase and SHP-2.^{26,54} In addition, tyrosine-phosphorylated EphA2 interacts with SHC, which in turn binds GRB2 to stimulate intracellular signaling and nuclear translocation of ERK kinases.⁶¹ In doing so, EphA2 rapidly translates signals from the cell surface to the nucleus.⁶¹ At first glance, these results seem to contradict a published study, which showed decreased ERK activity following Eph kinase stimulation.⁶⁵ However, this earlier study used reagents that cross-reacted with multiple EphA family members and these differences, along with other technical features (eg, choice of cell system, timing of treatment) appear to be responsible for the differences between the two reports.⁶¹

Cellular Consequences of EphA2 Signaling

Ligand stimulation of EphA2 causes powerful changes in tumor cell behavior. Activated EphA2 negatively regulates tumor cell growth, survival, migration, and invasion (Figure 2).^{28,29,34,54,55} The biological consequences of EphA2 stimulation seem to be most apparent when modeled using three-dimensional assay systems.⁵⁵ For example, artificial stimulation of malignant cells with EphrinA1-Fc or specific monoclonal antibodies prevents tumor cell colonization of soft agar.^{29,55} These same conditions cause highly aggressive tumor cells to adopt a more benign and differentiated phenotype when suspended within reconstituted three-dimensional basement membranes, such as Matrigel.⁵⁵ Notably, ligand or antibody stimulation does not alter two-dimensional (i.e., monolayer) growth or differentiation.⁵⁵ Similarly, ectopic overexpression of EphA2 in non-transformed epithelial cells does not increase their growth or invasiveness in monolayer culture but dramatically increases the growth and invasiveness of these same cells when measured using three-dimensional assays (soft agar or Matrigel) or

in vivo.²⁹ These results are consistent with the emerging concept that three-dimensional modeling can provide additional information about cell behavior that may not be conveyed by monolayer systems.⁵⁵

The mechanisms by which EphA2 can regulate cell behavior remain largely unknown, although a variety of different studies lead us to speculate that EphA2 regulates tumor cell interaction with its local microenvironment. For example, ligand or antibody stimulation of EphA2 causes a rapid and dramatic decrease in cell-extracellular matrix attachments.^{28,54} The longer-term consequences of EphA2 stimulation include decreased production of vital cell-adhesion proteins, which perpetuates the decrease in cell-extracellular matrix adhesions (M.S. Kinch et al, unpublished).

EphA2 and Melanoma Cell Vasculogenic Mimicry

In order for tumors to grow and metastasize, they must obtain a blood supply, which is commonly believed to occur by way of angiogenesis.⁶⁶ Vasculogenic mimicry has recently been described as a process whereby highly aggressive melanoma cells, but not poorly aggressive melanoma cells, form vasculogenic-like networks when cultured on three-dimensional matrices through a process that is reminiscent of embryonic vasculogenesis.^{67,68} These vasculogenic-like networks may provide an additional means for tumor perfusion and potentially contribute to tumor metastasis.^{69–71} EphA2 has been recently identified as an important mediator of vasculogenic mimicry *in vitro*.³⁴ Both highly aggressive uveal and cutaneous melanoma cells, but not poorly aggressive melanoma cells, express EphA2.^{34,67} Moreover, down-regulation of EphA2 expression, using anti-sense oligonucleotide technology, inhibits vasculogenic mimicry by aggressive melanoma cells.³⁴

The importance of EphA2 in mediating vasculogenic mimicry has sparked investigation into the downstream signaling pathways that are activated by EphA2 and which contribute to the formation of vasculogenic-like networks. Recent work has implicated VE-cadherin, a vascular endothelial cadherin expressed by the aggressive melanoma cells, as an important regulator of EphA2 function.^{72,73} VE-cadherin is also a critical component in melanoma vasculogenic mimicry, and thus, the linkage of EphA2 and VE-cadherin in the formation of vasculogenic-like networks by tumor cells may provide an important new pathway for therapeutic intervention.^{72,73} EphA2 may also interact with cellular signaling molecules such as PI 3-kinase and focal adhesion kinase (FAK) during vasculogenic mimicry.⁷³ By decreasing EphA2 expression using anti-sense oligonucleotides, there is a subsequent decrease in FAK phosphorylation during vasculogenic mimicry, which is notable since this effector is important for the formation of vasculogenic-like networks.⁷³ Furthermore, inhibition of PI 3-kinase by LY294002 (a PI 3-kinase-specific inhibitor) results in complete mitigation of vasculogenic mimicry, thus suggesting a possible relationship between PI 3-kinase and EphA2, which are two critical components of tumor cell

Figure 3. Model for the signal transduction pathways activated by EphA2 and VE-cadherin resulting in vasculogenic mimicry. Calcium-dependent homotypic binding of VE-cadherin molecules on adjacent tumor cells promotes the recruitment of EphA2 to the membrane. Once at the membrane, EphA2 is able to interact with its membrane-bound ligand, EphrinA1, resulting in phosphorylation on tyrosines. Phosphorylated EphA2 recruits both FAK and PI 3-kinase, either directly or indirectly, thus activating these two important cytoplasmic protein kinases. This signaling complex then activates other unidentified signaling molecules, which act in concert to promote vasculogenic mimicry *in vitro*.

vasculogenic mimicry. (A.R. Hess, unpublished observation). Therefore, EphA2 may activate PI 3-kinase and FAK, via direct or indirect mechanisms, thus facilitating the formation of vasculogenic-like networks by highly aggressive melanoma cells.^{67,68}

A hypothetical signaling model representing a potential signaling pathway initiated by EphA2 and critical for vasculogenic mimicry is presented in Figure 3. In this model, VE-cadherin mediates the recruitment of EphA2 to the cell membrane, where it may interact with its membrane-bound ligand EphrinA1, resulting in phosphorylation of the receptor. This step initiates the cascade, involving activation of both PI 3-kinase and FAK, leading to the formation of vasculogenic-like networks. Collectively, these data provide the first signaling pathway characterizing key molecules involved in tumor cell vasculogenic mimicry.

Therapeutic Opportunities

The prevalence of EphA2 overexpression and functional changes in cancer may provide a much-needed target for therapeutic intervention. In particular, much recent interest has focused on approaches to mimic the actions of EphA2 ligands (ephrins) using monoclonal antibodies and peptide-based mimetics.^{55,74} These reagents increase the phosphotyrosine content of EphA2 as measured *in vitro*.^{55,74} In the case of EphA2 monoclonal antibodies, the mechanism of action appears to require receptor degradation,⁵⁵ which is consistent with evidence that antisense-based targeting of EphA2 can also inhibit the growth and invasiveness of malignant cells.^{34,55} Altogether, these properties may provide a powerful means for selective targeting of the many cancers that overexpress EphA2.

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