THE ROLE OF EPHA2 RTK IN BREAST CANCER CELL MALIGNANCY AND TUMOR ANGIOGENESIS

By

Wei Bin Fang

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Approved:

Prof. Albert B. Reynolds

Prof. Ann W. Richmond

Prof. Steven K. Hanks

Prof. Jin Chen

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LIST OF ABBREVIATIONS

2D-DIGE	Differential gel electrophoresis
2D-TLC	2-dimensional thin layer chromatography
aa	amino acid
DMEM	Dulbecco’s modified Eagle’s media
E-cadherin	epithelial cadherin
ECD	extracellular domain
ECM	extracellular matrix
Eck	epithelial cell receptor tyrosine kinase
ECL	enhanced chemiluminscence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EphA2	erythopoietin producing hepatoma cells A2
Ephexin	Eph exchange interacting protein
Erk	extracellular signal-regulated kinase
FAK	Focal Adhesion Kinase
FBS	fetal bovine serum
GPI	glycosylphosphatidylinositol
h	hours
IgG	immunoglobulin G
IRES	internal ribosomal entry site

LMW-PTP	Low Molecular Weight Phosphatase
LC-MS	liquid chromatography-mass spectrometry
MAPK	mitogen activated protein kinase
Min	minutes
N-cadherin	neural cadherin
NP-40	noniodet P-40
p120	p120 catenin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	Platelet Derived Growth Factor
PI3K	phosphoinositide 3-kinase
PVDF	polyvinylidene fluoride
ROCK	Rho-associated kinase
RT	room temperature
RTK	receptor tyrosine kinase
SAM	sterile alpha motif
SDS	sodium dodecyl suflate
SH2	src homology 2 domains
siRNA	small interfering RNA
TBS	tris buffered saline
VE-cadherin	Vascular Endothelial cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

WT	wild type

CHAPTER I

INTRODUCTION

The Eph family is the largest subfamily of receptor tyrosine kinases, with 14 members. Since the first *eph* gene was cloned in 1987 (1), the Eph family of receptor tyrosine kinases (RTK) has been implicated in many physiological and pathological processes. The first Eph receptor identified, EphA1, was found to be expressed in erythropoietin producing hepatoma cells (eph). The Eph family of receptors can be divided into two classes, an EphA class and an EphB class based on the sequence homology and binding affinity to the ephrin ligands. There are at least nine EphA receptors which generally bind with different affinities to five ephrinA ligands. There are five EphB receptors which interact with three ephrinB ligands. There are exceptions, EphA4 can also interact with ephrinB2 and ephrinB3, and EphB2 can also interact with ephrinA5 (Fig 1). One unique feature of Eph/ephrin signaling is that both the receptor and ligand are membrane bound, making it possible to have bi-directional signaling. EphrinA proteins are anchored to the plasma membrane through glycosylphosphatidylinositol linkage (GPI), while ephrinB proteins contain a transmembrane domain and cytoplasmic tail. When the Eph receptor interacts with an ephrin ligand on a neighboring cell, the Eph receptor clusters and trans-phosphorylates an adjacent Eph receptor to transduce a cellular signal (forward signaling). Eph/ephrin interactions also cause clustering of ephrin ligands on neighboring cells to activate reverse signaling [reviewed in(2, 3)].

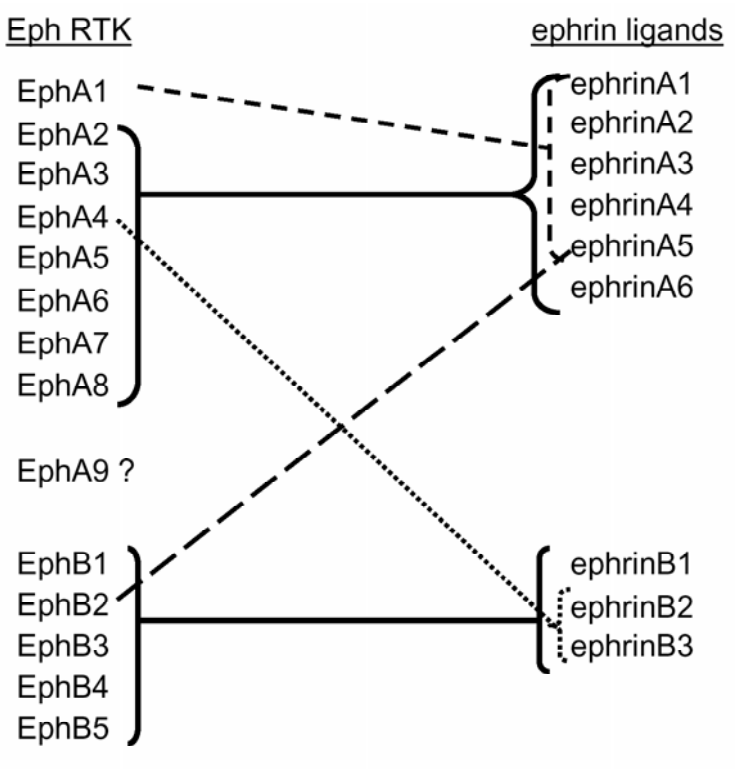


Figure 1. Eph family of receptor tyrosine kinases and ephrin ligands. There are nine EphA receptors and five EphB receptors. Generally, EphA receptors interact with ephrinA ligands and EphB receptors binds ephrinB ligands. There are exceptions, as EphA4 can bind ephrinB2/3 and EphB2 can bind ephrinA5.

Similar to other RTKs, the Eph receptors contain an extracellular, transmembrane, and cytoplasmic domain. The extracellular portion of the Eph receptor consists of three distinct regions: a ligand binding domain, a cysteine rich region, and fibronectin repeats (Fig 2)(4-7). Interestingly, it has been reported that each of these domains is capable of interacting with ephrin ligands. The ligand binding domain binds ephrins with high affinity. The cysteine rich region can also associate with ephrins but with a lower binding affinity, and promotes clustering of the receptor (5, 8, 9). The fibronectin repeats have been reported to bind ephrins as well, but only in a cis-manner when both the receptor and ligand are on the same cell, and is also believed to facilitate receptor clustering. The cytoplasmic portion of Eph is divided into three distinct regions: juxtamembrane region, the kinase domain, and SAM domain (Fig 2) (2, 10). Phosphorylation of tyrosines within the cytoplasmic region is critical in Eph kinase activity and interactions with signaling proteins. This will be discussed further in relation to EphA2 signaling.

EphA2 function in angiogenesis

One Eph family member, EphA2, has been shown to be critical in many physiological and pathological processes. EphA2 was identified from a screen of epithelial cells for conserved receptor tyrosine kinases in 1990, original termed Epithelial Cell Receptor Protein-Tyrosine Kinase (eck) (11). Since its discovery, there has been a wealth of research on EphA2 signaling and its role in development and disease.

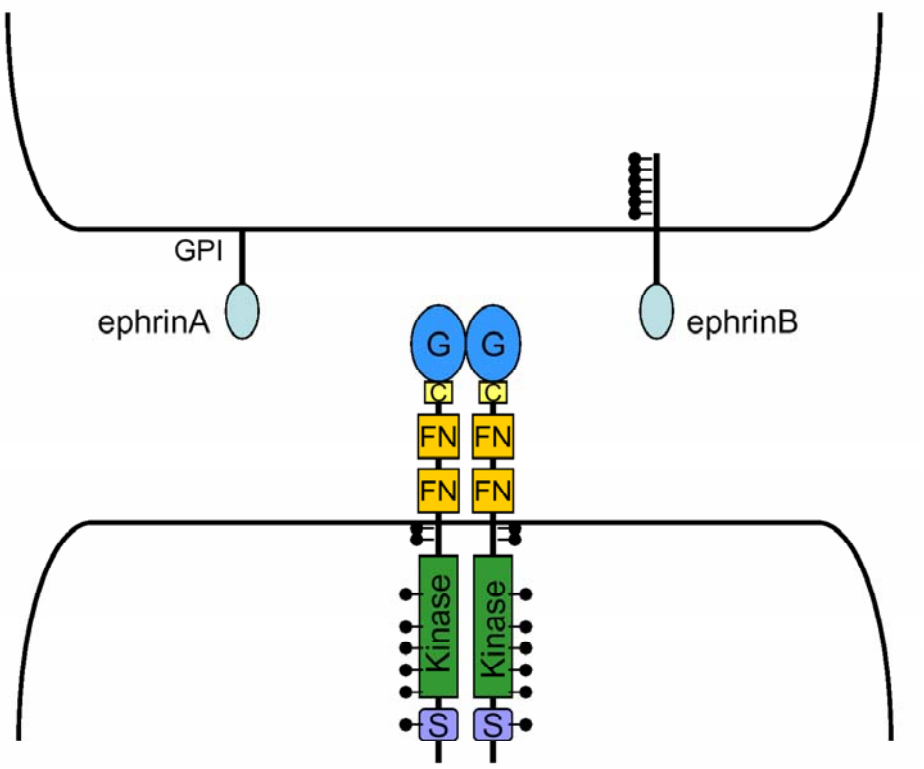


Figure 2. Eph receptor and ephrin ligand structures. The Eph receptor has three conserved extracellular domains: a globular/ephrin binding domain (G), a cysteine rich region (C), and two fibronectin repeats. The intracellular region of the receptor contains a juxtamembrane region, a kinase domain, and a sterile alpha motif (S). The ephrinA ligands are tethered to the membrane by a glycosylphosphatidylinositol (GPI) linkage, while ephrinB ligands contain a cytoplasmic tail. Eph receptors and ephrinB ligands contain numerous tyrosines within the cytoplasmic region that serve as potential phosphorylation and protein binding sites (●–).

Blood vessel formation is a fundamental process, that is categorized into two types: vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* synthesis of blood vessels. The process of vasculogenesis involves differentiation of angioblasts, originally derived from mesoderm, into endothelial cells. The endothelial cells assemble into a luminal network that undergoes remodeling during vasculogenesis. Recruitment of supporting cells such as pericytes and smooth muscle cells forms the mature and functional blood vessel (12). Angiogenesis, by contrast, involves sprouting of new blood vessel branches from pre-existing vasculature and this process also involves endothelial cell proliferation, migration, tubulogenesis, and recruitment of perivascular supporting cells (12, 13). Several of the Eph receptors are reported to be expressed in the vascular system. EphrinB2 is expressed in arterial endothelial cells and EphB4 is expressed almost exclusively on venous endothelial cells (14). These were among the first markers to distinguish arteries from veins, but it is unclear what the functional relevance is of this arteriovenous-specific expression pattern. Targeted disruption of either molecule results in embryonic lethality, at E10.5. These mice exhibit defects in vessel remodeling. Inactivation of either gene affected formation of both arteries and veins (14-16). EphrinB1 and EphB1 were detected in the developing kidney endothelium (17). Similar to EphB4, EphB3 is predominantly expressed in veins (15). EphB2 expression was detected in embryonic CNS, heart, and peripheral nerves (15, 18). EphrinB2 and EphB2 expression is also found in mesenchymal supporting cells (15). Inactivation of EphB2 or EphB3 alone in mice did not reveal any overt phenotype. In EphB2 /EphB3 double knockout mice, about 30% of these animals displayed a vessel defect and were embryonic lethal (15). In

situ hybridization of ephrinA1 exhibited expression in mesoderm and pre-endocardial cells at E7.25, the dorsal aorta at E8.5, the primary head vein, intersomatic vessels, and limb bud vasculature at E9.5 (19). EphA receptor expression was detected in many blood vessels, such as the umbilical vein and the blood vessels lining the aorta (20). These results would suggest that the Eph family of receptors play a critical function in vascular development.

In adult tissue, Eph receptors also play a pivotal role in angiogenesis. EphA2, in particular, has been linked to pathological angiogenesis, including tumor neovascularization. The first functional evidence that EphA2 participates in angiogenesis came from work in corneal angiogenesis. Soluble EphA2-Fc was able to suppress ephrinA1 or VEGF induced endothelial cell survival, migration, and corneal angiogenesis (21). Similarly, inhibition of EphA2 expression with EphA2 antisense oligonucleotides also suppressed ephrinA1 and VEGF induced migration of endothelial cells (21). EphA2 signaling has also been demonstrated to be important in tumor angiogenesis. Tumor xenografts of MDA-MB-435 human breast cancer cells or KS1767 Kaposi's sarcoma cells in nude mice demonstrated that both EphA2 and ephrinA1 were expressed in endothelial cells and tumor cells (22). Soluble EphA2-Fc receptor treatment suppressed tumor growth and tumor angiogenesis in murine 4T1 mammary carcinomas (23). Endothelial cells deficient in EphA2 expression showed impaired migration in response to 4T1 breast cancer cells (24). EphA2 deficiency also displayed defects in endothelial cell assembly and migration in response to ephrinA1 (25). EphrinB2 and EphB4 have also been reported to be involved in angiogenesis. Stimulation of endothelial cells with either soluble dimeric

forms of ephrinB ligand or EphB receptor promoted angiogenesis (15, 26). These results would suggest that both forward signaling, through the receptor, and reverse signaling, though the ligand, play essential roles in angiogenesis. EphrinB2 expression has also been detected in tumor vasculature in many types of cancer. Expression of the extracellular domain (ECD) of EphB4 on tumor cells has been reported to increase tumor growth due to its effects on tumor vascularization (27, 28). These data suggest that Eph receptors and the ephrin ligands play a pivotal role in cancer, at least in part by tumor angiogenesis. I identified several tyrosines that are phosphorylated on EphA2 in vascular endothelial cells. The phosphorylated tyrosine residues are important in mediating EphA2 interaction with signaling molecules involved in endothelial cell function. These data are presented in Chapter V.

EphA2 in Cancer

EphA2 has been investigated in many types of cancers including prostate, colon, lung, colorectal, cervical, ovarian, and breast cancer. Generally, these studies found that EphA2 expression correlated with tumor progression. EphA2 was reported to be upregulated by as much as 60-92% in clinical breast cancer specimens [Brantley-Sieders and Chen, unpublished data; (29, 30)]. Interestingly, an independent group reported that EphA2 expression was increased in both breast tumors as well as tumor endothelium (22, 23). In addition, high EphA2 expression was detected in 93% of prostate cancer specimens (31), 88.8% of cervical cancers (32), 74.1% of non-small cell lung carcinomas (33), and 92.8% of renal cell carcinomas (34). These studies indicate that EphA2 is overexpressed in many types of human cancers.

Several studies have shown that EphA2 is upregulated in both tumor cells and tumor associated endothelium. In a study by Kataoka et al, they found that EphA2 was expressed in colorectal tumors and tumor microvessels (35). Ogawa et al reported that EphA2 was upregulated in tumor cells and tumor vasculature in breast cancer (22). These data are consistent with previous reports that indicate EphA2 plays an important role in angiogenesis. Overall, the data would suggest that EphA2 may have a role in both tumor cells and tumor microenvironment.

EphA2 is believed to play an important role in tumor progression, as EphA2 expression has been correlated with tumor malignancy. Elevated EphA2 expression was correlated with disease stage in colorectal, lung, ovarian, and renal cancers (33-36). High EphA2 expression correlated with poor patient survival in cervical cancer, ovarian cancer, and melanomas (32, 37). Several studies have also shown that elevated EphA2 expression correlated with tumor metastasis. In non-transformed MCF-10A mammary epithelial cells, overexpression of EphA2 was sufficient to transform those cells (30). Overexpression of EphA2 in these cells induced colony formation in soft agar, increased invasion in Matrigel, and, when implanted in mice, formed tumors (30). In pancreatic cancer cells, overexpression of EphA2 was found to increase resistance to anoikis (38). Conversely, downregulation of EphA2 levels by siRNA, knockdown by antisense oligonucleotides, or antibody mediated degradation resulted in a decrease in tumorigenicity in cancer cell lines. Knockdown of EphA2 levels in several pancreatic cancer cells by siRNA, resulted in decreased Matrigel invasion, increased anoikis, and increased apoptosis (38). Treatment with EphA2-Fc, which binds and sequesters the ephrinA ligand, prevents endogenous

EphA2 activation and impairs tumor progression in vivo. Inhibition of EphA2 activation by EphA2-Fc treatment has been reported to inhibit tumor growth in mouse models of breast cancer and pancreatic cancer (23, 39). Interestingly, it has been reported that although EphA2 is overexpressed in many types of cancers, the EphA2 receptor is underphosphorylated in these cases (40). However, the underphosphorylated EphA2 receptor still retains kinase activity (40). These data would suggest that EphA2 plays an important role in tumor progression. Although, it remains unclear whether EphA2 kinase activity is essential for this role. The work in my dissertation shows EphA2 kinase functions to promote tumor progression. These data are presented in Chapter III.

EphA2 Signaling

EphA2 plays an important role in cellular function as indicated by its ubiquitous expression. Unlike other receptor tyrosine kinases, EphA2 is primarily thought to regulate cell adhesion, cell migration, and cell survival, although EphA2 forward signaling has been shown to inhibit tumor cell proliferation in a number of cancer cell lines (41, 42). EphA2 activation can promote multiple signaling pathways (Fig 3). Two major common downstream signaling pathways that are influenced by EphA2 signaling are the Rho family of GTPases and Ras-Raf-MAPK pathways.

EphA2 and the Rho family of GTPases

Eph signaling has been shown to affect cell morphology and cell migration. The Rho family of GTPases are a class of molecules that have emerged as downstream

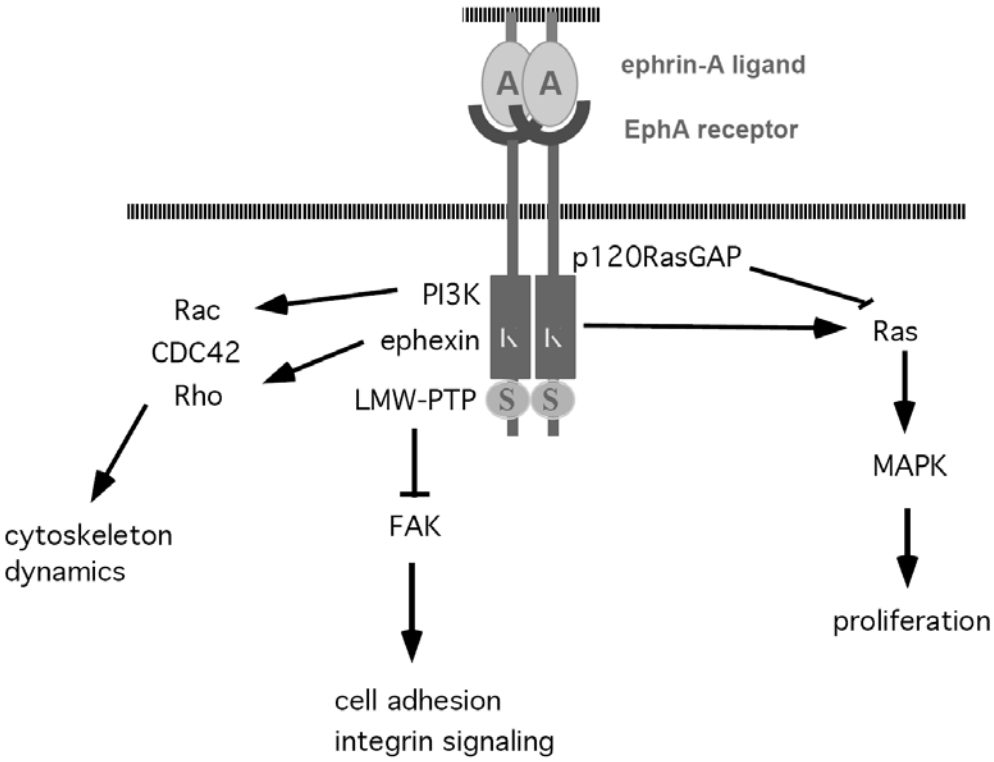


Figure 3. EphA receptor signaling. Many proteins have been currently identified to interact with the Eph receptors. Many of the proteins converge on similar signaling cascades. Some of the common downstream signaling pathways of Eph receptor activation are activation or inhibition of the Rho family of GTPases, activation or inhibition of the Ras-MAPK pathway, and downstream effectors of FAK signaling.

signaling proteins of Eph receptor signaling. Rho proteins cycle between an inactive (GDP bound) and active (GTP bound) conformation. Cycling between GTP-bound Rho and GDP-bound Rho is catalyzed by two classes of proteins, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Rho GTPases regulate cell shape and cell movement by regulating actin cytoskeleton dynamics (43-45). The three most well characterized members are: RhoA, Rac1, and Cdc42. RhoA promotes the formation of stress fibers and focal adhesions. Rac1 controls the formation of lamellipodis and Cdc42 regulates filopodia formation (46). Activation of the Eph receptors has been shown to activate or repress Rho GTPases depending on the cell type.

In neuronal cells, Rho activation stimulates growth cone collapse and inhibits axon regeneration (47-49). It has been reported that Eph receptor activation promotes cell repulsion in the developing nervous system, which mediates growth cone collapse (50). For example, activation of EphA2 receptors in retinal ganglion cells promotes growth cone collapse. This process is mediated by ephrinA5 treatment. Stimulation of these cells with ephrinA5 ligand, increases levels of GTP bound RhoA and induces activation of Rho-associated kinase (ROCK), a downstream effector of RhoA (50). Although levels of activated RhoA are increased, the levels of activated Rac1 are decreased(50). The Eph mediated activation of Rho family GTPases is not restricted to neuronal cells. In vascular smooth muscle cells, stimulation with ephrinA1 resulted in RhoA activation and stress fiber formation suggesting that ephrinA1 might regulate vascular contractility (51). Consistent with the results from neuronal cells, activation of RhoA and inactivation of Rac was observed in the

vascular smooth muscle cells (52). In endothelial cells, activation of EphA2 leads to the activation of Rac1 and not RhoA, and was found to be important in endothelial cell assembly and migration (25). These data would suggest that Eph receptor signaling may have differential effects based on cell type. However, it appears that Rho family GTPase signaling is a pivotal pathway downstream of Eph activation.

There are several ways by which the Eph receptors can influence Rho activation. RhoGEFs are proteins that catalyze the exchange of GDP for GTP on the Rho protein. There have been several RhoGEFs that have been characterized to interact with EphA receptors (Table 1). One of the first RhoGEFs identified as interacting with EphA4 was Ephexin. Ephexin is primarily expressed in the nervous system, but is also expressed at lower levels in the kidney, liver and testes (53, 54). Ephexin has been reported to activate RhoA and to a lesser extent Cdc42, but not Rac1. Although, overexpression of Ephexin in neuronal cells enhanced RhoA activation, Cdc42 activation, and only moderately activated Rac1 (54, 55). Another member of the Ephexin family of exchange factors also interacts with EphA4. Vsm-RhoGEF is closely related to Ephexin. Ephexin and Vsm-RhoGEF both contain tandem Dbl homology and pleckstrin homology domains. However, Vsm-RhoGEF is only expressed in vascular smooth muscle cells. Vsm-RhoGEF activates RhoA, but inactivates Rac1 (51, 52). Another family of RhoGEFs that associates with EphA2 is the Vav family exchange factors. The association of Vav3 with EphA2 participates in endothelial cell function, as Vav2/3 deficient endothelial cells fail to migrate or assemble in response to ephrinA1 treatment (56). The defect in migration and assembly is at least in part due to defective Rac1 activation (56).

Interestingly, Ephexin has been shown to interact with EphA4 in the kinase domain (54). However, the association of Vav3 with EphA2 occurs within the juxtamembrane domain of EphA2 (56). It is currently unclear how the association of these RhoGEFs with the Eph receptors affects Rho activation. One possibility is that it localizes the exchange factors at the cell membrane to produce localized activation of the Rho proteins. Another possibility is that the Eph receptors can phosphorylate these RhoGEFs to affect its catalytic activity. EphA4 has been reported to directly phosphorylate Vsm-RhoGEF (51). In the case of Vav2, ephrinA1 stimulation of neuronal cells induced tyrosine phosphorylation of Vav2 (57). The signaling pathways that mediate these effects are not completely known. It has been reported that SHIP2, a phosphatase, can influence Rho activation by production of lipid products to relieve the inhibition of the RhoGEF's catalytic activity. Zhuang et al demonstrated SHIP2 can associate with EphA2. SHIP2 interaction with EphA2 increased in PI-3kinase activity, which ultimately lead to an increase in Rac1 activation (58). CrkII has been shown to interact with activated EphA3 receptor in 293T cells overexpressing EphA3 (59). Stimulation of EphA3 with ephrinA5 caused RhoA activation, retraction of cellular protrusions, and decreased cell adhesion (59). It remains unclear how Eph receptor signaling can activate or inactivate the Rho family of GTPases. I identified several phosphorylated tyrosine residues that are important in mediating interactions with several of these proteins and the functional relevance of these interactions. The data are presented in Chapter V.

EphA2 and MAPK pathway

Another prominent pathway that has been shown to be downstream of EphA2 signaling is the MAPK pathway. Interestingly, until recently it was generally believed that Eph receptors do not regulate cell proliferation. However, several different publications have demonstrated that Eph receptor signaling can influence the MAPK signaling cascade. As there have been published reports of EphA2 signaling activating, as well as inhibiting MAPK activation, it remains to be determined exactly how EphA2 is linked to the MAPK pathway.

One of the first studies showing the relationship between EphA signaling and MAPK activation was performed using several different cell types. This study demonstrated ephrinA1 treatment of these cells inhibited Erk activation. In prostate epithelial cells (PRNS-1-1 and PC-3), mouse embryonic fibroblasts, and bovine endothelial cells, ephrinA1 treatment of these cells rapidly reduced Erk phosphorylation (41). This affect was likely mediated by inhibition of Ras-Raf signaling. Activation of EphA resulted in a decrease in activated Ras and could be compensated for by overexpression of Ras (41). In several recent publications, EphA2 has been reported to activate MAPK signaling. Pratt et al reported that MDA-MB-231 human breast cancer cells treated with ephrinA1-Fc induced Erk phosphorylation (60). These results were confirmed in several other breast cancer cell lines and also in PC-3 prostate cancer cells. The activation of the MAPK kinase cascade in this situation was mediated by SHC and Grb2 association with the EphA2 receptor, as expression of a dominant negative SHC could inhibit MAPK activation (60). Interestingly in a recent report, Macrae et al reported that the Ras-Raf-MAPK

pathway could upregulate transcription of EphA2 (42). Analysis of mRNA derived from NIH3T3 cells that expressed a constitutively activated Raf revealed that EphA2 was a target of Ras-MAPK activation, and these results were also confirmed by protein analysis. EphA2 signaling can attenuate EGF mediated Erk activation in several different breast cancer cell lines. Interestingly, analysis of a wide array of breast cancer cell lines revealed an inverse relationship between EphA2 and ephrinA1 expression (42). In general, malignant breast cancer cells that lack E-cadherin expression exhibited high expression of EphA2, while the highest level of ephrinA1 expression were found on cells that retained epithelial cell characteristics. Recent studies from our laboratory revealed that EphA2 cooperates with Neu/ErbB2 receptor tyrosine kinase to enhance the activity of both RhoA and Ras/MAPK in tumors and cells derived from MMTV-Neu transgenic mice, as well as in MCF-10A cells overexpressing the human homolog of ErbB2, Her2 (61).

EphA2 signaling has been clearly demonstrated to affect MAPK activation. However, it uncertain how EphA2 may affect activation, as in the case of breast cancer cells, or inhibition, in prostate cancer cells, of MAPK. One possible rationalization between these two opposing observations is that EphA2 signaling could mediate both activation and inhibition of MAPK. The signaling pathways involved may be quite different, as SHC association with EphA2 resulted in activation of MAPK, while association of p120 RasGAP could mediate inhibition of MAPK activation as observed in EphB2 (62). One of the aims of this study is to identify the different phosphorylated tyrosine sites on EphA2 that could interact with different signaling molecules, so as to provide the molecular and biochemical tools to

address these questions within the field. I identified several phosphorylated tyrosine residues that are important in mediating interactions with different signaling proteins and the functional relevance of these interactions. The data are presented in Chapter V.

EphA2 signaling and cell-cell adhesion

One of the hallmarks of cancer progression and invasiveness is downregulation of E-cadherin. As mentioned earlier, Macrae et al reported that EphA2 expression inversely correlated with E-cadherin expression in breast cancer cells (42). There have been several studies that have linked EphA2 signaling to cell-cell adhesion molecules.

One of the first studies that demonstrated a relationship between EphA2 and E-cadherin showed that EphA2 and E-cadherin colocalized at sites of cell-cell contacts. Disruption of cell-cell contacts by treatment with a calcium chelator dramatically reduced EphA2 phosphorylation (40). This study demonstrated that although EphA2 is upregulated in many cancer cells, it is underphosphorylated in comparison to normal mammary epithelium. This study suggests that loss of E-cadherin could mediate this effect in cancer cells. Orsulic et al reported that loss of E-cadherin could downregulate EphA2 mRNA and protein levels, and this could be rescued by re-expression of E-cadherin (63). Overexpression of E-cadherin in NIH3T3 cells regulated the localization of EphA2 to the surface membrane and overexpression of a dominant negative E-cadherin construct in HT-29, colon cancer cells, would relocalize EphA2 into a perinuclear region (63). These studies identify the functional

dependence of EphA2 on E-cadherin expression. It is unclear whether EphA2 can affect the adherens junction proteins, i.e. E-cadherin, β-catenin, α-catenin, and p120 catenin. Since E-cadherin and EphA2 colocalize to the plasma membrane, it is possible that these proteins can directly interact. However, Zantek et al and Orsulic et al were unable to show co-immunoprecipitation of these proteins with EphA2. In a recent publication, Potla et al reported that stimulation of HT-29 cells with ephrinA1-Fc could induce EphA2, E-cadherin, and β-catenin tyrosine phosphorylation (64). The significance in the increase phosphorylation of the adherens junction proteins are not known, tyrosine phosphorylation of these proteins could potentially disrupt cell-cell adhesion. Tanaka et al recently reported that EphA2 could directly associate with and phosphorylate Claudin-4 (65). The interaction and phosphorylation of Claudin-4 and EphA2 resulted in decreased interaction of ZO-1 with Claudin-4. The decreased association of Claudin-4 and ZO-1 resulted in a reduction in tight junction function and an increase in intracellular permeability of these cells (65). However, these results have not been observed for EphA2 and adherens junction proteins. In this dissertation, I show for the first time that EphA2 signaling through RhoA leads to weakening of cell-cell adhesion. The data are presented in Chapter IV.

Summary

Since its discovery, there has been a wealth of research on Eph signaling. Eph signaling affects many different signaling pathways. Ephrin ligand binding induces Eph receptor phosphorylation. Many phosphorylated tyrosines have been mapped to the juxtamembrane and kinase domain of the Eph receptor. These phosphorylated

tyrosines on the Eph receptor serve as docking sites for a number SH2/PTP domain containing proteins, such as Ras-GTPase-activating protein (RasGAP), Src family of tyrosine kinases, low molecular weight phosphotyrosine phosphatase (LMW-PTP), phospholipase Cγ, phosphatidylinositol 3-kinase, SLAP, Grb2, Grb10, and Nck (Table 1). One of the other goals of this dissertation was to dissect the signaling mechanisms underlying EphA2 phosphorylation for transduction of its cellular signal. To thoroughly address EphA2 signaling, it will be essential to identify important EphA2 phosphorylated tyrosine residues and to map the interactions of these signaling molecules to EphA2. I identified several phosphorylated tyrosine residues that are important in mediating interactions with several of these proteins and the functional relevance of these interactions are presented in Chapter V.

Table 1. EphA receptor interacting proteins.

Eph Receptor	Interacting protein	Eph binding region	Interacting domain	Reference
EphA2	c-Cbl	NT	NT	(66)
	FAK	NT	NT	(67)
	LMW-PTP	NT	NT	(68)
	P85β-PI3K	kinase	SH2	(69)
	SHP2	NT	NT	(67)
	SHIP2	NT	NT	(58)
	SLAP	NT	NT	(70)
	p120RasGAP	NT	NT	(71)
	Src	NT	NT	(72)
	SHC	NT	NT	(60)
	Vav3	juxtamembrane	SH2	(56)
EphA3	Abl	NT	SH2	(73)
	Fyn, Src, Yes	NT	SH2	(74)
	CrkII	juxtamembrane	SH2	(52)
EphA4	Abl	NT	NT	(73)
	Ephexin	kinase	DH-PH	(54)
	Vsm-RhoGEF	NT	DH-PH	(51)
	p59Fyn	juxtamembrane	SH2	(75)
	SHEP1	NT	SH2	(76)
	Src	NT	SH2	(74)
EphA7	AF6	PDZ	PDZ	(77)
	GRIP,PICK	PDZ	PDZ	(78)
EphA8	Fyn	juxtamembrane	SH2	(79)
	P110γ-PI3K	juxtamembrane	NT	(80)

Modified from (81).

CHAPTER II

MATERIALS AND METHODS

Antibodies and reagents. Antibodies used for immunoblot include anti-EphA2 (1:1000, Upstate Biotechnology), anti-phosphotyrosine (1:250, Santa Cruz Biotechnology), anti-myc (1 : 500; Cell Signaling Technology), anti-ephrin-A1 (1 : 500), anti-cleaved caspase-3 (1 : 100, Cell signaling), anti-PCNA (0.5 μg/ml, Neomarkers), anti-p190 RhoGAP (1:1000, Transduction Laboratory), anti-E-cadherin (0.1 μg/ml, Transduction Laboratory), anti-p120 (0.1 μg/ml, Transduction Laboratory), anti-β catenin (1:5000, Sigma), anti-α catenin (1:5000, Sigma), anti-tubulin (1:1000, Sigma), anti-phospho-Src (Y416) (1:1000, Cell Signaling Technology), anti-src (1:1000, Cell Signaling Technology), anti-Rac1 and anti-Cdc42 (1:1000; Transduction Laboratories), and anti-RhoA (1:200, Santa Cruz Biotechnology). Anti-LMW-PTP (1:2000) was a kind gift from Dr. Takamune Takahashi. Recombinant ephrinA1-Fc proteins were purchased from R&D Systems (Minneapolis, MN). ROCK inhibitor, Y27632, and Src inhibitor, PP2, were purchased from Calbiochem.

Immunoprecipitation of EphA2 from cell lysates was performed with anti-EphA2 antibody (2 μg; Upstate Biotechnology), and p85 was immunoprecipitated by anti-FLAG (M2)-agarose beads (Sigma). Recombinant ephrin-A1-Fc proteins were purchased from R&D Systems (Minneapolis, MN). Growth factor-reduced Matrigel

was purchased from BD Biosciences. Transient transfection was performed using Lipofectamine 2000 (Invitrogen).

Plasmids and viruses. EphA2/LZRS and EphA2-ΔC/LZRS were generated from existing plasmids. EphA2 was digested from EphA2/pcDNA3.0 with EcoRI. The EphA2 fragment was ligated into the LZRS EcoRI site. EphA2-ΔC/LZRS was generated by digesting EphA2-ΔC/pcDNA3.1/hisB with PmeI and EcoRI. The LZRS vector was digested with AfeI and EcoRI and EphA2-ΔC was ligated into LZRS. P190 RhoGAP/LZRS and p190 30-1/LZRS were described previously. LMW-PTP C12S/pBabe and LMW-PTP/pGEX were subcloned from plasmids that were described previously. Adenovirus expressing consitutively active RhoA (Q63L) was purchased from Cell Biolabs (San Diego, CA). Adenoviruses expressing control β-galactosidase was described previously.

EphA2 Mutagenesis. EphA2 mutations were generated by PCR amplification using EphA2 specific primers containing tyrosine to phenylalanine mutations (mutations shown in lower case). The fragments were digested with AgeI and BsiWI (for tyrosine mutations 593-846) and BamHI and BlpI (for tyrosine mutations 921-959) and ligated into the digested plasmids pcDNA3.0-EphA2 and LZRS-EphA2. All mutations were verified by DNA sequencing. The dominant-negative EphA2 receptor mutant, W42-EphA2, carries a D738N point mutation that is analogous to that identified in the spontaneously occurring dominant-negative W42 mutant allele of c-kit (Reith et al., 1990). Mutation of this conserved residue has previously been shown

to confer dominant-negative signaling properties in c-Kit and other RTKs in cell-based assays. The myc-tagged-dominant negative EphA2 cytoplasmic domain truncation mutant (EphA2-DeltaC) was generated by PCR amplification of the extracellular and transmembrane domain of EphA2 and subcloned into pcDNA 3.1 (Invitrogen).

Cell culture and retroviral infection. MCF-10A is a spontaneously immortalized, but non-transformed human mammary epithelial cell line that was derived from the breast tissue of a patient with fibrocystic changes. MCF-10A cells exhibit numerous features of normal breast epithelium, including the lack of tumorigenicity in nude mice, the lack of anchorage-independent growth, and the dependence on growth factors and hormones for proliferation and survival. MCF-10A cells were obtained from American Type Culture Collection and cultured in 5% horse serum (Hyclone), 20 ng/ml EGF (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml Cholera Toxin (Calbiochem), 10 μg/ml insulin (Sigma), and 1% penicillin-streptomycin (Life Technologies, Inc) in 50:50 DMEM/F12. MCF-10A cells were infected with LZRS retroviruses co-expressing EphA2 (wildtype or ΔC)-IRES-GFP and FACS sorted for comparable EphA2 receptor levels.

Endothelial Cell Culture and Retroviral Infection—Wild-type or EphA2-deficient primary murine pulmonary microvascular endothelial cells were isolated from 1-3-month-old mice derived from H-2Kb-tsA58 transgenic "Immorto-mouse" background. These cells were grown at 33 °C in EGM-2 medium supplemented with

interferon-γ (10 ng/ml), a permissive condition that allows the expression of SV40 T-antigen (TAg). The EphA2-deficient endothelial cells were infected with LZRS retroviruses co-expressing IRES-EphA2 (wild-type or mutant)-green fluorescent protein and sorted by a fluorescence-activated cell sorter for comparable EphA2 receptor levels. Cells were placed at physiologic temperature (37 °C) for 4 days to down-regulate thermolabile TAg before experiments.

Hanging drop aggregation assay. MCF-10A cells were trypsinized and resuspended at a concentration of 500,000 cells/ml of growth media. Approximately 15,000 cells (30 μl of cell suspension) were pipetted on the lid of a 24 well plate and the corresponding wells were filled with 1 ml of growth media. The culture dish lid was then carefully inverted over the wells, causing the cells to be suspended from the lid as hanging drops. Cells were incubated overnight at 37°C. Cell-cell adhesion was assessed by microscopy both before and after vigorous pipetting (20 times with a 200 μl Gilson pipette tip).

Calcium switch assay. MCF-10A cells were grown to approximately 80% confluence. The cells were then serum starved overnight with OptiMem (Invitrogen). The following day, the media was aspirated and the cells were washed once with PBS and DMEM containing no calcium or magnesium (Invitrogen) was added. The cells were cultured in calcium free media for 8 hrs and 24 hrs (to ensure complete lost of surface E-cadherin). For the 24 hr time point, the calcium free DMEM media was aspirated and the cells were washed once with PBS. Calcium free DMEM containing

1.8 mM $CaCl_2$ (Sigma) was added to the cells for 1 hr and 3 hr. For the experiments using the ROCK inhibitor (Y27632), cells were treated with 10 μM Y27632 for the duration of the calcium depletion and/or when the calcium was added back. The cells were then fixed with ice-cold methanol for visualization by immunofluorescence staining.

Rhotekin pull-down Assay. For RhoA activation assays, MCF-10A or MCF-10A cells expressing mutated EphA2 were serum starved overnight with serum free Optimem medium followed by stimulation with ephrinA1 (1 μg/ml). Lysates were prepared and incubated with Rhotekin-GST beads as previously described (25). Activated RhoA (or total RhoA in lysates) were detected by immunoblot using anti-RhoA antibodies (Santa Cruz). Relative levels of GTP-bound RhoA were quantified by densitometry using Scion Image 1.62c software analysis.

Immunoprecipitation and immunofluorescence. MCF-10A cells were lysed with RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholic Acid, 0.1% SDS, pH=7.4) or Brij Lysis Buffer (10 mM Tris, pH=7.5, 150 mM NaCl, 2 mM EDTA, 0.88% Brij 97, 1.25% NP-40) for MBP-EphA2, GST-LMW-PTP pulldown experiments, and co-immunoprecipitation of EphA2 and Src. For E-cadherin co-immunoprecipitations, cells were lysed in CSK lysis buffer (10 mM PIPES, pH=6.8, 10 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, and 0.5% NP-40 (solution pH=7.4)). All lysis buffers contained the following concentrations of protease inhibitors: 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1mM Sodium

Orthovanadate, and 1 mM EDTA. EphA2, E-cadherin, and p190 RhoGAP were immunoprecipitated using anti-EphA2 (2 μg, Upstate Biotechnology), anti-E-cadherin (4 μg, Transduction Laboratory), and anti-p190 (2 μg, Transduction Laboratory) antibodies. In brief, MCF10A cell lysates were incubated with antibody (Protein A/G Sepharose) or fusion protein (GST or MBP beads) for 2 hrs at 4 degrees. The samples were washed three times with PBS. Samples were resolved by SDS-PAGE and western blotted for respective proteins. For immunofluorescence, cells were washed with PBS and fixed in methanol for 7 minutes at -20 degrees. Cells were stained with anti-E cadherin and anti-p120 antibodies (0.1 μg/ml and 0.5 μg/ml, respectively, Transduction Laboratory). Images were taken on LSM 310 META confocal microscope.

LMW-PTP Phosphatase Assay. Cell lysates were obtained as described above. The samples were immunoprecipitated for LMW-PTP using an anti-LMW-PTP antibody that was generously provided by Dr. Takamune Takahashi. The samples were washed twice with PBS and once with phosphatase buffer (50 mM HEPES, pH=4.5, 1 mM DTT, 0.1% BSA). The samples were resuspended in phosphatase reaction buffer. 200 μM 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP, Invitrogen) was dissolved in phosphatase buffer and was added to each sample. An aliquot of the supernatant was transferred to a 96 well plate and the samples were read with an excitation of 350 nm and emission of 450 nm following a time course.

LC-MS Analysis. LC-MS was performed by the Proteomics Laboratory in the Vanderbilt Mass Spectrometry Research Center. Resolved mouse EphA2 was excised from SDS-polyacrylamide gels for in-gel digestion with trypsin (82). The resulting peptides were separated by reverse phase high pressure liquid chromatography that is coupled directly with automatic tandem MS (LC-MS) using a ThermoFinnigan LTQ ion trap mass spectrometer equipped with a Thermo surveyor autosampler and Thermo Surveyor HPLC pump, nanospray source, and Xcalibur 1.4 instrument control. HPLC separation of the tryptic peptides was achieved with a 100 mm x 11-cm C-18 capillary column (Monitor C18, 5 µm, 100 Å; Column Engineering), at a 0.7 µl min-1 flow rate. Solvent A was H2O with 0.1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid. The gradient program was as follows: 0-3 min, linear gradient from 0-5% B; 3-5 min, 5% B; 5-50 min, linear gradient to 50% B; 50-52 min, linear gradient to 80% B; 52-55 min, linear gradient to 90% B; 55-56 min, 90% B in solvent A. MS/MS scans were acquired using an isolation width of 2 m/z, an activation time of 30 ms, and activation Q of 0.250 and 30% normalized collision energy using one microscan and an ion time of 100 for each scan. The mass spectrometer was tuned prior to analysis using the synthetic peptide TpepK (AVAGKAGAR). Typical tune parameters were as follows: spray voltage of 1.8 kV, a capillary temperature of 160 °C, a capillary voltage of 60 V, and tube lens 120 V. Initial analysis was performed using data-dependent scanning in which one full MS spectrum, using a full mass range of 400-2000 atomic mass units, was followed by three MS/MS spectra. Incorporated into the method was a data-dependent scan for the neutral loss of phosphoric acid or phosphate (-98, -80), such that if these masses were

found, an MS/MS/MS of the neutral loss ion was performed. Peptides were identified using a cluster-compatible version of the SEQUEST algorithm (83, 84), using a mouse subset of proteins from the nonredundant data base from NCBI downloaded in January, 2004 containing 90, 197 sequences. Sequest searches were done on a high speed, multiprocessor Linux cluster in the Advanced Computing Center for Research. In addition to using the SEQUEST algorithm to search for phosphorylation on serines, threonines, or tyrosines, the data were also analyzed using the Pmod algorithm (85). All possible modified peptides were verified by manual inspection of the spectra.

Phosphopeptide Mapping by Two-dimensional Chromatography. EphA2-null murine pulmonary microvascular endothelial cells reconstituted with either wild-type or mutant EphA2 were stimulated with ephrin-A1 for 15 min. Cells were lysed and EphA2 was immunoprecipitated and phosphorylated in the presence of [γ-32P]ATP, as described under "Immunoprecipitation, Western Blot Analysis, and Kinase Assay." Immunoprecipitates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Polyvinylidene difluoride membrane containing 32P-labeled EphA2 receptor was excised, and proteins were digested in membrane with 1 mg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. The resulting peptide mixture was resolved in two dimensions on 20 cm x 20-cm thin layer cellulose plates by electrophoresis followed by ascending chromatography. Electrophoresis was performed at pH 1.9 in 10:1:189 acetic acid/pyridine/water for 3 h at 250 V with ~10 p.s.i. of pressure. Ascending chromatography was carried out in

625:19:48:29: 279 isobutyric acid/n-butanol/pyridine/acetic acid/water for 11 h or until the buffer was about 1 cm from the top of the TLC plate. The plates were dried and subjected to autoradiography overnight at -70 °C with an intensifying screen.

Kinase assay. Wild-type and EphA2-deficient lung microvascular endothelial cells were isolated from EphA2 knockout mice and were infected with LZRS retrovirus expressing wild-type or W42 mutant form of EphA2 receptor. Parental and infected cells were serum starved for 24 h in serum-free Optimum medium followed by stimulation with ephrin-A1 (1 mug/ml). Cells were lysed and EphA2 were immunoprecipitated with an anti-EphA2 antibody (2 mug, C-20, Santa Cruz) in Triton X-100 buffer (20 mM Tris-Cl, 150 mM NaCl, 25 mM beta-glycerophosphate, 2 mM EDTA, 10% glycerol,1% Triton X-1000, 1 mM DTT, 1 mM sodium orthovanadate, 10 mug/ml Leupeptin, and 1 mM PMSF). The samples were resuspended in 25 mul kinase buffer [20 mM HEPES pH=7.6, 20 mM beta-glycerophosphate, 0.1 mM sodium orthovanadate, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, 200 mM ATP, and 20 muCi [γ-32P] ATP (NEN, NEG502A500UC)], incubated at 30°C for 25 mins, resolved on a 8% SDS-polyacrylamide gel, and transferred to nylon membrane for autoradiography. The blot was stripped and re-probed with anti-EphA2 to ensure equal loading.

Orthotopic tumor transplantation. BALB/c mice, 10 weeks of age, were injected with 1 times 105 4T1 cells in the left inguinal mammary gland. The primary tumors were harvested 1 week after injection. Tumor size was assessed by caliper

measurements, and the tumor volume was calculated by the following formula: Tumor volume=0.52 times width2 times length. BALB/c female animals (10 weeks old) were obtained from Harlan Sprague–Dawley and housed under pathogen-free conditions. All experiments were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval.

Histologic analyses. Tumors isolated from BALB/c female mice were processed for histology by overnight fixation in 10% neutral-buffered formalin (Fisher Scientific) at 4°C followed by paraffin embedding and preparation of 7 mum sections. Apoptosis was assessed by TUNEL assay using an Apotag red in situ apoptosis detection kit (Serologicals Corporation, Norcross, GA, USA) as described previously. Images were captured using an Olympus BX60 microscope and digital camera. The percentage of apoptotic nuclei was calculated based on the number of TUNEL+nuclei divided by the number of DAPI+nuclei (total nuclei) in four random 20 times fields/section. Proliferation was assessed by quantification of PCNA-positive nuclei as described previously (Brantley et al., 2002; data not shown).

Metastasis assay. For the experimental metastasis studies, BALB/c mice were administered with 1 times 104 4T1 cells intravenously. The lungs were harvested 12 days after injection. The lungs were fixed by intratracheal infusion of 10% neutral formalin and embedded as described above for primary tumors. Surface lung metastases were scored in a blind fashion under the dissecting microscope. For

spontaneous metastasis studies, 10-week-old BALB/c females were injected with 5 times 105 4T1 cells in the left inguinal mammary gland as described (Yang et al., 2004). The primary tumors and lungs were harvested after 21 days. Lungs were fixed for 24 h in 10% neutral-buffered formalin at 4°C, dehydrated, and cleared by washing three times, 1 h each, in xylenes (Fisher Scientific). Lungs were rehydrated and stained in Mayer's hematoxylin (Sigma-Aldrich) for 1 h at room temperature. After briefly washing in tap water, lungs were destained in 15-min washes with 1% v/v HCl, dehydrated and cleared in xylenes. The lungs were then equilibrated into methyl salicylate (Sigma) for enumeration of metastases.

Wound closure assay. The wound closure assay was described previously (Cheng et al., 2002). Briefly, the 4T1 cells were grown to confluence in six-well tissue culture plates. Replicate circular 'wounds' were generated in confluent 4T1 cell monolayers. Residual fractional 'wound' areas were measured at 2, 4, 6, 8, and 24 h. Pictures were taken with an Olympus BX60 microscope and digital camera. The area of the wound was calculated using Scion Image software 1.62c (National Institute of Health, Bethesda, MD) for each time point.

Statistical analyses. Statistical analyses were performed using Microsoft Excel software using analysis of variance (ANOVA) for comparison of data among 4T1 cells or 4T1 cells expressing wild-type or mutant forms of EphA2 receptor; or unpaired Student's t-tests for comparison of data from control 4T1 cells with 4T1 cells expressing DeltaC or W42 EphA2 mutants. All tests of significance were two

sided, and differences were considered statistically significant when P-value<0.05. All data were expressed as means plus minus s.e.m.

CHAPTER III

A KINASE-DEPENDENT ROLE FOR EPHA2 RECEPTOR IN PROMOTING TUMOR GROWTH AND METASTASIS

Introduction

Receptor tyrosine kinases (RTKs) play a diverse role in cell growth and differentiation during normal physiologic responses and in oncogenic transformation and tumor progression. These cell surface receptors transmit extracellular signals by activation of intrinsic kinase activity, which phosphorylates cytoplasmic domains of clustered receptor complexes and other target proteins, thus initiating a cascade of signaling events that modulate cellular responses. Mutations leading to production of constitutively active receptors or overexpression of a normal receptor results in increased kinase activity and oncogenic transformation. Consequently, small molecule inhibitors of receptor tyrosine kinases are proving to be efficacious in the clinic for several cancer types [reviewed in (86)].

Eph receptors represent the largest family of receptor tyrosine kinases in the genome, consisting at least 16 receptors that interact with nine membrane-bound ephrin ligands [reviewed in (3, 13)]. They can be further divided into two groups, class A and B, based on sequence homology and binding affinity (87). Class A Eph receptors interact with multiple ligands of the ephrin-A family, a group of glycosyl-phosphoatidylinositol (GPI)-linked membrane proteins, while class B Eph receptors binds to ephrin-B ligands, a family of transmembrane proteins. Binding of Eph

receptors to their ligands induces receptor clustering, activation of kinase activity, and subsequent trans-phosphorylation of the cytoplasmic domains, creating docking sites for a number of signaling proteins [reviewed in (88, 89)]. In addition to kinase-dependent signaling, binding of certain members of the Eph family, such as EphA8 or EphB3, can also trigger kinase-independent signaling (80, 90). In contrast to other families of receptor tyrosine kinases, Eph receptor signaling does not promote proliferation but leads to regulation of cell–cell, cell–matrix adhesion and cell motility.

The Eph family RTKs and their ligands initially attracted interest as modulators of axonal guidance, angiogenesis, and embryonic patterning during development [reviewed in (88, 91)]. It is now clear that the Eph molecules also play a role in adult tissues under physiological conditions and in disease states such as cancer. Eph RTKs and their ligands, the ephrins, are frequently overexpressed in different types of cancer [reviewed in (13, 92, 93)]. One family member in particular, the EphA2 receptor, has been linked to breast, prostate, lung and colon cancer, as well as melanoma (22, 94). In cell lines, EphA2 overexpression is associated with increased cell growth in soft agar (30), increased invasion into Matrigel (30, 38), increased resistance to anoikis (38), and increased tumor growth when these cells were implanted into nude mice (30). Conversely, inhibition of EphA2 expression in tumor cells by siRNA (38) or induction of EphA2 degradation by ligand- or activating antibody-induced endocytosis (66) resulted in decreased cell invasion into matrigel, increased anoikis, and inhibition of tumor growth in vivo (95, 96). However, despite

the strong correlation of EphA2 receptor expression with malignant phenotypes, the mechanisms by which EphA2 contributes to tumor cell malignancy are far from clear.

One important mechanistic question is whether EphA2 receptor phosphorylation and kinase activity play a role in malignant transformation/progression. In certain tumor cell lines, EphA2 receptor is underphosphorylated (40). However, despite the low phosphorylation level, EphA2 kinase activity was not affected (40). Ligand-induced EphA2 phosphorylation induces receptor endocytosis and degradation, reducing malignant behavior of the cells and tumor growth in vivo (66). Such evidence supports the idea that EphA2 receptor phosphorylation is not necessary to confer kinase activity and tumorigenicity, but leaves question unanswered as to whether kinase activity is required for tumor malignancy. Other data have emerged showing that EphA2 receptor phosphorylation may be important in conferring oncogenic potential. Dobrzanski et al. (2004) observed high EphA2 receptor phosphorylation levels in xenografts of ASPC-1, U87MG, and SVP cell lines (97). Ogawa also reported EphA2 receptor phosphorylation in human mammary carcinoma xenography (22). In addition, studies using the 4T1 model of tumorigenesis have found that blocking EphA2 receptor activation through EphA2-Fc results in a decrease in phosphorylation that was concurrent with decreased tumor volume (23, 39, 97).

To directly test the role of EphA2 receptor phosphorylation/kinase activity, we overexpressed EphA2 variants, either lacking the cytoplasmic domain or carrying a point mutation that abolishes its kinase activity, in breast cancer cells. Expression of these EphA2 mutants in breast cancer cells resulted in decreased tumor volume and

increased tumor apoptosis in primary tumors, and significantly reduced the number of lung metastases. These data suggest that phosphorylation and kinase activity of the EphA2 receptor, at least in part, contributes to tumor malignancy.

Results

EphA2 receptor phosphorylation in tumor cells is regulated by cell density

Prior studies have shown that EphA2 receptors are under-phosphorylated in certain breast tumor cell lines (40) but phosphorylated in other tumor cells (97). As 4T1 tumor cells express both endogenous ephrin-A1 ligand and EphA2 receptor (23), we determined whether EphA2 receptors are tyrosine phosphorylated by engaging endogenous ephrin-As in a cell-cell contact-dependent manner, and whether the level of EphA2 tyrosine phosphorylation can be further elevated by exogenous ephrin-A1 ligand. We used two independent approaches to address this issue. First, cells were plated onto 10-cm dishes with 100%, 50%, or 25% confluency in the presence or absence of 1 μg/ml exogenous ephrin-A1 ligand for 15 minutes. Cell lysates were immunoprecipitated by anti-EphA2 and blotted for phosphotyrosine level. As shown in Figure 4A, phosphorylation of EphA2 receptor is up-regulated with increased cell density at basal level, and phosphorylation of EphA2 is further enhanced by exogenous ephrin-A1 ligand stimulation. In a second approach, identical numbers of 4T1 tumor cells were plated on culture dishes of different surface areas (3.5 cm, 6 cm, and 10 cm) so that cell density decreases with the increase of dish size (Fig. 4B). In

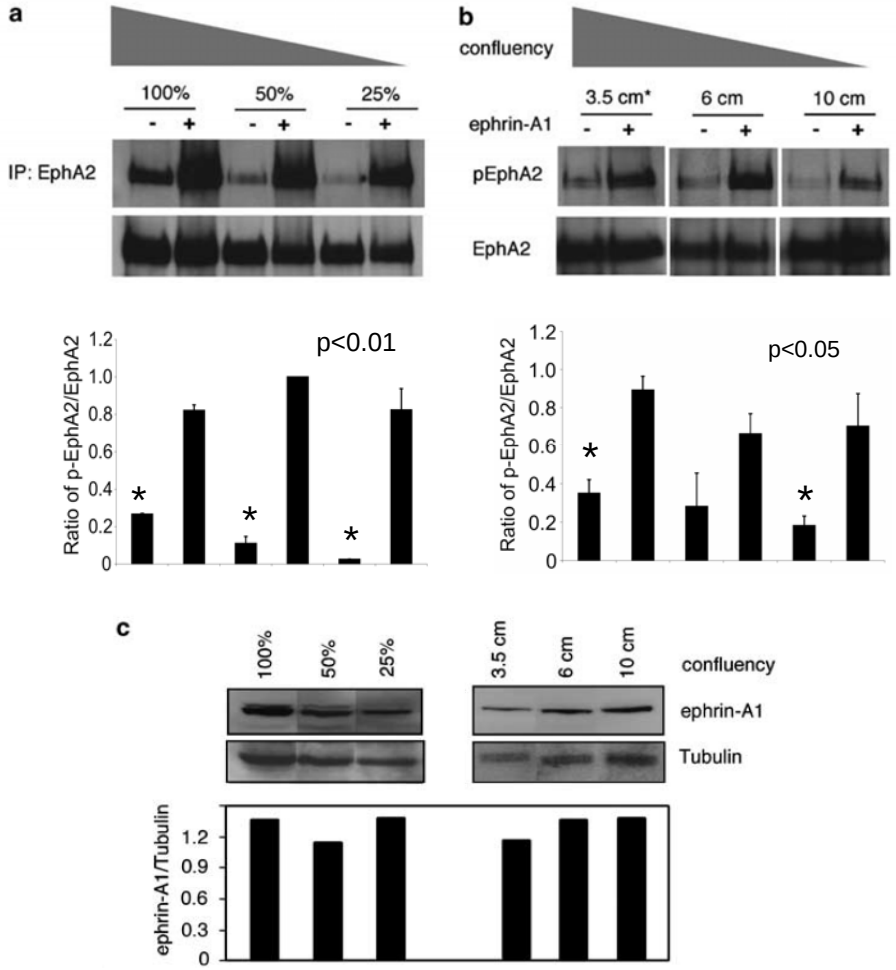


Figure 4. Cell density regulates the phosphorylation of EphA2 receptors. (A) Metastatic 4T1 mammary carcinoma cells were plated onto 10-cm dish with 100%, 50%, or 25% confluency. (B) Identical numbers (5 x 10^5) of 4T1 cells were plated on culture dishes of different surface areas (3.5 cm, 6 cm, and 10 cm). Cells were serum-starved overnight and stimulated with 1 μg/ml of ephrin-A1 ligand for 15 minutes. EphA2 proteins were immunoprecipitated from cell lysates and blotted for tyrosine phosphorylation and for total EphA2. Relative levels of pEphA2 and total EphA2 were quantified by densitometry using Scion image 1.62 software analysis. 3.5cm*, 5 dishes of 3.5 cm plate were pooled for western blot analysis. (C) Western blot analysis of ephrin-A1 ligand.

cells on 10 cm dish where there is no significant level of cell-cell contact, EphA2 is barely activated in the absence of ligands, yet became tyrosine phosphorylated upon exposure to exogenous ephrin-A1. With increasing cell density (6 cm and 3.5 cm), EphA2 receptor expression level gradually decreases. However, when adjusted to similar level of EphA2 expression by combining cells in 5 dishes of 3.5 cm plate, the EphA2 tyrosine phosphorylation level increases in high-density cell culture (Fig. 4B, 3.5 cm*). Ephrin-A1 levels did not change significantly with different cell density (Fig. 4C). These results revealed that high cell density up-regulates basal level of tyrosine phosphorylation of EphA2 receptor and phosphorylation of EphA2 receptor can be further increased by stimulation of exogenous ephrin-A1 ligand.

Signaling-defective forms of EphA2 mutants inhibit 4T1 mammary tumor progression in vivo

To test directly the role of EphA2 receptor phosphorylation/kinase activity, we generated EphA2 mutants that were either lacking the cytoplasmic domain (ΔC) or carrying a D738N point mutation (W42) (Figure 5A). W42 mutation is analogous to a spontaneously occuring loss of function point mutation identified in the W^{42} dominant negative allele of the c-kit receptor tyrosine kinase (98, 99) that serves as a dominant negative mutation for other RTKs in cell culture models (100). EphA2-deficient cells were infected with LZRS retrovirus expressing wild-type EphA2 receptor (WT), W42 mutant, or vector control and kinase activity of each receptor variant was determined by in vitro kinase assay. As shown in Figure 5B, EphA2-deficient cells have no detectable level of kinase activity. While overexpression of wild-type EphA2 receptor restored kinase activity, overexpression of W42 mutant

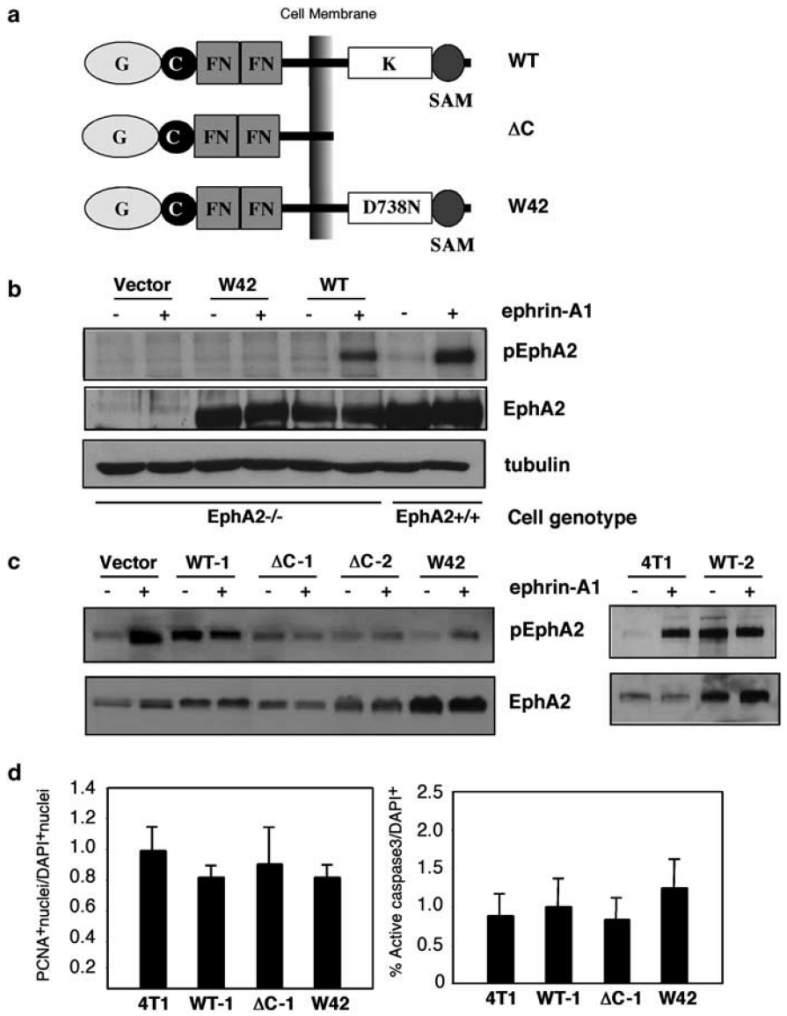


Figure 5. Signaling defective EphA2 mutants act in a dominant negative fashion. (A) A diagram of domain structures of wild-type and mutant forms of EphA2 receptor. (B) EphA2-deficient endothelial cells were infected with retrovirus LZRS expressing wild-type EphA2, W42 mutant, or vector control. Cells were serum-starved overnight and stimulated with 1 μg/ml of ephrin-A1 ligand for 15 minutes. EphA2 proteins were immunoprecipitated and subjected to in vitro kinase assay. W42 mutant is defective in kinase activity. (C) 4T1 clones expressing wild-type and mutant forms of EphA2 receptors were subject to immunoprecipitation /western blot analysis for EphA2 and phosphorylated tyrosine as in Figure 1. EphA2-ΔC and W42 mutants act in a dominant negative fashion to inhibit endogenous EphA2 receptor phosphorylation in response to ephrin-A1 ligand. (D) In vitro analyses of proliferation and apoptosis in 4T1 cells expressing wild-type or mutant forms of EphA2. Cell proliferation and apoptosis were assessed by expression of proliferating cell nuclear antigen (PCNA) and activated caspase-3, respectively. There is no significant difference in proliferation or apoptosis between 4T1 expression wildtype or mutant EphA2 proteins.

protein failed to confer kinase activity in EphA2-deficient cells, suggesting that W42 is a kinase-dead mutant.

Multiple 4T1 clones expressing either wild-type EphA2 receptor (WT), signaling-defective mutant forms of EphA2, ΔC or W42, or control vector were generated. As shown in Figure 5C, compared to vector control, overexpression of wild-type EphA2 leads to a constitutive phosphorylation of the receptor. In contrast, overexpression of mutant ΔC or W42 forms of EphA2 significantly inhibited ligand-induced receptor phosphorylation, suggesting that these mutants act in a dominant negative fashion to inhibit endogenous receptor signaling. As expected, transfected wild-type or mutant EphA2 proteins localized to the cell surface of culture cells (data not shown). When cultured in vitro, there are no significant changes in either cell proliferation or apoptosis among multiple 4T1 clones expressing wild-type or signaling-defective mutant forms of EphA2 receptor (Fig. 5D).

To assess whether EphA2 function in tumor growth and metastasis is kinase-dependent, 4T1 clones expressing either wild-type or mutant EphA2 proteins (100,000 cells) were injected in the mammary fat pad of syngeneic Balb/C recipient mice, and tumors were harvested one week later for analysis. While tumors expressing wild-type EphA2 have similar tumor volume as control tumors, tumors expressing either ΔC or W42 mutants (Figure 6A) have a much smaller tumor volume ($p<0.01$, ANOVA; $p<0.01$, unpaired student's *t* test, ΔC-1, ΔC-2, or W42 versus Vector, WT-1, or WT-2). We next determined whether decreased tumor volume in tumors expressing EphA2 signaling-defective mutants is due to decreased proliferation or increased apoptosis. Tumor growth was assessed by quantifying

nuclear expression of proliferating cell nuclear antigen (PCNA), a marker for actively dividing cells; and cell death was determined by in situ TUNEL stain in tumor sections. As shown in Figure 6B, compared to wild-type EphA2 tumors, there is an approximately two-fold increase in TUNEL positive nuclei in tumors expressing signaling-defective EphA2 mutants ($p<0.01$, ANOVA; $p<0.01$ Student's *t* test. Vector or WT versus ΔC-1, ΔC-2, or W42). However, there is no significant difference in tumor cell proliferation between tumors expressing wild-type EphA2 and those expressing EphA2 mutants (data not shown), consistent with studies suggesting that Eph RTKs do not promote proliferation directly (23). Taken together, these data suggest that blockade of EphA2 forward signaling in tumor cells by signaling-defective EphA2 mutants inhibits tumor progression by promoting tumor cell apoptosis.

Signaling-defective forms of EphA2 mutants inhibit lung metastasis

To determine whether blocking of EphA2 signaling in tumor cells could suppress metastatic progression, 10,000 4T1 cells expressing either wild-type or mutant forms of EphA2 proteins were administered intravenously into Balb/C recipient mice. Twelve days after injection, lungs were harvested, fixed, and processed for histological analyses. Gross examination revealed numerous lung surface metastases in mice injected with 4T1 cells expressing wild-type EphA2, compared to 4T1 cells containing vector alone (Figure 7A, top panels). In contrast, there were significantly lower numbers of visible lung surface metastases in mice injected with 4T1 cells expressing signaling-defective EphA2 mutants (Figure 7A, bottom panels), compared

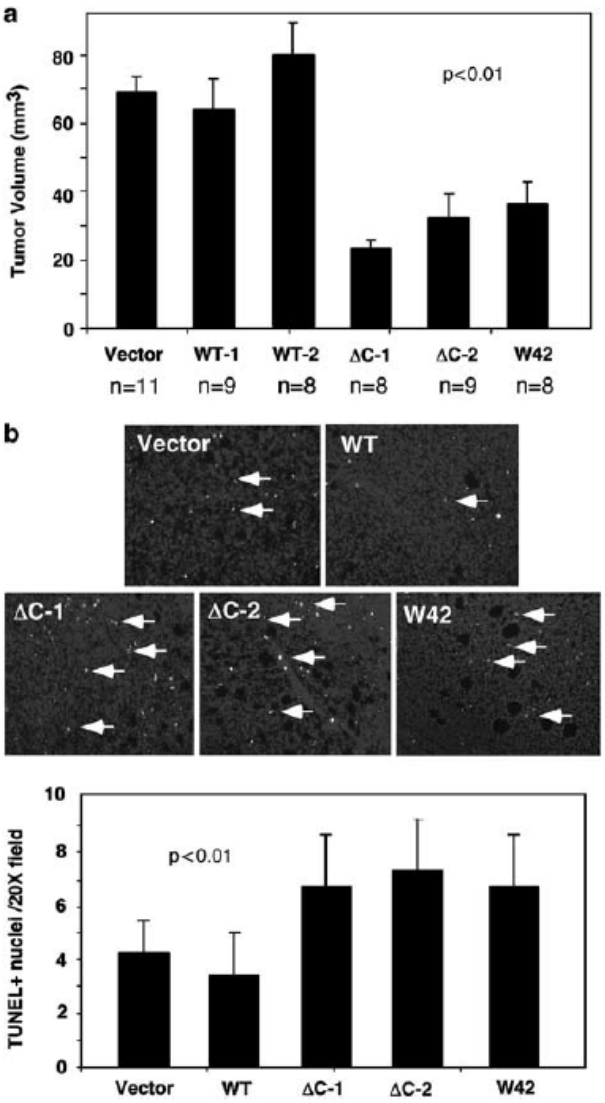


Figure 6. Dominant negative EphA2 mutants inhibit 4T1 mammary tumor progression in vivo. (A) A hundred thousand 4T1 cells or 4T1 cells expressing wild-type or mutant EphA2 proteins were orthotopically transplanted into the mammary gland of syngeneic Balb/C female mice. Tumors were harvested one week after transplantation and tumor volume was measured as described in the Materials and Methods. Tumors expressing ΔC or W42 EphA2 mutants exhibit significantly smaller tumor volume than those of vector or WT control tumors. (B) Tumor paraffin sections were subjected to TUNEL assay to evaluate the level of apoptosis. Arrows indicate TUNEL positive nuclei. A significant increase in apoptosis was observed in tumors expressing ΔC or W42 EphA2 mutants.

to 4T1 cells expressing wild-type EphA2. Histological examination of hematoxylin/eosin-stained lung sections revealed numerous large pulmonary metastases in lungs from mice harboring wild-type EphA2 tumors, while only small avascular metastases could be seen in lungs from mice harboring either ΔC-EphA2 or W42-EphA2 tumors (data not shown). Comparison of tumor metastasis by counting the lung surface metastases confirmed that mice injected with 4T1/WT-EphA2 cells carried a significantly greater metastatic burden than mice injected with cells expressing either vector control, ΔC, or W42 (Figure 7B) ($p<0.01$, ANOVA; $p<0.05$, unpaired student's *t* test, ΔC-1, ΔC-2, or W42 versus WT-1, or WT-2). These results suggest that signaling-defective forms of EphA2 mutants inhibit the colonization of tumor cells in the lung.

To determine whether signaling-defective forms of EphA2 mutants could also inhibit spontaneous metastases, 500,000 parental 4T1 cells or 4T1 cells expressing wild-type or mutant EphA2 receptors were implanted into mammary gland fat pads of recipient Balb/C females and tumors were grown for 21 days to permit spontaneous lung metastases. Consistent with data derived from 1-week tumors, we observed significantly decreased tumor volume in tumors expressing ΔC or W42 at 3 week (Figure 7A) ($p<0.01$, ANOVA; $p<0.01$, unpaired student's *t* test, ΔC-1, or W42 versus 4T1, or WT-1). The number of surface lung lesions in EphA2 signaling-defective tumor recipients was also reduced compared to controls (data not shown). Quantification of total lesions in cleared lungs by staining with hematoxylin revealed a significant decrease in EphA2-deficient tumor recipients relative to controls (Figure 8B) ($p<0.01$, ANOVA; $p<0.01$, unpaired student's *t* test, ΔC-1, or W42 versus WT-1).

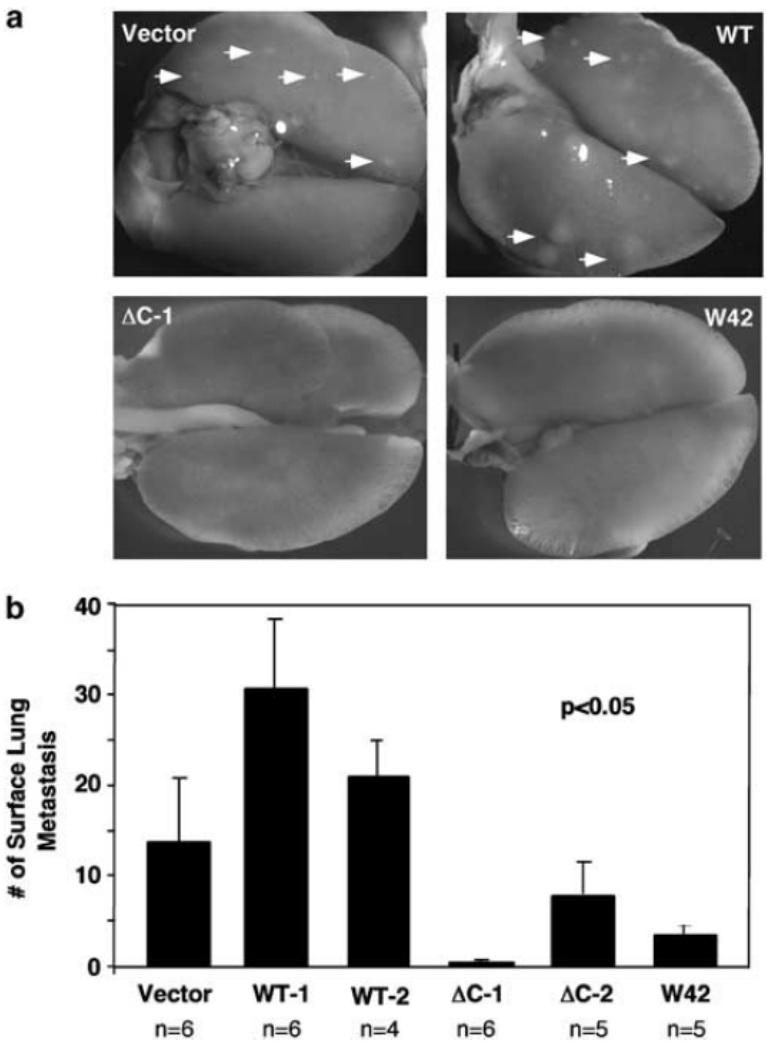


Figure 7. Decreased lung metastasis in 4T1 cells expressing ΔC or W42 EphA2 mutants. Ten thousand 4T1 cells expressing wild-type or mutant EphA2 receptors, or vector control were administered intravenously and lungs were harvested 12 days later. (A) Gross examination of lungs from mice injected with 4T1 cells expressing ΔC or W42 mutants revealed fewer surface lesions than in those collected from mice injected with 4T1 cells expressing wild-type EphA2 or vector control. Arrows indicate surface metastatic lesions. (B) Surface metastases were enumerated, and a significantly lower number of lesions was detected in lungs from mice injected with 4T1 cells expressing ΔC or W42 mutants relative to controls.

These data demonstrate that EphA2 signaling-defective mutants inhibit tumor metastasis in vivo.

Signaling-defective EphA2 mutants do not affect tumor angiogenesis but inhibit tumor cell motility

It has become increasing clear that multiple factors could influence tumor metastasis, including tumor angiogenesis and increased tumor cell motility. As EphA2 receptor and ephrin-A ligands were previously shown to be involved in tumor neovascularization (23, 39, 97), we first determined the abundance of microvessels in tumors expressing either wild-type or mutant EphA2 receptors. No significant difference of tumor microvascular density was observed between tumors expressing wild-type and mutant EphA2 receptors (data not shown), suggesting that tumor angiogenesis is not a determining factor for decreased tumor metastasis in tumors expressing mutant EphA2 receptors.

Next we determined the ability of cells to migrate, as cell motility contributes to tumor invasion and metastasis. 4T1 cells expressing WT, W42, ΔC, or vector control were grown to confluency and monolayer of cells was then scratched with a pipette tip. As shown in Figure 9A, the wound closing process was significantly retarded in 4T1 cells expressing mutant W42 or ΔC receptor, compared to those expressing WT receptor, vector control, or parental 4T1 cells, suggesting that blocking of EphA2 receptor signaling inhibits tumor cell motility. As dynamic regulation of the actin cytoskeleton is critical in cell migration and Rho family GTPases are known to be key regulators of this process [reviewed in (101-103)], we examined the level of activated Rho family GTPases in 4T1 clones expressing wild-type or mutant EphA2 by GST-

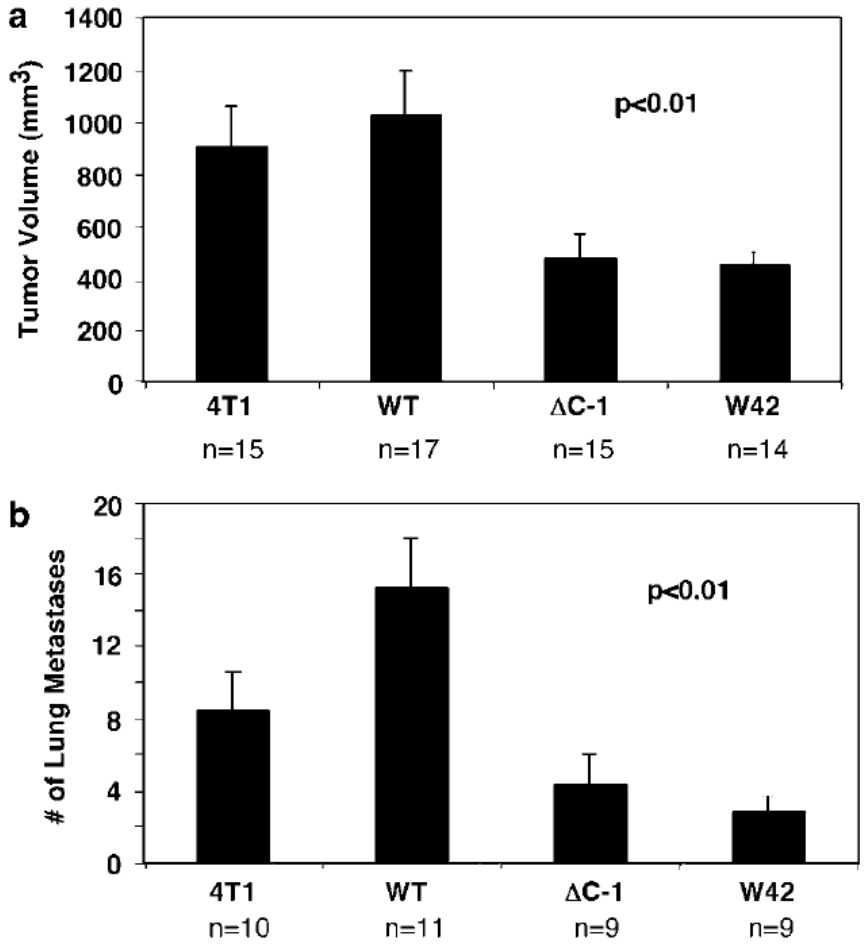


Figure 8. EphA2-ΔC or W42 mutants inhibit spontaneous lung metastasis. Five hundred thousand tumor cells were orthotopically implanted into mammary gland of syngeneic recipient Balb/C female mice. Primary breast tumors and lungs were collected 21 days later. (A) Tumors expressing ΔC or W42 mutants exhibit significantly smaller tumor volume than those of 4T1 parental or wild-type control tumors. (B) Quantification of total lung metastases revealed a significant decrease in lung lesions from mice harboring tumors expressing ΔC or W42 mutants relative to controls.

Rhotekin or GST-PAK pull-down assays. As shown in Figure 9B, there is no significant difference in Rac activity between cells expressing wildtype EphA2 and those expressing mutant EphA2 proteins, although Rac activity appears to increase in cells expressing EphA2 proteins for unknown reasons. In contrast, ephrin-A1 stimulation induced RhoA activation within 2.5 minutes in both vector-transfected 4T1 cells and 4T1 cells expressing WT-EphA2. RhoA activation was undetectable in cells expressing W42-EphA2 or ΔC-EphA2 mutant proteins. Thus, it appears that the changes in RhoA activity is more relevant to phenotypes we observed. Taken together, these data suggest W42-EphA2 or ΔC-EphA2 mutants may suppress tumor cell migration through inhibition of RhoA activation.

Discussion

Classic oncogenic transformation by receptor tyrosine kinases and their growth factor ligands involves elevated levels of receptor autophosphorylation and tyrosine kinase activity. In fact, an abnormally high level of tyrosine kinase activity is the major determinant of the oncogenic potential of an RTK, as is the case for ErbB2/Neu (104). However, it is not clear whether receptor autophosphorylation and kinase activity is required for EphA2 receptor-mediated oncogenic transformation or in the development of metastatic potential. siRNA knock down of EphA2 receptor tyrosine kinase expression in pancreatic cancer cells inhibits tumor growth and metastasis (38), but this study did not address whether the kinase activity is required for tumor malignancy. Soluble EphA2-Fc receptor can effectively inhibit EphA2 receptor phosphorylation and tumor growth and metastasis in vivo (23, 39, 97). While this

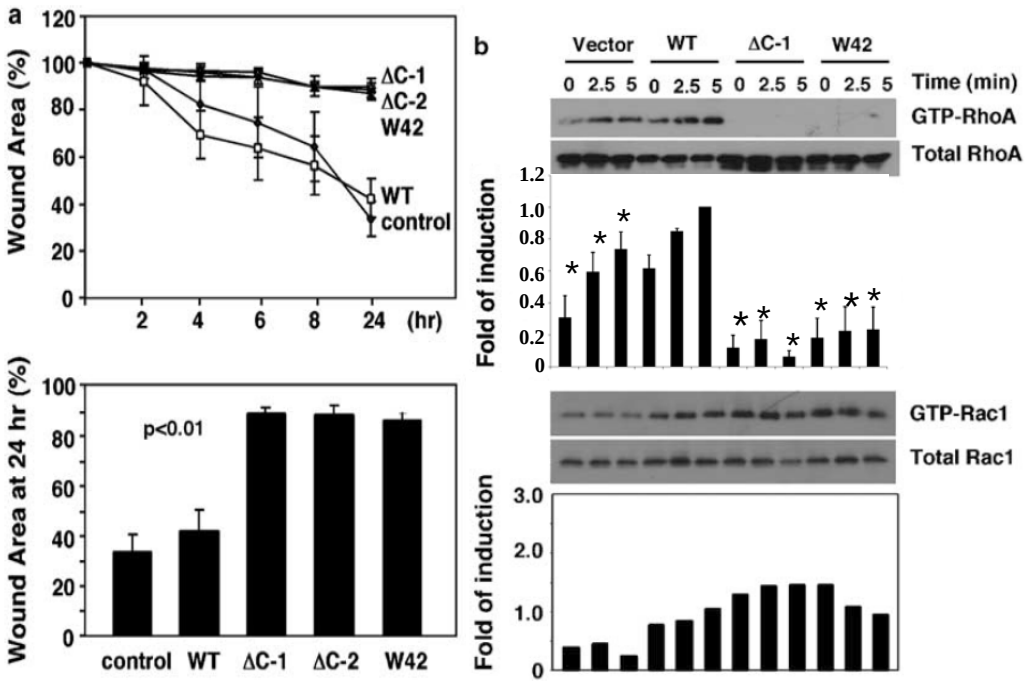


Figure 9. Reduced cell migration in 4T1 cells expressing ΔC or W42 EphA2 mutants. Cells were cultured in 12-well plates until confluency. The monolayer was wounded with a fine pipette tip to create a circular wound. (A) Pictures were taken at 2, 4, 6, 8 and 24 hours after wounding. Residual "wound" areas were measured and quantified by Scion Image software. Percentage of remaining wounded area in 4T1 cells expressing ΔC or W42 EphA2 mutants at 24 hours is significantly greater than controls, indicating reduced migration. (B) Active GTP-bound forms of RhoA and Rac1 were analyzed by Rhotekin-RBD or Pak-PBD pull-down followed by immunoblot in lysates from 4T1 cells overexpressing wild-type or mutant forms of EphA2 receptors or vector control. Expression of ΔC or W42 EphA2 mutants blocked basal and ephrin-A1-induced RhoA activation ($p<0.01$) but had no significant effect on Rac1 activation.

study supported the notion that EphA2 receptor phosphorylation/activation is required for tumor progression, these in vivo studies cannot differentiate the effect of soluble EphA2-Fc receptor on tumor cells from other cell types in the tumor microenvironment. Finally, Zantak et al showed that EphA2 receptor is not phosphorylated in some human breast cancer cell lines, although the unphosphorylated receptor still has kinase activity (40). Overexpression of EphA2 receptor in the immortalized breast epithelial cell line MCF10A induces oncogenic transformation, but again the overexpressed EphA2 receptor is not phosphorylated (30). These data argue for a phosphorylation-independent role of EphA2 in oncogenic transformation. In this report, we found that EphA2 level and phosphorylation status are cell-density dependent in culture. Inhibition of receptor phosphorylation by EphA2 mutants that either contain a cytoplasmic deletion (ΔC) or a point mutation rendering "kinase-dead" (W42) suppressed the activity of RhoA GTPase and tumor cell motility. Furthermore, blocking of EphA2 receptor signaling by ΔC or W42 mutants inhibited tumor growth and lung metastasis in vivo. Taken together, our results suggest a kinase-dependent role of EphA2 in tumor progression.

In addition to a kinase-dependent role of EphA2 in tumor progression, EphA2 may also have kinase-independent functions. Genetic studies in vivo have shown that Eph receptors have both kinase-dependent and kinase-independent functions (105-108). In addition, some Eph receptors either have kinase-deficient variant forms generated by alternative splicing (EphA6, 7, and B1) (109) or have kinase domains with defective catalytic activity (EphB6 and A10) (110, 111). Activation of EphA8 induce extracellular matrix adhesion through a PI3Kγ-mediated regulation of integrin

activity in a kinase-independent manner (80). More recently, Miao et al showed that inhibition of integrin-mediated cell adhesion but not directional cell migration requires EphB3 tyrosine kinase activity (90). Finally, the phenotypes of ΔC tumors are more severe than W42 tumors, suggesting an additional function of cytoplasmic domain of EphA2 receptor independent of its kinase activity.

Aside from EphA2 receptor, the expression of EphB4 receptor is also elevated in breast carcinomas with a high grade of malignancy (112). In transgenic mouse models of mammary carcinogenesis, overexpression of EphB4 in mammary epithelial cells leads to accelerated tumor growth and metastatic progression caused by the Neu oncogene (113). Interestingly, tumor promotion effects of the EphB4 overexpression may not be due exclusively to EphB4 forward signaling, as overexpression of a EphB4 cytoplasmic trunction mutant, EphB4ΔC-EGFP, also increases tumor growth (114). The tumor growth promotion effects of EphB4ΔC-EGFP are apparently due to reverse signaling through ephrin-B2 via enhanced tumor angiogenesis (114). Although there is precedence for reverse signaling through class A ephrin ligands (115-117), we do not think the tumor suppression effect of ΔC-EphA2 or W42-EphA2 is due to reverse signaling because WT-EphA2 retains the capability to signal through ephrin-As but have the opposite biologic outcome. As both WT-EphA2 and signaling-defective EphA2 mutants are specifically expressed in the tumor cells, we believe the reduced tumor volume and decreased metastasis in ΔC-EphA2 or W42-EphA2 tumors is due to defective forward signaling in tumor cells.

What is the mechanism of EphA2 signaling-defective mutant-mediated inhibition of tumor progression in vivo? Inhibition of primary tumor growth could result from

suppression of tumor cell proliferation or enhancement of tumor cell death directly, or reduction of tumor blood vessels indirectly. As we did not observe any changes in tumor cell proliferation or tumor vessel density, increased apoptosis is likely to play a major role in diminishing tumor volume in primary tumors expressing EphA2 signaling-defective mutant proteins. It is interesting to note that while program cell death is increased in tumors expressing signaling mutant forms of EphA2 receptor (Fig. 6B), we did not observe a significant change between wildtype and mutant forms of EphA2 in apoptosis in cultured cells under serum starvation in vitro (Fig. 5D). As tumor in vivo interacts with its complex microenvironment, apoptosis can occur in response to stimuli (e.g. Fas ligand) other than cytokine/nutrient deprivation. Further experiments will be needed to dissect the role of EphA2 receptor in cell survival in vivo.

Tumor metastasis is a complex multi-step process involving tumor cell invasion, intravasation, survival in the circulation, extravasation, local migration and colonization of secondary organs. Decreased cell motility and increased apoptosis in tumors expressing EphA2 signaling-defective mutant proteins could affect multiple steps of the metastatic process, leading to decreased metastatic lesions in vivo. Comparison of the results from experimental metastasis with those from spontaneous metastasis suggests that in addition to a possible role in tumor cell invasion and intravasation, EphA2 receptor is also essential in later steps of metastasis involving colonization of lung tissue. In addition to the effect of EphA2 signaling on metastasis, recent data from multiple laboratories showed that the level of EphA2 expression correlates with the degree of tumor malignancy and metastasis (33, 36,

118), suggesting high level of EphA2 protein promotes metastasis. In support of this hypothesis, we observed that mice received 4T1 expressing wildtype EphA2 exhibited elevated number of lung metastases compared to those received parental 4T1 cells or 4T1 vector controls (Fig. 7B and 8B). Although there is no significant difference in cell migration between WT-1 and parental 4T1 cells, it is conceivable that WT-1 has advantage over parental 4T1 in other steps of metastasis. For example, EphA2 has been shown to regulate the expression of MMP-2 (96). Thus, in principle, one mechanism by which elevated EphA2 receptor could promote tumor cell invasion and colonization is via an MMP-dependent mechanism.

Cell migration can be divided into separate steps: lamellipodium extension, formation of new adhesions, cell body contraction and tail detachment. Rho family small GTPases are well-established as mediators in these steps: Rac 1 is required for lamellipodium extension and formation of new adhesion, while RhoA is primarily involved in stress fiber formation and cell body contraction [reviewed in (103)]. In general, Rac1 activation promotes cell migration. However, RhoA activation can either inhibit or promote cell migration depending on cell types and experimental conditions(103). In less adherent cells such as macrophages, neutrophils and various cancer cell lines, RhoA activity has been correlated to cell polarization and migration (119). Under our experimental condition, we did not observe a significant decrease in Rac1 activity. Rather, a dramatic inhibition of RhoA activity is seen in tumor cells expressing EphA2 signaling-defective mutants. As 4T1 cells are very malignant metastatic cancer cells, activation of RhoA in this cell type may resemble to that of non-adherent cells to promote cell migration. This hypothesis is consistent with the

fact that elevated expression of RhoA has been correlated with advanced tumor stage or enhanced metastasis in tumors, including breast cancer, melanomas, pancreatic ductal adenocarcinoma and testicular germ cell tumors (120).

Because EphA2 receptor is frequently overexpressed in human cancers and the level of EphA2 expression has been correlated with tumor malignancy, therapeutic strategies are currently being developed to inhibit EphA2 in cancer. These includes down-regulation of EphA2 expression by siRNA (38), activating antibodies or ligand mimetic peptides to induce EphA2 endocytosis and degradation (121, 122), or blocking EphA2 receptor activation by soluble EphA-Fc receptor (23, 39, 97). This study provides a mechanism for effectiveness of soluble EphA-Fc receptor and lays foundation for future development of EphA2-specific kinase inhibitors in cancer therapeutics.

CHAPTER IV

OVEREXPRESSION OF EPHA2 RECEPTOR DESTABILIZES ADHERENS JUNCTIONS VIA A RHOA-DEPENDENT MECHANISM

Introduction

Protein tyrosine phosphorylation is a powerful signal that regulates cell proliferation, cell invasion, and cell migration. Mutation, gene amplification, or aberrant regulation of protein tyrosine kinase has been linked to tumor initiation and progression. Specific tyrosine kinase inhibitors, such as inhibitors of the Her-2/Neu receptor and EGFR, have been developed for cancer therapeutics with varying degrees of success. While exciting in their clinical effectiveness, these inhibitors are only suitable for treating a small subset of cancer types. The hope remains that other receptor tyrosine kinases will be identified and that their inhibition will have broader efficacy in cancer treatment.

A new family of receptor tyrosine kinases, the Eph family, plays a critical role in cancer. Originally discovered as modulators of axonal guidance and embryonic patterning during development, subsequent studies have shown that many Eph receptors are overexpressed in a large number of cancers (3, 81). One family member in particular, the EphA2 receptor, has been linked to breast cancer, prostate cancer, lung cancer, ovarian and cervical cancer, esophageal and colorectal cancer, as well as malignant melanoma (92). Furthermore, the level of EphA2 receptor expressed on tumor cells correlates with the degree of tumor malignancy (123). Overexpression of the EphA2 receptor in MCF-10A cells is associated with increased cell growth in soft

agar and increased tumor growth when these cells were implanted into nude mice (30). However, despite the strong correlation of EphA2 receptor expression with malignant phenotypes, the mechanisms by which EphA2 contributes to tumor cell malignancy are not completely understood.

One hallmark of malignancy in tumor cells is the loss of cell-cell adhesion. In non-malignant circumstaces, cell-cell adhesion connects epithelial cells in their normal polarized position. In mammals, adhesion between epithelial cells is generally mediated by three types of junctions: tight junctions, adherens junctions, and desmosomes. Adherens junctions are cadherin-dependent adhesive structures that are intimately linked to the cytoskeleton. The extracellular domain of classical cadherins mediates calcium-dependent homophilic interactions, whereas the intracellular domain interacts with several catenins. In general, the cadherin-bound catenins function to either anchor the adhesion complex to the actin cytoskeleton (β- and α-catenin) or regulate the cadherin stability at the junction (p120 catenin). Cadherins and their associating catenins are collectively known as the cadherin complex. The stability of the epithelial cadherin, E-cadherin, and its associated complex dictates both the polarity and the motility of epithelial cells (124). Several studies have shown that the loss of E-cadherin down-regulates EphA2 receptor levels and induces EphA2 mis-localization in tumor cells and mouse embryonic stem cells (40, 63). Likewise, VE-cadherin, another type of cadherin, appears to regulate EphA2 localization in melanoma cells that undergo vascular mimicry (125). However, the effect of EphA2 overexpression on adherens junctions remains unclear.

Cell-cell adhesion is regulated by multiple mechanisms. Rho family of GTPases have been increasingly recognized as key players in regulation of adherens junction [reviewed in (120, 124, 126)]. Rho proteins are small GTPases that cycle between an active, GTP-bound, conformation and an inactive, GDP-bound conformation. In response to extracellular cues through cell surface receptor, Rho proteins can be activated by guanine nucleotide exchange factor (GEF) or inactivated by GTPase activating protein (GAP). Multiple Eph receptors have been shown to modulate Rho family GTPase activity [reviewed in (89)]. EphA2 receptor activation leads to elevation of GTP-Rac1 via Vav GEFs in vascular endothelial cells to regulate angiogenesis (25, 56). In epithelial and tumor cells, stimulation of EphA2 receptor induces activation of RhoA GTPase and affects cell migration (127, 128).

In addition to activation of Rho family GTPases, EphA receptors have been shown to both regulate low molecular weight phosphotyrosine phosphatase (LMW-PTP) activity and serve as substrates for the same phosphatase. Park S. showed that the EphA8 receptor phosphorylates and activates LMW-PTP in vitro (129). Conversely, LMW-PTP negatively regulates EphA receptors by dephosphorylation of EphA2 and EphA8 receptors (68, 71, 129). Another substrate of the LMW-PTP is the p190RhoGAP. In fibroblasts, LMW-PTP regulates adherens junction stability by modulating phosphorylation levels of p190RhoGAP (130). However, the role of LMW-PTP in regulating receptor tyrosine kinases and/or cell-cell adhesion remains to be determined in epithelial cells.

To determine whether the EphA2 receptor can regulate adherens junctions, we expressed wild-type and mutant EphA2 in the immortalized, non-transformed MCF-

10A breast epithelial cell line. We found that overexpression of wild-type EphA2 de-stabilizes adherens junctions. Interestingly, EphA2 overexpression does not affect overall levels or phosphorylation status of E-cadherin, p120 catenin, β-catenin, and α-catenin, but appears to weaken the adherens junction by upregulating RhoA GTPase activity via p190 RhoGAP and LMW-PTP. Thus, in addition to regulating tumor cell motility, the increased EphA2 receptor levels in tumors also promote destabilization of cell-cell adhesion through regulating RhoA GTPase activity.

Results

Overexpression of EphA2 receptor weakens cell-cell adhesion

Prior studies showed that loss of E-cadherin down-regulates EphA2 receptor levels and induces mis-localization of EphA2 in both tumor cells and mouse embryonic stem cells (40, 63, 125). However, the reciprocal effect of EphA2 on adherens junction components remains unclear. Because EphA2 levels are often elevated in tumor cells [reviewed in (92)], we studied the effects of overexpression of EphA2 receptor tyrosine kinase in the immortalized non-transformed MCF-10A breast epithelial cell line. MCF-10A cells were infected with LZRS retrovirus overexpressing moderate levels of either full length, wild type EphA2-IRES-GFP or an EphA2 cytoplasmic truncation mutant, ΔC-IRES-GFP. Pools of transduced cells were FACS sorted for comparable levels of EphA2 receptor expression and subjected to assays that measure adhesion strength and stability.

To study the impact of increased EphA2 receptor levels on cell-cell adhesion, we first performed a hanging drop aggregation assay, an assay that was designed to

assess the strength of cell-cell adhesion without the influence of cellular adhesion to the plate. As shown in Figure 10A, A431 cells expressing wild-type E-cadherin induced tightly compacted cell aggregates that could not be dissociated by shear force. In contrast, the control A431D cells, which do not express E-cadherin, exhibited loose association and immediate separation of cells when subjected to shear force. MCF-10A cells grown in hanging drop suspension culture do not form compact cellular masses as A431 cells, but they do form a cell spheroid structure that was resistant to shear force separation. Stimulation of ephrin-A1, however, resulted in cell-cell dissociation in MCF10A spheroids. While vector transduced MCF-10A cells behave similarly to parental MCF-10A, overexpression of wild-type EphA2 in MCF-10A cells greatly facilitated the dissociation of cells by shear force. In contrast, MCF-10A cells expressing the EphA2ΔC mutant form a tightly compact cell spheroid that was resistant to dissociation (Fig 10B). These results suggest that EphA2 receptor signaling is required for regulating the strength of cell-cell adhesion.

Overexpression of EphA2 does not affect the expression and phosphorylation of cadherin/catenin proteins nor the composition of adherens junction complexes

To dissect the mechanisms by which overexpression of EphA2 promote dissociation of cell-cell contacts, we examined whether the altered cell-cell adhesion mediated by EphA2 receptor activation involved modulation of the adherens junction complex. We observed no alteration in E-cadherin protein levels in stable junctions from cells cultured to confluence, as judged either by western blot analysis (Fig. 11A) or immunofluorescence (Figure 11C-F). Since EphA2 activation does not result in cadherin loss, we evaluated whether it could compromise cell adhesion by modulating

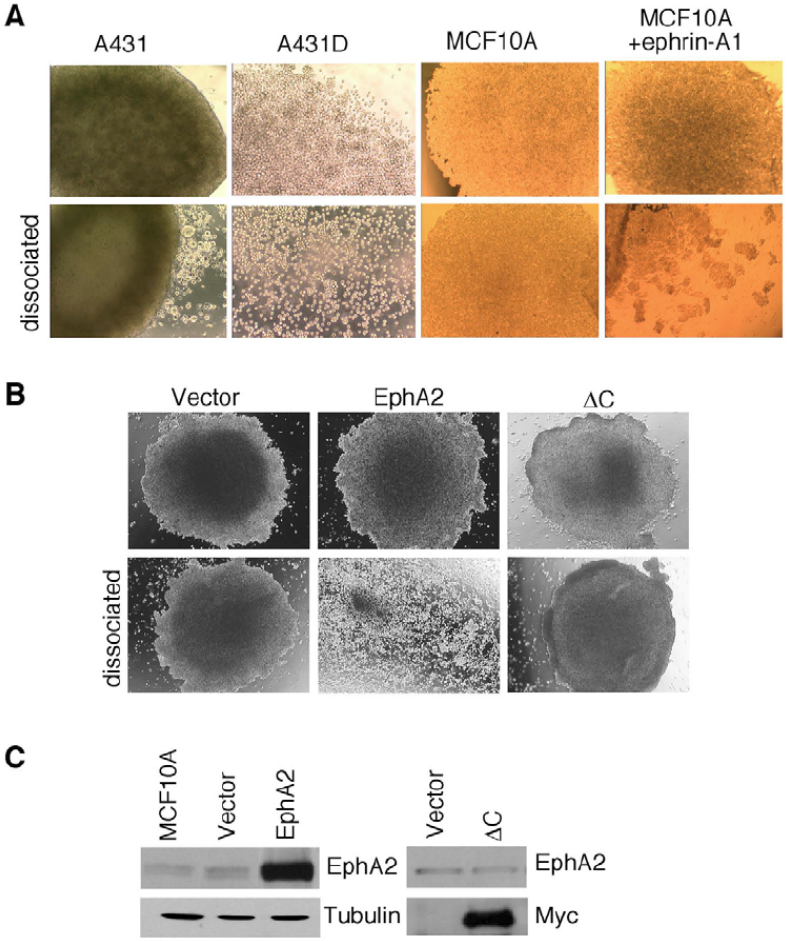


Figure 10. EphA2 overexpression in MCF10A cells impairs cell-cell adhesion. (A) A431, A431D, and MCF10A cells were subjected to a hanging drop aggregation assay, as described in Material & Methods. A431D cells, which lack E-cadherin expression, exhibited a defect in cell-cell adhesion. Ephrin-A1 stimulation also induced dissociation of MCF10A cells. (B) MCF10A-EphA2, but not MCF10A-ΔC cells, exhibited decreased cell-cell adhesion. (C) Western blot analysis of EphA2 and Myc-tagged mutant ΔC protein in MCF10A cells.

the composition of adherens junctional complexes. Adherens junctions consist of E-cadherin molecules, the cytoplasmic domains of which interact directly with p120 catenin and β-catenin. α-catenin interacts with E-cadherin indirectly through binding with β-catenin and links the adherens junction to the actin cytoskeleton. Thus, any member of the catenin family could impact the adhesive capacity and association with the cytoskeleton (124, 131). However, the levels of p120 catenin, β-catenin, and α-catenin were not altered in MCF-10A/WT-EphA2 cells compared to the control cell lines (Fig. 11A). When E-cadherin was immunoprecipitated from MCF-10A/WT-EphA2 cells, the levels of associated catenins were likewise unaltered in these cells compared to the immunoprecipitations from the control cell lines (Fig. 11B). Nor were tyrosine phosphorylation levels of cadherin/catenin protein significantly changed among parental MCF-10A, MCF-10A/EphA2, or MCF-10A/ΔC cells (Fig 11G). Together, these data suggest that EphA2 receptor activation alone is unable to initiate the disruption of adherens junctions. However, as shown in Figure 10, overexpression of EphA2 weakens the strength of cell-cell adhesion and may accelerate dissociation of cells in a dynamic environment.

Overexpression of EphA2 accelerates adherens junction disruption by decreasing the localization of E-cadherin at the adherens junction

To test whether EphA2 can accelerate the dissociation of cell-cell adhesion, we performed a calcium depletion assay. Cells were cultured in low calcium medium and disappearance of cellular junctions was monitored by immunofluorescence with anti-E-cadherin. The rate of junction dissociation was compared in cells expressing either wild-type or mutant EphA2 receptor. As shown in Figure 12E, 8 hours after

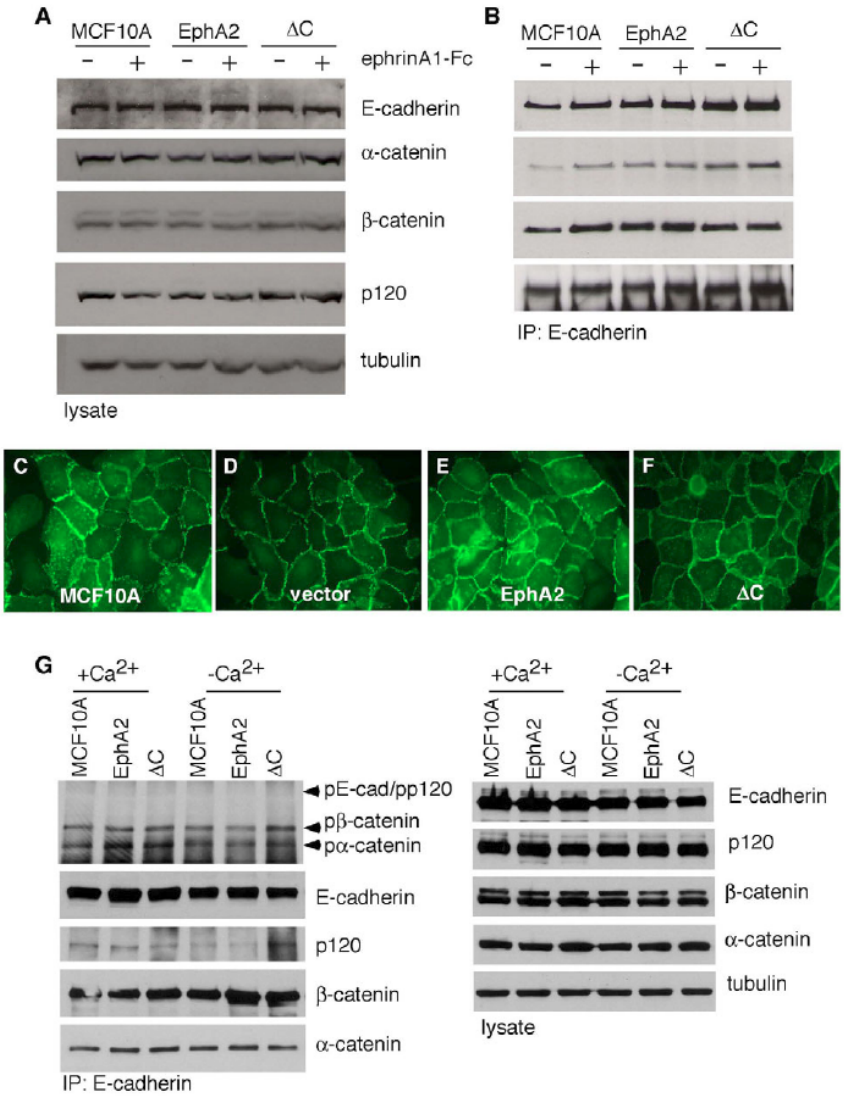


Figure 11. Effects of EphA2 expression on level and phosphorylation of cadherin/catenin proteins and the composition of adherens junction complexes. (A) Total levels of E-cadherin, p120 catenin, β-catenin, and α-catenin were assayed by western blot analysis. No significant differences in expression of these proteins were detected in the respective cell types in the presence or absence of ephrin-A1 stimulation. (B) Association of the catenin proteins to E-cadherin was assessed by co-immunoprecipitation of p120 catenin, β-catenin, or α-catenin with E-cadherin. The ability of these proteins to associate with E-cadherin was unchanged regardless of ephrin-A1 stimulation. (C-F) E-cadherin is localized at cell-cell junctions in confluent MCF10A, vector control, MCF10A-EphA2, or MCF10A-ΔC cells. (G) Association of catenin proteins to E-cadherin was assayed by co-immunoprecipitation in the presence of normal or low calcium medium. Tyrosine phosphorylation of cadherin/catenin proteins was determined by western blot analysis.

calcium depletion, E-cadherin localization was substantially reduced at the junction in cells expressing wild-type EphA2 receptor. In contrast, MCF-10A expressing mutant EphA2ΔC still retained E-cadherin localization at levels similar to those observed in parental MCF-10A cells (Fig. 12F and 12D, respectively). Reduced levels of E-cadherin at cell-cell junctions could be due to a decrease in E-cadherin synthesis, an increase in degradation, a change in cellular localization, or a re-distribution on cell surface. However, total levels of E-cadherin were not changed among parental MCF-10A, MCF-10A/EphA2, or MCF-10A/ΔC cells either in normal or low calcium medium (Fig 11G), indicating that expression or degradation of E-cadherin was not affected in these cells. To determine whether reduced E-cadherin levels at cell-cell contact in MCF10A/EphA2 cells was due to internalization of E-cadherin molecule, we performed a biotinylation assay. No significant alterations of E-cadherin internalization were observed, suggesting that expression of EphA2 did not affect E-cadherin endocytosis (data not shown). Taken together, these data suggest that EphA2-dependent destabilization of cell-cell adhesion is probably mediated by a re-distribution of E-cadherin on the cell surface.

RhoA activity is required for EphA2-mediated destabilization of cell-cell adhesion

Adherens junctions are regulated by multiple mechanisms. In addition to regulation of cadherin/catenin protein levels, phosphorylation status, and composition of adherens junction complex, Rho family GTPases are known to modulate adherens junction stability. As Eph family RTKs are often capable of activating Rho family

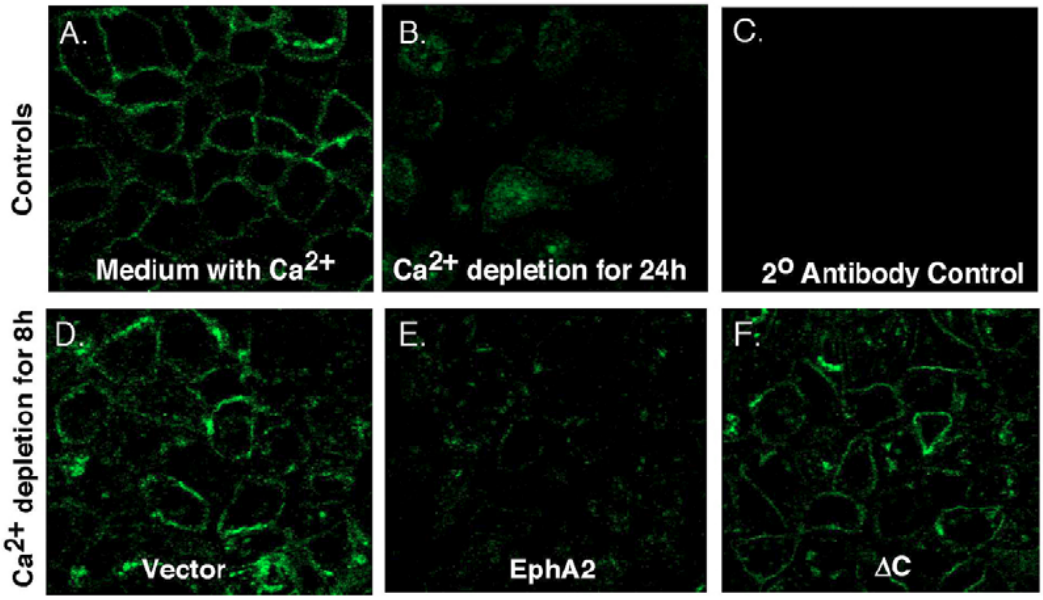


Figure 12. EphA2 overexpression destabilizes adherens junctions. MCF10A cells were cultured in the presence of normal (A&C) or low calcium (B) media for 24 hours. Cells were subsequently fixed and stained for anti-E-cadherin (A&B) or secondary antibody alone (C). MCF10A cells carrying LZRS control vector (D), LZRS-EphA2 (E), or LZRS-ΔC (F) were subjected to calcium depletion for 8 hours and stained for E-cadherin. EphA2 overexpression destabilized adherens junctions, as E-cadherin was barely detectable in MCF10A-EphA2 cells (E).

GTPases (89), we investigated whether EphA2-induced destabilization of cell-cell adhesion is mediated by Rho proteins. We have previously shown that ephrin-A1 stimulation of 4T1 mammary tumor cells induced activation of RhoA GTPase (127). Accordingly, we used a Rhotekin pull-down assay to test whether RhoA GTPase is also activated by EphA2 receptor signaling in MCF-10A cells. As shown in Figure 13A, ephrin-A1 stimulation of MCF10A cells induced RhoA activation within 5 minutes. The basal levels of RhoA activity was elevated in cells expressing WT-EphA2, but was diminished in cells expressing the ΔC-EphA2 mutation. Although RhoA activity is often regulated by Rac (130), we did not observe significant changes in the level of activated Rac1 between cells expressing WT-EphA2 and cells expressing the ΔC-EphA2 mutant.

To test the functional role of EphA2-activated RhoA GTPase in cell-cell adhesion, we assessed the effect of inhibition of RhoA activity on cell adhesion. Rho proteins can regulate adherens junctions by signaling through ROCK kinase (132). Therefore, we performed a calcium depletion assay in the presence of ROCK inhibitor Y-27632 to specifically block the actin-myosin contraction pathway. As shown in Figure 13B and D, 8 hours after calcium depletion, E-cadherin was substantially reduced in cells expressing wild-type EphA2 receptor (Fig. 13D) relative to parental MCF10A (Fig. 13B). However, inhibition of ROCK activity in EphA2 overexpressing cells (Fig. 13E) partially restored the expression of E-cadherin to a level similar to the expression in parental MCF-10A cells (Fig. 13B) and cells expressing the ΔC mutation (Fig. 13C). These data suggest that RhoA activity is required for EphA2-mediated destabilization of cell-cell adhesion.

To investigate whether increased RhoA activation is sufficient to disrupt cell-cell adhesion in MCF10A and MCF10A-ΔC cells, these cells were transduced with control Ade-LacZ or a constitutively activated Rho mutant Ade-Rho (Q63L) (133, 134). As shown in Figure 13F and H, the MCF10A and MCF10A-ΔC cells infected with control viruses exhibited stable adherens junctions. In contrast, expression of activated Rho in the MCF10A and MCF10A-ΔC (Fig. 13G and I) cells destabilized adherens junctions, consistent with the notion that activation of RhoA GTPase is sufficient to regulate the stability of cell-cell adhesion [reviewed in (120, 124, 126)].

EphA2-dependent activation of RhoA is regulated by p190 RhoGAP

Rho GTPases cycle between an inactive, GDP-bound, conformation and an active, GTP-bound, conformation. The Rho proteins can exchange nucleotide and hydrolyse GTP at slow rates in vitro, and these reactions are catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Recent studies showed that p190RhoGAP binds to p120-catenin and regulates cell-cell adhesion via inhibition of Rho activity (135). To investigate whether EphA2-dependent activation of RhoA is regulated by GAP proteins, we measured the phosphorylation state of p190RhoGAP. As shown in Figure 14A, tyrosine phosphorylation of p190RhoGAP in MCF-10A cells overexpressing the EphA2 ΔC mutant was significantly elevated, compared to cells overexpressing wildtype EphA2.

To assess the functional role of p190 RhoGAP in EphA2-dependent destabilization of adherens junctions, MCF-10A and MCF-10A-ΔC cells were

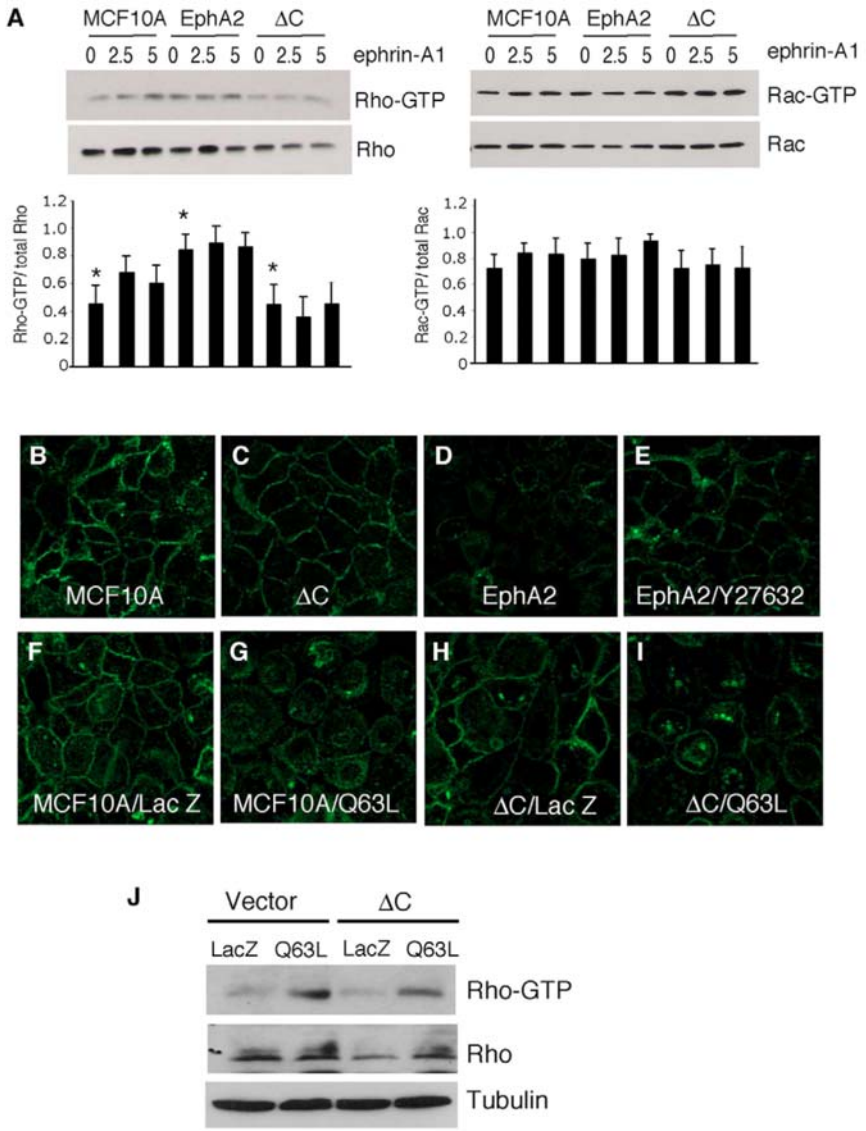


Figure 13. EphA2 regulates adherens junction stability via modulation of RhoA activity. (A) Activated Rho and Rac GTPases in MCF10A, MCF10A-EphA2, or MCF10A-ΔC cells in response to ephrin-A1 stimulation were measured by GST-Rhotekin binding domain and GST-Pak binding domain pull-down assays, respectively. Total levels of Rho and Rac proteins were assay by western blot analysis. * $p<0.05$. (B-E) MCF10A, MCF10A-ΔC, MCF10A-EphA2, or MCF10A-EphA2 treated with ROCK kinase inhibitor, Y27632, were subjected to calcium depletion for 8 hours, followed by detection of E-cadherin. (F-I) MCF10A and MCF10A-ΔC cells expressing a constitutively active Rho (Q63L) or control β-galactosidase (LacZ) were assayed for adherens junction stability by calcium depletion assay. (J) The expression and activity of Q63L in control and MCF10A-ΔC cells were confirmed by Rhotekin pull-down assays and western blot analysis.

transduced with LZRS retrovirus expressing wild type p190 or p190/30-1, a p190 mutant that is defective in GTPase activating enzymatic activity. As expected, in the MCF-10A and MCF-10A-ΔC cells, E-cadherin remains localized to cell-cell contacts even after calcium depletion of 8 hrs (Fig. 14B and E). Expression of wild-type p190 did not affect the localization of E-cadherin (Fig. 14C and F). However, while overexpression of EphA2 resulted in loss of E-cadherin expression at cell-cell contacts (Fig. 14H), expression of wild-type p190 in these cells completely restored E-cadherin expression (Figure 14I). Conversely, expression of the p190/30-1 in the MCF10A and MCF10A-ΔC cells resulted in a destabilization of the adherens junctions, as shown by loss of E-cadherin localization at the cell-cell contacts (Fig. 14D and G). These data suggest that EphA2 destabilizes the adherens junction by regulating p190 RhoGAP activity.

LMW-PTP acts downstream of the EphA2 receptor to regulate p190 RhoGAP

Next, we wanted to determine how p190 RhoGAP is regulated by EphA2. A plausible candidate is the ubiquitously expressed Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP), because p190Rho-GAP has been shown to be a target of LMW-PTP (130, 136). In addition, Eph receptors are known to recruit LMW-PTP upon receptor activation (40, 137). To investigate whether LMW-PTP function can be regulated by EphA2 receptor, we first measured LMW-PTP phosphatase activity in MCF-10A-EphA2 and MCF-10A-ΔC cells using the synthetic substrate DiFMUP (138-140). As shown in Figure 15A and B, LMW-PTP activity was significantly higher in MCF-10A-EphA2 cells than that in MCF-10A-ΔC cells.

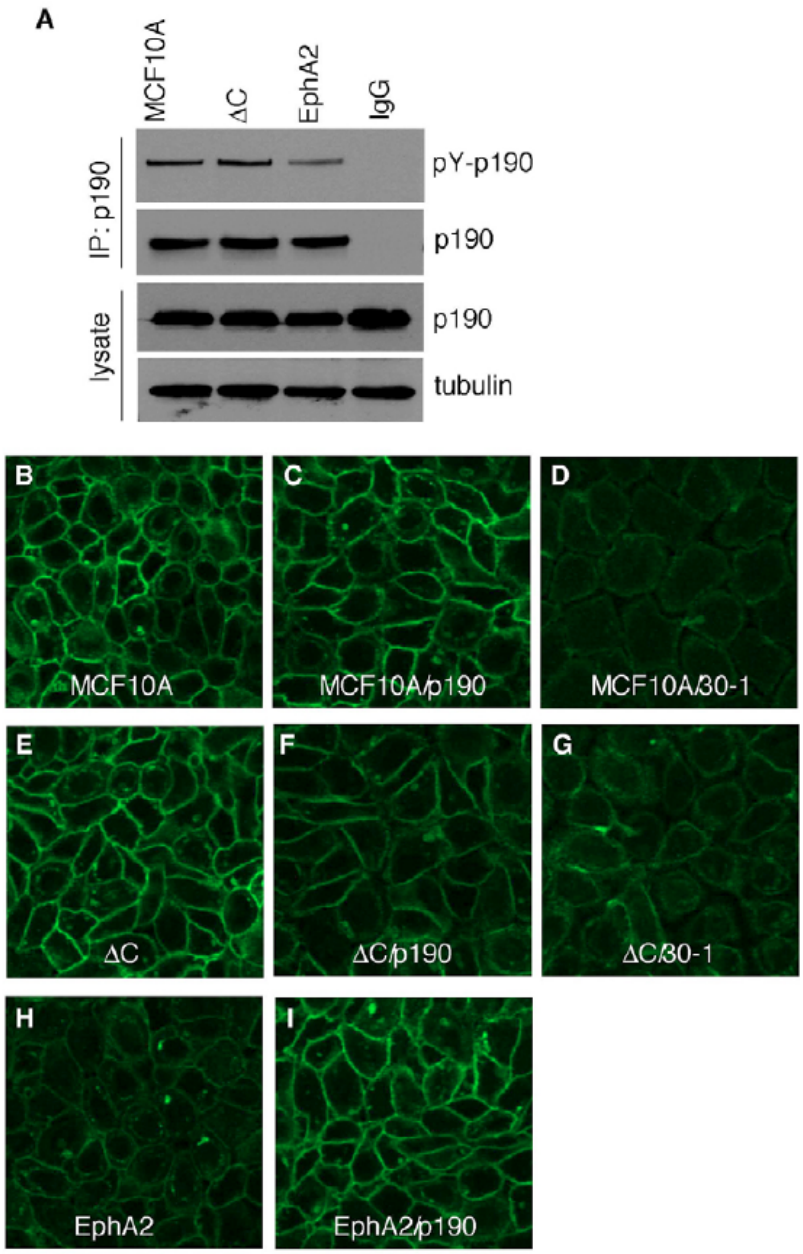


Figure 14. EphA2 regulates adherens junction stability through p190 RhoGAP. (A) p190 RhoGAP were immunoprecipitated from MCF10A, MCF10A-ΔC, or MCF10A-EphA2 cell lysates and blotted for tyrosine phosphorylation. Tyrosine phosphorylation of p190 was decreased in MCF10A-EphA2 cells. (B-I) MCF10A, MCF10A-EphA2, and MCF10A-ΔC cells expressing p190 or the dominant negative p190 mutant, 30-1, were assayed for adherens junction stability by calcium depletion assay. Expression of wild-type p190 rescued the adherens junctions in MCF10A-EphA2 cells (I), whereas expression of 30-1 mutant destabilized cell-cell adhesion in parental MCF-10 cells and MCF10A-ΔC cells (D&G).

To determine the mechanisms by which EphA2 regulates LMW-PTP activity, we investigated whether EphA2 affects LMW-PTP protein levels, phosphorylation states, or recruitment of LMW-PTP to EphA2 receptor. Neither the total levels of LMW-PTP protein nor the tyrosine phosphorylation state were affected by overexpression of EphA2 or inhibition of EphA2 signaling (data not shown). However, higher levels of the interaction between EphA2 and LMW-PTP were detected in MCF-10A-EphA2 cells (Fig. 15C), suggesting that EphA2 receptor recruits LMW-PTP. We reasoned that if EphA2 is signaling through LMW-PTP to destabilize the adherens junctions, this phenotype should be rescued by overexpression of the phosphatase inactive mutant of LMW-PTP, C12S (141). To test this possibility, MCF-10A-EphA2 cells were transduced with retrovirus expressing C12S and assayed for tyrosine phosphorylation of p190 RhoGAP and stability of the adherens junction. Consistent with data shown in Figure 14A, p190 RhoGAP tyrosine phosphorylation is decreased in cells overexpressing EphA2, and expression of the dominant negative C12S mutant restores RhoGAP tyrosine phosphorylation levels (Fig 15D). In addition, while MCF-10A-EphA2 cells transduced with control virus exhibited reduced E-cadherin staining at sites of cell-cell contact (Fig. 15E), expression of the LMW-PTP-C12S mutant stabilized the adherens junctions (Fig. 15F). Taken together, these data suggest that LMW-PTP provides a molecular link between EphA2 receptor activation and the inhibition of p190 RhoGAP, leading to activation of Rho GTPase and destabilization of the adherens junctions.

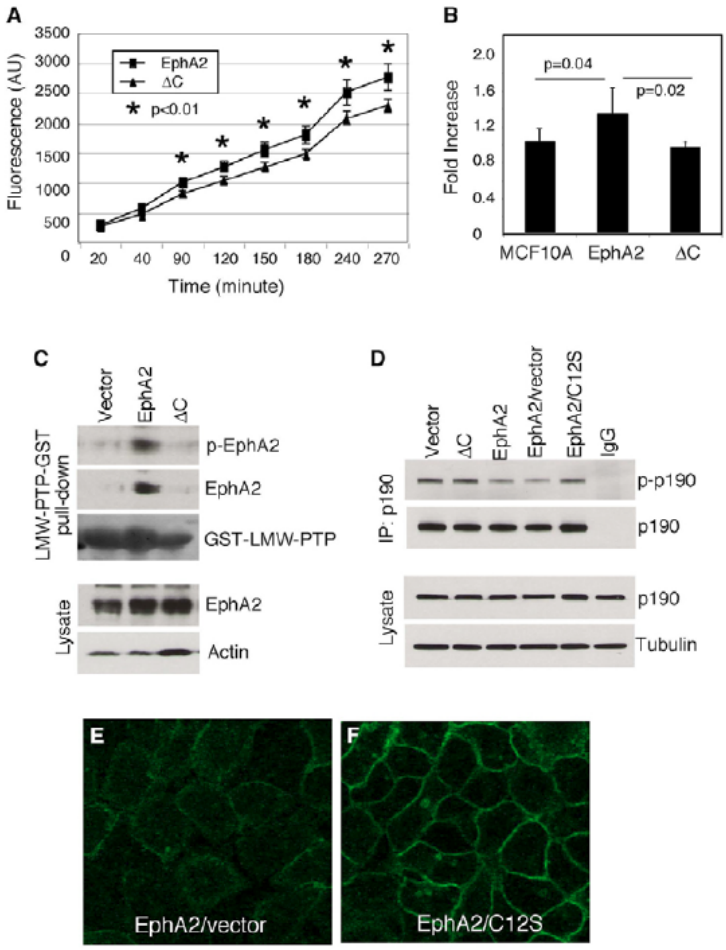


Figure 15. LMW-PTP interacts with EphA2 and regulates adherens junction stability. (A) LMW-PTP was immunoprecipitated from MCF10A, MCF10A-EphA2, or MCF10A-ΔC cells and incubated with phosphatase substrate, DiFMUP, following a time course (as described in Material & Methods). LMW-PTP phosphatase activity was elevated in MCF10A-EphA2 cells, compared to the levels in MCF10A-ΔC cells. (B) Average fold increase of LMW-PTP phophatase activity in different cell types after incubation with substrate for 2.5 hours. (C) The association of LMW-PTP and EphA2 was assessed by GST-LMW-PTP pull-down assay. The levels of EphA2 that bound to GST-LMW-PTP were significantly higher in cells overexpressing EphA2 than those expressing ΔC or vector control. (D) MCF10A-EphA2 cells were transduced with control retrovirus pBABE (vector) or pBABE-LMW-PTP-C12S (C12S), a dominant negative mutant of LMW-PTP. Tyrosine phosphorylation of p190 RhoGAP was decreased in cells expressing EphA2 and EphA2/vector cells. Expression of C12S restored phosphorylated tyrosine levels in MCF10A-EphA2 cells, compared to parental or cells expressing ΔC. (E-F) MCF10A-EphA2 cells were infected with retrovirus carrying either vector or the C12S mutant. Stability of the adherens junctions in these cells was determined by calcium depletion. C12S rescued cell-cell contact in MCF10A-EphA2 cells.

Src kinase interacts with EphA2 to alter adherens junction stability

In addition to LMW-PTP, Src kinase was shown to regulate p190 RhoGAP function (142-144). Moreover, several EphA receptors were found to interact with Src family kinases (72, 145). To determine if Src is involved in EphA2-mediated destabilization of the adherens junctions, total Src and tyrosine phosphorylated Src levels, as well as Src association with EphA2 receptor were assayed in cells overexpressing EphA2 or ΔC. Src expression levels and tyrosine phosphorylation were not significantly changed in the cells overexpressing EphA2 or ΔC (data not shown). In contrast, the levels of Src associated with the EphA2 receptor were significantly higher in MCF-10A-EphA2 cells than those in MCF-10A or MCF-10A-ΔC cells (Fig. 16A and B). Differential recruitment of Src kinase by EphA2 receptor in these cells suggests a possibility of Src kinase-dependent destabilization of cell-cell adhesion. To investigate this possibility, MCF-10A cells overexpressing EphA2 were treated with the Src kinase inhibitor, PP2. MCF-10A-EphA2 cells treated with vehicle control lost E-cadherin expression after calcium depletion for 8 hours (Fig. 16C). In contrast, treatment of cells with PP2 stabilized adherens junction (Fig. 16D), as determined by the increase E-cadherin levels at cell-cell contacts.

To test specifically whether association of EphA2 with Src is required for mediating the destabilization of the adherens junctions, we generated a series of tyrosine (Y) to phenylalanine (F) mutants in the juxtamembrane and kinase domains of EphA2 receptor. These mutants, or control wild-type EphA2, were co-transfected with Src into COS7 cells, and the ability of Src to interact with these mutants was assessed by co-immunoprecipitation and western blot analysis. While the majority of

EphA2 mutants were capable of interacting with Src, two kinase domain mutants, Y812F and Y816F, exhibited significantly decreased association with Src kinase (Fig. 16E). To determine the requirement of EphA2 interaction with Src in mediating the destabilization of the adherens junctions, Y812F, Y816F, or control wild-type EphA2 were expressed in MCF-10A cells. As before, overexpression of wild-type EphA2 destabilized cell-cell adhesion (Fig. 16F). However, EphA2 "uncoupling" mutants that are defective in binding Src, Y812F or Y816F, were unable to destabilize cell-cell adhesion, as evident by strong E-cadherin staining at the cellular junction in calcium depletion assay (Fig. 16G&H). These results suggest that EphA2 recruitment of Src kinase is critical for destabilization of cell-cell adhesion.

To determine whether Src acts upstream or downstream of LMW-PTP in the EphA2 signaling cascade, MCF-10A/Y812F or MCF-10A/Y816F cells were transduced with retrovirus expressing LMW-PTP and assayed for E-cadherin localization at cell-cell contact in low calcium medium. As shown in Figure 16I-L, expression of LMW-PTP in either MCF10-A/Y812F or MCF10-A/Y816F cells destabilizes adherens junction, suggesting that Src kinase functions upstream of LMW-PTP (see diagram in Figure 18).

High levels of EphA2 expression correlate with accelerated destabilization of the adherens junction

EphA2 receptor tyrosine kinase overexpression is common in malignant tumor cells, and the level of EphA2 receptor expressed on tumor cells correlates with the degree of tumor malignancy (123). To determine how the level of EphA2 expression

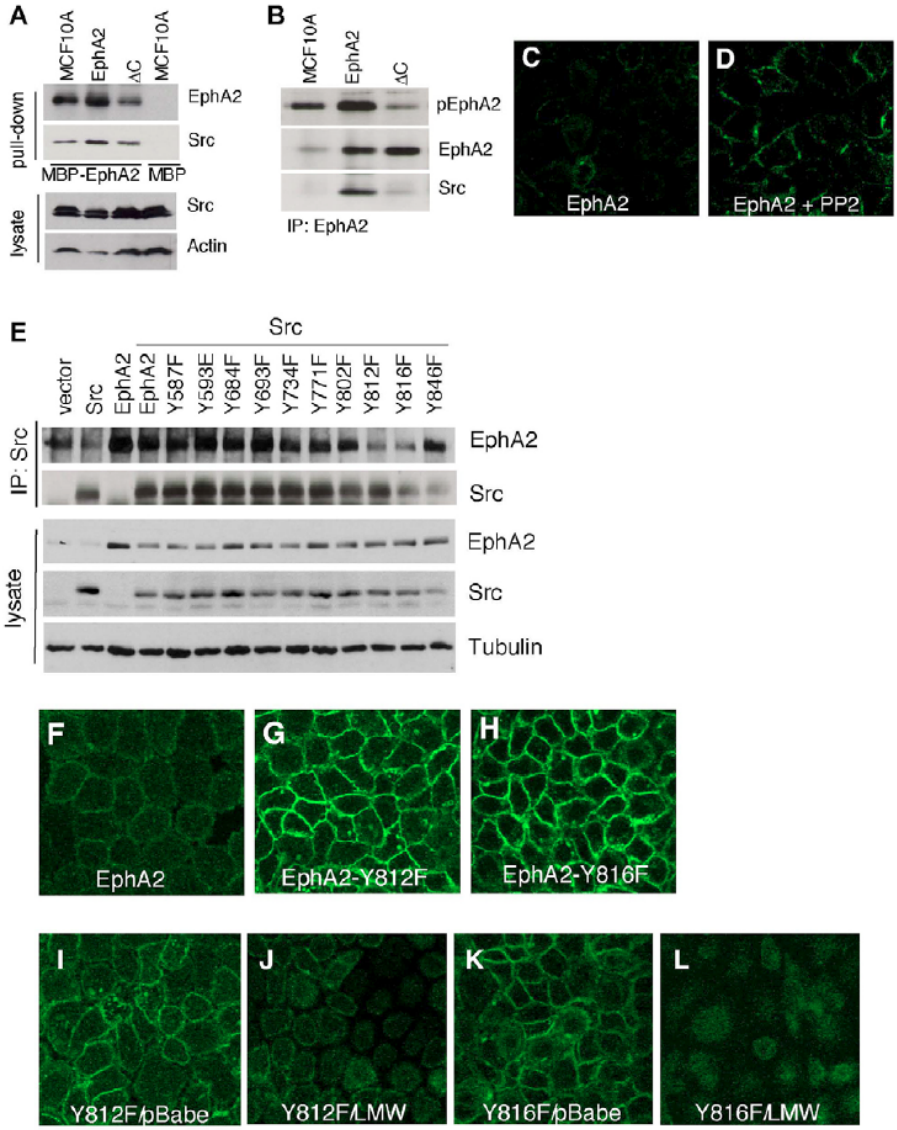


Figure 16. EphA2 recruits Src kinase to regulate adherens junction stability. Association of EphA2 with Src kinase was measured by an MBP-EphA2 pull-down assay (A) and co-immunoprecipitation/western blot analysis (B). (C-D) Inhibition of Src activity restores adherens junction stability in MCF10A-EphA2 cells. (E) Co-immunoprecipitation of EphA2 and Src from COS7 cells transfected with Src and wild-type or mutant EphA2. (F-G) Overexpression of EphA2-Y812F and EphA2-Y816F mutants in MCF10A cells fails to destabilize adherens junction, even after 8 hrs of calcium depletion. (I-L) Overexpression of wild type LMW-PTP is sufficient to destabilize adherens junctions in MCF10A/ Y812F and MCF10A/ Y816F cells.

affects cell-cell adhesion, we first examined the EphA2 expression levels in MCF-10A, MCF-10A/LZRS-EphA2, MCF-10A/Ad-EphA2, and two commonly used human breast cancer cell lines, BT-549 and MDA-MB-231. As shown in Figure 17, EphA2 is expressed at low levels in MCF10A, a non-transformed breast epithelial cell line. Expression of exogenous EphA2 receptor via LZRS retroviral transduction increased EphA2 expression modestly above endogenous level, but the EphA2 levels in theses cells are still considerably lower that those observed in two tumor cell lines or cells infected with adenoviruses (Fig. 17A). Interestingly, although levels of EphA2 were significantly higher in MDA-MB-231 or BT-549 cells, the phosphorylation state of the receptor was dramatically lower in the tumor cells, possibly reflecting the fact that the tumor cells have lost cell-cell contact and were unable to interact with ephrin ligands on adjacent cells.

To determine the effect of EphA2 expression on cell-cell adhesion, we compared E-cadherin expression in low calcium medium among MCF-10A, MCF-10A/LZRS-EphA2, or MCF-10A/Ad-EphA2. Compared to MCF-10A or MCF-10A/LZRS-EphA2, MCF-10A/Ade-EphA2 cells depleted of calcium exhibited a dramatic increase in rate at which the adherens junctions were disrupted (Fig. 17B). Within 2 hours of calcium depletion, overexpression of EphA2 in MCF-10A/Ade-EphA2 cells resulted in a complete loss of E-cadherin staining at cell-cell contacts. In contrast, destabilization of the adherens junctions was only detected at 8 hours of calcium depletion in cells expressing moderate amounts of EphA2 (MCF-10A/LZRS-EphA2). Taken together, these data suggest that EphA2 overexpression can destabilize the

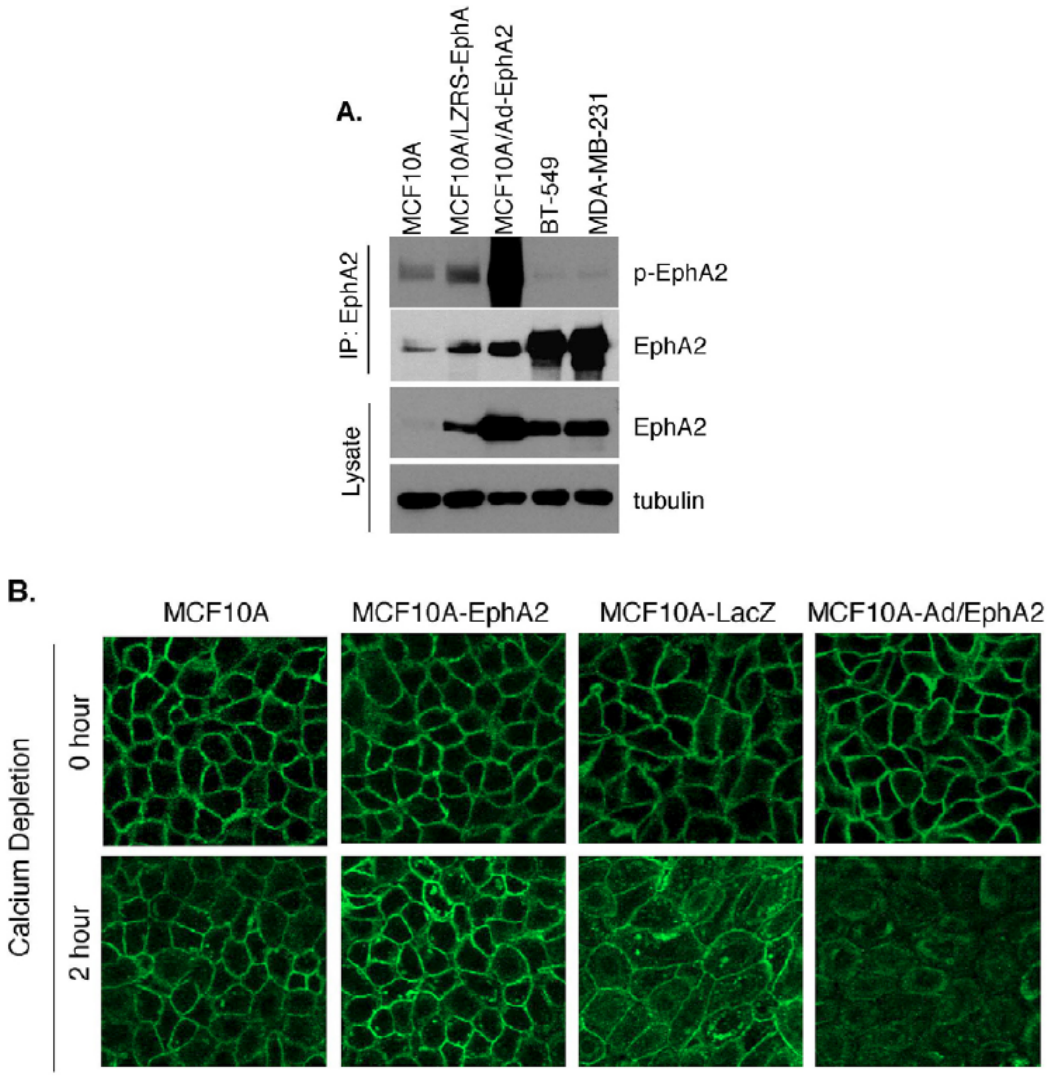


Figure 17. Level of EphA2 overexpression determines rate of adherens junction disassembly. (A) Expression and tyrosine phosphorylation of EphA2 receptor in various MCF10A cells and two breast cancer cell lines, BT-549, and MDA-MB-231, were determined by immunoprecipitation and western blot analysis. (B-I) MCF10A, MCF10A/LZRS-EphA2, MCF10A/LacZ, or MCF10A/Ad-EphA2 were immunostained for E-cadherin localization in the presence of normal or low calcium medium for 2 hours.

adherens junctions and the rate of destabilization of the adherens junctions is dependent upon EphA2 levels.

Discussion

Despite significant evidence supporting a role for EphA2 receptor tyrosine kinase in tumorigenesis, the mechanisms by which EphA2 overexpression contributes to tumor progression are not completely understood. Adherens junctions play a fundamental role in embryonic development and the in the maintenance of tissue architecture in adults. Loss of cadherin function has been associated with migratory behavior in vitro and is a hallmark of invasive carcinoma in vivo. Previous studies showed that loss of E-cadherin inhibits the expression of Eph receptors in embryonic stem cells (63), suggesting EphA2 expression is regulated by E-cadherin. However, many malignant tumor cells that have lost E-cadherin expression exhibited elevated EphA2 receptor levels (40, 92), raising the question of whether EphA2 levels affect the adherens junctions. In this report, we studied how cell-cell adhesion is affected by EphA2 overexpression. We found that overexpression of EphA2 does not induce epithelial to mesenchymal transition directly; however, elevated EphA2 expression destabilizes adherens junctions and accelerates dissociation of cell-cell contact. The rate of destabilization of the adherens junctions is dependent upon EphA2 levels; higher EphA2 expression leads to more rapid down-regulation of E-cadherin localization at cell-cell contacts. The fact that overexpression of EphA2 also affects E-cadherin suggests that a reciprocal regulation exists between E-cadherin and EphA2. A delicate balance of relative levels of E-cadherin and EphA2 receptor

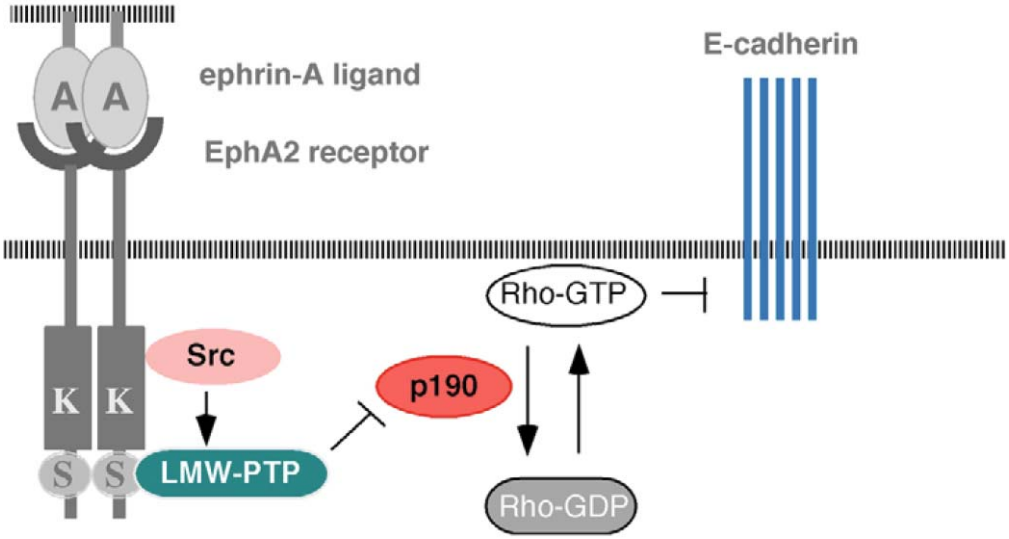


Figure 18. A model for how EphA2 overexpression promotes destabilization of adherens junctions. Overexpression of EphA2 increases the recruitment of LMW-PTP and Src kinase. Increased LMW-PTP phosphatase activity dephosphorylates p190 RhoGAP and inhibits p190 RhoGAP activity. Decreased p190 RhoGAP activity in turn upregulates activated Rho-GTP levels. Rho signaling through ROCK destabilizes the adherens junction.

appears to ensure normal cellular architecture and function.

Several cellular mechanisms have been proposed to perturb adherens junctions. These include cleavage of the cadherin extracellular domain, phosphorylation of the cadherin complexes, increased turnover of cadherin receptors, and regulation of cytoskeletal attachment to cadherin complexes (120, 126, 146). Activation of receptor tyrosine kinases (RTKs) has been shown to destabilize adherens junctions through phosphorylation of cadherin/catenin junctional proteins (146) and ephrin-A1 stimulation is reported to induce β-catenin phosphorylation in HT-29 colon carcinoma cells (64). However, we did not observe changes in tyrosine phosphorylation of E-cadherin, p120, and α– or β-catenin among MCF-10A, MCF-10A/EphA2, or MCF-10A/ΔC cells, a result that is consistent with the data reported by Orsulic et al (63) and by Kinch et al (personal communication). Nor did we detect any alternations of cadherin expression or composition of adherens junction complex (Fig 11). In addition, no significant changes in cortical actin cytoskeleton were observed, suggesting that the effects of EphA2 on adhesive strength are probably not due to gross disruption of the cytoskeleton.

A growing number of studies have linked Rho family small GTPases with cadherin-dependent cell-cell contacts [reviewed in (120, 124, 126)]. Rho family GTPases can regulate cell-cell adhesion by acting on the cadherin-catenin complex or on the actin cytoskeleton and other components. Rac1 and Cdc42 have been shown to directly affect the cadherin-catenin complex by modulating the interaction of IQGAP1, a GTPase activating protein, with β-catenin (147). In contrast, activation of RhoA has been implicated in regulating E-cadherin-mediated adhesive activity

through the actin cytoskeleton (126, 148). Here we show that EphA2 signaling activates RhoA GTPase. As the ROCK kinase inhibitor suppresses EphA2-induced destabilization of cell-cell adhesion, these data suggest a critical role of EphA2-induced RhoA activation in regulating the strength of adherens junctions. In fibroblasts, Rho activity can be regulated by Rac via a signaling pathway involving ROS, LMW-PTP, and p190 RhoGAP (130). More recently, Wildenberg et al showed that a p120 catenin-p190 RhoGAP interaction is required for Rac inhibition of Rho in the stabilization of adherens junctions (135). Although EphA2 receptor activation in vascular endothelial cells upregulates Rac1 activity (25), EphA2 receptor overexpression in mammary epithelial cells does not appear to affect Rac-GTP levels (Fig 13A). Enhanced RhoA activity in MCF-10A-EphA2 cells is apparently regulated by enhanced LMW-PTP phosphatase activity and inhibition of tyrosine phosphorylation of p190 RhoGAP, ultimately leading to the destabilization of cell-cell adhesion (diagramed in Fig 18).

It is interesting to note that while LMW-PTP can be activated by EphA receptors [this report and (129)], EphA2 receptor has also been shown to be a substrate for the same phosphatase (68, 71). However, in our system, phosphorylation of EphA2 does not appear to be affected by LMW-PTP activity. While a general PTP inhibitor, pervanadate, increased EphA2 phosphorylation, overexpression of wild-type or a C12S mutant of LMW-PTP did not change the EphA2 phosphorylation level significantly (data not shown), suggesting that EphA2 may not be a major substrate of LMW-PTP in MCF10A cells.

How does EphA receptor activation enhance LMW-PTP activity? In the case of EphA8, the kinase activity of the receptor directly phosphorylates LMW-PTP (129). However, direct phosphorylation of LMW-PTP by EphA2 was not observed in these cells, indicating that EphA2 regulates LMW-PTP activity by alternative mechanism(s). One possible mechanism is through Src kinase. Although Src can phosphorylate both LMW-PTP and p190 RhoGAP (142-144, 149), the phenotype of MCF-10A-EphA2 cells suggest that p190 RhoGAP is most likely not a candidate substrate for Src. As EphA2 can physically associate with both Src kinase and LMW-PTP, Src could possibly regulate cell-cell adhesion through modulating LMW-PTP activity. Alternatively, Src could also affect adherens junctions by upregulating Rho GEF activity. Further experiments are needed to dissect these possibilities.

In addition to adherens junctions, tight junctions also contribute to intercellular junctional complexes (124). Members of Eph family have also been implicated in regulating tight junctions. Ephrin-B1 binds to Claudin-1 and Claudin-4, which are components of tight junction complexes. In addition, ephrin-B1 is phosphorylated in a Src kinase-dependent manner upon cell-cell contact (150). Furthermore, Tanaka et al reported that EphA2 phosphorylates the cytoplasmic tail of Claudin-4 and mediates paracellular permeability in MDCK cells (65). Whether EphA2 overexpression in MCF-10A cells also affects tight junctions in addition to regulation of adherens junctions still remains to be determined. Furthermore, it will be interesting to determine whether other members of Eph RTK family also play a role in regulating adherens junctions.

In summary, our results support a role of EphA2 receptor in regulation of cell-cell adhesion. EphA2 overexpression likely promotes destabilization of adherens junction through a signaling pathway of recruitment of Src kinase, enhanced LMW-PTP activity, inhibition of p190 RhoGAP, and activation of RhoA GTPase. As EphA2 level is linked to tumor malignancy, these studies provide a foundation for investigating EphA2 as a potential target for therapeutic intervention.

CHAPTER V

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF PHOSPHORYLATED TYROSINE RESIDUES WITHIN EPHA2 RECEPTOR TYROSINE KINASE

Introduction

The Eph receptors belong to a large family of receptor tyrosine kinases (RTKs) that regulate a variety of physiological processes during development and contribute to the pathogenesis of diseases such as cancer (3, 81). One of the key events important both in embryogenesis and pathogenesis in adult organisms is angiogenesis, the process by which new blood vessels are formed from pre-existing vasculature. On the basis of sequence homology and binding affinity, the Eph receptors are divided into two subclasses. EphA receptors bind preferentially to the glycosylphosphatidylinositol (GPI)-linked ephrin-A ligands, while EphB receptors bind preferentially to the transmembrane ephrin-B ligands (87). Both class A and class B Eph receptors have been implicated in regulation of vascular remodeling and angiogenesis. Targeted disruption of several class B RTKs and ephrin-B ligands resulted in defects in angiogenic remodeling of the rudimentary embryonic vasculature (12, 13, 16, 151). Manipulation of the level of one receptor, EphB4, in tumor cells also affected tumor angiogenesis in adult animals (114, 152). In the A class, ephrin-A1 stimulates endothelial cell migration and assembly in culture (17, 22) and induces corneal angiogenesis in vivo (21, 153). More recently, Eph receptors have been detected in tumor blood vessel endothelial cells (13, 81). Inhibition of

class A Eph receptor signaling by soluble EphA2-Fc or EphA3-Fc receptors decreased tumor volume, tumor angiogenesis, and metastatic progression in vivo (23, 39, 97). A main target of soluble EphA receptors appears to be EphA2, as EphA2-deficient endothelial cells fail to migrate and assemble in vitro (25) and loss of EphA2 receptor resulted in impaired tumor growth and metastasis in vivo (24).

The binding of ephrin ligands to Eph receptors induces the transphosphorylation of the cytoplasmic domains and initiates kinase activity. Extensive tyrosine phosphorylation of the activated Eph receptor is not only induced by auto/trans-phosphorylation, but also elicited by receptor associated protein tyrosine kinases such as Src family kinases (3). Many phosphorylated tyrosine residues in the EphB receptors and ephrin-B ligands in neuronal cells/tissues have been mapped by both phosphopeptide mapping using two-dimensional chromatography, and by MALDI mass spectrometry (154-156). Several tyrosine-phosphorylation sites in EphA3 and EphA4 have also been identified by mutational analysis on sites homologous to those in EphB receptors (59, 156). However, as these phosphorylated tyrosine residues are not mapped in endothelial cells, their role in signal transduction leading to angiogenic responses is not clear. Moreover, phosphorylated tyrosine residues have not been mapped in EphA2, a major EphA receptor that is critical in mediating tumor angiogenesis.

We have previously shown that activation of EphA2 receptor in endothelial cells recruits Vav GEFs, resulting in upregulation of GTP-bound activated Rac1 GTPase and endothelial cell migration (56). The Vav GEF/Rac1 pathway appears to be regulated by PI3 kinase, as PI3 kinase-specific inhibitors wortmanin and LY294002,

or a dominant negative p85 subunit of PI3 kinase, block ephrin-A1-induced Rac1 activation and endothelial cell migration (25). As the SH2 domains of both Vav GEFs and p85 subunit of the PI3 kinase are capable of binding to phosphorylated EphA2 receptor (56, 69), we sought to identify critical phosphorylated tyrosine residues that mediate the recruitment of Vav GEFs and p85. As a first step, we have used a combination of mass spectrometry analysis and traditional phosphopeptide mapping to identify the phosphorylated tyrosine residues within the EphA2 receptor. Four phosphorylated tyrosine residues in the cytoplasmic domain of the EphA2 receptor were identified. Changing three of these sites to phenylalanine or glutamic acid resulted in an EphA2 mutant that could not be phosphorylated, failed to interact with p85 or Vav GEFs, and was unable to rescue defects in endothelial assembly in EphA2-deficient cells in vitro and in vivo. Our results suggest that phosphorylation of tyrosine Y587/593 and Y734 is critical in recruitment of Vav and p85, respectively. Phosphorylation of these tyrosines is also essential in activation of Rac1 GTPase and promoting angiogenic responses and tumor neovascularization.

Results

Mapping tyrosine phosphorylation sites in EphA2 receptor

To identify the phosphorylated tyrosine residues in the cytoplasmic domain of EphA2 receptor induced upon binding to ephrin-A1 ligand, we initially expressed EphA2 in COS7 cells. Immunoprecipitated EphA2 proteins were digested with trypsin and subjected to LC-MS mass spectrometric analysis. Greater than 50% of the tryptic peptides were not detected and were therefore not analyzed. Among the

Table 2. Tryptic peptides from in vivo phosphorylated EphA2 identified by Mass Spectrometry

Tyrosine	$Mass_{calc}$	$Mass_{measured}$	Δ	Peptide
Y593	2068.9	2071.1	2.2	TYVDPHTY$_{PO4}$EDPNQAVLK
Y734	1375.6	1375.4	0.2	YLANM$_{OX}$NY$_{PO4}$VHR
Y771	1761.7	1762.3	0.6	VLEDDPEATY$_{PO4}$TTSGGK

$Mass_{calc}$, calculated mass. $Mass_{measured}$, measured mass. Δ, difference between measured mass and calculated mass. OX, oxidized methionine

remaining tryptic peptides analyzed, three phosphorylated peptides were identified which contained Y593 in the juxtamembrane domain, as well as Y734 and Y771 in the kinase domain (Figure 19 and Table 2).

To verify phosphorylation sites mapped by mass spectrometry and to identify additional sites not covered by mass spectrometric analysis, we performed phosphopeptide mapping by two-dimensional chromatography in conjunction with site-directed mutagenesis. We chose to use immortalized EphA2-null and wild-type control endothelial cell lines for our analysis, as the EphA2-null background facilitates mutational analysis and subsequent functional assays. These endothelial cells were isolated from EphA2-deficient mice that were bred into the *H-2K*^{b}-tsA58 transgenic "Immorto-mouse" background (157). These immorto-mice harbor a temperature sensitive SV40 **T a**nti**g**en (TAg) cassette driven by the mouse major histocompatibility complex *H-2K*^{b} promoter, which permits expression in a wide array of tissues. In addition, the promoter is responsive to interferon-γ, permitting elevated expression of the TAg in cells derived from these mice when cultured at 33ºC in the presence of interferon-γ. Once cells are placed at physiologic temperature (37ºC), protein levels of the thermolabile TAg are downregulated and cells are restored to a non-transformed state over the course of several days (157).

Wild-type and a panel of Y to F mutant EphA2 constructs were stably expressed in endothelial cells via retroviral transduction using the LZRS retroviral system (127). In vitro kinase assays using an exogenous substrate revealed that Y593E, Y587/593EE, Y734F and Y771F mutations do no affect kinase activity significantly. However, Y587F and Y929F inhibited, and Y593F abolished EphA2 kinase activity

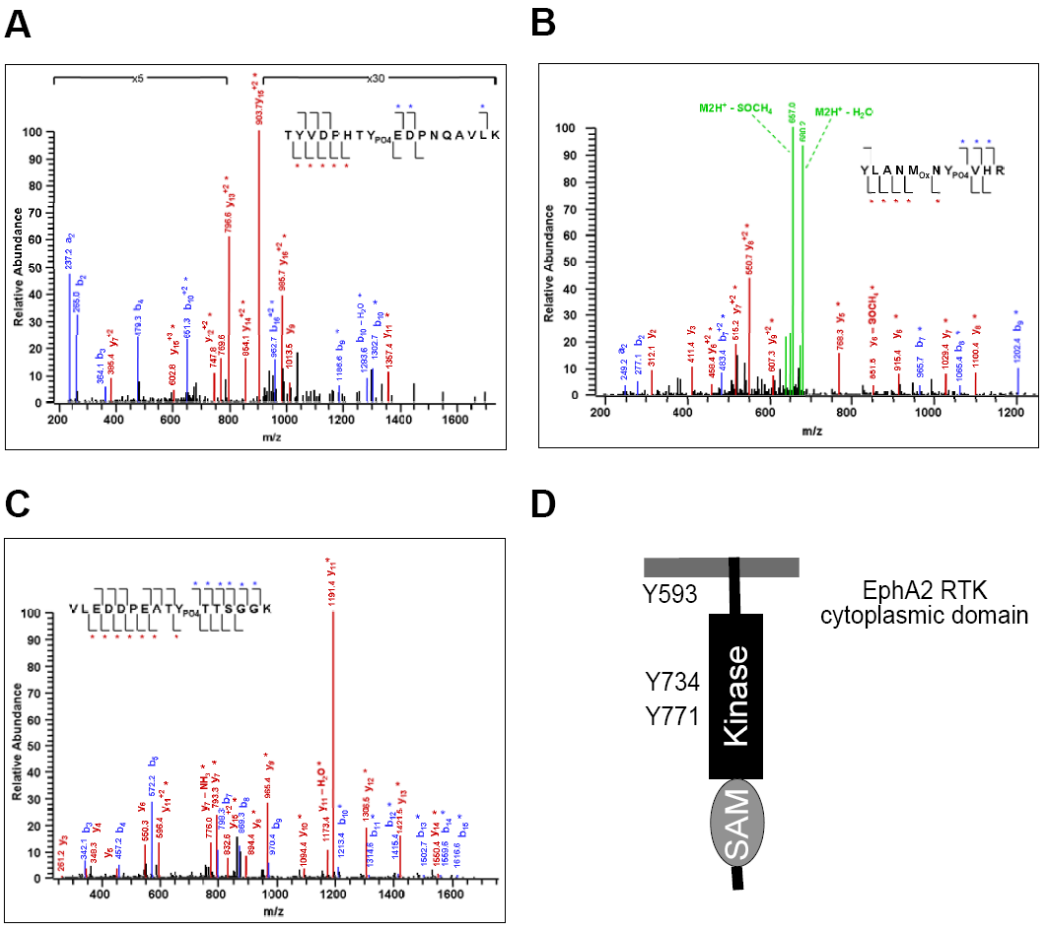


Figure 19. In vivo tyrosine phosphorylation sites of EphA2 in transfected COS7 cells. (A-C) LC-MS mass spectra of tyrosine-phosphorylated tryptic peptides from immunoprecipitated EphA2. Tyrosine-phosphorylated peptides were isolated from the resulting peptide mixture and purified by reverse phase chromatography. Peaks corresponding to tyrosine-phosphorylated peptides of EphA2 are denoted by their masses and sequences. (A) Triply charged peptide with m/z 691.4. (B) Doubly charged peptide with m/z 688.69. (C) Doubly charged peptide with m/z 882.14. (D) Schematic diagram of phosphorylated tyrosine residues in the cytoplasmic domains of the EphA2 receptor.

(Figure 20A). Y587/593 and Y771 appeared to be major tyrosine phosphorylation sites in the EphA2 receptor, as phosphorylation of EphA2 was markedly reduced in Y587/593EE and Y771F mutants (Figure 20B), despite the observation that these mutants retained kinase activity.

Phosphopeptide mapping by two dimensional chromatography detected five distinct phosphopeptides in activated wild-type EphA2 (Figure 21, Experiment #1). To identify the phosphorylated tyrosines within the tryptic peptides, the phosphorylated tyrosines identified by mass spectrometry analysis or those tyrosine residues that were not covered were mutated to phenylalanine. These include tyrosine residues in the juxtamembrane region (Y587F, Y593F), kinase domain (Y685F, Y693F, Y734F, Y771F, Y802F, Y812F, Y816F, Y846F), and the carboxy terminal SAM domain (Y921F, Y929F, Y959F). Because the Y593F could not be analyzed due to defective kinase activity resulting in insufficient γ-^{32}P incorporation, a tyrosine to glutamic acid mutant, Y593E, that retained kinase activity was used for further analysis. Tryptic phosphopeptide maps of wild-type microvascular endothelial cells were similar to those EphA2-null cells reconstituted with wild-type EphA2 receptor (Figure 21, Experiment #1). Each of the EphA2 mutants was deficient in certain γ-^{32}P-labeled phosphopeptides. The Y587F mutant lacks two major phosphopeptides (**a** & **b**). Phosphopeptide **c** was absent in the Y593E mutant, and phosphopeptide **d** was absent in the Y771F mutant. Phosphorylation of Y734 was identified in a separate experiment when the first dimension chromatography was performed in the reverse direction (Figure 21, Experiment #2). Taken together, these

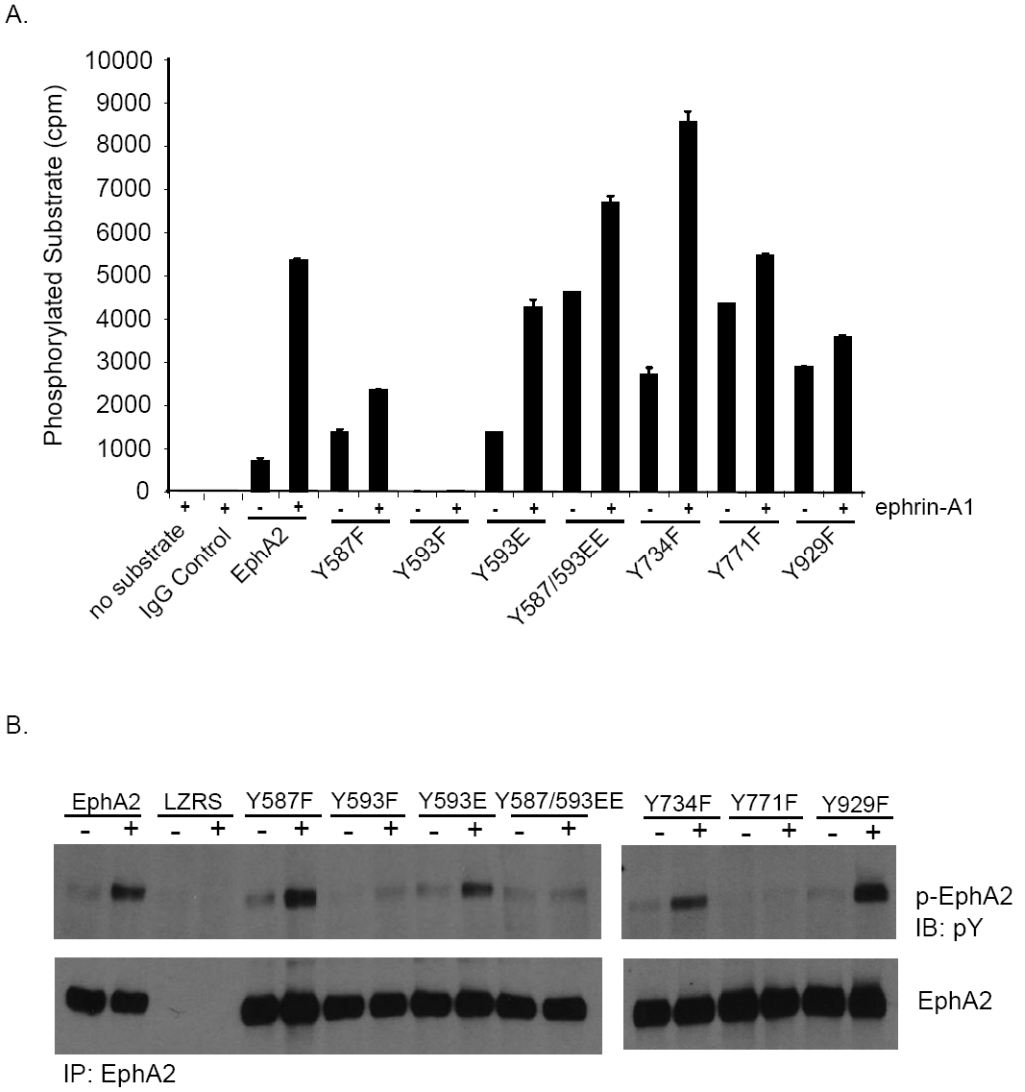


Figure 20. Wild type and mutant EphA2 kinase activity. (A) In vitro kinase assay was performed on EphA2 immunoprecipitated from EphA2-null endothelial cells reconstituted with wild-type or mutant EphA2 via LZRS retroviral transduction. EphA2 kinase activity was measured by its ability to phosphorylate the synthetic substrate, poly (glu:tyr) (4:1). (B) Phosphorylated EphA2 levels were assayed in EphA2-null endothelial cells reconstituted with wild type or mutant EphA2. EphA2 was immunoprecipitated by an anti-EphA2 antibody, and tyrosine phosphorylation was detected by a mixture of anti-pY20 and anti-pY99 antibodies.

results suggest that Y587, Y593, Y771, and Y734 are likely to be autophosphorylated in vascular endothelial cells.

Vav GEFs binds to pY587/Y593 in the juxtamembrane region and p85 interacts to pY734 in the EphA2 kinase domain

We have previously shown that guanine nucleotide exchange factors Vav2 and Vav3 are recruited to phosphorylated EphA2 receptor, and the binding is significantly reduced in Y587F/Y593F double mutants (56). To assess which phosphorylated tyrosine residue, or whether both pY sites, in the juxtamembrane region of the EphA2 is/are required for interaction with Vav proteins, we performed a series of co-immunoprecipitation experiments coupled with western blot analysis. As shown in Figure 22A, mutation at either Y587 or Y593 inhibited binding of EphA2 receptor to Vav2 and Vav3 exchange factors, suggesting that both sites are required for optimal binding to Vav GEFs. Interestingly, Y929 in the SAM domain also appears to affect binding to Vav3, but not Vav2 GEF. As phosphorylation of Y929 was not detected by in vitro kinase assay, this site might be phosphorylated by another tyrosine kinase in vivo.

In addition to binding to Vav GEFs, we and others have shown that activated EphA2 receptor also recruits the p85 subunit of the PI3 kinase, and PI3 kinase activity is required for ephrin-A1 induced Rac1 GTPase activation and endothelial cell migration (25, 69). However, although the SH2 domain of the p85 was shown to interact with the kinase domain of the EphA2 receptor (69), the precise phosphotyrosine residue that mediates this interaction is unknown. Thus, a panel of

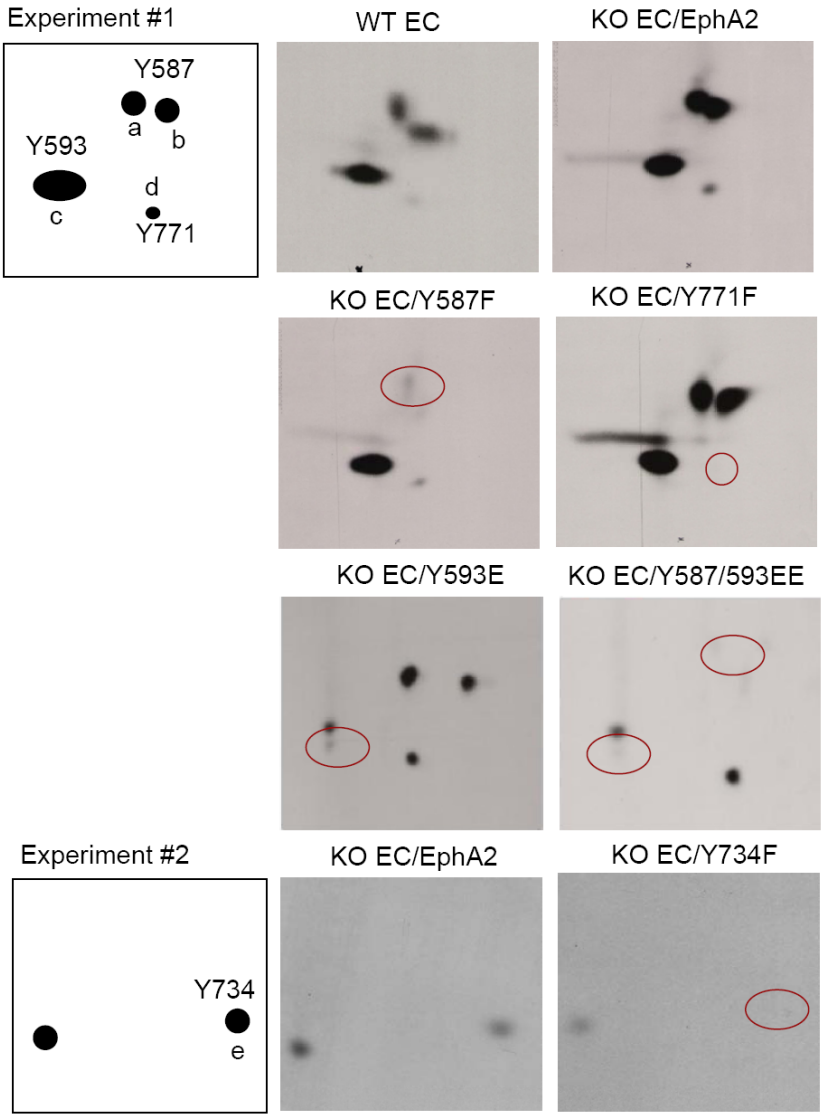


Figure 21. Tryptic phosphopeptide maps of wild-type and mutant forms of EphA2. Wild-type and mutant forms of EphA2 were expressed in EphA2-null endothelial cells via LZRS retroviral transduction. EphA2 receptors were immunoprecipitated, phosphorylated in the presence of [γ-^{32}P] ATP, and resolved by SDS-PAGE. Radioactive bands representing phosphorylated forms of EphA2 were excised, digested with trypsin, and subjected to two-dimensional chromatography. Four peptides were detected (labeled a-d) in both wild-type and EphA2-null reconstituted with wild-type EphA2 in Experiment #1. An additional phosphopeptide (labeled e) was identified in Experimental #2 when the first dimension chromatography was performed in the reverse direction. Peptides containing identified phosphorylated tyrosines are indicated on the left in two schematic representations, one for each set of experiments.

EphA2 mutants containing Y to F mutations was tested for the ability to bind to p85. As shown in Figure 22B, while p85 binds to Y587F, Y593E, Y587/593EE, and Y771F as well as wild-type EphA2, it fails to bind to Y734F and Y929F, suggesting that p85 interacts with phosphorylated Y734 in the kinase domain and Y929 in the SAM domain.

We have previously shown that the SH2 domain of Vav3 binds to EphA2 in a yeast two-hybrid system and in an in vitro binding assay (56). To determine the mechanism of interaction between EphA2 and Vav/p85 in mammalian cells, we performed co-immunoprecipitation assays using the SH2 domain of p85 or Vav3, or a Vav2ΔSH2 mutant. As shown in Figure 22C, the SH2 domain of p85 or Vav3 were capable of binding to EphA2 receptor, whereas the Vav2ΔSH2 mutant fails to interact with EphA2, suggesting that interaction between EphA2 and Vav/p85 is mediated by binding between SH2 domains and pY sites in EphA2. Taken together, our data suggest that VavGEFs and p85 are major (but perhaps not the sole) binding partners of EphA2 receptor.

Mutations that uncouple EphA2 receptor with Vav or p85 inhibit ephrin-A1-induced Rac1 GTPase activation and migration

Dynamic regulation of the actin cytoskeleton is critical in cell migration, and Rho family GTPases are known to be key regulators of this process and have been shown to be necessary for endothelial cell migration (103). We have previously reported that ephrin-A1 stimulation of endothelial cells induces activation of Rac1 GTPase through activation of guanine nucleotide exchange factors Vav2/Vav3 (56). In

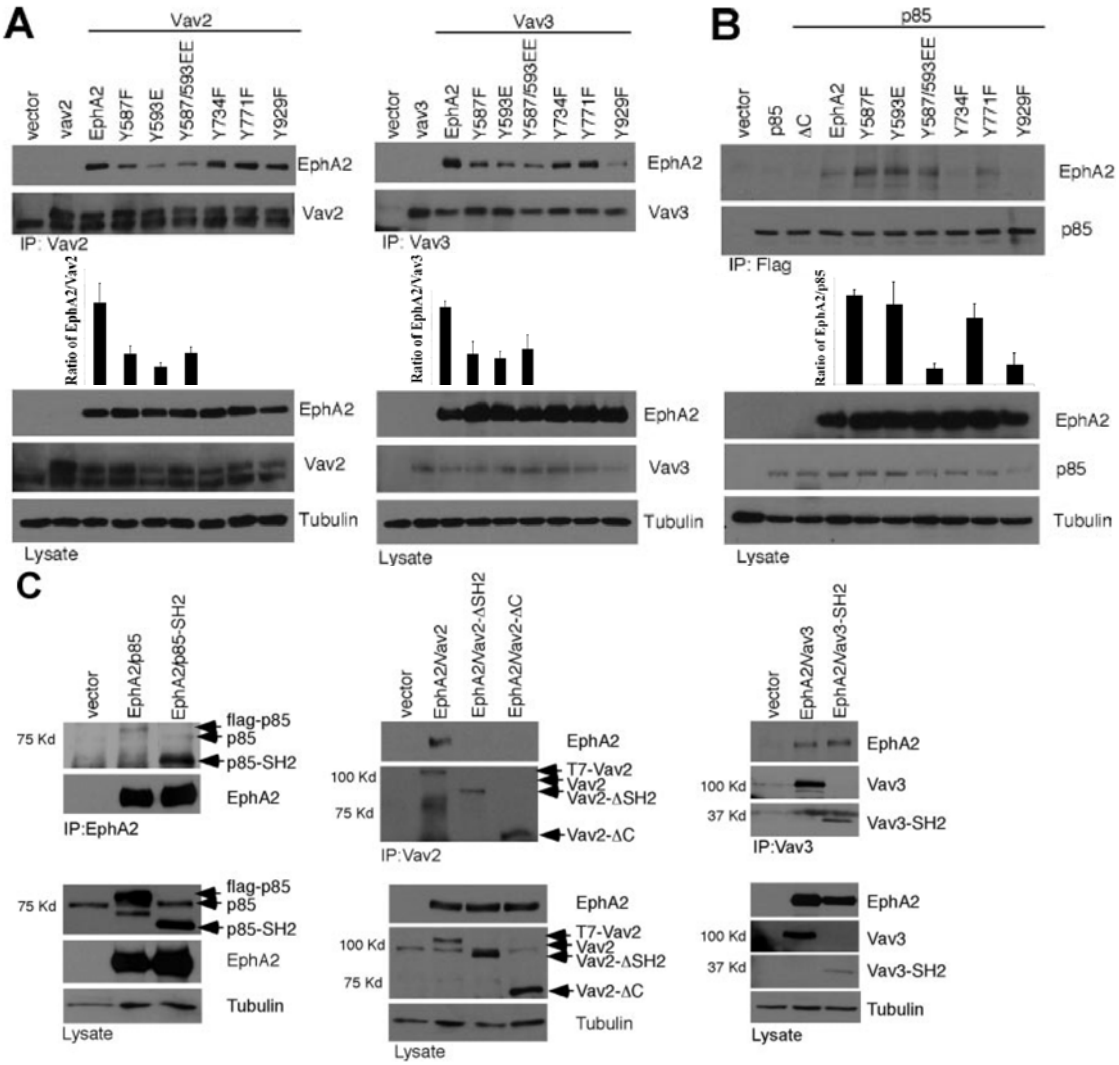


Figure 22. Mapping of Vav and p85 binding sites in EphA2 receptor. (A) The wild-type or mutant EphA2 and Vav2 or Vav3 were co-expressed in the COS 7 cells. The Vav2 or Vav3 proteins were immunoprecipitated and western blotted using antibodies against EphA2. The blots were stripped and reprobed using antibodies against Vav2 or Vav3 for confirmation of equal loading (for Vav2 and Vav3 $p<0.01$). (B) The wild-type or mutant EphA2 and Flag-tagged p85 were co-expressed in the COS 7 cells. The p85 proteins were immunoprecipitated and western blotted using antibodies against EphA2. The blots were stripped and reprobed by anti-Flag for equal loading. EE, a double Y to E mutation (Y587E/Y593E) in the juxtamembrane domain ($p<0.01$). (C) Wild-type or mutant Vav2, Vav3, or p85 were co-expressed with EphA2 in COS7 cells. EphA2 or Vav proteins were immunoprecipitated by appropriate antibodies, and western blotted by antibodies against p85 and EphA2, respectively.

addition, ephrin-A1 induced Rac1 activation is dependent on the activity of PI3 kinase (25). As Y587/593 and Y734 are required for recruitment of Vav GEFs and p85, respectively (Figure 22), we tested whether Y587/593EE and Y734F could affect ephrin-A1-induced Rac1 activation in endothelial cells. Cells were stimulated with ephrin-A1 and activated GTP-bound Rac1 or Cdc42 was isolated from lysates by precipitation with Pak1 p21-binding domain (PBD)-GST fusion proteins. As shown in Figure 23A, consistent with our previous findings (25), ephrin-A1 induced Rac1 activation in EphA2-null endothelial cells reconstituted with wild-type EphA2, but not in control LZRS infected cells. In contrast, Y587/593EE, Y734F, and Y929F mutants fail to restore Rac1-GTP level, suggesting that recruitment of p85 and Vav proteins to EphA2 receptor is critical for ephrin-A1-induced Rac1 activation. Activated Cdc42 was not significantly changed in response to ephrin-A1 stimulation in either wild-type or EphA2 mutants (Figure 23B), indicating that EphA2 is not directly involved in regulating Cdc42 activity.

As activation of Rac1 is critical for ephrin-A1-induced endothelial cell migration (25), we tested whether cell migration is impaired in EphA2-null endothelial cells reconstituted with uncoupling EphA2 mutants. As shown in Figure 23C, EphA2-null endothelial cells exhibit a defect in ephrin-A1-induced cell migration. Re-expression of wild-type EphA2 receptor rescued migration defects in EphA2 knock out endothelial cells. In contrast, expression of Y587/593EE or Y734F mutants fail to promote ephrin-A1-induced cell migration, suggesting that phosphorylation of Y587/Y593 and Y734 is critical for recruitment of p85 subunit of PI3 kinase and Vav

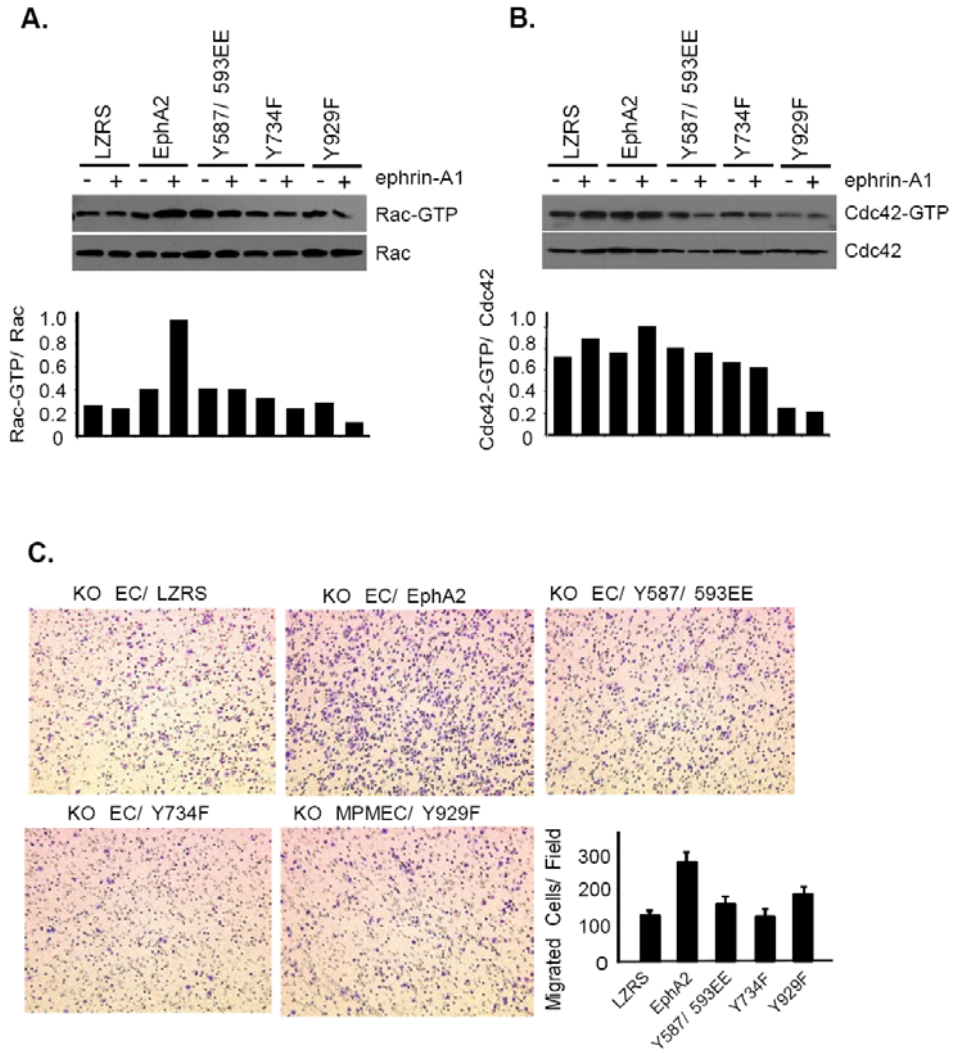


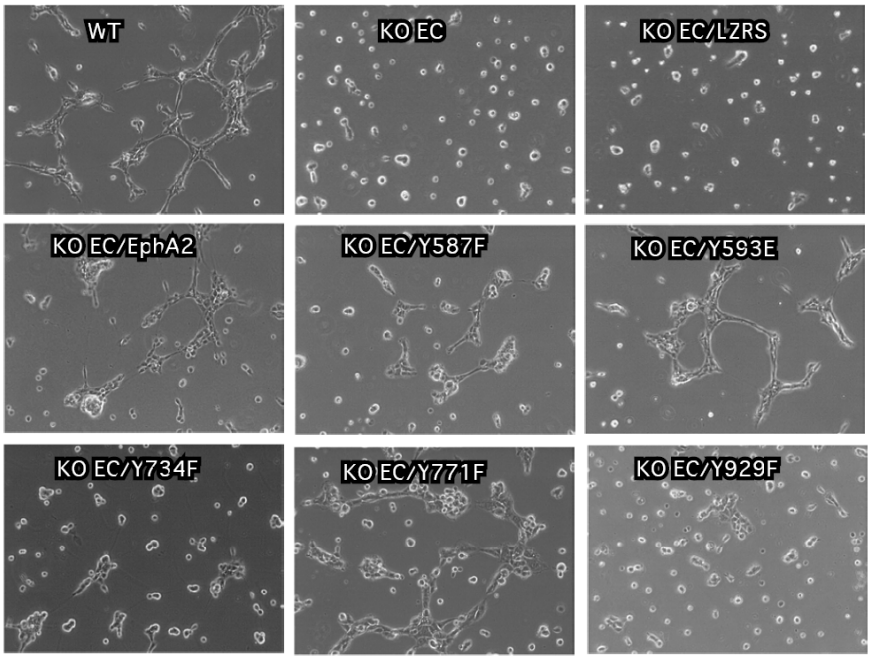
Figure 23. Phosphorylation of Y587/593 and Y734 in the EphA2 receptor is required for EphA2-dependent Rac1 activation and cell migration. Active GTP-bound forms of Rac1 (A) and Cdc42 (B) were analyzed by Pak-PBD pull-down followed by immunoblot in lysates from EphA2-null endothelial cells reconstituted with wild-type or mutant EphA2 in response to ephrin-A1 stimulation. Total Rac1 and Cdc42 levels within the lysates prior to PBD-pulldown were detected by immunoblot. Data are a representation of 4 independent experiments. (C) EphA2-null endothelial cells reconstituted Y587/593EE or Y734F mutant displayed significant reduced migration in response to ephrin-A1 stimulation in transwell migration assays ($p<0.01$, EphA2 versus Y587/593EE or Y734, two-tailed paired student *t* test).

GEFs, which transduce downstream signaling to activate Rac1 GTPase and cell migration.

Functions of EphA2 phosphorylated tyrosine residues in vascular assembly and tumor angiogenesis

Angiogenesis is a complex, multi-stage process by which new blood vessels are formed from pre-existing vasculature. Two critical steps in this process are endothelial cell migration and assembly into new tubules. To test the functional roles of phosphorylated tyrosine residues of EphA2 receptor in ephrin-A1-induced angiogenic responses, we measured the vascular assembly in EphA2-null endothelial cells reconstituted with EphA2 mutants. Ephrin-A1 stimulation induced wild-type, but not EphA2-deficient, endothelial cell assembly into an interconnected vascular network on a thin layer of Matrigel. Re-expression of wild-type EphA2, but not empty control vector, by LZRS retrovirus-mediated infection rescued defects in EphA2-null endothelial cells. Likewise, expression of mutant Y593E or Y771F in EphA2-null cells restored the ability of cells to assemble and form interconnecting cellular network on Matrigel. In contrast, EphA2 mutations in the juxtamembrane (Y587F, Y587/Y593EE), kinase domain (Y734F), or SAM domain (Y929F) inhibited ephrin-A1-induced vascular assembly (Figure 24).

To test whether phosphorylated tyrosines important in mediating vascular assembly in vitro are also critical in tumor angiogenesis in vivo, we performed tumor cell/endothelial cell co-transplantation experiments using EphA2-null endothelial cell reconstituted with wild-type or mutant EphA2 receptors. For co-transplantation,



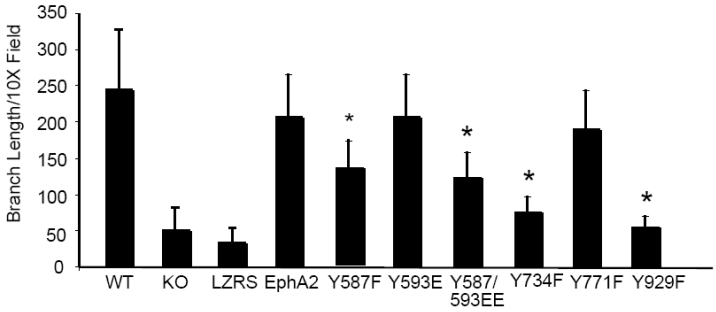


Figure 24. Phosphorylation of Y587/593, Y734, and Y929 in the EphA2 receptor is required for vascular assembly in vitro. (A) EphA2-null endothelial cells reconstituted with wild-type or mutant EphA2 were plated on a thin layer of growth-factor reduced Matrigel in the presence of ephrin-A1 to examine and quantify vascular assembly. After 9 hours, the endothelial cells were photographed. (B) Average branch length was scored using morphometric software analysis. Four fields per culture were scored for each condition and data are means +/- S.D. of 3 independent experiments ($p<0.05$, EphA2 versus Y587F, Y593F, Y587/593EE, Y734F or Y929F, two-tailed paired student *t* test).

endothelial cells were infected with adenoviruses encoding nuclear β-galactosidase (Ad-βgal) in order to distinguish them from endogenous host endothelium. These labeled endothelial cells were then co-transplanted with 4T1 mammary carcinoma cells in Matrigel into the subcutaneous dorsal flank of nude female mice. After 7 days, tumor-endothelial cell Matrigel plugs were harvested, sectioned, and double stained with X-gal and CD31 to identify donor endothelial cells. As shown in Figure 25, significant numbers of LacZ positive donor endothelial cells reconstituted with either wild-type or Y921F control EphA2 mutant have incorporated into tumor (panel A) or peripheral vessels (panel B). In contrast, EphA2-deficient donor endothelial cells, as well as cells reconstituted with Y587/Y593EE, Y734F, or Y929F, remained isolated and failed to incorporate into tumor vasculature. In addition, tumor volume was significantly increased in tumors harboring donor endothelial cell reconstituted with wild-type or Y921F control EphA2, relative to tumors containing EphA2-deficient endothelial cells, or cells reconstitute with Y587/593EE, Y734F, or Y929F (Figure 25C). Taken together, these data suggest that phosphorylated tyrosine residues, Y587/593, Y734, and Y929, are critical in EphA2 signal transduction and tumor angiogenesis in vivo.

Discussion

A wealth of evidence demonstrated that ephrin-A ligand stimulation of EphA2 receptors activates a signaling cascade that modulates actin cytoskeleton dynamics and regulates cell-cell and cell-matrix adhesion and cell motility (25, 56, 128). Phosphorylated tyrosine residues on the EphA2 receptor were thought to play a

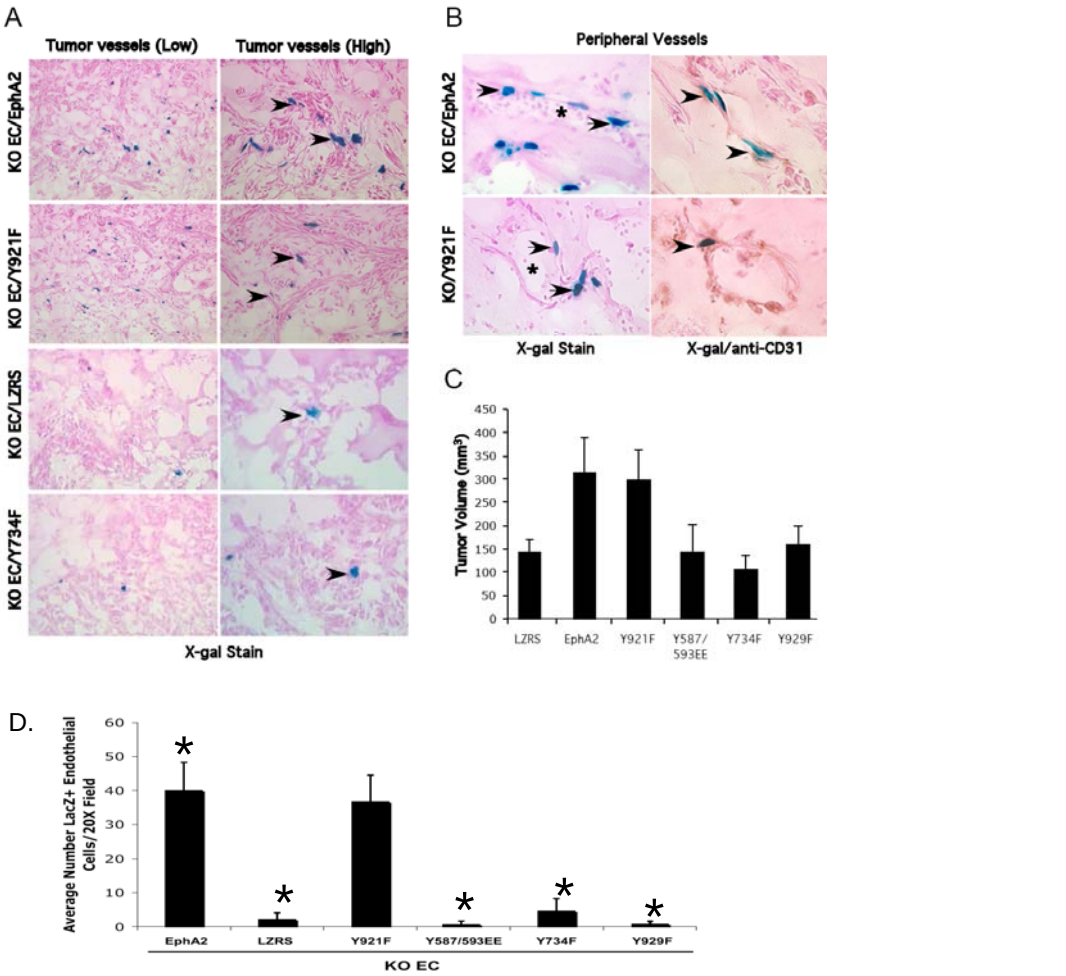


Figure 25. Phosphorylation of Y587/593, Y734, and Y929 in the EphA2 receptor is required for efficient incorporation of endothelial cells into tumor vasculature in vivo. 4T1 tumor cells were mixed with Ad-LacZ transduced EphA2-null endothelial cells reconstituted with wild-type or mutant EphA2 in Matrigel and co-transplanted subcutaneously into Balb/c nude female mice. Tumors were collected 7 days post-transplantation. (A and B) Tumor sections were co-stained with X-gal (blue) and anti-CD31 antibodies (brown in panel B) to visualize donor endothelium and counterstained with eosin to visualize tumor cells (pink). Donor endothelial cells derived from EphA2-null reconstituted with wild-type EphA2 or control Y921F mutant coalesced around tumor cell clusters and displayed an elongated phenotype typical of endothelial cells. In contrast, endothelial cells derived from EphA2-null cells expressing Y734F mutant or control vector LZRS remained isolated and failed to incorporate into tumor vessels. Arrowheads indicate exogenous endothelial cells, and * indicates central lumen of chimeric vessels in panel B. (C) Tumor volume was significantly decreased for tumors harboring EphA2-null endothelial cells expressing Y587/593EE, Y734F, or Y929 mutant or control LZRS vector, relative to tumors co-transplanted with those expressing wild-type EphA2 or control Y921F mutant ($p<0.05$, EphA2 versus Y587/593EE, Y734F or Y929F, two-tailed paired student *t* test). Data are representative of three independent experiments. (D) Quantitation of LacZ positive cells from tumor sections ($p<0.001$).

critical role in the recruitment of SH2- or PTB-domain containing signaling molecules such as the p85 subunit of PI3 kinase (69), adaptor proteins SLAP (70) and Shc (60), tyrosine phosphatase SHP-2 (67) and LMW-PTP (68, 141), ubiquitin ligase c-Cbl (66, 158), and guanine nucleotide exchange factors Vav2 and Vav3 (56). In the endothelial cells, we have previously shown that either PI3 kinase inhibitors or a dominant negative p85 mutant significantly inhibited ephrin-A1 ligand-induced endothelial cell migration (25). Likewise, Vav2/3-deficient endothelial cells were incapable of mediating cell migration and assembly upon ephrin-A1 stimulation (56). These data indicate important roles of PI3 kinase and Vav GEFs in ephrin-A-elicited angiogenic responses. However, as these signaling molecules also act downstream of many receptor tyrosine kinases, it remains unclear whether recruitment of these proteins by EphA2 receptor is critical for endothelial cell function. As a first step to dissect the specific function of different phosphorylated tyrosine residues in the activated EphA2 receptor, we undertook mapping major tyrosine-phosphorylation sites on the EphA2 receptor. We found that 4 of the 15 tyrosines (Y587, Y593, Y734, and Y771) in the EphA2 cytoplasmic domain were phosphorylated in vascular endothelial cells.

Two tyrosine residues in the juxtamembrane region of the EphB receptor were previously shown to be phosphorylated in vivo and are important in regulating kinase activity (10, 156). Of the corresponding two juxtamembrane tyrosine residues in the EphA2 receptor, we found that both Y587 and Y593 were phosphorylated in our in vitro kinase assay. However, only the phosphorylation of Y593 was detected in vivo in COS7 cells by LC-MS mass spectrometric analysis. This apparent discrepancy is

most likely due to dephosphorylation of Y587 in vivo upon ephrin-A1 stimulation for 15 minutes. As predicted, Y587 plays a critical role in endothelial cell assembly in vitro and in endothelial cell incorporation into tumor vasculature in vivo (Figures 24 & 25). Y593 was phosphorylated in COS7 cells and in vascular endothelial cells (Figures 19-21). Consistent with data shown in EphB receptor (10, 156), the Y593F mutation abolished kinase activity. Although the Y593E mutation inhibited binding of EphA2 to Vav GEFs (Fig. 22), it is somewhat surprising that it did not affect ephrin-A1-induced vascular assembly (Fig. 24). It is conceivable that residual levels of EphA2 binding to Vav GEFs through Y587 in vascular endothelial cells may be sufficient to transduce signals and regulate angiogenic responses.

For tyrosine residues in the kinase domain, Y771 resides in the activation loop of the kinase and was phosphorylated in both the in vitro kinase assay and in vivo as shown by mass spectrometric analysis (Figures 19-21). Interestingly, the Y771F mutant retains kinase activity and no obvious phenotype was detected in our assays. Mass spectrometric analysis and two dimensional phosphopeptide mapping also revealed a novel phosphorylated tyrosine residue, Y734. The significance of Y734 phosphorylation appears to recruit the p85 subunit of PI3 kinase (Figure 22), as the Y734F mutant failed to rescue cell migration and vascular assembly in EphA2-deficient endothelial cells.

Of the three tyrosines in the SAM domain, Y921, Y929, Y959, none of them was identified to be phosphorylated by either mass spectrometry or phosphopeptide mapping analysis. Yet, Y929F inhibited ephrin-A1 induced vascular assembly and endothelial cell incorporation into tumor vasculature in vivo (Figures 24 & 25). As

Y929F also displays reduced kinase activity, the phenotype could be attributed to either lack of phosphorylation at the Y929 site or insufficient kinase activity, or a combination of both deficiencies. It is also possible that Y929 was not phosphorylated within 15 minutes of stimulation by ephrin-A1, but was phosphorylated in a different time frame. Alternatively, Y929 may not be phosphorylated in COS7 cells, but may be phosphorylated in endothelial cells in vivo by protein tyrosine kinases other than EphA2. Our binding data suggest that Vav3 and p85 interact with pY929 (Fig. 22). In addition, Stein et al., reported that LMW-PTP and Grb10 can bind to corresponding site in EphB1 receptor (137, 159). If LMW-PTP also interacts with pY929 in EphA2 receptor, it may attenuate its signaling by dephosphorylation of EphA2 receptor, as demonstrated in tumor cells (68, 141). Alternatively, LMW-PTP may transduce EphA2 signaling by interacting with p190RhoGAP to regulate the activity of Rho GTPases (Fang et al., 2008, in press). It remains to be determined whether LMW-PTP can interact with EphA2 and modulate ephrin-A1-induced angiogenic responses.

Our results revealed that phosphorylated tyrosine residues in the EphA2 receptor are not only critical for signal transduction in cultured microvascular endothelial cells in vitro, but also important for these cells to participate in tumor angiogenesis in vivo. It is interesting to note that tumors grow better in the presence of wild-type donor endothelial cells than EphA2-null cells or null cells reconstituted with Y587/593EE, Y734F, or Y929F mutants. This may not be entirely due to extra oxygen and nutrients supplied by new blood vessels, as tumor blood flow appeared to be restricted to peripheral vessels distant from the tumor mass. It is possible that there

could be paracrine signaling from donor endothelial cells to tumor cells to promote tumor growth, and this signal(s) is absent or diminished in EphA2-null endothelial cells. Indeed, we have previously shown that ephrin-A1 regulates soluble growth factor production in tumor cells (160). The growth factors/signaling molecules that are modulated by EphA2 receptor activation in vascular endothelial cells remain to be determined.

In summary, mapping of phosphorylated tyrosine residues in the EphA2 receptor allowed us to generate tyrosine to phenylalanine mutants that were used to identify binding sites to key EphA2 downstream signaling molecules such as the p85 subunit of PI3 kinase and Vav guanine nucleotide exchange factors. These uncoupling mutants permit the possibility to test whether recruitment of these proteins by EphA2 receptor is critical for endothelial cell function in vivo. As shown in Figures 22 and 23, Y587/593 and Y734 are major sites for recruitment of Vav2/3 GEFs and p85, respectively, and the recruitment of both Vav proteins and p85 to the activated EphA2 receptor is critical for ephrin-A1 induced endothelial cell migration and assembly. It should be now feasible to screen for other putative EphA2 phosphotyrosine-dependent interacting proteins such as Shc, SHP2, c-Cbl, and SLAP, or to use phosphopeptides as ligands in chromatography analysis to identify novel binding partners. Moreover, EphA2 tyrosine phospho-specific monoclonal antibodies can be developed to facilitate the identification of downstream signaling events associated with EphA2 tyrosine phosphorylation.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Ephrins and Eph receptors are key regulators of physiological and pathological processes in development and disease. Expression of Eph receptors is often elevated in many types of malignant tumors, yet precise role of these molecules in cancer is not well understood. The work in my thesis revealed for the first time a kinase-dependent role of EphA2 receptor tyrosine kinase in promoting tumor malignancy. I have shown that EphA2 functions in tumor promotion in part, through destabilization of cell-cell adhesion by a RhoA GTPase-dependent mechanism. In addition to promoting tumor malignancy, EphA2 also plays critical roles in tumor angiogenesis. My work in vascular endothelial cells contributed to our understanding of how Eph receptor transduces signals in angiogenic responses and in tumor neovascularization. I found that phosphorylation of key tyrosine residues in the EphA2 receptor cytoplasmic domain recruits Vav family guanine nucleotide exchange factors and the p85 subunit of PI3 kinase, resulting in Rac1 GTPase activation, endothelial cell migration and assembly in vitro and incorporation of vascular endothelial cells into tumor vasculature in vivo. Taken together, my thesis work showed that EphA2 promotes tumor progression through both enhancing tumor malignancy and increase tumor angiogenesis.

EphA2: Oncogene or Tumor Suppressor?

Recently, there is a growing debate as to whether *EphA2* is an oncogene or tumor suppressor, as there is evidence to support both roles. Guo et al recently reported that loss of *EphA2* increased susceptibility to skin carcinogenesis in *EphA2* deficient gene trap mice treated with DMBA/TPA, a chemical carcinogen (161). This would suggest that *EphA2* functions as a tumor suppressor. Another line of evidence is provided by work demonstrating EphB4 can also act as tumor suppressor. Like EphA2, expression of EphB4 has been correlated with tumor malignancy in several cancer types. Similar to EphA2, EphB4 is upregulated in cancer cells, but in cancer cells levels of phosphorylated EphB4 are reduced in comparison to normal tissue or cells. Noren et al demonstrated that, in several different breast cancer cell lines, treatment with ephrinB2-Fc inhibited proliferation and increased apoptosis. Treatment with ephrinB2-Fc also inhibited cell motility and invasion (162). These results would argue that the Eph receptor can function as a tumor suppressor.

There are published reports, however, demonstrating that *EphA2* functions as an oncogene. Zelinski et al reported that overexpression of EphA2 in MCF-10A cells was sufficient to transform those cells (30). These transformed MCF-10A cells exhibited many properties consistent with those found in cancer cells. EphA2 overexpressing MCF-10A cells formed colonies on soft agar, and demonstrated increased cell migration. In addition, inhibition of EphA2 by siRNA knockdown or receptor degradation reduced tumor volume and metastasis. Brantley-Sieders et al reported that EphA2 deficiency decreased tumor burden in the MMTV-Neu mouse model of breast cancer (61). In this model, overexpression of the oncogene, *ErbB2*, is

restricted to the breast epithelium. These mice will form spontaneous mammary tumors within 3 months. In MMTV-Neu mice also deficient in *EphA2*, tumor growth was significantly decreased. Decreased tumor volume and metastasis was due, in part, to cooperative signaling between EphA2 and ErbB2. It was demonstrated that EphA2 and ErbB2 could be co-immunoprecipitated. Association of EphA2 and ErbB2 could enhance MAPK signaling (61). My work suggests that in the context of breast cancer, *EphA2* functions as an oncogene. Inhibition of EphA2 signaling by overexpression of a dominant negative EphA2 mutant decreased tumor growth in vivo, while overexpression of EphA2 weakened cell-cell adhesion. In my experimental system, overexpression of EphA2 in the MCF-10A cells did not appear to transform these cells. When MCF-10A/EphA2 cells were cultured in a 3D-Matrigel they behaved like parental MCF-10A cells, which is contrary to the results observed by Zelinski et al. The discrepancy in results I believe is due to differences in the level of EphA2 overexpression. I was not able to achieve EphA2 expression level comparable to those in the previous study, using the LZRS retroviral system. As described in Chapter IV, I demonstrated that high overexpression of EphA2 using an adenoviral vector resulted in more rapid destabilization of cell-cell adhesion, in comparison to the MCF-10A or MCF-10A/LZRS-EphA2 cells. These data would argue that *EphA2* acts as an oncogene.

To reconcile the dual roles of Eph receptors in tumor promotion and tumor suppression, we proposed a working model (Fig 26). Under physiologic conditions, epithelial cells form cell-cell junctions, permitting ephrins to interact with Eph receptors in trans on adjacent cells (28, 163). Ligand stimulation inhibits the

activation of Ras-MAPK pathway, the RhoA GTPase, and the function of the adaptor Crk through Abl kinase activity, keeping cells quiescent and non-invasive (41, 42). Upon tumor initiation, Eph receptor expression is up-regulated by oncogenic signaling pathways such as the Ras-MAPK pathway or the Wnt-β-catenin pathway, whereas their ephrin ligands are often downregulated (42, 164). Cross talk between elevated Eph receptors and other oncogenes, such as the ErbB family of receptor tyrosine kinases (61, 165) leads to enhanced cell proliferation and tumorigenesis, presumably independently of ephrin stimulation. Thus, ligand-dependent Eph receptor signaling functions in tumor suppression, whereas ligand-independent Eph receptor activities appear to promote tumor progression. It remains to be determined whether the differences in signaling in the above two scenarios are achieved by differential receptor phosphorylation or mediated through phosphorylation-independent mechanisms, such as signaling through the SAM domain or PDZ binding motif at the cytoplasmic domain. The precise role of Eph receptor kinase activity also remains to be determined.

EphA2 phosphorylation and signaling

Biochemical anaylsis of the phosphorylated tyrosines on EphA2 only identified three tyrosine residues that were phosphorylated on EphA2 by both mass spectrometry and 2D-TLC coupled with phosphopeptide mapping. Characterization of EphA4, EphB2, and EphB5 using in vitro and in vivo labeling revealed tyrosines in similar positions were also phosphorylated (154, 156). They identified the juxtamembrane tyrosines and the corresponding tyrosine in the activation loop, these

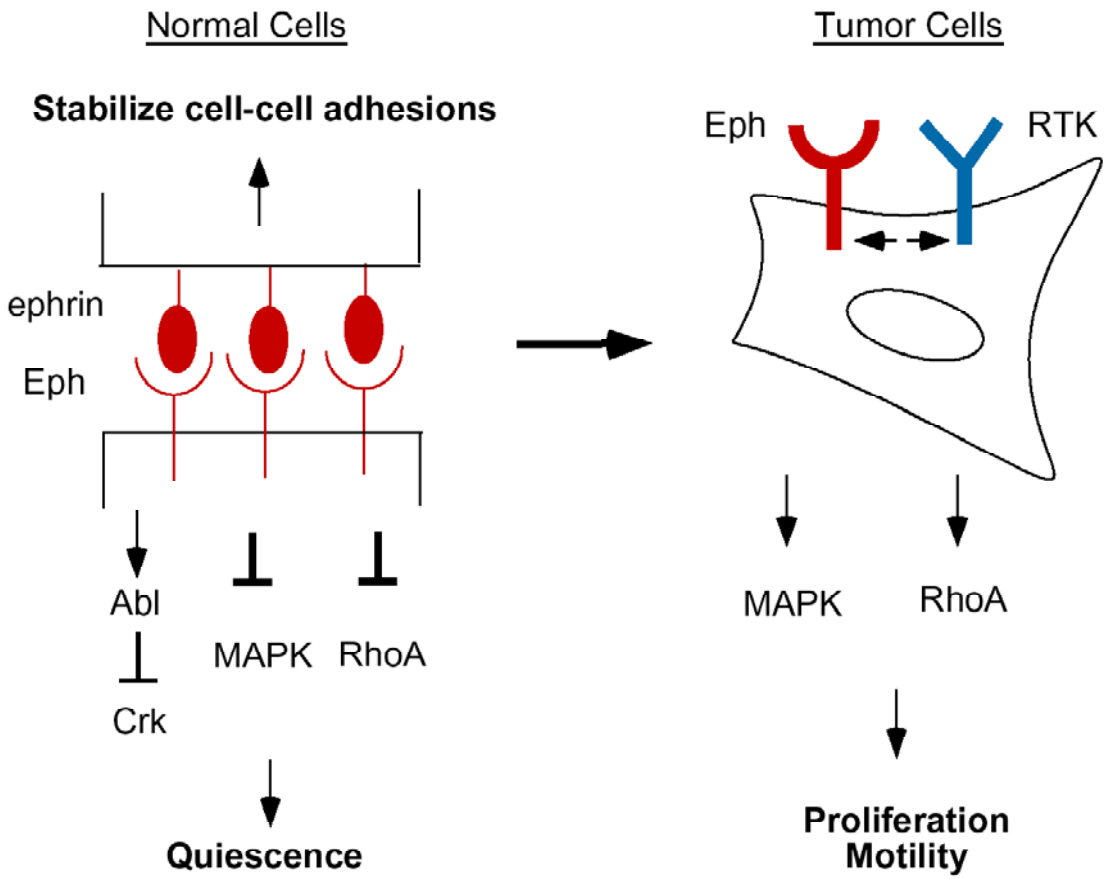


Figure 26. A working model for the dual roles of Eph receptors in tumor promotion and tumor suppression. In normal cells, engagement of Eph receptors with ephrins on adjacent cells in trans induces receptor forward signaling, leading to inhibition of Ras/MAPK activity or RhoA GTPase. Reverse signaling through ephrin-B stabilizes cell-cell adhesion. In tumor cells, disruption of cell-cell junctions inhibits Eph receptor interaction with endogenous ephrins in trans. In addition, some tumor cells have low ephrin levels. Elevated Eph receptors cross talk with other receptor tyrosine kinases can result in increased activity of the Ras-MAPK pathway, the RhoA GTPase, and enhanced tumor malignancy.

are Y587, Y593, and Y771 on EphA2. However, these analyses also identified several other tyrosines, predominantly found in the kinase domain. I believe the lack of sensitivity of our assays maybe responsible for the differences. To gain better insight into EphA2 phosphorylation, we would need to perform similar experiments using in vivo labeling of EphA2 phosphorylation as opposed to the in vitro kinase assay. I would predict that many more of the 15 possible tyrosine residues found within the cytoplasmic domain of EphA2 would be phosphorylated.

To date, there have been numerous proteins that have been demonstrated to interact with EphA2, generally mediated by SH2/PTP domains. In my work, I have identified four proteins that interact with EphA2 and mapped their association on EphA2. This work has generated an extensive library of EphA2 tyrosine mutants. As a result of my work, it is now be possible to map interactions with other proteins, e.g. LMW-PTP, p120 RasGAP, Src family of kinases, adaptor proteins (such as Grb2 and Nck), and other Rho GEFs (such as ephexin and Vsm-RhoGEF) to obtain a greater understanding of downstream signaling events.

It is still unclear what signaling pathways are active due to EphA2 upregulation. In the context of cancer, there appears to be several pathways that are commonly affected by EphA2 activation. It has been reported in many systems that upregulation of EphA2 signaling affects cell adhesion, cell migration, and cell proliferation. One pathway that consistently is shown to be upregulated by EphA2 signaling is the Rho family of GTPases. EphA2 signaling functions through several different pathways to alter Rho activation. Zhuang et al demonstrated that SHIP2 association with EphA2 activated Rac1 through phosphoinositide 3-kinase (58). Parri et al reported that

activation of EphA2 increased RhoA activation by increasing Src activity and subsequent FAK phosphorylation (72). These findings are consistent with my work. In 4T1 cells, I observed a decrease in RhoA activation when EphA2 signaling was inhibited by overexpression of a dominant negative EphA2 mutant, while overexpression of EphA2 in MCF-10A cells increased RhoA activation. Another pathway that is commonly affected by EphA2 signaling is the MAPK pathway. Interestingly, Ras-Raf signaling was reported to upregulate EphA2 expression through activation of the MAPK pathway (42, 166). Conversely, EphA2 has been shown to affect Ras-Raf signaling as well. EphA2 can inhibit MAPK activation. Miao et al reported that stimulation of PC-3 cells with ephrinA1 could dramatically decrease levels of activated Erk (41). However, EphA2 can also activate the MAPK pathway. Pratt et al reported that stimulation of EphA2 with ephrinA1-Fc increased Erk activation and cell proliferation in prostate cancer cells (60). EphA2 signaling can also affect cell adhesion. As mentioned previously, Parri et al reported that EphA2 signaling can increase FAK phosphorylation through Src to cause cell retraction in prostate cancer cells (72). Miao et al also reported that EphA2 signaling can affect the phosphorylation status of FAK to decrease cell adhesion. Miao et al demonstrated that in the absence of ephrinA1 stimulation, FAK and EphA2 can associate. However, in the presence of ephrinA1 binding to EphA2, FAK becomes dissociated from EphA2 and is dephosphorylated by a tyrosine phosphatase (41). In my work, I did not observe any difference in levels of FAK phosphorylation. Similar to Parri et al, I did observe cell retraction with ephrinA1-FC stimulation of MCF-10A

cells that overexpressed EphA2. It is currently unclear how there can be two opposing results in response to EphA2 signaling.

There is a growing body of work that has demonstrated Eph receptors can interact with other RTKs. Larsen et al reported that EphA2 and EGFR can be co-immunoprecipitated (165). The interaction between EGFR and EphA2 increased cell motility. Warner et al recently reported using an in vitro systematic analysis of EphA4 peptide substrates that EphA4 can phosphorylate EGFR (167). Brantley-Sieders et al demonstrated that EphA2 can physically interact with ErbB2 (61). EphA2 interaction with ErbB2 could enhance MAPK activation, while EphA2 deficiency could decrease tumor size in the MMTV-Neu transgenic mice (61). These results would suggest that EphA2 can phosphorylate other RTKs and other RTKs can phosphorylate EphA2 to facilitate different signaling events. It will now be possible to investigate which tyrosines can be phosphorylated by other RTKs and what signaling pathways these interactions activate.

EphA2 activation is dependent upon ligand binding of the receptor and clustering of the receptor. Recent work on the ephrins has demonstrated that the ephrin ligands can be cleaved by presenilin and metalloproteases. There have been two proposed mechanisms for termination of Eph forward signaling. Ligand engagement with the Eph receptor is thought to cause internalization of the receptor/ligand complex. Another possible mechanism is proteolysis of the ephrin ligand by metalloproteases. Georgakopoulosa et al reported that ephrinB2 can be cleaved by metalloproteases to produce a 14 kd fragment (168). Similarly, ephrinA5 and ephrinA2 can be cleaved by ADAM10. This cleavage occurred in a trans manner, occurring when ephrin

expressing cells engage and bind to the Eph receptor(169). In the original studies on ephrinA1, Bartley et al demonstrated that soluble monomeric ephrinA1 was able to induce EphA2 phosphorylation (170). These studies would suggest that the ephrins can be cleaved and exist as soluble factors. It is unclear what ramification this would have for Eph receptor signaling, as EphA2 activation is generally mediated by receptor clustering and Eph phosphorylation. It is clear that Eph signaling is quite complicated. Eph receptors can signal through kinase dependent and kinase independent, ligand dependent and ligand independent pathways, and they can cooperatively associate with other receptor tyrosine kinases to facilitate different signaling cascades. In my work, I addressed the contribution of kinase dependent EphA2 signaling in breast cancer cells. EphA2 activation promotes tumor progression. 4T1 mammary carcinoma cells express high levels of EphA2, and I reported that EphA2 phosphorylation and activation was dependent on cell density or cell-cell contact. Inhibition of EphA2 signaling by overexpression of an EphA2 truncation mutant decreased tumor volume and metastasis. In MCF-10A cells, overexpression of EphA2 weakened cell-cell adhesion. It would be interestingly to do a comparative study on the many different forms of Eph signaling and whether different tyrosines are phosphorylated in response to the different forms of Eph signaling. This point is addressed further in the perspectives section.

Therapeutics

An ultimate goal of most biomedical research is to identify novel information that can be utilized to develop therapies targeting certain molecules in different diseases.

In the field of Eph research, there have been several studies that have show promise for targeting EphA2 in relation to cancer therapy. There are wide array of strategies that have been used including monoclonal antibody conjugates, ligand based cytotoxins, siRNA, antangonistic peptides, and small molecule inhibitors. Wykosky et al reported that a portion of ephrinA1 ligand conjugated with a modified bacterial toxin was a potent inhibitor of glioblastoma multiforme cells that overexpressed EphA2 (171). Several groups have shown the effectiveness of using siRNA methods to inhibit tumor progression. Landen et al demonstrated that knockdown of EphA2 could dramatically reduce tumor growth in a mouse model of ovarian cancer (172). Consistent with these findings, Duxbury et al also showed that knockdown of EphA2 in prostate cancers could do the same (38). These results are consistent with my findings in 4T1 mammary adenocarcinoma cells, as inhibition of EphA2 exhibited a statistically significant reduction in tumor growth and metastasis. Chencik et al and Koolpe et al have demonstrated that an antagonistic peptide for ephrinB2 could inhibit endothelial cell function (122, 173). These findings would suggest that therapeutic strategies targeting Eph receptors are promising for both tumor cells and tumor vasculature.

A growing area of research involves the interaction of Eph receptors with other families of receptors. Current work in this area has demonstrated that Eph receptors can potentiate signaling from other RTKs, including EGFR family of receptors, FGF receptors, or HGF signaling (61, 128, 165, 167). Eph receptor can also coordinate signaling with chemokine receptors and with integrin signaling . Signaling from these proteins has also been reported to be important in the context of cancer. This

area of research holds great promise for novel treatment in cancer therapeutics. It would be possible to use combinatorial therapy, where treatment would target multiple proteins or pathways, to produce greater efficacy in cancer treatment. However, the mechanisms by which the Eph receptors could crosstalk with other receptors or proteins are not well understood. It will be necessary to dissect how Eph receptor signaling can influence other receptor signaling and how other receptors can influence Eph receptor signaling in cancer cells. More research will have to be done to address this, but this will be of great importance in terms of cancer treatment.

Perspective

Since the discovery of the first Eph receptor some 20 years ago, there has been tremendous progress in understanding this family of receptor tyrosine kinases. My work has demonstrated that EphA2 signaling is important in tumor progression. However, it is evident that there are still many unanswered questions in the field. Firstly, how can EphA2 function as both a tumor suppressor and oncogene? Studies on EphA2 deficient mice showed that these mice exhibited no overt phenotype under physiological conditions. However, when the mice were challenged with disease the results were contradictory. Guo et al demonstrated that EphA2 deficiency in a gene trap model promoted skin carcinogenesis (161). Brantley-Sieders et al reported that EphA2 deficiency in a conventional knockout decreased tumor progression in the MMTV-Neu mouse model of breast cancer. Although in this work, it was reported that the role of EphA2 in breast cancer maybe dependent on the context of the tumors, as EphA2 deficiency did not affect tumor volume in the MMTV-PyV-MT mouse

model of breast cancer (61). One approach to address the dual functions of EphA2 in cancer would be to look at how in vivo overexpression of EphA2 can affect tumor formation and progression. It would be advantageous to generate an EphA2 transgenic mouse model and examine how EphA2 overexpression affects tumor onset and progression. We would predict that if EphA2 functioned as a tumor suppressor in skin cancer, then treatment of these EphA2 transgenic mice with chemical carcinogens, as described Guo et al, would result in a decrease incidence of cancer. It is unclear whether overexpression of only EphA2 is sufficient to induce tumor formation. It is possible that the ephrinA ligands also play a role in this. It has been reported that EphA2 and ephrinA1 expression are inversely related. In early cancer cells, ephrinA1 levels are elevated but ephrinA1 expression is reduced in cells with a high degree of malignancy. EphA2 levels, on the other hand, are lower in early stage tumors than in later stage tumors. To investigate the role of ephrin/Eph in tumor initiation, we will need to dissect the mechanism of ligand mediated EphA2 signaling.

Eph receptor signaling is very complex. Eph receptors can signal through a ligand dependent and independent mechanisms, as well as kinase dependent and independent processes. It is also possible to have reverse signaling though the ephrin ligands. To investigate ligand dependent versus independent signaling, it would be interesting to see what difference in tyrosine phosphorylation there is between the two groups. Are different tyrosine residues phosphorylated on EphA2 in the presence of ligand versus absence of ligand? Mass spectrometry analysis could be used to address this. A complementary approach would be to identify different signaling proteins that are upregulated or downregulated in the presence or absence of ligand.

2D-DIGE is a powerful tool that can address this question. The combination of these approaches would identify tyrosine phosphorylation sites that are relevant to ligand activation and signaling pathways that are ligand dependent and ones that are independent. This experiment would be vital in reconciling whether EphA2 is a tumor suppressor or oncogene in cancer. According to our model, we would expect that proteins involved in Rho and MAPK signaling pathways would be downregulated in the presence of ephrinA ligand stimulation, while in the absence of ligand these pathways would be upregulated. It would also be possible to address the differences in proteins that interact with EphA2 in a ligand dependent and ligand independent fashion. We currently have a large panel of EphA2 tyrosine to phenylalanine mutants that could be used to address and verify the results seen from the proteomic analysis of EphA2 signaling with and without ligand treatment.

This report and other studies on the Eph receptor family have shown us that we are at an exciting time in this area of research. There has been tremendous progress, but many questions remain unanswered. There is great potential that future work will lead to effective new therapies for cancer treatment. Therapeutic strategies specifically targeting Eph receptors will not only target cancer cells but also tumor specific stromal cells, including tumor endothelium. There are still many unresolved dilemmas that will need to be addressed, but this work has provided a step forward to achieving this.

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