PRO AND ANTI TUMORIGENIC EFFECTS OF EPHA RECEPTOR SIGNALING IN BREAST AND LUNG CANCER

By

Guanglei Zhuang

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Approved:

Prof. Ambra Pozzi

Prof. Ann Richmond

Prof. Chang Chung

Prof. Jin Chen

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2. Hunter S.G., **Zhuang G.**, Brantley-Sieders D.M., Swat W., Cowan C.W., and Chen J. Essential role of Vav family guanine nucleotide exchange factors in EphA receptor-mediated angiogenesis. *Mol Cell Biol.* 2006 Jul; 26 (13), 4830-42.
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Dedicated to

my grandfather, Shulin He, my father, Jianhong Zhuang, and my mother, Zhaolan He,

who tremendously contributed to this work from the very beginning,

even though they cannot read it.

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LIST OF ABBREVIATIONS

ADAM10	a disintegrin and metallopeptidase 10
ANOVA	analysis of variance
BrdU	Bromodeoxyuridine
CFP	cyan fluorescent protein
CHCl3	chloroform
CXCR4	CXC-chemokine receptor 4
DAB	diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminscence
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosomal antigen 1
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
Eph	erythopoietin producing hepatoma
Ephexin	Eph exchange interacting protein
ephrin	Eph family receptor interacting proteins
ErbB2	erythroblastic leukemia viral oncogene homolog 2
Erk	extracellular signal-regulated kinase
EXALT	expression signature analysis tool
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
FRET	fluorescence resonance energy transfer
GBM	glioblastoma multiforme
GPI	glycosylphosphatidlinositol
GST	glutathione-S-transferase
HCl	hydrochloric acid
HER2	human epidermal growth factor receptor 2
MAPK	mitogen activated protein kinase
MEK	mitogen-activated protein kinase kinase
MeOH	methanol
MMTV	mouse mammary tumor virus
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
NMDAR	N-methyl-D-aspartate receptor
NSCLC	non-small cell lung cancer
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDZ	PSD95/Dlg/ZO1

PI3K	phosphatidylinositol 3 kinase
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PMEC	primary mammary epithelial cells
PMTC	primary mammary tumor cell
PTPRO	protein tyrosine phosphatase receptor type O
PyV-mT	polyoma virus middle T
RHBDL2	rhomboid, veinlet-like 2
RTK	receptor tyrosine kinase
SAM	sterile-α-motif
SD	standard deviation
SDS	sodium dodecyl suflate
SEM	standard error of the mean
SH2	src homology 2
SHIP2	Src homology 2 domain-containing phosphoinositide 5-phosphatase 2
SHP2	Src homology 2-containing tyrosine phosphatase 2
siRNA	small interfering RNA
SNP	single-nucleotide polymorphism
TMA	tissue microarray
TMB	tetramethylbenzidine
TSC2	tuberous sclerosis 2
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand factor
YFP	yellow fluorescent protein

CHAPTER I

INTRODUCTION

Overview

Since its discovery two decades ago, the Eph family of receptor tyrosine kinases has been implicated in an accumulating number of physiological and pathological processes in many cell types and various organs. Members of the Eph family of RTKs have recently received lots of attention, given the important roles for their bidirectional signals in multiple aspects of cancer development and progression. Substantial advances have been made in understanding the key roles of Eph in the signaling pathways that govern fundamental cellular processes in cancer, such as proliferation, survival, migration and invasion, as well as those that regulate intercellular communication during tumorigenesis. Common themes and controversies of Eph-ephrin interactions in cancer biology have been gleaned from intensive research efforts in the field, and continue to be uncovered. As part of them, our current results, presented herein, primarily explore the role of EphA receptor signaling in breast and lung cancer progression, which has not been elucidated systematically and comprehensively. We show pro-tumorigenic effects of EphA2 receptor in breast cancer by crosstalk with HER2 signaling, and anti-tumorigenic effects of EphA3 receptor in lung cancer by inhibiting the mTOR pathway. Our increased understanding of the genetic, molecular, and biological characteristics of the Eph-ephrin system is essential for the rational development of novel anti-cancer therapies.

The Eph receptors and ephrin ligands

The Eph receptors with 15 members constitute the largest family of receptor tyrosine kinases (RTK) that are discovered in human genome [1]. Their ligands, the ephrins, are divided into two subclasses: the A-subclass (ephrinA1-ephrinA6), which is tethered to the cell membrane by a glycosylphosphatidlinositol (GPI) anchor, and the B-subclass (ephrinB1-ephrinB3), which contains a transmembrane domain followed by a short cytoplasmic region (Figure 1.1). On the basis of extracellular sequence similarity and ligand-binding affinity, the Eph receptors are also grouped into the A-subclass (EphA1-EphA10) and the B-subclass (EphB1-EphB4, EphB6).

The extracellular portion of Eph receptors contains a highly conserved N-terminal ephrin-binding domain, a cysteine-rich region (including an epidermal growth factor-like motif) and two fibronectin type-III repeats. The Eph receptor cytoplasmic side is composed of a juxtamembrane segment, a classical protein tyrosine kinase domain, a sterile-α-motif (SAM) domain and a PSD95/Dlg/ZO1 (PDZ)-binding motif (Figure 1.1).

Recent structural and biophysical studies of Eph receptors and ephrin ligands have provided molecular insights into how Eph-ephrin complexes assemble to initiate signaling (Table 1). The first step is the monovalent interaction between an Eph receptor and an ephrin ligand on juxtaposed cell surfaces [2]. Upon binding, the N-terminal ephrin-binding domain of the Eph receptor forms an interaction surface complementary to a protruding hydrophobic loop from the ephrin. Both Eph receptor and ephrin ligand undergo structural rearrangements to induce the interaction. Interestingly, the A-class

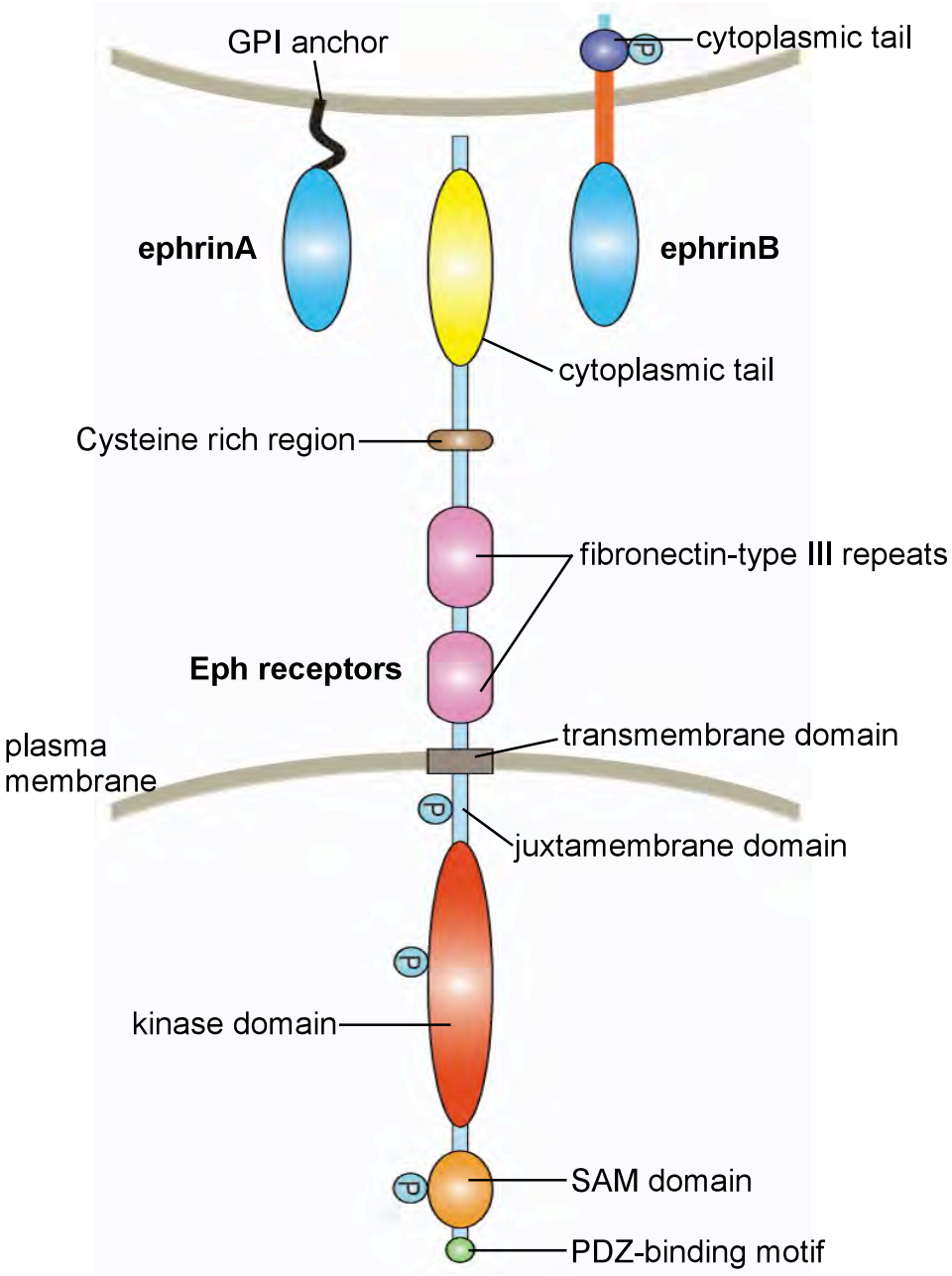

Figure 1.1. Eph receptors and ephrin ligands.

A schematic diagram of Eph receptors and ephrin ligands, which shows an ephrin-expressing cell (top) interacting with an Eph-expressing cell (bottom).

Eph/ephrin interactions involve smaller rearrangements in the interacting partners, better described by a "lock-and-key" mechanism, in contrast to the "induced fit" mechanism defining the B-class molecules [3].

In addition to the high-affinity interface, the Eph receptor and ephrin ligand contain a second, lower-affinity interface on the opposite side, which can mediate the dimerization of two Eph-ephrin heterodimers into a tetramer that comprises two Eph receptors and two ephrin ligands [2]. The Eph-ephrin tetramer complexes can further progressively aggregate into larger clusters, dependent on the densities of Eph and ephrin molecules on the cell surface. Several protein-protein interaction domains can potentially mediate this process including the juxtamembrane segment of the ephrinB cytoplasmic tail, the ephrin-binding domain, cysteine-rich region and cytoplasmic SAM domain of Eph receptors [4].

On ligand engagement, each Eph receptor of the Eph-ephrin complexes autophosphorylates tyrosine residues that are located in the partner Eph receptors. The activation of the protein kinase domain of Eph receptors is regulated via autorepression by their juxtamembrane region, which contains two autophosphorylation sites [5, 6]. When these tyrosine residues are phosphorylated, the juxtamembrane domain is uncoupled from the interaction with the kinase domain, which allows the kinase domain to convert into its active state. This conformational change also releases the phosphorylated juxtamembrane domain to bind to phosphotyrosine-binding proteins [1].

Table 1. Structure analysis of Eph receptors and ephrin ligands

Eph/ephrins	Species	Domain/Complex	Reference
EphB2	Murine	Ligand binding domain	[7]
EphB2	Murine	Juxtamembrane and kinase domains	[5]
EphB2	Murine	Kinase domain	[8]
EphB2	Human	Sterile alpha motif (SAM)	[9]
ephrinB1	Murine	Ectodomain	[10]
ephrinB2	Human	Ectodomain	[11]
EphA2	Human	Kinase domain	[12]
EphA4	Murine	Sterile alpha motif (SAM)	[13]
EphA4	Murine	Juxtamembrane and kinase domains with Y604/610F, Y750A	[8]
EphA3	Human	Kinase domain with and without juxtamembrane region	[6]
EphA1	Human	Transmembrane domain	[14]
ephrinA5	Human	Ectodomain	[15]
EphB2-ephrinB2	Murine	Ligand binding domain of EphB2 in complex with ectodomain of ephrinB2	[2]
EphB4-ephrinB2	Human	Ligand binding domain of EphB4 in complex with ectodomain of ephrinB2	[16]
EphB2-ephrinA5	Murine Human	Ligand binding domain of EphB2 in complex with ectodomain of ephrinA5	[17]
EphA2-ephrinA1	Human	Ligand binding domain of EphA2 in complex with ectodomain of ephrinA1	[3]
EphA4-ephrinA2	Human	Ligand binding domain of EphA4 in complex with ectodomain of ephrinA2	[18]
EphA4-ephrinB2	Human	Ligand binding domain of EphA4 in complex with ectodomain of ephrinB2	[19]
EphA2-SHIP2	Human	Heterotypic SAM-SAM domain association	[20]
EphB4-peptide	Human	Ligand bind domain of EphB4 in complex with TNYL-RAW peptide	[21]
EphB2-peptide	Human	Ligand binding domain of EphB2 in complex with SNEW peptide	[22]
EphA4-small inhibitors	Human	Ligand binding domain of EphA4 in complex with small molecule antagonists	[23]
ephrinB2-NiV-G or HeV-G	Human	Ectodomain of ephrinB2 in complex with Nipah or Hendra viral G proteins	[24]

Signaling mechanisms of Eph-ephrin system

Eph receptors and ephrin ligands activate a variety of signal transduction pathways via binding many cytoplasmic proteins. Eph-ephrin system participates in a wide spectrum of developmental processes and diseases, and the complexity of its signaling networks underlies the diverse functions of Eph-ephrin in human. Compared with other RTK families, Eph-ephrin signaling displays several distinctive and intriguing features, which are just now beginning to be uncovered.

Forward versus reverse signaling

A unique characteristic of Eph-ephrin complexes is their ability to transduce bidirectional signals that affect both the Eph-expressing and ephrin-expressing cells [25]. Eph receptor "forward" signaling depends on the tyrosine kinase domain, which mediates autophosphorylation as well as phosphorylation of other proteins, and on the association of the receptor with various adaptor molecules. A growing number of signaling pathways has been identified downstream of Eph receptors, including Rho family of GTPases, PI3K-Akt, Abl-Crk, and Ras-Raf-MAPK pathways [26, 27], which have been shown to affect numerous cell behaviors (Figure 1.2).

EphrinB "reverse" signaling is dependent on tyrosine phosphorylation of the cytoplasmic region mediated by Src family kinases or other receptor tyrosine kinases, and on associated effector proteins [28]. EphrinA ligands can also mediate their own signaling cascades [29, 30]. However, the mechanisms of reverse signaling for ephrinA are less understood since they are GPI-linked proteins without cytoplasmic domain. Presumably, EphrinA-mediated reverse signaling requires the association of a transmembrane

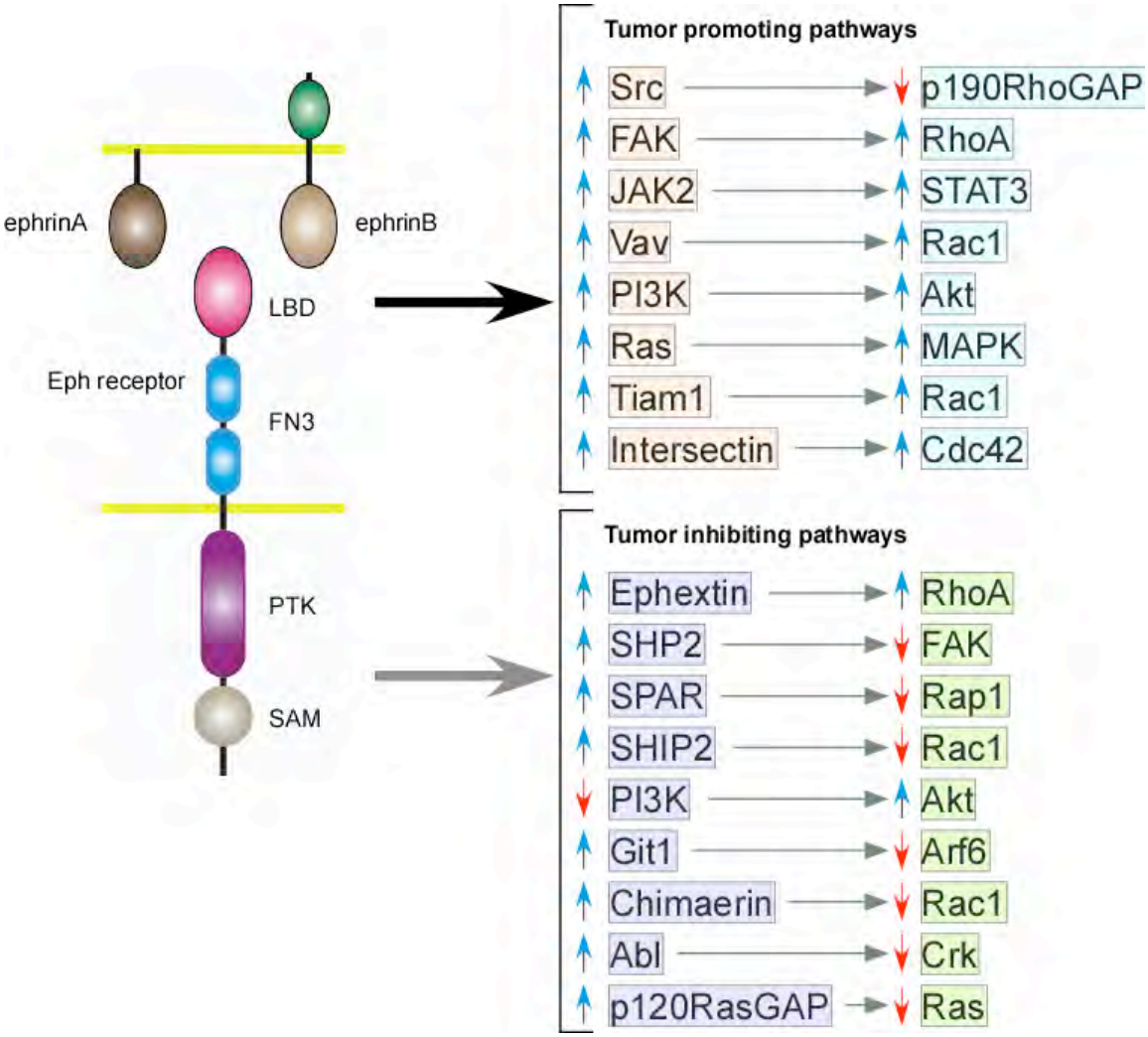

Figure 1.2. Simplified diagram of Eph signaling.

Eph receptors interact with ephrin ligands on adjacent cell membrane. Activated Eph transduce both tumor promoting and inhibiting pathways depending on ligand stimulation, signaling cross-talk, or other contextual factors. A growing number of signaling pathways has been identified to be downstream of Eph receptors, including Rho family of GTPases, PI3K-Akt, Abl-Crk, Ras-Raf-MAPK pathways and many others. LBD, ligand binding domain; FN3, fibronectin-type III repeats; PTK, protein tyrosine kinase domain; SAM, sterile α-motif.

signaling partner. The p75 neurotrophin receptor was recently shown to serve this role during retinotopic mapping and p75 may be a candidate co-receptor for ephrinAs [31].

Ligand-dependent versus ligand-independent signaling

The complexity of Eph-ephrin signaling also lies in that both ligand-dependent and ligand-independent signaling pathways exist. The most studied activity of Eph receptors and ephrin ligands is in the establishment of topographically organized neuronal connections in many regions of the developing nervous system by Eph-mediated repulsive responses at excessive ephrin concentration [32]. This was one of the first clues that ephrin-dependent Eph signaling may be detrimental to cell functions. Indeed, ligand stimulation of Eph receptors inhibits the Abl-Crk pathway [33], Ras-Raf-MAPK cascade [34], PI3K-Akt activity [35], integrin signaling [36] and Rac GTPase activation [37], which are correlated with inhibition of cell proliferation and migration. Consistently, the Eph receptors appear to be ubiquitously expressed but poorly activated in many human cancers [25, 38]. Forcing Eph activation with exogenous ephrin ligands inhibits tumor cell proliferation, survival, migration and invasion in cell culture and several mouse models [33, 39, 40].

There is now mounting evidence that this classical RTK activation does not account for all Eph/ephrin signalling and indeed that ligand-independent signalling processes can occur [27]. Ephrin independent function has been demonstrated in C. elegans [41], suggesting that this mechanism is evolutionally conserved. A hypothesis for ligand-independent Eph function is that high levels of Eph receptors may function in concert

with other cell-surface communication systems (Table 2). For example, recent studies have proposed that the EGF receptor can cooperate with EphA2 as an effector to promote cell motility independent of ephrin stimulation [42]. Other studies have reported a direct interaction and synergistic responses of fibroblast growth factor receptor (FGFR) and EphA4 [43, 44]. Using genetic mouse models and *in vitro* biochemical analyses, we discovered the crosstalk between EphA2 and human epidermal growth factor receptor 2 (HER2), which is required for breast tumor initiation and metastatic progression. These data are presented in Chapter III.

An emerging theme is that ligand-dependent and ligand-independent Eph signaling pathways often include common intracellular effectors with opposite effects. This partially explains why differences in cellular and oncogenic context can dramatically alter the outcome of Eph receptors in tumorigenesis. Miao et al. demonstrated that EphA2 receptor associates in a ligand-independent manner with focal adhesion kinase (FAK). Activation of EphA2 with ephrinA1 results in recruitment of the protein tyrosine phosphatase SHP-2, which dephosphorylates and inactivates FAK [36]. Another example is the recently discovered reciprocal regulatory loop between EphA2 and Akt, a serine/threonine kinase frequently activated in cancer cells [35]. While activation of EphA2 with its ligand ephrinA1 suppresses Akt activation and inhibits chemotactic migration of glioma and prostate cancer cells, EphA2 overexpression promotes migration in a ligand-independent manner. The latter effects require phosphorylation of EphA2 on serine 897 by Akt. Thus, EphA2 is both an upstream negative regulator and a downstream effector of Akt, dependent on the presence or absence of ephrin ligands.

Table 2. Crosstalk between Eph and other receptors

Eph receptor	Other receptors	Signaling outcome	Reference
EphA2	EGF receptor	modulate cell motility	[42]
EphA4	FGF receptors	MAPK activation	[43]
EphA	CXCR4 receptor	Cdc42 inhibition	[45]
EphB2, EphB4	CXCR4 receptor	Akt activation	[46]
EphA2	integrins	FAK inhibition	[36]
EphA4	integrins	integrin activation	[47]
EphA8	integrins	PI3K activation	[48]
EphA	integrins	Rac1 inhibition	[49]
EphA2	claudin-4	claudin-4 phosphorylation	[50]
EphA2	E-cadherin	EphA2 activation	[51]
EphB	E-cadherin	E-cadherin redistribution	[52]
EphB2	Syndecan-2	Syndecan-2 phosphorylation	[53]
EphB2	L1	L1 phosphorylation	[54]
EphB2, EphB3	Ryk receptor	unknown	[55]
EphB	NMDA receptor	NMDAR activation	[56]
EphB6	T cell receptor	T cell activation	[57]

Enzymatic cleavage versus transendocytosis

The Eph receptors and ephrin ligands were initially described as modulators of neuronal repulsion and only later were discovered that they can also promote cell adhesion in certain circumstances [58-60]. The cell-cell repulsive response following Eph-ephrin contact is particularly important for axon guidance and sorting of Eph-expressing cells from ephrin-expressing cells during development. Cell separation is not a trivial issue as the Eph-ephrin interaction has a very high affinity and these molecules are abundant at the cell surface, making dissociation of the Eph-ephrin complexes difficult [1, 61]. Two general mechanisms have been proposed to allow the separation of two cell surfaces that adhere to each other through Eph-ephrin contacts.

One strategy involves regulated proteolytic cleavage of the ephrin by transmembrane proteases. The first evidence for proteolytic ephrin regulation identified ephrinB3 as a substrate for the rhomboid transmembrane protease RHBDL2 [62]. Flanagan and colleagues documented that interaction of ephrinA2 with EphA3 activates metalloprotease ADAM10 (a disintegrin and metalloprotease 10), which cleaves the extracellular domain of the ephrin [63]. It remains to be determined whether Eph receptor ectodomains might also be cleaved. Interestingly, similar regulatory mechanism also exists in glioblastoma multiforme (GBM) and breast adenocarcinoma cells, which could cleave and release soluble monomeric ephrinA1 detected in conditioned media [64].

The other strategy utilizes rapid trans-endocytosis which removes the adhesive Eph-ephrin complexes from the cell surfaces, allowing the cells to disengage. Two parallel

studies showed that the internalized vesicles contain intact EphB4-ephrinB2 complexes and both of their surrounding plasma membranes [65, 66]. Importantly, it was found that EphB4-ephrinB2 endocytosis requires cytoskeletal rearrangement and Rac1 activity. We identified a lipid phosphatase SHIP2 (Src homology 2 domain-containing phosphoinositide 5-phosphatase 2), which binds with EphA2 receptor and regulates EphA2 endocytosis via inhibiting phosphatidylinositol 3-kinase-dependent Rac1 activation. These data are presented in Chapter II.

Role of Eph-ephrin in tumor promotion

The Eph receptors and ephrin ligands have intriguing expression patterns in cancer cells and tumor blood vessels, which suggest important roles for Eph signaling in multiple aspects of cancer development and progression [67, 68]. The activities of the Eph system in cancer are complex in their paradoxical effects. There is good evidence that Eph receptors and ephrin ligands can both promote and inhibit tumorigenicity. However, multiple Ephs and/or ephrins are present, and often dysregulated, in essentially all types of cancer cells and tumor microenvironment, suggestive of their role in tumor promotion.

Eph receptors in tumor progression

The high abundance of Eph receptors in many human cancers, including various carcinomas, melanoma, sarcoma, leukemia, renal and brain tumors, has been very well documented [69-71]. In some cases, there is a clear link between this increased expression and tumor progression. A number of studies have shown a correlation between the degree of tumor malignancy and the levels of Eph receptors. For example,

esophageal squamous cell carcinomas that exhibit the highest degree of lymph node metastasis have the highest levels of EphA2 expression [72]. In human brain tumors, invading glioblastomas have higher EphB2 expression than do low-grade astrocytomas [73].

In addition to dysregulation of Eph expression, many studies have demonstrated a causal role of Eph receptors in cancer, which is perhaps best exemplified by EphA2 receptor in breast cancer. Experimentally induced overexpression of EphA2 resulted in malignant transformation of nontransformed MCF10A breast epithelial cells and enhanced malignancy of pancreatic carcinoma cells [74, 75]. Conversely, siRNA-mediated inhibition of EphA2 expression impaired malignant progression of pancreatic, ovarian, and mesothelioma tumor cell lines, and overexpression of dominant-negative EphA2 constructs suppressed growth and metastasis of 4T1 metastatic mouse mammary adenocarcinoma cells in vivo [75-78]. Similarly, over-expression of EphB4 in the mammary epithelium accelerates tumor onset and lung metastasis in MMTV-Neu animals [79], while EphB4 knockdown inhibited breast cancer survival, migration, and invasion *in vitro* and tumor growth in a xenograft model *in vivo* [80].

The mechanisms by which Eph receptors exhibit oncogenic effects are not entirely clear. Highly expressed Eph receptors could be hijacked by other oncogenes, such as EGFR and FGFR1 [42, 44], to maximize downstream oncogenic signaling pathways. In certain cellular contexts, Eph receptors also activate particular effectors to acquire specific oncogenic ability. For example, RRas phosphorylation downstream of EphB2 can

enhance glioma cell invasiveness but reduce cell proliferation [81]. Additionally, activation of RhoA downstream of EphA2 and EphB4 promotes ameboid-type migration of tumor cells and destabilizes epithelial adherens junctions in various cancer cell lines, even though RhoA activity inhibits mesenchymal-type cell migration [78, 82, 83]. Consistent with these controversial observations, a recent study revealed that EphB2 both promotes proliferation and inhibits migration in intestinal cells through distinct pathways [84]. Therefore, Eph signaling in tumor progression is extremely dynamic and capable of contrasting effects.

Eph receptors in tumor angiogenesis

Besides being expressed in cancer cells, Eph receptors and ephrin ligands are also present in the tumor vasculature [85]. In contrast to the complex effects of Eph signaling in tumor cells, Ephs and ephrins have been well characterized to play a vital role to promote angiogenesis in tumor vascular endothelial cells [27]. Because tumor associated blood vessels are critical for tumor growth and metastatic dissemination, this represents an important aspect of the oncogenic effects of Eph-ephrin bidirectional signaling. The main roles of Eph-ephrin in tumor angiogenesis have so far been attributed to EphB4-ephrinB2 reverse signaling and ephrinA1-EphA2 forward signaling based on a series of *in vitro* and *in vivo* experiments with mouse tumor models.

EphB4 and ephrinB2 play a prominent role in both developmental and tumorigenic angiogenesis. During development, they are characteristically expressed in the endothelial cells of veins and arteries, respectively, and enable arterial-venous vessel

segregation and vascular remodeling [86, 87]. The results of tumor angiogenesis studies are consistent with the importance of ephrinB2 reverse signaling, while little is known about the role of EphB4 forward signaling. Reverse signaling by ephrinB2 in tumor endothelial cells, pericytes and smooth muscle cells is triggered by EphB receptors expressed in vascular and/or tumor cells, and has been shown to be crucial for blood vessel assembly, maturation and maintenance both in cell culture and in mouse models [88-90]. EphrinB2 signaling also promotes the interaction between endothelial cells and pericytes or vascular smooth muscle cells [91], suggesting that upregulation of ephrinB2 may stabilize the vessels of tumors recurring after anti-VEGF therapy.

Extensive evidence, including analysis of tumor growth in EphA2 knockout mice or mice systematically treated with inhibitory EphA-Fc fusion proteins, shows that EphA2 forward signaling promotes tumor angiogenesis [92-95]. In contrast, EphA2 does not seem to play a critical role in developmental angiogenesis or in the adult quiescent vasculature. Interaction with ephrinA1 in tumor endothelial cells as well as tumor cells is responsible for activating endothelial EphA2. Activated EphA2 regulates endothelial cell migration and assembly through PI3K-dependent Rac1 GTPase activity [94]. Furthermore, a yeast two-hybrid screen identified Vav guanine nucleotide exchange factors to be recruited to active EphA2 receptors and subsequently elevate Rac1-GTP levels [96]. The upregulation of EphA2 and ephrinA1 observed in late-stage pancreatic tumors of mice treated with VEGF inhibitors suggests that EphA2-mediated angiogenesis may also contribute to the development of resistance to anti-VEGF therapies [97].

Eph receptors in resistance to anti-cancer therapies

One arising challenge in cancer therapy is to overcome intrinsic resistance, which leads to tumor progression regardless of treatments, and acquired resistance, which is induced by therapeutic reagents and results in cancer recurrence after initial response. Because Eph receptors are frequently overexpressed in various cancers and exhibit intimate interplay with other oncogenic pathways, they are also likely to contribute to regulating drug sensitivity to targeted therapeutics. However, characterization of the Eph system in resistance to anti-cancer therapies is still at an early stage. Kinch and colleagues reported that EphA2 overexpression decreases estrogen dependence as defined by both *in vitro* and *in vivo* criteria, and impairs the ability of tamoxifen to inhibit breast cancer cell growth and tumorigenesis [98]. Therapeutic intervention using EphA2 antibodies can resensitize EphA2-overexpressing breast tumor cells to tamoxifen treatment. In another study, EphB4 was related to imatinib resistance in Philadelphia chromosome-positive acute lymphoblastic leukemia cells [99]. We provide evidence that elevated level of EphA2 mediates resistance to trastuzumab therapy and propose strategies to target EphA2 as a tactic to reverse trastuzumab resistance. These data are presented in Chapter IV.

Eph receptors in cancer prognosis

Eph expression promises to be a powerful predictor of prognosis. EphA2, for example, has been linked to increased malignancy and poor clinical prognosis in breast cancer [100], non-small cell lung cancer [101], esophageal squamous cell carcinoma [72], cervical squamous cell carcinoma [102], renal cell carcinoma [103], glioblastoma

multiforme [104], and endometrial cancer [105]. Overexpression of EphA4 in gastric cancer is associated with significantly short overall survival periods [106]. Increased expression of EphA7 associates with adverse outcome in primary and recurrent glioblastoma multiforme patients [107]. EphB4 overexpression predicts poor overall survival in patients with ovarian cancer and head and neck squamous cell carcinoma [108-110]. Conversely, colorectal cancer patients with EphB2 expression exhibit significantly prolonged overall survival [111]. Therefore, there is a need for a comprehensive assessment of Eph expression in large cohorts of human tumors in correlation with stages of malignancy and clinical outcome. Carefully validated antibodies and quantitative proteomics approaches are necessary to ensure the reliability of such studies.

Role of Eph-ephrin in tumor suppression

Eph-ephrin system does not necessarily aid the tumorigenic process. Tumor suppressor activities for Eph signaling have been reported in colorectal, breast, prostate and skin cancers both *in vitro* and *in vivo* [34, 112, 113]. Accumulating evidence implicates that the complexities of the Eph system and dichotomous Eph activities in different tumor components partially explain the many confusing and contradictory phenotype. To dissect the oncogenic or suppressive role of Eph signaling in tumor pathogenesis and advance our understanding of Eph cancer biology, it will be imperative to examine the effects of Eph or ephrin deletion, overexpression, and cancer relevant mutations in genetically engineered mouse models that mimic the progression of human cancers. Such *in vivo* models are key for studying the Eph system, given its penchant for regulating

communication between different cell types, which is difficult to accurately recapitulate *in vitro*. We combine gene knockout mice and transgenic mouse tumor models and demonstrate that the role of EphA2 in tumor progression is dependent upon the oncogene/tumor suppressor context within which it functions. These data are presented in Chapter III.

Inhibition of tumorigenicity by Eph receptors

EphB receptors have been extensively characterized in suppressing colorectal cancer. In normal intestine, EphB receptors and ephrinB ligands are expressed in complementary gradients along the crypts under the control of the Wnt/β-catenin/Tcf pathway, which upregulates EphB and downregulates ephrinB expression [114]. EphB activities, which determine progenitor cell positioning and promote cell proliferation, play an important role in intestinal homeostasis [115]. In the transition from normal cells to intestinal adenoma, EphB receptors are usually upregulated and ephrinB downregulated by the constitutive activation of the β-catenin/Tcf pathway [114]. The EphB proliferative effects may have some tumor-promoting ability, which is responsible for about half of the proliferation in adenomas [115], but adenoma growth is restricted by repulsion from ephrinB in the surrounding differentiated epithelium [52, 112]. Therefore, EphB receptors have to be lost in tumor cells, enabling invasiveness as well as tumor expansion. This EphB downregulation represents a critical step in the progression to malignant stages and correlates with a poor prognosis.

Another example is EphA2 in susceptibility to chemically induced keratinocyte transformation. Despite the observed upregulation of EphA2 in mouse as well as human keratinocyte-derived skin carcinomas, the tumors in EphA2 knockout mice grow faster and are more invasive [113]. Similar to the EphB-ephrinB interplay in colorectal cancer, ephrinA1 expression in the surrounding skin appears to restrict expansion of the EphA2-positive tumor cells. Deletion of EphA2 may eliminate EphA2-ephrinA1 interactions, which leads to increased tumor susceptibility.

Eph signaling in tumor suppression

A prominent pathway that may explain these tumor suppressor effects of Eph receptors is the Ras-Raf-MAPK signaling cascade. One of the first studies showing the relationship between Eph signaling and MAPK activity was performed in several different cell types [34]. The study showed ephrinA1 activated EphA signaling inhibits MAPK activation in prostate epithelial cells, mouse embryonic fibroblasts, and bovine endothelial cells. This effect is likely Ras-dependent, as Ras activity is also decreased and overexpression of Ras could compensate for ephrinA-EphA2 induced MAPK inhibition. Recently, a conditional feedback loop has been identified, whereby Ras-Raf-MAPK activation promotes EphA2 expression, which in turn negatively regulates MAPK activities upon ligand stimulation in human breast cancer cell lines [116].

The PI3K-Akt is another signaling cascade involved in the tumor suppression by Eph receptors. Phosphorylation by Akt of a single serine site (S897) in EphA2 appears to promote cancer cell migration and invasion, an effect independent of catalytic activity of

EphA2. Ephrin-A1 stimulation of EphA2 negates Akt activation by growth factors and causes EphA2 dephosphorylation on S897. These results identified a reciprocal regulatory loop between EphA2 and Akt, with unligated EphA2 functioning as a downstream substrate and effector of Akt kinase, but with the ligand-activated EphA2 functioning as an upstream negative regulator to turn off Akt and cause dephosphorylation of EphA2 at the Akt substrate site [35].

Elegant work by Pasquale and colleagues found that ephrinB2 stimulated EphB4 signaling results in tyrosine phosphorylation of Crk on Tyr221, which induces a conformational change that blocks the ability of Crk to function as an adaptor protein [33]. Tyr 221 of Crk is a major target for the Abl family of non-receptor tyrosine kinases, and the treatment of breast cancer cells with the Abl inhibitor imatinib or Abl small interfering RNA block Crk phosphorylation in response to ephrinB2 stimulation, without affecting EphB4 tyrosine phosphorylation. Thus, the Abl–Crk pathway mediates inhibition of breast cancer cell proliferation, survival and motility downstream of EphB4.

Silencing of Eph signaling in cancer cells

In general, ephrin induced Eph receptor forward signaling appears to transduce an inhibitory signal that keep cells quiescent and non-invasive. Therefore, cancer cells have developed a variety of mechanisms to minimize the tumor suppressor effects of Eph signaling. A common phenomenon is the differential expression Eph receptors and ephrin ligands in tumors, resulting in low bidirectional signaling [33, 116]. Eph overexpression in cancer often is due to oncogenic signaling pathways, hypoxia, or inflammatory

cytokines. The Wnt/β-catenin/Tcf pathway promotes EphB expression in colorectal cancer cells and the Ras-Raf-MAPK pathway promotes EphA2 expression in breast cancer cells. Surprisingly, activation of these two pathways also leads to ephrin downregulation and, as a consequence, low Eph receptor activation [114, 116]. Chromosomal alterations also regulate Eph and ephrin expression in tumor cells, and a number of Eph receptors and ephrin ligands are located in chromosomal regions frequently amplified or deleted in human cancers. By analyzing SNP array-based genetic maps with gene expression signatures, we found that EphA3 resided in a frequently deleted focal locus on chromosome 3p11.2. Interestingly, genes encoding EphA3 ligands, ephrinA1, A3, and A5, on chromosome 1q21-q22 are frequently amplified in these tumors. These data are presented in Chapter V.

Other mechanisms to keep Eph receptors under-phosphorylated in spite of overexpression include disruption of cell-cell contacts in tumor cells and increased activity of phosphotyrosine phosphatase. Indeed, loss of E-cadherin or VE-cadherin impairs endogenous EphA2-ephrinA1 interactions, which silence their signaling function, in malignant breast cancer and melanoma cells, respectively [51, 117]. Phosphotyrosine phosphatases could also negatively regulate Eph receptor forward signaling in cancer cells [118]. For example, the low molecular weight phosphotyrosine protein phosphatase (LMW-PTP) has been implicated in cell transformation through dephosphorylating EphA2, thus counteracting ephrin stimulated activation [119]. The protein tyrosine phosphatase receptor type O (PTPRO) also dephosphorylates Eph receptors in neurons, although it is unknown whether this plays a role in cancer [120].

Somatic mutations in Eph receptors have recently been identified in multiple sequencing efforts of tumor specimens to identify cancer genes [121-126], whereas cancer-related ephrin mutations have not been reported so far perhaps because most screens have focused on the human kinome. These Eph mutations may also contribute to disrupting forward signaling by impairing ephrin binding or kinase activity. For instance, the EphA3 E53K mutation found in a melanoma cell line abrogates ephrin binding [127], and the EphB2 G787R mutation found in colorectal cancer impairs kinase activity [128]. We systematically investigated the EphA3 mutations identified in non-small cell lung cancer and discovered that they are loss-of-function mutations. These data are presented in Chapter V.

Eph-based therapeutics in cancer

Encouraged by recent significant successes of various molecular targeted therapies in cancer treatment, as well as a more comprehensive understanding of Eph-ephrin in tumor progression as discussed above, Eph receptors and ephrin ligands represent promising new therapeutic targets in cancer. A wide range of strategies have been proposed and are under evaluation to minimize their tumor-promoting effects and maximize their tumor-suppressing roles (Table 3). These strategies include directly interfering with Eph-ephrin functions, using Eph receptors as delivery vehicles for drugs, and Eph-based immunotherapy.

Interfering with Eph-ephrin function

Downregulation of EphA2 or EphB4 with siRNAs or antisense oligonucleotides has been shown to inhibit tumor growth in breast cancer, colon cancer, pancreatic cancer and ovarian cancer [75, 76, 129, 130]. Ephrin ligands and agonistic Eph antibodies have also been successful to inhibit tumor progression, probably by stimulating both forward signaling and receptor degradation in the cancer cells [131-134]. Additionally, antibody-dependent cell-mediated cytotoxicity may also contribute to the anti-cancer effects *in vivo* [135]. Efforts to identify small molecules that target the Eph kinase domain have begun to yield some high affinity inhibitors [136-138]. Furthermore, a number of inhibitors designed to target other kinases also inhibit Eph receptors. Dasatinib, an Abl and Src inhibitor for the treatment of chronic myelogenous leukemia, potently inhibits EphA2 [139], and XL647, an EGF and VEGF receptor inhibitor being evaluated in lung cancer, also targets EphB4.

Another strategy that shows promise for anti-angiogenic therapy is to inhibit Eph-ephrin interactions. The dimeric EphA2 ectodomain fused to Fc, which inhibits Eph forward signaling but promotes reverse signaling, and the monomeric EphB4 ectodomain, which inhibits both forward and reverse signaling, can both reduce tumor growth by inhibiting tumor angiogenesis [95, 140]. Pasquale's laboratory has developed antagonistic peptides by phage display which selectively inhibit specific Eph-ephrin binding [142, 147, 148]. imilar strategy is also suitable for chemical compounds to specifically target Eph receptors, and two isomeric small molecules that preferentially inhibit ephrin binding to EphA2 and EphA4 have been identified [149]. Structural characterization of these

Table 3. Eph-ephrin based therapeutics

Targets	Molecules	Activity	Reference
EphA2	activating antibodies	Eph activation/degradation	[131]
EphA3	activating antibodies	Eph activation/degradation	[141]
EphA	ephrinA1-Fc	Eph activation/degradation	[40]
EphB4	ephrinB2-Fc	Eph activation/degradation	[33]
EphA2	peptides	Eph activation/degradation	[142]
EphA2	siRNA or oligonucleotides	Eph downregulation	[75, 76]
EphB4	siRNA or oligonucleotides	Eph downregulation	[143, 144]
ephrinA	EphA2-Fc, EphA3-Fc	Eph competitor	[95, 145]
ephrinB	sEphB4	Eph competitor	[140, 146]
EphA4	peptides	ephrin competitor	[147]
EphB2	peptides	ephrin competitor	[148]
EphB4	peptides	ephrin competitor	[148]
EphA2	small inhibitors	ephrin competitor	[149]
EphA4	small inhibitors	ephrin competitor	[149]
EphB2	antibody	ephrin competitor	[132]
EphB4	kinase inhibitors	ATP competitors	[150, 151]
Eph	kinase inhibitors	ATP competitors	[137, 138]
EphA2	antibody conjugate	internalization of microtubule inhibitors	[152, 153]
EphA2	bispecific antibody	CD3-mediated T cell recruitment	[154]
EphA	ephrinA1-exotoxin A	internalization of exotoxin A	[155]
EphA	ephrinA1-nanoshells	photothermal ablation of tumor cells	[156]
EphB2	antibody conjugate	internalization of microtubule inhibitors	[132]
EphA2	Cu-DOTA-1C1 antibody	radioimmunoPET	[157]
EphA2	peptide-nanoparticles	tumor cell extraction	[158]
EphA3	^{111}Indium antibody	tumor detection	[141]

peptides and small molecules in complex with Eph receptors may assist to optimize the specificity and affinity [21-23].

Targeted delivery of toxins and imaging agents

Eph functions are redundant in normal tissues, and Eph receptors are often upregulated in many tumors, making them attractive targets for the delivery of toxins or imaging agents into cancer cells. Several chemotherapeutic toxins conjugated with Eph antibodies or ephrin ligands, which cause receptor-mediated drug internalization, appear promising in preclinical studies. EphA2 antibodies coupled to the microtubule inhibitor exhibited significant antitumor activity in several cancers [152, 153]. Another potential application is the targeted delivery of gold-coated nanoshells conjugated to ephrin for photothermal destruction of Eph-positive cancer cells [156]. Eph receptors can also be used to deliver imaging agents for diagnostic purposes. Promising results have been obtained in animal models by using an EphA2 antibody coupled to Cu-DOTA for radioimmunoPET imaging and an EphA3 antibody coupled to Indium for gamma camera imaging [141, 157].

Eph-based immunotherapy

In addition to the immune cell-mediated cytotoxicity elicited by Eph-targeted antibodies, a bispcific single-chain antibody that simultaneously binds both EphA2 on tumor cells and the T cell receptor-CD3 complex on T cells causes T cell-mediated destruction of EphA2-positive tumor cells *in vitro* and decreases tumor growth *in vivo* [154]. Furthermore, Eph receptor isoform has been identified as sources of tumor-associated peptide antigens that are recognized by effector T cells [159-162]. Eph receptors that are

preferentially expressed in tumors compared with normal tissues are also attractive targets for cancer vaccines, and vaccination with EphA2 derived epitopes shows promise as a strategy to elicit tumor rejection [163-165].

Summary

Eph receptors and ephrin ligands form an important cell communication system in cancer pathogenesis. Eph-ephrin has a clear role in cancer cell autonomous regulation of tumorigenesis, but it also functions in the tumor microenvironment that contributes to the progression of the disease. The unique mechanisms of Eph signaling enable it to either potentiate the activities of oncogenic signaling networks or repress them, making Eph receptors associated with both tumor promotion and suppression. Due to this puzzling dichotomy, designing the therapeutic targeting strategy that involves modification of this pathway remains a challenge and the balance of pro- versus anti-tumorigenic effects should be considered. As an example, EphA2 agonists would be expected to activate tumor suppressor signaling pathways and induce receptor degradation in cancer cells, which leads to tumor inhibition. Conversely, tumor angiogenesis may be increased by EphA2 stimulation in endothelia cells and this could result in subsequent tumor progression. Therefore, Eph-targeting agents likely act through a combination of multiple effects on cancer cells and tumor microenvironment, and in order to develop optimal strategies to interfere with Eph function, we must better understand the differential roles of this pathway in the different cellular compartments of specific tumors.

Increasing evidence suggests that Eph receptors function as an oncogene. They are upregulated in a wide variety of cancers and their expression has been linked to increased malignancy and poor clinical outcome. In addition, it is well-documented that Eph receptors play a causal role in neoplasia and tumor angiogenesis. Other evidence, however, suggests that Eph may function as a tumor suppressor. Ligand-dependent Eph signaling is detrimental to several oncogenic pathways including Ras-Raf-MAPK, PI3K-Akt, and Abl-Crk. One possible reason for this paradox may reside in that the experimental systems *in vitro* may not accurately recapitulate human cancers *in vivo*. We have now integrated genetically engineered mouse models as well as biochemical analyses in cell culture, and provided one possible explanation to these controversial observations. More importantly, we have used these mouse models for preclinical evaluation of new Eph-based therapies.

In addition to dysregulation in Eph expression, Eph gene mutations likely also contribute to cancer pathogenesis. Indeed, somatic mutations in Eph receptors have recently been identified in multiple sequencing projects of human cancers. Elucidating the effects of the mutations will provide important insights into the functional roles of the Eph system in cancer. Nevertheless, the significance of these mutations is not yet known. Here, we comprehensively analyzed EphA3 mutations discovered in lung adenocarcinoma. These information, together with the functional analysis of EphA3 both *in vitro* and *in vivo*, has expanded our understanding of Eph receptors in cancer biology and paved the way for future therapeutic strategies targeting Eph receptors.

Eph receptors promise to be a powerful predictor of prognosis and perhaps drug sensitivity, which have only started to be appreciated. We have undertaken the approach of analyzing clinical cohorts of breast cancer and lung adenocarcinomas, and found that EphA2 and EphA3 are associated with patient survival in breast and lung cancers, respectively. Additionally, elevated EphA2 expression in breast tumor cells appears to mediate resistance to trastuzumab, an approved targeted therapy for women with HER2 positive breast cancer. Although these findings remain to be clinically validated, they undoubtedly hold the promise that Eph receptors can potentially be utilized to predict whether and how we should treat cancer patients.

The thesis projects

Extensive evidence implicates the Eph receptor family of tyrosine kinases in cancer development, but it remains incompletely understood how Eph receptors affect cancer progression. Both tumor-promoting and tumor-suppressing effects have been described, sometimes for the same Eph receptor in the same type of cance. Understanding the mechanisms responsible for these divergent activities has potential therapeutic implications for targeting Eph receptors. We therefore integrated genetically engineered mouse models, biochemical analyses in cell culture and genomic datamining in clinical specimens to systematically investigate the functions of Eph receptors in physiological contexts. The thesis projects presented here include several significant findings. We discovered the crosstalk between EphA2 and human epidermal growth factor receptor 2 (HER2), which is required for breast tumor initiation and metastatic progression. Consequently, elevated levels of EphA2 in a subset of HER2-positive breast cancers

mediate resistance to trastuzumab therapy that targets HER2 receptor. One caveat is that the oncogenic role of EphA2 in tumor progression is dependent upon the oncogene context such as the presence of HER2. On the other hand, EphA3 is frequently deleted, downregulated or mutated in non-small cell lung cancer, resulting in the loss of suppressive role of EphA3 receptor which inhibits the mTOR pathway. These work revealed one possible mechanism responsible for the hitherto conflicting roles attributed to Eph receptors in tumorigenesis, and dissociated two modes of Eph signaling for development of novel cancer therapies.

CHAPTER II

REGULATION OF EPHA2 RECEPTOR ENDOCYTOSIS BY SHIP2 LIPID PHOSPHATASE VIA PHOSPHATIDYLINOSITOL 3-KINASE-DEPENDENT RAC1 ACTIVATION

The work presented in this chapter is published with the same title in the *Journal of Biological Chemistry*, Jan 2007 [166].

Abstract

Endocytosis of Eph receptors is critical for a number of biological processes, including modulating axon growth cone collapse response and regulating cell surface levels of receptor in epithelial cells. In particular, ephrin-A ligand stimulation of tumor cells induces EphA2 receptor internalization and degradation, a process that has been explored as a means to reduce tumor malignancy. However, the mechanism and regulation of ligand-induced Eph receptor internalization are not well understood. Here we show that SHIP2 (Src homology 2 domain-containing phosphoinositide 5-phosphatase 2) is recruited to activated EphA2 via a heterotypic sterile α motif (SAM)-SAM domain interaction, leading to regulation of EphA2 internalization. Overexpression of SHIP2 inhibits EphA2 receptor endocytosis, whereas suppression of SHIP2 expression by small interfering RNA-mediated gene silencing promotes ligand-induced EphA2 internalization and degradation. SHIP2 regulates EphA2 endocytosis via phosphatidylinositol 3-kinase-dependent Rac1 activation. Phosphatidylinositol 3,4,5-trisphosphate levels are significantly elevated in *SHIP2* knockdown cells, phosphatidylinositol 3-kinase inhibitor decreases phosphatidylinositol 3,4,5-trisphosphate levels and suppresses increased

EphA2 endocytosis. Ephrin-A1 stimulation activates Rac1 GTPase, and the Rac1-GTP levels are further increased in *SHIP2* knockdown cells. A dominant negative Rac1 GTPase effectively inhibited ephrin-A1-induced EphA2 endocytosis. Together, our findings provide evidence that recruitment of SHIP2 to EphA2 attenuates a positive signal to receptor endocytosis mediated by phosphatidylinositol 3-kinase and Rac1 GTPase.

Introduction

The Eph family of receptor tyrosine kinases (Ephs) and their ligands, the ephrins, regulate a diverse array of biological responses in development and disease (reviewed in Refs. [1, 25, 69]). These receptors represent the largest family of receptor tyrosine kinases in the genome, consisting of at least 16 receptors that interact with nine membrane-bound ephrin ligands. They can be further divided into two groups, class A and class B, based on sequence homology and binding affinity [167]. Class A Eph receptors interact with multiple ligands of the ephrin-A family, a group of glycosyl-phosphatidylinositol-linked membrane proteins, whereas class B Eph receptors bind to ephrin-B ligands, a family of transmembrane proteins. Binding of Eph receptors to their ligands induces receptor clustering, receptor transphosphorylation, and activation of kinase activity, followed by activation of signaling cascades that mediate multiple cellular responses [1, 25, 69].

Major advances have been made in recent years to dissect the molecular mechanisms by which Ephs/ephrins regulate biological processes. In particular, ligand-induced receptor

endocytosis has been studied in a number of biological systems. Upon juxtacrine interaction of cell surface ephrin-B ligand and EphB receptor, ligand-receptor complexes are internalized bidirectionally [65, 66, 168]. This bidirectional endocytosis of ephrin-B and EphB complexes appears to be sufficient to promote cell detachment in axon withdrawal during growth cone collapse. Endocytosis of EphA receptor also appears to be critical in converting an initial adhesive interaction into a repulsive event in growth cone collapse response [169]. Outside of the nervous system, ligand-induced phosphorylation of the Eph receptors in tumor cells has been shown to result in receptor endocytosis and down-regulation of surface receptors [170, 171]. Enhanced EphA2 endocytosis and subsequent degradation are associated with decreased malignant cell behavior. Activating EphA2 monoclonal antibodies [134], ephrin-mimetic peptides [142], or adenovirus-expressing ephrin-A1 ligand [40] have been developed to induce receptor endocytosis as a means to reduce EphA2 activity. Despite the important roles that Eph receptor endocytosis plays in biological responses, relatively little is known about how this pathway is regulated.

We have previously shown that activation of the EphA2 receptor regulates Rac1 GTPase activity through a PI3-kinase-dependent pathway [94]. Through a yeast two-hybrid screen, we identified two proteins that interact with the activated EphA2 receptor and are capable of regulating the level of phosphatidylinositol 3,4,5-trisphosphate (PIP3): the p85 subunit of PI3-kinase and SHIP2 (SH2-containing phosphoinositide 5-phosphatase 2). SHIP2 belongs to the SHIP family of lipid phosphatases that dephosphorylates PIP3 produced by PI 3-kinase [172]. PIP3 has been shown to interact with pleckstrin homology

domain-containing proteins, such as the Ras and Rho family guanine nucleotide exchange factors, leading to the membrane recruitment and activation of these proteins [173]. Although expression of SHIP1 is restricted to the hematopoietic cell lineage, SHIP2 is expressed in many cell types [174]. These data suggest that SHIP2, together with PI 3-kinase, may function downstream of the EphA2 receptor to regulate Rho family GTPases.

In this study, we investigate the molecular mechanisms that regulate ephrin-Eph endocytosis. We show that ligand stimulation induces EphA2 receptor internalization in MDA-MB-231 mammary carcinoma cells. In response to ephrin binding to EphA2, SHIP2 is recruited to the sterile α motif (SAM) domain of EphA2. Overexpression of SHIP2 inhibited ligand-induced receptor endocytosis. Conversely, suppression of SHIP2 expression by small interfering RNA (siRNA)-mediated gene silencing increased EphA2 endocytosis and subsequent degradation. The mechanism of regulation of EphA2 endocytosis by SHIP2 involves down-regulation of cellular PIP3 levels and inhibition of Rac1 GTPase activity. These findings suggest that SHIP2 plays a central role in regulation of EphA2 receptor endocytosis.

Methods

Yeast two-hybrid screening

The mouse EphA2 cytoplasmic domain was cloned into pBridge-LexA (BD Biosciences) (pSGS2) as a bait to screen a human placenta library consisting of 3.5×10^6 independent clones (Clontech) as described [96]. Briefly, yeast strain L40 (MATa his3D200 trp1–901

leu2–3112 ade2 LYS2::(4lexAop-HIS3) URA3::(8lexAop-lacZ) GAL4) was transformed with pSGS2 and the placenta cDNA library. The resulting transformants were screened for histidine prototrophy and expression of LacZ. The His+/LacZ+ clones that did not interact with lamin C were subjected to PCR analyses to eliminate duplicate clones. Among 14 unique His+/LacZ+ clones, six overlapping clones encompassing the SAM domain of the *SHIP2* gene were identified.

Antibodies

Antibodies used for immunoblot or immunocytochemistry include anti-Myc (1:500; BD Biosciences), anti-FLAG (1:1000; Sigma), anti-EphA2 (1:1000; Santa Cruz Biotechnology), anti-phosphotyrosine (1:400; Santa Cruz Biotechnology), anti-tubulin (1:1000; Sigma), anti-Rac1 and anti-Cdc42 antibodies (1:250; Pharmingen), anti-PIP3 (1:250; Echelon), and anti-EEA1 (1:1000; BD Biosciences). For immunoprecipitation, 1.5 μg of polyclonal rabbit anti-SHIP2 antibody (Santa Cruz Biotechnology) was used. Anti-SHIP2 polyclonal chick antibodies were made by Zymed Laboratories Inc., using the purified GST-SAM domain of the SHIP2 protein as antigen, and used in Western blot analysis.

In vitro binding assay

MBP-EphA2-SAM, the fusion of the intracellular portion of mouse EphA2 SAM domain and maltose-binding protein, was created from pMAL-c2X (New England Biolabs) and purified on amylose resin according to the manufacturer's instructions. *Escherichia coli* lysate containing GST-SHIP2-SAM domains was incubated with amylose-bound MBP-

EphA2-SAM or control MBP alone. After extensive washing, bound proteins were eluted and subjected to Western blot analyses using anti-EphA2 and anti-SHIP2 antibodies.

Co-immunoprecipitation and western blot analyses

COS7 cells were co-transfected with 1 μg each of FLAG-tagged *SHIP2* and *EphA2* or Myc-tagged *EphA2* mutants using Lipofectamine 2000. Cells were lysed in 1% Nonidet P-40 buffer (10 mm Tris-HCl, pH 7.5, 150 mm NaCl, 2 mm EDTA, 1% Nonidet P-40 plus 50 mm protease inhibitors). Anti-FLAG antibody (Sigma) was used to immunoprecipitate SHIP2. The resulting proteins were resolved on SDS-PAGE and subjected to Western blot using anti-EphA2. 1.5 μg of anti-EphA2 antibody (sc-924; Santa Cruz Biotechnology) was also used in immunoprecipitation, and precipitated proteins were subjected to SDS-PAGE and Western blot analysis by SHIP2 antibodies. For coimmunoprecipitation between SHIP2 and EphA2ΔSAM or SAM domain alone, Myc-agarose (Sigma) was used to immunoprecipitate EphA2 mutants.

siRNA-mediated silencing of SHIP2 in MDA-MB-231 cells

SHIP2 knockdown was achieved by siRNA-mediated stable silencing of *SHIP2* via retroviral transduction, as described [92]. Briefly, human *SHIP2* siRNAs and control siRNA were designed using Invitrogen software according to the manufacturer's instructions. A 64-base pair oligonucleotide linker containing *SHIP2*-specific sense and corresponding antisense sequences, flanking a 6-base hairpin, was generated, PAGE-purified, and subcloned into retroviral vector pRS (a gift of R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands). pRS *SHIP2* siRNA or control siRNA

retrovirus were produced in the Phoenix cell packaging line. For siRNA expression, MDA-MB-231 cells were infected with pRS *SHIP2* or control siRNA retroviruses and selected in the presence of 5 μg/ml puromycin. Pooled clones of MDA-MB-231, number 1, 2, 3, and 4, or single clone 2A expressing either *SHIP2* or control siRNAs were analyzed. The level of *SHIP2* knockdown in pooled clones was assessed by quantifying band intensity of SHIP2 over tubulin using Scion Image software.

Confocal microscopy analysis

Control or *SHIP2* knockdown cells were plated on coverslips in 6-well dishes and cultured to 50% confluence. Growth medium was replaced with 1 ml of starvation medium (Dulbecco's modified Eagle's medium plus 1% bovine serum albumin or Opti-MEM) per well. For immunofluorescence assays, cells were stimulated with 1 μg/ml ephrin-A1 for 30 min and fixed with 4% paraformaldehyde. Following fixation, cells were incubated with primary antibodies (anti-EphA2, 1:1000 (Santa Cruz Biotechnology); monoclonal anti-EEA1, 1:1000 (BD Biosciences); monoclonal anti-Myc, 1:1000 (Cell Signaling); monoclonal anti-FLAG, 1:1000 (Sigma)) for 2 h at 25 °C or overnight at 4 °C, followed by secondary antibodies (Alexa594-conjugated goat anti-mouse (1:3000) or Alexa 488-conjugated goat anti-rabbit (1:3000) from Molecular Probes). Images were recorded by confocal microscopy. Internalization was quantified by drawing an area under the cell membrane (within the cell but excluding cell membrane) of each cell on the confocal image, and pixels of internalized vesicles within the area were counted. Pixel density is calculated as total pixels per cell within the circled area using Metamorph computer software. Experiments were repeated three times, and 20–40 cells/experiment

were analyzed. Data are presented as mean ± S.D., and statistical significance was assessed by a two-tailed, paired Student's *t* test.

Biotinylation assay for endocytosis

MDA-MB-231 cells were incubated with 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) for 30 min at 4 °C followed by washing to quench free biotin. Cells were then incubated in normal medium at 37 °C for the indicated times in the presence of ephrin-A1-Fc or control IgG. Biotinylated cell surface proteins were removed by 0.01% trypsinization. Remaining biotinylated proteins were sequestered inside cells by endocytosis and were protected from trypsinization. Cell lysates were immunoprecipitated with streptavidin beads, and biotinylated EphA2 proteins were visualized by anti-EphA2 coupled chemiluminescence detection using an ECL kit (Amersham Biosciences).

PIP3 ELISA

Confluent cells were serum-starved overnight and treated with ephrin-A1-Fc or control IgG for 10 min. PIP3 was extracted from cells and subjected to ELISA assay using a PIP3 mass ELISA kit (Echelon, Salt Lake City, UT). Briefly, lipids were extracted with 2.25 ml of MeOH, CHCl3,12 m HCl (80:40:1) for 15 min at room temperature and partitioned by centrifugation after the addition of 0.75 ml of CHCl3 and 1.35 ml of 0.1 m HCl. The lower phase was vacuum-dried and dissolved in PIP3 buffer. Controls, standards and samples were incubated with PIP3 detector, secondary detection reagent, and 3,3′,5,5′-tetramethylbenzidine (TMB) solution sequentially. The reaction was terminated by adding stop solution (0.5 m H_2SO_4), and the absorbance was measured at

450 nm. Experiments were repeated twice, and all controls, standards, and samples were run in triplicate per experiment. Data are presented as mean ± S.D., and statistical significance was assessed by two-tailed, paired Student's *t* test.

Guanine nucleotide exchange assays

For Rac1 and Cdc42 activation assays, cells were serum-starved for 24 h in 1% fetal bovine serum followed by stimulation with ephrin-A1 (1 μg/ml). Lysates were prepared and incubated with Pak-1 binding domain-GST beads (Upstate Biotechnology, Inc.) as described by the manufacturer's protocol to pull down GTP-bound Rac1 and/or Cdc42. Activated Rac1 and Cdc42 (or total Rac1 and Cdc42 in lysates) were detected by immunoblot using anti-Rac1 or anti-Cdc42 antibodies. Active Rac1-GTP or Cdc42-GTP levels were quantified by densitometry (Rac1-GTP/total Rac1) using Scion Image software. Experiments were repeated three times, and data are presented as means ± S.D. Statistical significance was assessed by a two-tailed, paired Student's *t* test.

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Results

Activated EphA2 receptor interacts with SHIP2

A yeast two-hybrid screen was used to identify EphA2 receptor-interacting proteins [96]. The bait construct consisted of the intracellular portion of mouse EphA2 fused to the DNA binding domain of LexA. Upon screening a cDNA library from human placenta, we obtained six independent but overlapping interacting clones that contained the SAM

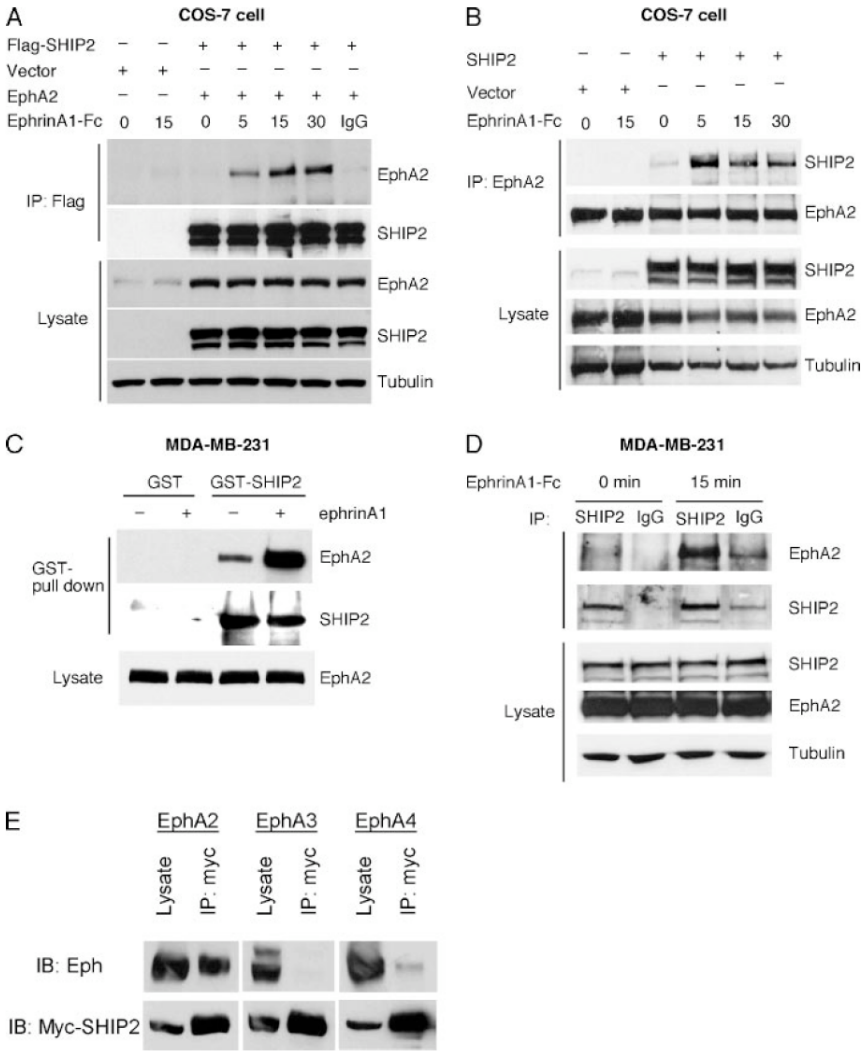


Figure 2.1. Activated EphA2 receptor recruits SHIP2 in mammalian cells. A. full-length EphA2 and FLAG-tagged *SHIP2* cDNA expression plasmids or vector alone were co-transfected into COS7 cells. Cells were stimulated with ephrin-A1 at the indicated times, and cell lysates were immunoprecipitated (*IP*) with anti-FLAG, followed by Western blot analysis with anti-EphA2 antibodies. Blots were stripped and reprobed for expression of SHIP2. **B.** FLAG-tagged *SHIP2* or vector were transfected into COS7 cells and stimulated in the presence or absence of ephrin-A1 following a time course. Endogenous EphA2 receptors were immunoprecipitated by anti-EphA2, followed by Western blot analyses with anti-SHIP2. **C.** MDA-MB-231 cell lysates were added to GST-SHIP2 or control GST resin, and bound proteins were eluted and analyzed by Western blot analysis using anti-EphA2 antibodies. **D.** MDA-MB-231 cells were stimulated with ephrin-A1 for the indicated time, and cell lysates were immunoprecipitated with anti-SHIP2 or control IgG, followed by Western blot analysis using anti-EphA2. *E*, Myc-tagged *SHIP2* and EphA2, A3, or A4 receptors were co-transfected into COS7 cells. SHIP2 was immunoprecipitated with anti-Myc-conjugated resins, followed by Western blot analysis (*IB*) using anti-EphA2, A3, or A4 antibodies.

domain of SHIP2. Interestingly, the binding of SHIP2 to the EphA2 receptor is quite specific, since it binds to neither EphB1 receptor nor other EphA receptors (data not shown).

The observation that the SHIP2 SAM domain interacts with the cytoplasmic domain of EphA2 in yeast raised the possibility that SHIP2 and EphA2 interact in mammalian cells. To test this hypothesis, we transfected COS7 cells with a FLAG-tagged full-length *SHIP2* expression construct and immunoprecipitated cell lysates with an anti-FLAG antibody. As shown in Fig. 2.1*A*, the EphA2 was readily detected in anti-FLAG immunoprecipitates. The co-immunoprecipitation of EphA2 with the anti-FLAG antibody was dependent on the expression of SHIP2 and ephrin-A1 stimulation, since EphA2 was undetectable in the unstimulated samples and in immunoprecipitates in which a control vector was expressed. In the reverse direction, SHIP2 was easily detected in anti-EphA2 immunoprecipitates from cells transfected with *SHIP2* and stimulated with ephrin-A1 (Fig. 2.1*B*). However, SHIP2 does not bind to EphA3 or EphA4 (Fig. 2.1*E*), demonstrating the binding specificity of SHIP2 to EphA2.

To determine whether endogenous EphA2 receptor can bind to SHIP2, MDA-MB-231 cells were stimulated with ephrin-A1, and cell lysates were subjected to a GST-SHIP2-SAM pulldown assay. As shown in Fig. 2.1*C*, GST-SHIP2, but not control GST, binds to endogenously expressed EphA2 in response to ephrin-A1 ligand stimulation. In addition, EphA2 was detected in anti-SHIP2 immunoprecipitates upon ephrin-A1 stimulation in

MDA-MB-231 cells (Fig. 2.1*D*). These findings indicate that SHIP2 is recruited to activated EphA2 receptors in breast cancer cells.

Mapping of interaction domains between EphA2 and SHIP2

To identify the domains within the EphA2 receptor that mediate the interaction with SHIP2, a panel of EphA2 constructs was generated in which portions of EphA2 were deleted. The resulting EphA2 mutants were expressed at comparable levels in the yeast two-hybrid assay and tested for their interaction with SHIP2 (Fig. 2.2A). EphA2 receptors lacking the juxtamembrane domain, kinase domain, or PDZ binding motif were capable of binding with SHIP2 as efficiently as the wild-type receptor. The interaction between the two proteins is disrupted in EphA2 mutants with a deletion of the SAM domain. The EphA2 SAM domain alone binds to SHIP2 as well as the wild-type receptor, indicating that the SAM-SAM interaction is required for binding to SHIP2. The original six independent and overlapping yeast two-hybrid interacting clones of *SHIP2* all contained the SAM domain. As shown in Fig. 2.2*B*, the SAM domain of SHIP2 alone can bind to the EphA2 cytoplasmic domain, whereas the SHIP2 SH2 domain fails to bind to EphA2.

The heterotypic interaction between the EphA2 SAM and the SHIP2 SAM domains was verified in mammalian cells. As predicted from the yeast two-hybrid assay, binding of EphA2 to SHIP2 is independent of EphA2 receptor phosphorylation, since a kinase-dead (D738N) mutant or three Tyr to Phe mutations (Y921F, Y929F, and Y959F) in the EphA2 SAM domain did not affect binding significantly (Fig. 2.2*C*); nor did the deletion

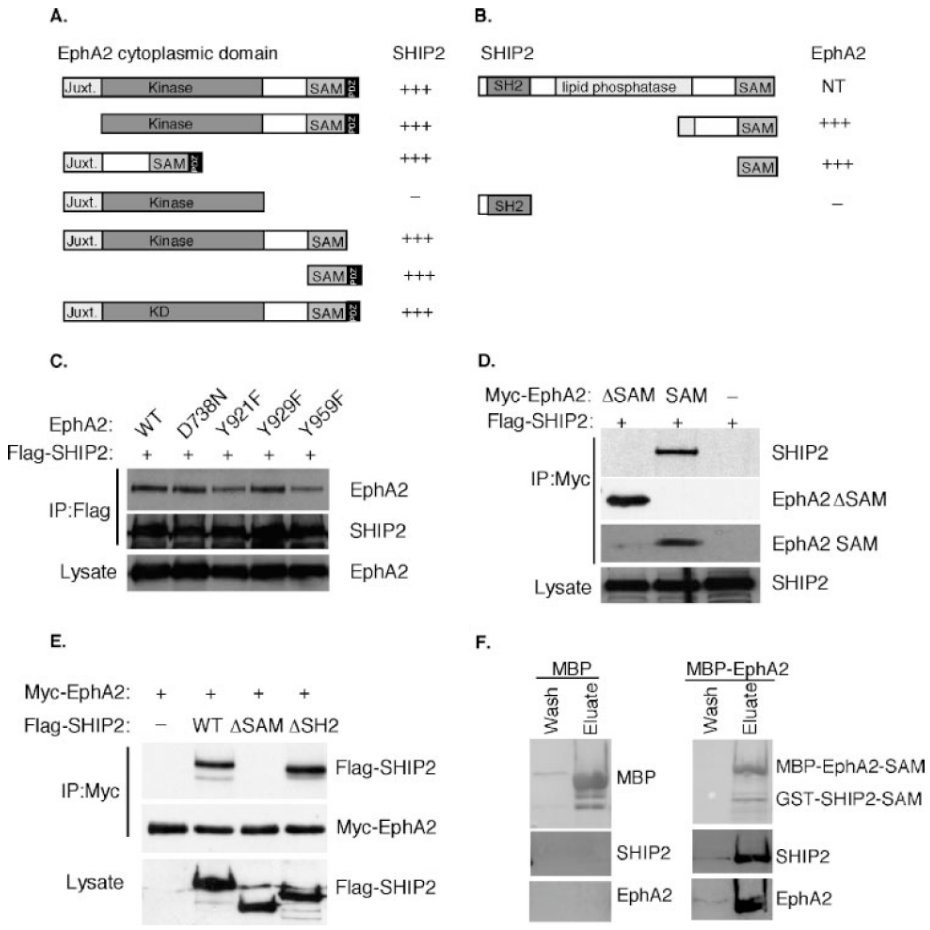


Figure 2.2. Interaction and domain mapping of EphA2 and SHIP2. A. a *SHIP2* cDNA fragment containing the SAM domain (amino acids 1117–1258) was co-expressed in the yeast two-hybrid assay with wild type or various mutants of the EphA2 cytoplasmic domain. *D738N*, a kinase dead mutation. **B.** the EphA2 cytoplasmic domain was co-expressed in the yeast two-hybrid assay with wild type or various deletion mutants of *SHIP2*. **C–E.** wild type and various mutants of EphA2 and *SHIP2* were transfected into COS7 cells and subjected to coimmunoprecipitation/Western blot analysis. **F.** the maltose-binding protein-EphA2 SAM domain fusion protein (MBP-EphA2-SAM) and the GST-SHIP2-SAM domain fusion protein were expressed in *E. coli*. Soluble GST-SHIP2-SAM was added to the MBP-EphA2-SAM or control MBP amylose column, and bound proteins were eluted and analyzed by SDS-PAGE followed by silver staining (*top panel*). Western blots of eluted fractions are shown in the *bottom panels*.

of the SH2 domain in SHIP2 protein (Fig. 2.2*E*). In contrast, deletion of the EphA2 SAM domain abolished the ability to bind to SHIP2, whereas the EphA2 SAM domain alone was capable of interacting with SHIP2 (Fig. 2.2*D*). Conversely, deletion of the SHIP2 SAM domain also inhibited binding to the EphA2 receptor (Fig. 2.2*E*), confirming the heterotypic interaction between the two SAM domains.

To test for a direct interaction between EphA2 and SHIP2, the SAM domains of SHIP2 and EphA2 were expressed as GST and MBP fusion proteins, respectively. As shown in Fig. 2.2*F*, GST-SHIP2-SAM bound to MBP-EphA2-SAM that was linked to amylose beads. After extensive washing, only GST-SHIP2-SAM and MBP-EphA2-SAM were eluted from the column. Although GST-SHIP2-SAM bound to MBP-EphA2-SAM, it failed to interact with MBP alone, indicating that the binding is specific to the EphA2-SAM *in vitro* and independent of phosphorylation. Taken together, these results suggest that the EphA2 receptor binds to the SHIP2 phosphatase through a SAM-SAM heterotypic interaction.

Overexpression of SHIP2 inhibits ligand-induced EphA2 receptor endocytosis

As a first step to determine the functional link between SHIP2 and EphA2 endocytosis, we analyzed the kinetics of ligand-induced EphA2 receptor internalization in MDA-MB-231 cells by confocal microscopy. As shown in Fig. 2.3*A*, upon stimulation with soluble ephrin-A1, EphA2 receptor (*green*) clusters rapidly and localizes in large patches, followed by internalization of the receptor. These internalized vesicles were costained with EEA1 (*red*), an early endosomal marker [175], suggesting that EphA2 receptor is

internalized by endocytosis. Next, we over-expressed wild-type Myc-tagged *SHIP2* in COS7 cells and examined ephrin-A1-induced receptor endocytosis. Anti-Myc antibodies detected cells expressing exogenous SHIP2 (*red*), whereas subcellular localization of the endogenous EphA2 receptor was detected by anti-EphA2 antibodies (*green*). Internalization of EphA2 receptor (*green*) was significantly inhibited in cells overexpressing SHIP2 (*red*) but not SHIP2ΔSAM (Fig. 2.3, *B* and *C*), suggesting that SHIP2 regulates ligand-induced EphA2 receptor endocytosis via its SAM domain.

Although SHIP2 can function directly as a phosphoinositide phosphatase, it is interesting to note that it also contains multiple functional domains/motifs that may mediate the recruitment of other signaling molecules. To determine whether SHIP2 regulates EphA2 endocytosis via its phosphatase activity or acting as an adaptor protein, we transfected COS7 cells with a catalytic-inactive SHIP2 mutant, D607A [176, 177]. As shown in Fig. 2.3, *B* and *C*, the D607A mutant did not inhibit ephrin-A1-induced EphA2 receptor internalization, suggesting that the enzymatic activity of SHIP2 is required for regulation of EphA2 endocytosis.

Enhanced EphA2 receptor endocytosis in SHIP2 knockdown cells

To understand the role of SHIP2 in EphA2 receptor endocytosis in breast cancer cells, we inhibited the endogenous SHIP2 expression by retrovirus-mediated siRNA knockdown. Four siRNA duplexes of sequence specific to SHIP2 were tested in MDA-MB-231 breast cancer cells. These siRNAs were stably expressed in MDA-MB-231 cells by retroviral transduction. As shown in Fig. 2.4A, MDA-MB-231 cells expressing siRNA2 inhibited

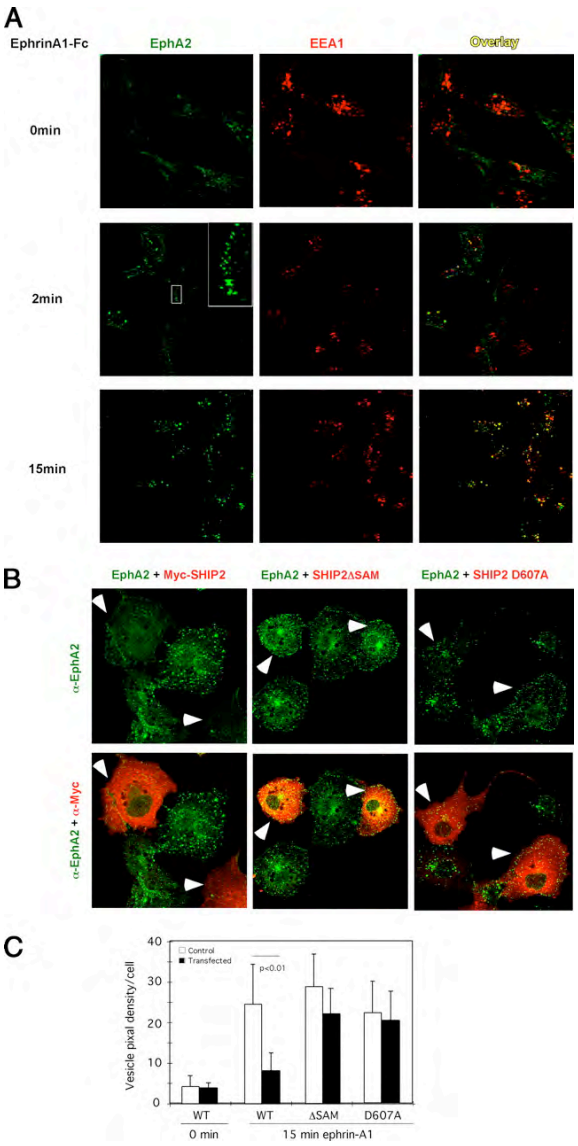


Figure 2.3. Overexpression of SHIP2 inhibits ligand-induced EphA2 receptor endocytosis. A. kinetics of ligand-induced EphA2 endocytosis. MDA-MB-231 cells were stimulated with ephrin-A1 ligand following a time course. EphA2 receptor (*green*) is localized diffusely at the cell border at 0 min. Ephrin-A1 stimulation results in EphA2 receptor clustering and internalization (see *insert*) at 2 min. At 15 min, extensive internalized EphA2 vesicles were observed, co-localizing with EEA1, an endosomal marker. **B.** EphA2 and a Myc-tagged *SHIP2*,a *SHIP2* mutant with SAM domain deletion (*SHIP2*Δ SAM), or a *SHIP2* mutant that lacks phosphatase activity (*SHIP2*D607A) were co-transfected into COS7 cells, and cells were stimulated with ephrin-A1 for 15 min. EphA2 and SHIP2 are detected by antibodies against EphA2 (*green*) and Myc (*red*), respectively. EphA2 endocytosis is significantly inhibited in SHIP2-overexpressing cells ($p < 0.01$) but not in SHIP2ΔSAM or D607A mutant-expressing cells. *Arrowhead*, transfected cells. **C.** internalized vesicles in each cell were quantified by counting vesicle pixel density using Metamorph software. Experiments were repeated three times, and ~30 cells/experiment were analyzed. Data are presented as mean ± S.D., and statistical significance was assessed by a two-tailed, paired Student's *t* test. *WT*, wild type.

the expression of endogenous SHIP2 to greater than 80%. siRNAs 1, 3, and 4 also produced a target protein suppression but to a lesser degree (~50%). A control siRNA with two mismatches in sequence 2 was completely inactive.

To investigate whether ephrin-A1-induced EphA2 receptor endocytosis is affected by knockdown of *SHIP2* in MDA-MB-231 cells, *SHIP2* knockdown or control cells were treated with ephrin-A1, and EphA2 receptor localization was followed by confocal immunofluorescence analysis. As shown in Fig. 2.4*B*,in *SHIP2* knockdown cells, ephrin-A1 stimulation enhanced the accumulation of internalized EphA2 vesicles, compared with parental MDA-MB-231 (data not shown) or control siRNA-expressing cells. Quantification of these vesicles revealed that there was an ~2-fold increase in vesicle pixel density in *SHIP2* knock down cells, compared with control siRNA-expressing cells, indicating that SHIP2 negatively regulates EphA2 endocytosis.

Internalization of cell surface EphA2 was also tracked using a surface biotinylation assay described by Le *et al.* [178]. MDA-MB-231 cells were surface-biotinylated at 4 °C and then returned to 37 °C following a time course to allow trafficking to resume. Cells were incubated briefly with a dilute trypsin solution to remove cell surface proteins. Internalized EphA2 was sequestered at 37 °C and therefore protected from trypsin digestion. Little if any EphA2 was detected in control cells (Fig. 2.4*C*, 0 min), confirming that under these conditions, biotinylated cell surface proteins were efficiently removed by trypsin. In contrast, after 10, 20, and 30 min at 37 °C, a biotinylated pool of EphA2 was detected in cells following trypsin treatment (Fig. 2.4*C*, 10, 20, and 30 min), indicating

that EphA2 was internalized and protected from typsinization. Ephrin-A1 induced EphA2 internalization in both control and *SHIP2* knockdown cells, but the level of internalized EphA2 was appreciably higher in *SHIP2* knockdown cells compared with that in control cells (Fig. 2.4*C*). We also observed a basal level of EphA2 internalization in the absence of ligand stimulation (Fig. 2.4*C*, *right*). However, this level is significantly lower than that with ephrin-A1 stimulation. These data provide independent evidence that ephrin-A1 stimulation induces EphA2 internalization, and this process is regulated by SHIP2.

To determine whether increased endocytic vesicles observed in *SHIP2* knockdown cells affected ligand-induced receptor degradation, we performed Western blot analysis of EphA2 following ephrin-A1 treatment. The total level of EphA2 receptor decreased with increasing length of ephrin-A1 treatment, and this process was enhanced in *SHIP2* knockdown cells (Fig. 2.4*D*), indicating that SHIP2 regulates EphA2 degradation.

SHIP2 regulates EphA2 endocytosis through modulation of cellular PIP3 levels

We next explored the mechanisms by which SHIP2 regulates EphA2 receptor endocytosis. As SHIP2 is a phosphoinositide 5-phosphatase, we determined the impact of SHIP2 knockdown on phospholipid PIP3 levels. We measured PIP3 levels by ELISA using an anti-PIP3 monoclonal antibody that is widely used in many studies [179-182]. As shown in Fig. 2.5C, compared with those in control cells, PIP3 levels in SHIP2 knockdown cells increased ~2-fold, either at resting phase or stimulated by ephrin-A1. Because phospholipid PIP3 is a product of PI 3-kinase, we tested whether inhibition of PI 3-kinase affects EphA2 endocytosis. The PI 3-kinase inhibitor LY294002 (50 μm)

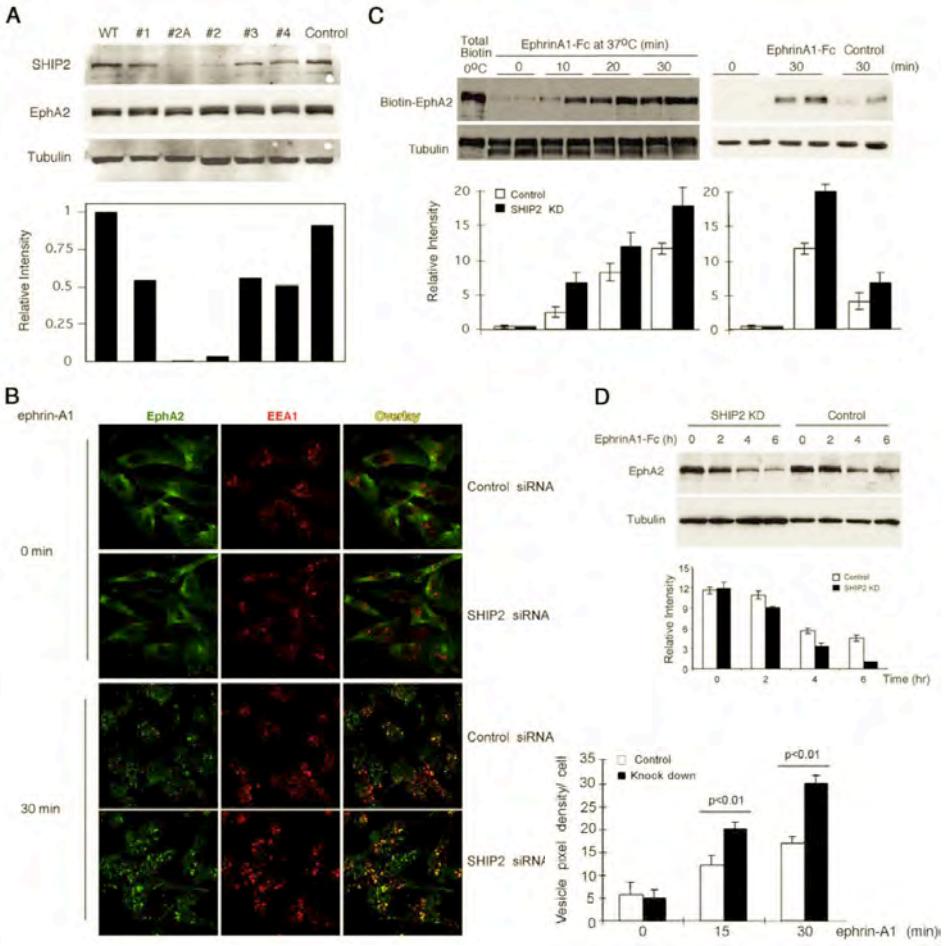


Figure 2.4. Inhibition of SHIP2 in cancer cells by siRNA-mediated silencing. A. Western blot analysis on *SHIP2* siRNA clones. MDA-MB-231 cells were infected with retroviruses expressing siRNA 1, 2, 2A, 3, and 4 and a control mutant 2 siRNA. Pooled clones were subjected to Western blot analysis using an anti-SHIP2 polyclonal antibody. The blot was stripped and reprobed for EphA2 and tubulin for a loading control. **B.** EphA2 endocytosis is documented by confocal imaging analysis and quantified by Metamorph analysis as described in the legend to Fig. 3*C*. MDA-MB-231 cells were co-stained by anti-EphA2 antibody (*green*) and endosomal marker EEA1 (*red*) in *SHIP2* knockdown and control cells. Enhanced endocytosis of EphA2 receptor was observed in *SHIP2* knockdown cells. **C.** MDA-MB-231 cells were biotinylated, and surface proteins were either removed immediately as indicated by the 0 time point or removed following a time course in the presence (*left panel*) or absence (*right panel*) of ephrin-A1 stimulation. Levels of internalized biotinylated EphA2 were then determined by immunoprecipitation with streptavidin beads followed by Western blot analysis using anti-EphA2 antibody. **D.** EphA2 protein level is detected by Western blot analysis following ephrin-A1 stimulation in control and *SHIP2* knockdown cells. Enhanced EphA2 degradation was observed in *SHIP2* knockdown cells.

significantly inhibited increased PIP3 levels (Fig. 2.5*C*) as well as ephrin-A1-induced EphA2 endocytosis (Fig. 2.5, *A* and *B*). Taken together, these data indicate that SHIP2 regulates EphA2 endocytosis via modulation of cellular PIP3 levels.

SHIP2 regulates EphA2 endocytosis through a Rac1 GTPase-dependent pathway

Previous studies have shown that Vav family guanine nucleotide exchange factors and Rac GTPase activity are required for endocytosis of Eph molecules [66, 169]. Since the activation status of guanine nucleotide exchange factors can be regulated by PIP3 levels [183, 184] and PIP3 is a major substrate of SHIP2 [172], we investigated whether ephrin-A1-induced Rac1 GTPase activation is affected in *SHIP2* knockdown cells. As shown in Fig. 2.6*A*, upon ephrin-A1 stimulation, we detected a transient activation of Rac1 GTPase, with a peak at 2.5–5 min. In *SHIP2* knockdown cells, the basal level of GTP-bound Rac1 is increased, and the Rac1 activity is further enhanced upon ephrin-A1 stimulation. In contrast, ephrin-A1 stimulation does not appear to affect Cdc42 activity. The enhanced basal and ephrin-A1-induced Rac1-GTP levels in *SHIP2* knockdown cells were blocked by PI 3-kinase inhibitor, LY294002 (Fig. 2.6*A*, *bottom*). These data suggest that, in contrast to PI 3-kinase, SHIP2 negatively regulates Rac1 GTPase activity.

To determine the functional relevance of Rac1 in ligand-induced EphA2 endocytosis, we expressed a wild-type Rac1, a constitutively active mutant of Rac1 (Rac1 V12), or a dominant negative mutant of Rac1 (Rac1 N-17) [185] in COS7 cells. As shown in Fig. 2.6*B*, expression of either wild-type Rac1 (*green, top*) or Rac1 V-12 (*red, middle*) did not affect EphA2 internalization significantly, but expression of Rac1 N-17 (*red, bottom*)

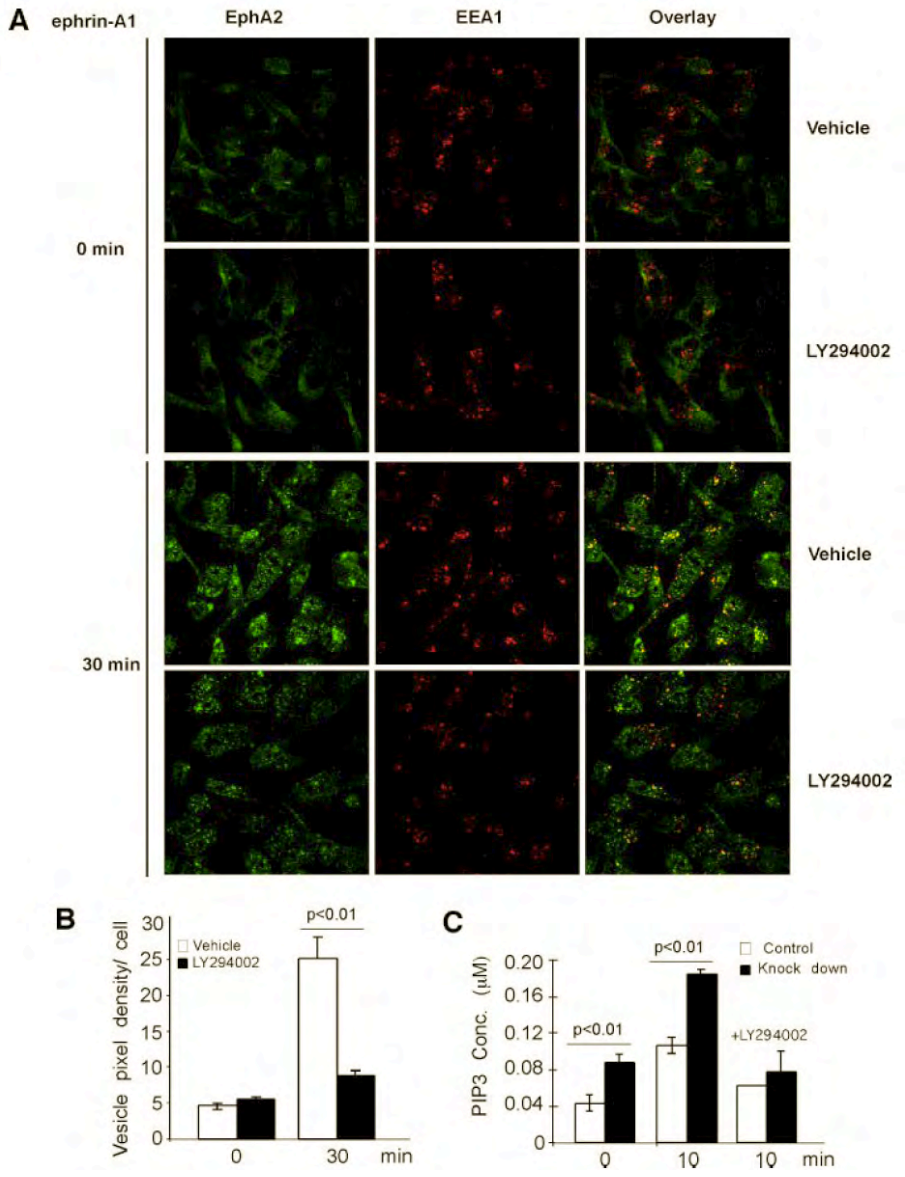


Figure 2.5. SHIP2 regulates EphA2 endocytosis through modulation of cellular PIP3. **A.** *SHIP2* knockdown cells were stimulated with ephrin-A1 in the presence of PI 3-kinase inhibitor LY294002 (50 μm) or control vehicle for the indicated time, fixed, permeabilized, and stained with anti-EphA2 and anti-EEA1 antibodies. LY294002 inhibits elevated EphA2 endocytosis in *SHIP2* knockdown cells. ***B.*** quantification of the EphA2 endocytosis using Metamorph software as described in the legend to Fig. 3*C*. **C.** control and *SHIP2* knockdown cells were stimulated with ephrin-A1 for the indicated time in the presence or absence of PI 3-kinase inhibitor LY294002. The PIP_3 levels in control and *SHIP2* knockdown cells were quantified by ELISA analysis.

markedly impaired EphA2 endocytosis, suggesting that Rac1 GTPase activity is required for ligand-induced EphA2 endocytosis.

Discussion

In this study, we show that when ephrins bind to Ephs the lipid phosphatase SHIP2 is recruited to the SAM domain of the activated EphA2 receptor through a heterotypic SAM-SAM interaction. We found that overexpression of SHIP2 significantly inhibited ligand-induced EphA2 receptor endocytosis. Silencing of *SHIP2* via siRNA-mediated knockdown enhanced the ephrin-A1 ligand-induced increase in PIP3 levels and Rac1 GTPase activity as well as ligand-induced EphA2 receptor endocytosis. Taken together, our data indicate an important role for SHIP2 in regulating phosphoinositol lipids to modulate Eph receptor function in cancer cells.

Recruitment of SHIP2 to the activated EphA2 receptor via a heterotypic SAM-SAM domain interaction

SHIP1 and SHIP2 have been shown to associate with a number of cell surface receptors [172]. Both SHIP1 and SHIP2 contain an N-terminal SH2 domain, a lipid phosphatase domain, proline-rich regions, and NP*X*Y motifs serving as potential protein-protein interaction sites. SHIP2 also possesses a C-terminal SAM domain that is not present in SHIP1. In the case of SHIP1, the amino-terminal SH2 domain binds to phosphotyrosine residues to mediate the interactions with a number of signal transduction proteins [172, 186, 187]. However, this is not the case in the interaction between SHIP2 and the EGFR. Pesesse *et al.* [188] reported that the SH2 domain of SHIP2 was unable to precipitate the

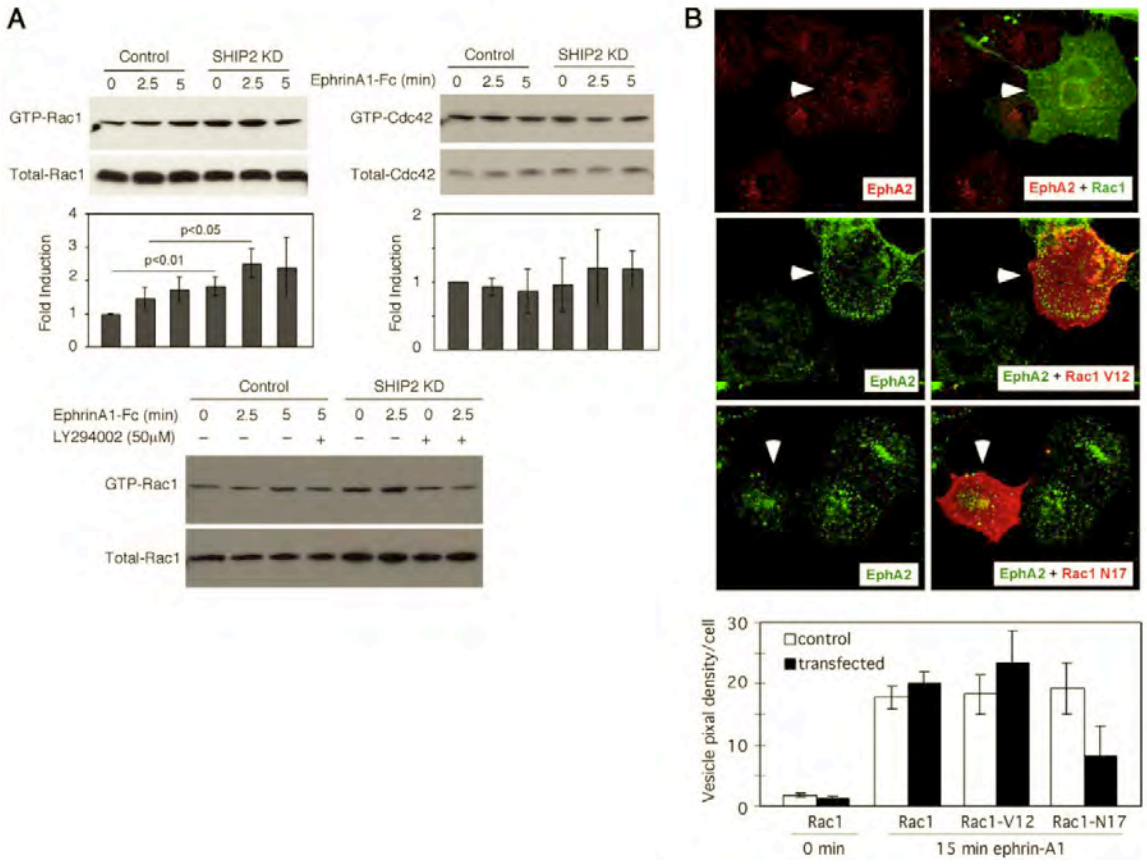


Figure 2.6. SHIP2 regulates EphA2 endocytosis via Rac1 GTPase. A. active GTP-bound forms of Rac1 and Cdc42 were analyzed by Pak-1 binding domain pull-down, followed by immunoblot in lysates from *SHIP2* knockdown or control MDA-MB-231 cells stimulated with ephrin-A1, in the presence or absence of PI 3-kinase inhibitor LY294002 (blots). Total Rac1 and Cdc42 levels within the lysate prior to Pak-1 binding domain pull-down were detected by immunoblot. Results from three independent experiments were quantified using Scion Image software and expressed as mean ± S.D. (*graphs*). **B.** a wild-type Rac1, a Myc-tagged constitutively active Rac1 mutant (Rac1 V-12), or a dominant negative N17 Rac1 expression construct was transfected into COS7 cells, and ligand-induced EphA2 endocytosis (at 15 min) was analyzed by confocal microscopy. Ephrin-A1-induced EphA2 endocytosis is not affected in wild type-or V12 Rac1-expressing cells but is inhibited in N17 Rac1 expressing cells (*red*). *Arrowhead*, transfected cells. Internalized vesicles in each cell were quantified by counting vesicle pixel density using Metamorph software, as detailed under “Methods”

EGFR, whereas a C-terminal truncated form of SHIP2 that lacks the last 366 amino acids was able to bind to EGFR in EGF-stimulated cells. These results suggest that neither the SH2 nor SAM domain of SHIP2 is capable of binding to the activated EGFR, and a specific EGFR binding domain in SHIP2 remains to be identified. Here we show that it is the SAM domain of SHIP2, rather than its SH2 domain (Fig. 2.2*E*), that is required for interacting with the activated EphA2 receptor.

SAM domains are protein-protein interaction motifs that can interact homotypically with identical SAM domains or heterotypically with other related SAM domains [189]. All known Eph proteins contain SAM domains at the C terminus, which are thought to play a role in receptor clustering. Crystal and solution structures of the EphA4-SAM domain and the EphB2-SAM domain have been resolved [13, 190, 191]. Despite the potential role of these SAM domains in promoting receptor oligomerization, homotypic SAM-SAM self-association in solution is weak (Kd > 1 mm). In light of our data, one major function of the EphA2 receptor SAM domain appears to be mediating heterotypic protein-protein interactions to transduce signals downstream of the Eph receptor. As the EphA2 SAM domain contains three tyrosine residues, one possibility is that the activated receptor phosphorylates its own SAM domain, leading to recruitment of SHIP2. However, our data do not favor this hypothesis. A kinase-dead (D738N) EphA2 mutant and Tyr to Phe mutations in the SAM domain are all still capable of binding to the SHIP2 SAM domain (Fig. 2.2, *A* and *C*). In addition, an SH2 domain deletion mutant of SHIP2 protein retains its ability to interact with the EphA2 receptor (Fig. 2.2*E*). The more likely model

is that ligand stimulation induces conformational changes in the EphA2 receptor, allowing SHIP2 to gain access to the EphA2 SAM domain.

SHIP2 and regulation of receptor endocytosis

Ligand-induced Eph receptor endocytosis has been previously reported [66, 192]. In these cases, Rac1 was shown to be required for endocytosis of the plasma membrane and reorganization of F-actin. More recently, Vav family proteins were recognized as Rho guanine nucleotide exchange factors to activate Rac GTPase in the growth cone collapse response [169]. Since Vav proteins can be regulated through tyrosine phosphorylation and/or binding to PIP3 via the pleckstrin homology domains, one way to regulate receptor endocytosis is to modulate PIP3 levels through PI 3-kinase and/or lipid phosphatases. In support of this idea, we found that the phosphorylated EphA2 receptor interacts with the p85 subunit of PI 3-kinase (data not shown), a result that is consistent with previous findings by Pandey *et al.* [193]. Activated EphA2 also recruits the SHIP2 phosphatase, providing negative feedback to reduce PIP3 levels. Indeed, knockdown of *SHIP2* in MDA-MB-231 cells led to an increase in EphA2 receptor endocytosis and degradation. This increased endocytosis of the EphA2 receptor was accompanied by increased ephrin-A1-induced PIP3 levels and activation of Rac1. A PI 3-kinase inhibitor, LY294002, blocked basal and ligand-induced Rac1 activation and significantly inhibited EphA2 endocytosis in *SHIP2* knockdown cells. Taken together, these results suggest a switch from a phosphotyrosine-dependent to a SAM-dependent signaling mechanism to regulate EphA2 endocytosis (Fig. 2.7).

Involvement of a phosphoinositide 5-phosphatase in receptor endocytosis has been described previously [194]. Irie *et al.* reported that activation of EphB2 in neurons induced tyrosine phosphorylation of synaptojanin 1, a phosphatidylinositol 5-phosphatase that is involved in clathrin-mediated endocytosis. Ephrin-induced phosphorylation of synaptojanin 1 inhibits both the interaction with endophilin and the 5-phosphatase activity of synaptojanin 1, resulting in inhibition of internalized vesicle uncoating and blocking entry to endosomes. This mechanism apparently is different from regulation of EphA2 endocytosis by SHIP2. Although we also observe increased vesicles in *SHIP2* knockdown cells, the elevated numbers of vesicles co-localize with EEA1, an endosomal marker. In addition, EphA2 receptor degradation is enhanced in *SHIP2* knockdown cells, suggesting that internalized vesicles reach endosomes for protein degradation. Thus, it is likely that SHIP2 acts at an early stage of EphA2 endocytosis, through modulation of Rac1-dependent cytoskeletal dynamics, to regulate EphA2 trafficking.

Aside from modulating Rac1 GTPase activity, SHIP2 may also regulate EphA2 receptor endocytosis via Cbl, a ubiquitinating ligase (E3). Cbl has been recently reported to interact with SHIP2 through the SHIP2 SH2 domain [195, 196] as well as several receptor tyrosine kinases, including the EphA2 and the EGF receptors [170, 171, 196]. Prasad and Decker proposed that SHIP2 may sequester Cbl, preventing it from binding to the EGF receptor, thereby inhibiting EGF receptor degradation. If SHIP2 also regulates EphA2 via a similar mechanism, the enzymatic activity may not be required for SHIP2 function. However, a SHIP2 mutation (D607A) that abolishes phosphatase activity did not affect EphA2 endocytosis (Fig. 2.3*B*). Our finding suggests that inhibition of EphA2

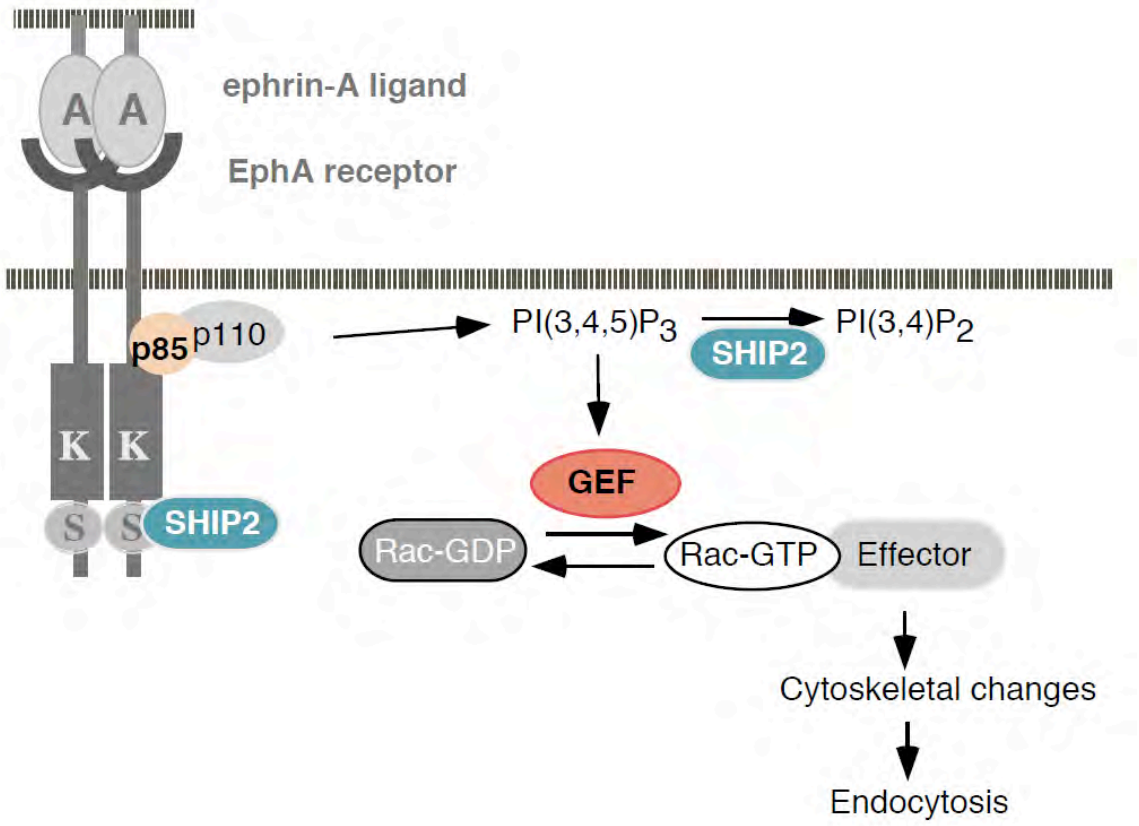


Figure 2.7. A model for how SHIP2 may regulate ephrin-induced EphA2 endocytosis. Upon binding to ephrins, the EphA2 receptor is tyrosine-phosphorylated. Through the recruitment of the p85 subunit of PI 3-kinase, EphA2 receptor up-regulates phospholipid PIP_3 levels and activates Rac1 GTPase to transduce signals as well as promoting EphA2 receptor endocytosis. EphA2 also recruits SHIP2 through a heterotypic SAM-SAM interaction to mediate negative feedback to reduce PIP_3 levels. Thus, the EphA2 receptor is capable of switching from phosphotyrosine-dependent to SAM-dependent signaling to regulate EphA2 endocytosis.

endocytosis by SHIP2 is unlikely to be due to sequestion of Cbl by SHIP2. Rather, SHIP2 may function as a lipid phosphatase to down-regulate PIP3 levels, which inhibits Rac1 GTPase activity and EphA2 receptor endocytosis.

Role of SHIP2 in tumor cell malignancy

SHIP2 belongs to a family of SH2-containing phosphoinositide 5-phosphatases. The closest relative to SHIP2 is SHIP1. SHIP1 expression is restricted to hematopoietic cells and developing spermatogonia, whereas SHIP2 is more widely expressed in many different tissues and cell types [172]. The role of SHIPs in the enzymatic conversion of PI 3,4,5-trisphosphate to PI 3,4-biphosphate raises the question of whether they can act as tumor suppressors like PTEN, which utilizes the same lipid substrate but produces a different lipid product, PI 4,5-biphosphate. Loss of SHIP1 in mice resulted in a myeloproliferative disease [197]. In addition, SHIP1 expression is reduced in both primary cells from leukemic patients and upon induction of BCR-ABL [198]. These data suggest that reduced SHIP1 activity may be a prerequisite for the proliferative advantage of some chronic and acute myelogenous leukemic clones. It is currently unclear whether SHIP2 may also play a tumor suppressor-like role. In an earlier study, loss of both SHIP2 and Phox2a in mice led to neonatal lethality and increased sensitivity to insulin [199]. Neonatal lethality in this strain renders it difficult to study the role of SHIP2 in cancer, and deletion of Phox2a would confound the results. More recently, deletion of *SHIP2* alone in mice resulted in resistance to dietary obesity [200]. Since this *SHIP2*-deficient mouse strain is viable, it provides an opportunity to investigate SHIP2 function in tumorigenesis and metastatic progression.

Our data in malignant breast cancer cells suggest that SHIP2 inhibits EphA2 receptor endocytosis. Since EphA2 level is correlated with tumor malignancy, it is possible that enhanced EphA2 receptor endocytosis and degradation in *SHIP2* knockdown cells may reduce tumor malignancy. However, it is important to note that SHIP2 also regulates other growth factor receptors, endocytosis of many of which is required for receptor signaling. Thus, it is also possible that ablation of *SHIP2* globally may enhance receptor signaling and cell proliferation/migration, resulting in increased cell malignancy. In *vivo* experiments using relevant animal models are required to resolve this issue.

In summary, our findings reported here implicate a critical role for SHIP2 in regulating ligand-induced EphA2 receptor endocytosis. Since EphA2 level is linked to tumor malignancy, these studies provide a foundation for investigating EphA2 as a potential target for therapeutic intervention.

CHAPTER III

THE RECEPTOR TYROSINE KINASE EPHA2 PROMOTES MAMMARY ADENOCARCINOMA TUMORIGENESIS AND METASTATIC PROGRESSION IN MICE BY AMPLIFYING ERBB2 SIGNALING

The work presented in this chapter is published with the same title in the *Journal of Clinical Investigation*, Jan 2008 [201].

Abstract

Overexpression of the receptor tyrosine kinase EPH receptor A2 (EphA2) is commonly observed in aggressive breast cancer and correlates with a poor prognosis. However, while EphA2 has been reported to enhance tumorigenesis, proliferation, and MAPK activation in several model systems, other studies suggest that EphA2 activation diminishes these processes and inhibits the activity of MAPK upon ligand stimulation. In this study, we eliminated EphA2 expression in 2 transgenic mouse models of mammary carcinoma. EphA2 deficiency impaired tumor initiation and metastatic progression in mice overexpressing ErbB2 (also known as Neu) in the mammary epithelium (MMTV-Neu mice), but not in mice overexpressing the polyomavirus middle T antigen in mammary epithelium (MMTV–PyV-mT mice). Histologic and ex vivo analyses of MMTV-Neu mouse mammary epithelium indicated that EphA2 enhanced tumor proliferation and motility. Biochemical analyses revealed that EphA2 formed a complex with ErbB2 in human and murine breast carcinoma cells, resulting in enhanced activation of Ras-MAPK signaling and RhoA GTPase. Additionally, MMTV-Neu, but not MMTV–

PyV-mT, tumors were sensitive to therapeutic inhibition of EphA2. These data suggest that EphA2 cooperates with ErbB2 to promote tumor progression in mice and may provide a novel therapeutic target for ErbB2-dependent tumors in humans. Moreover, EphA2 function in tumor progression appeared to depend on oncogene context, an important consideration for the application of therapies targeting EphA2.

Introduction

Malignant progression of solid tumors is a complex process that involves the activation of oncogenic signaling and downregulation of tumor suppressor pathways. In addition, modulation of the tumor microenvironment, for example through neovascularization, enhances tumor cell growth and survival, promoting invasion and metastatic spread (reviewed in refs. [202-204]). Oncogenic conversion, amplification, or overexpression of protooncogenes, such as those encoding cell surface receptor tyrosine kinases (RTKs) like the EGF receptor family member ErbB2, are frequently observed in human cancers and contribute to malignancy. Other pathways, such as p53 transcription factor/genome surveillance factor, negatively regulate growth, and loss of these pathway components also contributes to tumorigenesis (reviewed in refs. [204, 205]). Recent evidence suggests that Eph RTKs play multiple roles in neoplastic progression, including regulation of processes intrinsic to tumor cells, and in the tumor microenvironment, such as tumor neovascularization (reviewed in refs. [69]).

The Eph RTK family is the largest family of RTKs identified in the genome, with at least 15 receptors and 9 ligands identified in vertebrates (reviewed in refs. [85, 206]). The

family is subdivided into class A and class B based on homology and binding affinity for 2 distinct types of membrane-anchored ephrin ligands. Class B receptors generally bind to class B ephrins that are attached to the cell membrane by a transmembrane-spanning domain, while A class receptors normally interact with glycosyl-phosphatidylinositol–linked class A ephrins, although interclass binding does occur among certain family members. These molecules function during embryogenesis to regulate angiogenic remodeling processes, axon guidance, and tissue boundary formation (reviewed in refs. [1, 32]). More recently, members of this RTK family, including EPH receptor A2 (EphA2), have been linked to tumor progression and neovascularization (reviewed in ref. [85]).

Increasing evidence suggests that EphA2 expression may be causally related to neoplasia. EphA2 RTK overexpression has been observed in several models of cancer, including primary and transplanted rodent tumors, human tumor xenografts, and primary human tumor biopsies (reviewed in refs. [69, 85, 207]). Experimentally induced overexpression of EphA2 resulted in malignant transformation of nontransformed MCF10A breast cells and enhanced malignancy of pancreatic carcinoma cells [74, 75]. Conversely, siRNA-mediated inhibition of EphA2 expression impaired malignant progression of pancreatic, ovarian, and mesothelioma tumor cell lines, and overexpression of dominant-negative EphA2 constructs suppressed growth and metastasis of 4T1 metastatic mouse mammary adenocarcinoma cells in vivo [74, 76-78]. EphA-Fc receptor proteins that disrupt endogenous receptor activation significantly inhibited growth and neovascularization of tumors in vivo [95, 145, 208]. Coupled with the observation that EphA2 signaling induces phosphorylation and activation of the pro-proliferative p42/44 MAPK family

member Erk in tumor cell lines [209, 210], these data suggest that EphA2 functions as an oncogene.

Other evidence, however, suggests that EphA2 may function as a tumor suppressor. EphA2–/– gene-trap mice displayed increased susceptibility to chemical carcinogen-induced skin cancer compared with control littermates, along with increased tumor cell proliferation and phosphorylation of Erk [113]. Stimulation of EphA receptors with soluble ephrin-A1–Fc ligand reduced Erk phosphorylation in tumor cell lines, fibroblasts, and primary aortic endothelial cells and suppressed growth of primary keratinocytes and prostate carcinoma cells [34, 113, 116]. Macrae et al. also reported that treatment of human breast cancer cell lines with ephrin-A1–Fc, which stimulated EphA2 phosphorylation, attenuated EGF-mediated phosphorylation of Erk and inhibited transformation of NIH3T3 cells expressing v-erbB2 [116]. In addition, EphA2 was reported to be a transcriptional target of the tumor suppressor p53 [211-214]. Overexpression of EphA2 in lung and breast cancer cell lines negatively regulated proliferation and induced apoptosis [211, 214]. These data suggest that EphA2 functions as a tumor suppressor.

Given the controversy surrounding the role of EphA2 in tumorigenesis, we investigated the consequences of EphA2 deficiency in transgenic mouse models of endogenous mammary tumor formation. We chose the MMTV-Neu and MMTV–PyV-mT transgenic models, as they each recapitulate the numerous stages of human breast tumor formation and progression. In these models, the mouse mammary tumor virus (MMTV) long-

terminal repeat drives expression of Neu, the rat homolog of ErbB2, or polyoma virus middle T (PyV-mT) antigen specifically in mammary gland epithelium. These models recapitulate multistage tumor progression in vivo in a similar fashion to that observed in human breast cancer, making them excellent models for analysis of endogenous tumor progression [215, 216]. Herein, we demonstrate that EphA2 enhanced tumor formation and proliferation in the context of Neu, both in vivo and in ex vivo molecular analyses of purified tumor cells. Host-derived EphA2 was required for maximal tumor vascularization in the MMTV-Neu model. Within MMTV-Neu tumor cells, maximal levels of Neu/ErbB2 signaling required EphA2, which promoted both tumor initiation and metastatic progression of MMTV-Neu–derived mammary tumors. Therapeutic inhibition of EphA2 impaired growth of MMTV-Neu tumors. By contrast, EphA2 deficiency or inhibition did not affect tumor formation or progression in the MMTV–PyV-mT model of breast cancer. These results demonstrate that the role of EphA2 in tumor progression is dependent upon the oncogene/tumor suppressor context within which it functions. Such considerations are likely to be important regarding the application of therapies targeting EphA2.

Methods

Reagents

Antibodies against the following proteins were used: EphA2 (Zymed Laboratories, Santa Cruz Biotechnology, and Upstate Biotechnology); EphA4 (Upstate Biotechnology); PCNA (BD Biosciences); anti-Erk, anti–phosphothreonine-202/tyrosine-204 Erk, Akt,

and phosphoserine-473 Akt (Cell Signaling Technology); anti-tubulin (Sigma-Aldrich); ErbB2 (Neomarkers/Lab Vision Corporation); anti–β-actin (Santa Cruz Biotechnology); Ras (BD Biosciences); RhoA (Santa Cruz Biotechnology and BD Biosciences); vWF (Zymed Laboratories); E-cadherin (BD Biosciences); Ki67 (Vision Biosystems Inc.); and normal rabbit IgG (Santa Cruz Biotechnology). Therapeutic anti-EphA2 (1C1) and control nonspecific IgG (R347) antibodies were provided by MedImmune Inc. Raf-1 RBD agarose Ras assay reagent was purchased from Upstate Biotechnology. BrdU was purchased from Sigma-Aldrich. BrdU detection and ApopTag Red In situ Apoptosis kits were purchased from Zymed Laboratories and Chemicon/Millipore, respectively. Avidin peroxidase reagents were from Vector Laboratories, and liquid 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit was from Zymed Laboratories. Ephrin-A1–Fc was from R&D Systems. Estrogen, progesterone, insulin, and EGF were from Sigma-Aldrich. DAPI was purchased from Sigma-Aldrich. TO-PRO-3 iodide nuclear stain, CellTracker orange CMTMR, and CellTracker green CMFDA dyes was purchased from Invitrogen. Growth factor–reduced Matrigel was purchased from BD Biosciences. AG825 ErbB2 kinase inhibitor was from Calbiochem (EMD Biosciences). Recombinant adenoviruses expressing constitutively active RhoA (Q63L) and Erk-1 were purchased from Cell Biolabs and Vector Biolabs, respectively. Control adenoviruses expressing β-gal and adenoviruses expressing EphA2 have been previously described [94, 217]. MEK inhibitor U0126 was purchased from Calbiochem.

Mice and in vivo tumor studies

All animals were housed under pathogen-free conditions, and experiments were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. EphA2–/– mice were backcrossed with FVB animals for 5–7 generations prior to crossing with MMTV-Neu or MMTV–PyV-mT mice on an inbred FVB background (Jackson Laboratories; refs. [215, 216]). MMTV-Neu– or MMTV–PyV-mT–positive transgenic animals that were EphA2+/+, EphA2+/–, or EphA2–/– [94]were identified by PCR analysis of genomic DNA from tail biopsy using the following primers: 5′-GGGTGCCAAAGTAGAACTGCG-3′ (forward), 5′-GACAGAATAAAACGCACGGGTG-3′ (neo), 5′-TTCAGCCAAGCCTATGTAGAAAGC-3′ (reverse). The neu and PyV-mT transgenes were detected by PCR using primers and conditions recommended by Jackson Laboratories. Age-matched littermates were monitored for tumor formation by weekly palpation.

Tumors and lungs were collected from 2 cohorts of MMTV-Neu hemizygous EphA2+/+, EphA2+/–, and EphA2–/– animals at 8 months and 1 year after birth. Tumors and lungs were collected from MMTV–PyV-mT hemizygous EphA1+/+, EphA1+/–, and EphA1–/– animals 100 days after birth. Tumors were enumerated, and dimensions were measured by caliper. Tumor volume was calculated as $l \times w^2 \times 0.52$, where l represents length and w width [218]. Lungs were fixed and dehydrated, and surface metastases were enumerated. For transplantation studies, the left inguinal mammary gland fat pad of 3-

week-old recipient EphA2+/+ or EphA2–/– FVB female animals was cleared of endogenous epithelium as described previously [219]and injected with 106 tumor cells derived from MMTV-Neu [220] or MMTV–PyV-mT [221] animals. Resulting tumors were harvested 4–5 weeks after injection for analysis. Where indicated, beginning at 2 weeks after tumor cell injection, recipient mice received intraperitoneal injections of 1C1 anti-EphA2 antibody or control IgG (10 mg/kg twice weekly for 3 weeks) prior to collection and analysis of primary tumors. At least 10 animals per condition were analyzed in 2–3 independent experiments. Statistical analysis of tumor development and metastasis frequency was assessed by c^2 test assuming that 50% of MMTV-Neu female mice should develop tumors within 7–8 months after birth, as was originally reported [216].

Histologic analyses

Mammary glands and tumors were harvested at the indicated time points and fixed in 10% neutral buffered formalin (Fisher Scientific) for 24 hours at 4°C. Whole-mount hematoxylin staining of mammary glands and H&E staining of 7-μm mammary gland tissue sections was performed as described previously [219]. Immunohistochemical staining for EphA2 and PCNA was performed as described previously [95], and proliferation was quantified by calculating the average percentage of PCNA+ nuclei relative to total nuclei (4 random fields of at least 4 independent mammary and tumor samples per genotype; original magnification, ×20). Apoptosis assays were performed using the Apoptag red in situ apoptosis detection kit per the manufacturer's protocol (Chemicon International). Apoptosis was calculated as the average percentage TUNEL+

nuclei relative to total nuclei (4 random fields of at least 4 independent mammary and tumor samples per genotype; original magnification, ×20). We detected p-Erk in tissue sections using rabbit monoclonal anti–p-Erk antibody clone 20G11 per the manufacturer's protocol (Cell Signaling Technology). Colorimetric immunohistochemical staining for vWF was performed by the Vanderbilt University Immunohistochemistry Core Facility, and immunofluorescence staining was performed as described previously [93]. Microvascular density was determined by counting the number of vWF+ vessels in 4 random fields per sample of at least 4 independent tumors per genotype (original magnification, ×20). ErbB2 immunohistochemistry was performed using 5 μg/ml rabbit anti-ErbB2 antibody (Neomarkers/Lab Vision Corporation).

Cell culture

PMECs were isolated from mice as described previously [219, 221, 222] and maintained in PMEC media (DMEM/F12 media [Mediatech] supplemented with 5 ng/ml estrogen, 5 ng/ml progesterone, 5 ng/ml EGF, and 5 μg/ml insulin [Sigma-Aldrich]) on growth factor–reduced Matrigel–coated (1:20 dilution) tissue culture dishes. Primary tumor cells were derived from EphA2+/+ or EphA2–/– MMTV-Neu animals as previously described [220]. Enrichment of tumor cells in cultures was verified by expression of the neu transgene. The MMTV-Neu tumor-derived cell line [220] and the MMTV–PyV-mT tumor-derived cell line [221] used in transplantation and signaling studies were cultured in PMEC media. For EphA2 degradation studies, tumor cells were cultured in the presence of 1C1 anti-EphA2 antibody or control IgG (MedImmune) at the indicated concentrations for 48 hours prior to harvesting lysates for immunoblot analysis. In vitro

proliferation and apoptosis analyses were performed as described previously [95, 222] using BrdU and TUNEL detection kits described above. For rescue experiments, EphA2–/– MMTV-Neu primary tumor cells were transduced with 1 × 108 pfu/ml adenovirus expressing Erk-1, EphA2, or control β-gal 48 hours prior to BrdU assay. For MEK inhibitor studies, cells were treated with 5 and 10 μM U0126 (Calbiochem) or DMSO vehicle control for the 12 hours during the BrdU labeling/serum stimulation time frame. Transwell migration assays were performed as described previously [94]. For rescue experiments, EphA2–/– MMTV-Neu primary tumor cells were transduced with 1×10^8 pfu/ml adenovirus expressing constitutively active RhoA (Q63L) or control β-gal 48 hours prior to transwell assay. Tumor-endothelial cell coculture migration assays were performed as described previously [92, 93].

siRNA sequences for mouse EphA2 or irrelevant control sequences were cloned into pRetroSuper viral vector and used to produce retroviruses for infection of MMTV-Neu tumor cells as previously described [92, 223]. The following sequences were used to target EphA2: siRNA no. 1, 5′-GCCAAAGTAGAACTGCGTT-3′ (aa 1,140–1,158); siRNA no. 2, 5′-GCGCTAGACAAGTTCCTTA-3′ (aa 2,211–2,229); control siRNA, 5′-GCACCAGTTCAGCAAGACT-3′. We established 3-dimensional spheroid cultures as described previously [224]. Cultures were maintained for 8 days prior to photodocumentation. Digital images were scored for spheroid culture area in 4 random fields, 3 cultures per field, using NIH ImageJ software. For confocal imaging, spheroid cultures were fixed in 10% neutral buffered formalin and subjected to immunohistochemistry for E-cadherin followed by nuclear staining with TO-PRO-3 as

previously described [225]. Tumor cells were transplanted into the cleared fat pads of recipient FVB mice as described above. At least 10 animals per condition were analyzed in 2–3 independent experiments.

Parental MCF10A and MCF10A cells stably overexpressing HER2 were maintained as described previously [226]. We established 3-dimensional spheroid cultures as described previously [224]. Cells were transduced with 1×10^8pfu/ml adenovirus expressing constitutively EphA2 or control β-gal 48 hours prior to analysis. Staining for confocal analysis was performed as described above.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot of EphA2 was performed as described previously [95]. ErbB2 was immunoprecipitated using 1 μg rabbit anti-ErbB2 plus 1 μg mouse anti-ErbB2 Ab-17 (Neomarkers/Lab Vision Corporation). Where indicated, 2.5×10^5 PMECs (for Western analyses) or 2.5×10^6 primary tumor cells (for GTP-Ras and –Rho/Rac pulldown assays) were cultured in DMEM:F12 media plus 2% FBS overnight. For analysis of EphA2 stability, MMTV-Neu or MMTV–PyV-mT tumor cells (2.5×10^6) were treated with EphA2-agonist monoclonal antibody 1C1 or control IgGs at the indicated doses and times. Lysates were harvested and used for immunoblot analysis as described previously [95]. Densitometric analysis was performed using NIH ImageJ software.

For Ras and Rho/Rac pulldown assays, tumor tissue was collected, weighed, mechanically homogenized in PBS, pelleted, and resuspended in manufacturer-recommended assay buffer (Upstate Biotechnology). Approximately 500 μg tumor lysate was used per assay. Ras assays were performed using Raf-1 Ras-binding domain–GST assay reagent (Upstate Biotechnology) per the manufacturer's protocol. Rho assays were performed using Rhotekin-binding domain–GST reagents as previously described [78]. For some coimmunoprecipitation assays, COS7 cells were cotransfected with 1 μg each of myc-tagged erbB2 (pcDNA3-erbB2) and ephA2 (pcDNA3-EphA2) using Lipofectamine 2000 (Invitrogen). Cells were lysed in 1% NP-40 buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2 mM EDTA; and 1% NP-40 plus 50 mM protease inhibitors). Lysates were used for immunoprecipitation with anti-myc (Sigma-Aldrich) or anti-EphA2 antibodies (catalog no. sc-924; Santa Cruz). Immune complexes were resolved on SDS-PAGE and Western blotted using anti-EphA2 or anti-myc antibodies. EphA2 was immunoprecipitated from MMTV-Neu cells, followed by treatment of half the samples with the 1 mM of the crosslinking agent DTSSP. Immunoprecipitates were subjected to Western blot analysis using anti-ErbB2 (1:2,000 dilution; Neomarkers). EphA2 and ErbB2 were immunoprecipitated from MCF10A and MCF10A.HER2 cells as described above. Where indicated, cells were incubated with 10 μg/ml AG825 ErbB2 kinase inhibitor for 24 or 48 hours prior to immunoprecipitation.

Statistics

Statistical differences among groups were determined by single-factor ANOVA, by 2-tailed, paired Student's t test; or by χ^2 test as indicated in the figure and table legends. A P value less than 0.05 was considered significant.

Results

EphA2 deficiency suppresses mammary epithelial hyperplasia, tumorigenesis, and metastasis in MMTV-Neu mice

MMTV-Neu–positive female mice that were EphA2+/+, EphA2+/–, or EphA2–/– were generated and monitored for tumor formation. Mammary gland tissue and/or tumors were collected from 2 cohorts of animals 8 months and 1 year after birth. Relative to EphA2+/+ and EphA2+/– controls, EphA2–/– MMTV-Neu females exhibited a significant decrease in epithelial hyperplasias and tumors of the mammary gland with a 2-to 3-fold reduction in frequency (Table 4). Whole-mount and histologic analysis revealed a reduction in mammary epithelial hyperplasia and epithelial cell content for EphA2–/– MMTV-Neu glands relative to controls (Figure 3.1B).

To examine premalignant changes within the epithelium of EphA2–/– versus EphA2+/+ MMTV-Neu mammary glands, we assessed proliferation and apoptosis in tissue sections by staining for proliferating cell nuclear antigen (PCNA) and by TUNEL assay, respectively. We observed a 5.5-fold reduction in epithelial cell proliferation in the EphA2–/– versus the EphA2+/+ MMTV-Neu mammary epithelium, while levels of

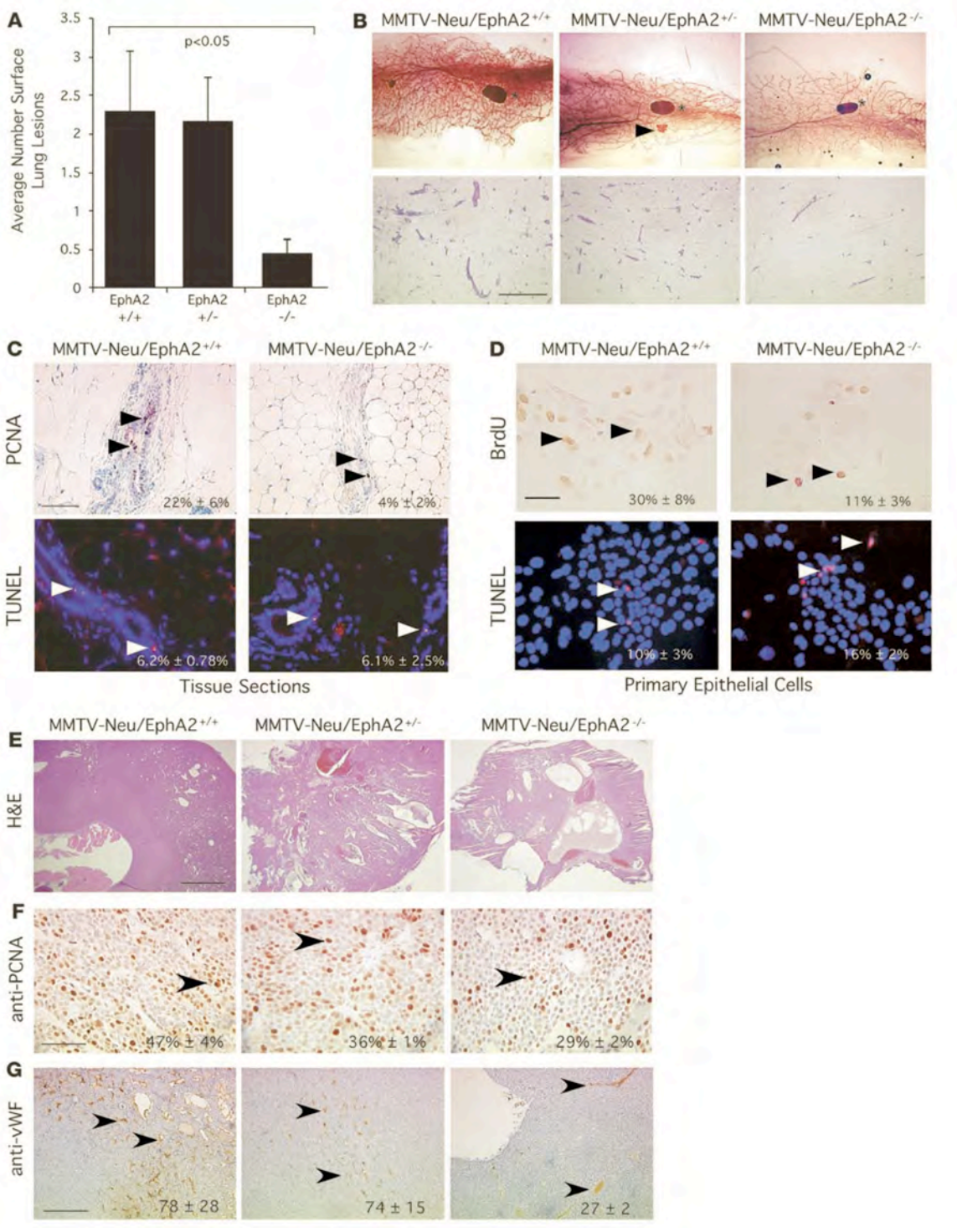
Table 4. Incidence of hyperplasia, tumorigenesis, and lung metastasis frequency in mice 8 months and 1 year after birth

Genotype	Hyperplasia	Tumors		Lung lesions
	8 mo	8 mo	1 yr	1 yr
EphA2$^{+/+}$ MMTV-Neu	71%	57%	72%	63%
EphA2$^{+/-}$ MMTV-Neu	64%	43%	76%	69%
EphA2$^{-/-}$ MMTV-Neu	23%	23%^{A}	40%^{A}	44%

n = 13–14 per genotype scored 8 months after birth; 10–13 per genotype scored 1 year after birth. $^{A}P < 0.05$ versus EphA2$^{+/+}$ MMTV-Neu; χ^2 test.

Figure 3.1. EphA2 deficiency reduces mammary tumorigenesis, metastasis, proliferation, and vascularity in MMTV-Neu mice.

A. Number of surface lung lesions was significantly reduced in EphA2$^{-/-}$ MMTV-Neu mice ($P < 0.05$; single-factor ANOVA). Data are mean ± SEM. **B.** Top: Whole-mount mammary gland preparations (8 mo) revealed diminished hyperplasia in EphA2$^{-/-}$ glands relative to controls. Shown are an EphA2$^{+/+}$ gland with pervasive epithelial hyperplasia (left) and an EphA2$^{+/-}$ gland with a small tumor (arrowhead; middle). Asterisks indicate inguinal lymph node. Bottom: H&E-stained mammary gland sections (8 mo) reveal reduced epithelial cell content in EphA2$^{-/-}$ MMTV-Neu tissue samples relative to controls. Scale bar: 250 μm. **C.** Top: Mammary epithelial proliferation (PCNA^{+} nuclei; arrowheads), was significantly reduced ($P < 0.05$; 2-tailed, paired Student's *t* test). Scale bar: 50 μm. Bottom: Mammary epithelial apoptosis (TUNEL^{+} nuclei; arrowheads) was not affected. **D.** Top: Proliferation of primary mammary epithelial cells from EphA2$^{-/-}$ animals (BrdU incorporation; arrowheads) was reduced relative to EphA2$^{+/+}$ cells ($P < 0.05$; 2-tailed, paired Student's *t* test). Bottom: Apoptosis (TUNEL^{+} nuclei; arrowheads) was significantly increased in EphA2$^{-/-}$ primary mammary epithelial cells relative to controls ($P < 0.05$; 2-tailed, paired Student's *t* test). Scale bar: 20 μm. **E.** H&E-stained tumor sections (1 yr) demonstrate increased cystic degeneration and lumen formation in EphA2$^{-/-}$ tumors. Scale bar: 250 μm. **F.** Decreased tumor cell proliferation (PCNA^{+} nuclei; arrowheads) was observed for EphA2$^{-/-}$ MMTV-Neu tumors compared with controls ($P < 0.05$; single-factor ANOVA). Scale bar: 50 μm. **G.** Microvascular density (CD31^{+} vessels; arrowheads) was significantly reduced in EphA2$^{-/-}$ MMTV-Neu tumors relative to controls ($P < 0.05$; single-factor ANOVA). Scale bar: 100 μm.


A
Average Number Surface Lung Lesions
3.5
3
2.5
2
1.5
1
0.5
0
p<0.05
EphA2 +/+
EphA2 +/-
EphA2 -/-
B
MMTV-Neu/EphA2+/+
MMTV-Neu/EphA2+/-
MMTV-Neu/EphA2-/-
C
MMTV-Neu/EphA2+/+
MMTV-Neu/EphA2-/-
PCNA
22% ± 6%
4% ± 2%
TUNEL
6.2% ± 0.78%
6.1% ± 2.5%
Tissue Sections
D
MMTV-Neu/EphA2+/+
MMTV-Neu/EphA2-/-
BrdU
30% ± 8%
11% ± 3%
TUNEL
10% ± 3%
16% ± 2%
Primary Epithelial Cells
E
MMTV-Neu/EphA2+/+
MMTV-Neu/EphA2+/-
MMTV-Neu/EphA2-/-
H&E
F
anti-PCNA
47% ± 4%
36% ± 1%
29% ± 2%
G
anti-vWF
78 ± 28
74 ± 15
27 ± 2

apoptosis were unaffected (Figure 3.1C). To determine whether proliferation defects were due to EphA2 deficiency in mammary epithelium versus surrounding host tissue, we analyzed proliferation and apoptosis in purified primary mammary epithelial cells (PMECs) isolated from EphA2+/+ or EphA2–/– animals. Proliferation, as measured by incorporation of BrdU, was reduced nearly 3-fold in serum-stimulated EphA2–/– cells relative to EphA2+/+ controls (Figure 3.1D), suggesting that EphA2-mediated effects on proliferation are, at least in part, intrinsic to the epithelial cell. Interestingly, unlike mammary epithelium in situ, we observed a modest yet significant increase in apoptosis for EphA2–/– versus EphA2+/+ PMECs (Figure 3.1D). Together, these data indicate that loss of EphA2 inhibits ErbB2-initiated mammary epithelial cell hyperplasia.

Among the EphA2–/– animals that actually developed tumors, no significant change in time of tumor onset was observed. However, we detected a nearly 3-fold decrease in tumor volume in EphA2–/– relative to EphA2+/+ mice (data not shown). In addition, EphA2+/+ and EphA2+/– controls displayed a higher overall tumor burden relative to EphA2–/– mice, as control animals developed 2 or more tumors 1 year after birth while EphA2–/– animals developed single tumors. At 1 year of age, lungs harvested from EphA2–/– MMTV-Neu mice displayed a nearly 5-fold reduction in the number of surface metastases compared with EphA2+/+ or EphA2+/– controls (Figure 3.1A). Moreover, the overall frequency of metastasis was decreased in EphA2–/– animals relative to EphA2+/+ and EphA2+/– controls (Table 4).

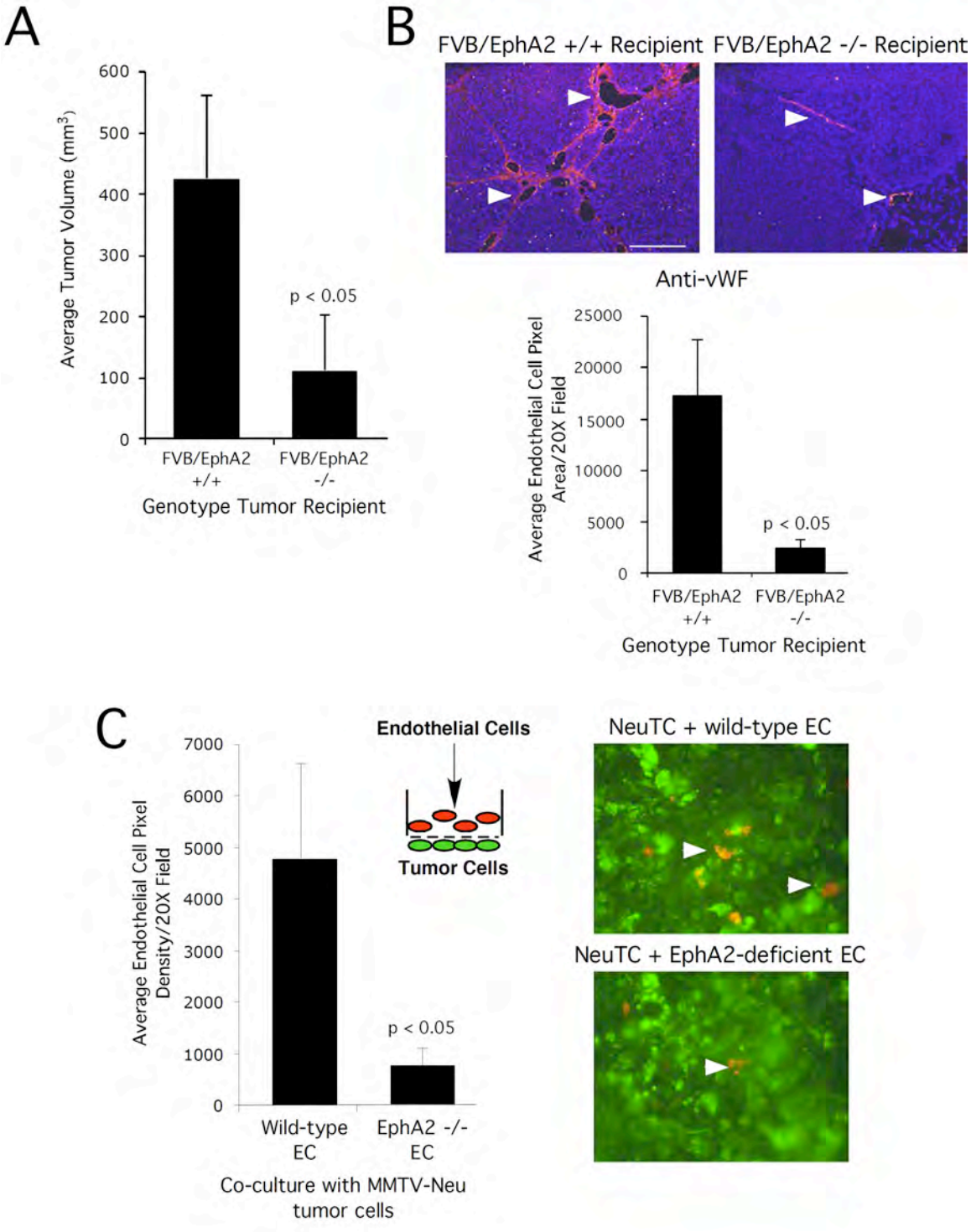
Histologic examination of tumors collected from each genotype 8 months after birth disclosed mainly well-circumscribed proliferations of invasive carcinoma with broad pushing, rather than infiltrating, borders. More infiltrative-appearing carcinomas were seen in animals 1 year after birth. Tumors isolated from EphA2–/– MMTV-Neu mice showed more areas of cystic degeneration and occasional lumen formation, suggestive of a more differentiated phenotype relative to the dense, solid sheet-like growth patterns seen in EphA2+/+ MMTV-Neu tumors (Figure 3.1E). PCNA staining of tumor tissue revealed a nearly 2-fold decrease in proliferation in EphA2–/– relative to EphA2+/+ MMTV-Neu tumors (Figure 3.1F). The tumor microvasculature was evaluated by immunohistochemical staining against vWF, which demonstrated that loss of EphA2 expression was associated with a significant 2.9-fold reduction in microvascular density (Figure 3.1G). Levels of apoptosis were unaltered in EphA2–/– MMTV-Neu tumors compared with controls (data not shown). These data suggest that EphA2 is required for both mammary tumor initiation and progression.

EphA2 is required in the host microenvironment for vascular recruitment in MMTV-Neu tumors

While the data presented herein suggest that EphA2 deficiency restrains epithelial proliferation in MMTV-Neu mammary glands, previously reported data suggest that EphA2 may be required for tumor vascularization (reviewed in ref. [85]). Indeed, decreased tumor vascularization was observed in EphA2–/– MMTV-Neu tumors (Figure 3.1G). To determine whether the defects in tumor microvascular density result from EphA2 deficiency in host tissue versus tumor cells, we orthotopically transplanted

Figure 3.2. Vascular defects observed in MMTV-Neu/EphA2-deficient tumors are due in part to loss of EphA2 expression in host endothelium.

A. Tumor cells derived from MMTV-Neu animals were orthotopically transplanted into cleared mammary fat pads wild-type or EphA2-deficient FVB host animals. Relative to wildtype controls, we observed a significant decrease in tumor volume in tumors collected from EphA2-deficient host animals 5 weeks after transplantation ($p<0.05$; single factor ANOVA). **B.** Consistent with previous studies, we observed significantly reduced ($p<0.05$; ANOVA) microvascular density in tumors isolated from EphA2-deficient hosts versus wild-type controls based on quantification of vWF immunofluorescence (arrowheads indicate vWF+ blood vessels). Scale bar = 100 μm. **C.** To determine if the defects observed in vascular recruitment were due to loss of EphA2 expression in host endothelium, we performed tumor cell-endothelial cell co-culture migration assays (see diagram). Wild-type MMTV-Neu tumor cells labeled with a green fluorescent marker were seeded on the lower surface of a Matrigel-coated transwell. Endothelial cells derived from wild-type or EphA2-deficient animals were labeled with a red fluorescent dye and added to the upper chamber of the transwell and recruitment of endothelial cells to the lower surface by tumor-derived signals was measured. After 5 hours, we observed significantly fewer ($p<0.05$; 2-tailed, paired student's T-test) EphA2-deficient endothelial cells on the lower surface of the transwell than control wild-type endothelial cells (arrows indicate endothelial cells that migrated to the lower surface of the transwell).


A
Average Tumor Volume (mm3)
600
500
400
300
200
100
0
p < 0.05
FVB/EphA2 +/+
FVB/EphA2 -/-
Genotype Tumor Recipient
B
FVB/EphA2 +/+ Recipient
FVB/EphA2 -/- Recipient
Anti-vWF
Average Endothelial Cell Pixel Area/20X Field
25000
20000
15000
10000
5000
0
p < 0.05
FVB/EphA2 +/+
FVB/EphA2 -/-
Genotype Tumor Recipient
C
Average Endothelial Cell Pixel Density/20X Field
7000
6000
5000
4000
3000
2000
1000
0
p < 0.05
Wild-type EC
EphA2 -/- EC
Co-culture with MMTV-Neu tumor cells
Endothelial Cells
Tumor Cells
NeuTC + wild-type EC
NeuTC + EphA2-deficient EC

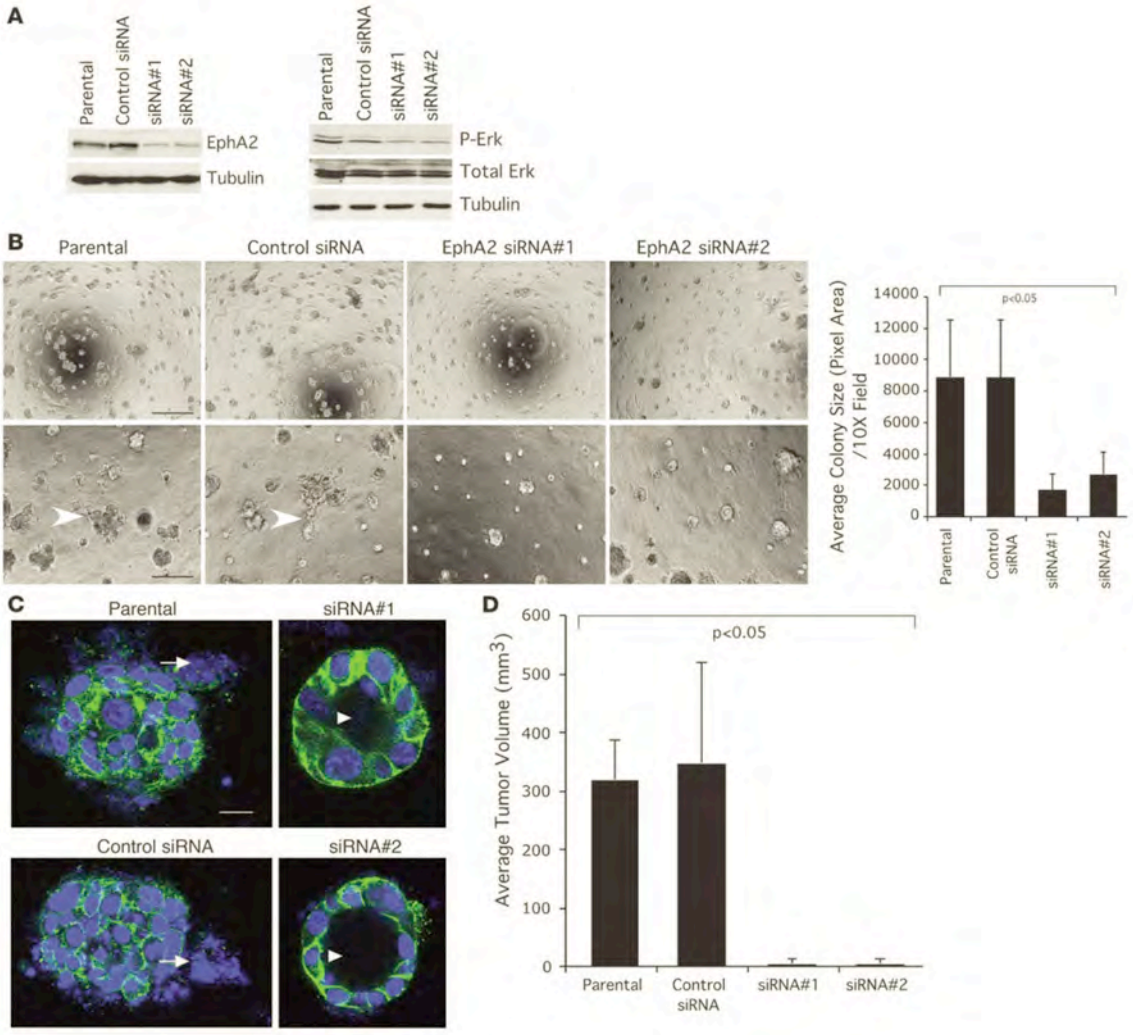
EphA2+/+ MMTV-Neu tumor cells into the cleared fat pads of syngeneic EphA2+/+ or EphA2–/– FVB host animals. EphA2+/+ tumor cells transplanted into EphA2–/– hosts produced significantly smaller tumors than those transplanted into EphA2+/+ hosts (Figure 3.2A). We also observed a 7-fold decrease in microvascular density of tumors isolated from EphA2–/– versus EphA2+/+ recipients (Figure 3.2B). Consistent with these data, microvascular endothelial cells isolated from EphA2–/– animals displayed a markedly decreased migratory response to MMTV-Neu tumor cells in coculture assays compared with the robust migratory response exhibited by endothelial cells isolated from EphA2+/+ mice (Figure 3.2C). Together, these data suggest that EphA2 signaling promotes tumorigenesis and progression through distinct processes both in the tumor microenvironment, including vascular endothelium, and within tumor cells.

Loss of EphA2 expression impairs tumor formation and invasiveness in MMTV-Neu tumor cells

In addition to analysis of EphA2 function in tumor initiation and progression within endogenous MMTV-Neu tumors in which EphA2 deficiency precedes tumorigenesis, we examined the effects of diminishing EphA2 expression in established tumor cells. Using an RNAi knockdown strategy in an established cell line derived from an MMTV-Neu tumor, stable expression of 2 independent siRNA sequences significantly reduced EphA2 expression in MMTV-Neu cells relative to parental cells and cells expressing control siRNA (Figure 3.3A). Pooled populations of cells in which EphA2 expression was diminished displayed slower growth rates than parental or control siRNA-expressing cells (data not shown). Consistent with the diminished growth rates, inhibition of EphA2

Figure 3.3. Loss of EphA2 expression impairs tumor formation and invasiveness in MMTV-Neu tumor cells.

A. EphA2 expression was significantly diminished in MMTV-Neu tumor cells transduced with retroviruses expressing EphA2 siRNA sequences versus control siRNAs. Erk phosphorylation was reduced upon EphA2 knockdown. **B.** Parental and control siRNA tumor cells formed large, irregularly shaped clusters with invasive protrusions (arrowheads) when cultured on Matrigel, whereas EphA2 siRNA–expressing cells formed smaller clusters with a rounded morphology and few protrusions, indicative of reduced invasiveness. Scale bar: 200 μm (top), 50 μm (bottom). We observed a significant decrease in colony size, as determined by calculating the average pixel area occupied by individual colonies, for cells expressing EphA2 siRNA relative to controls ($P < 0.05$; single-factor ANOVA). **C.** Cultures stained with TO-PRO-3 iodide nuclear stain (blue) and anti–E-cadherin (green) were imaged by confocal microscopy. Control tumor cells formed multiacinar structures with invasive protrusions (arrowheads), whereas tumor cells expressing EphA2 siRNA sequences formed round, uniform acinar structures composed of a single layer of epithelial cells surrounding a central lumen (arrows). Scale bar: 20 μm. **D.** Upon orthotopic transplantation into cleared fat pads of FVB recipient female mice, tumor cells expressing control siRNA sequences produced tumors of comparable volume to those generated by transplantation of parental cells at 5 weeks. Tumor cells expressing EphA2 siRNA sequences, however, either failed to form tumors or formed very small, nonpalpable tumors in a small fraction of animals ($P < 0.05$; single-factor ANOVA). Data are mean ± SEM.


A
Parental
Control siRNA
siRNA#1
siRNA#2
EphA2
Tubulin
P-Erk
Total Erk
Tubulin
B
Parental
Control siRNA
EphA2 siRNA#1
EphA2 siRNA#2
Average Colony Size (Pixel Area) /10X Field
p<0.05
C
Parental
siRNA#1
Control siRNA
siRNA#2
D
Average Tumor Volume (mm3)
p<0.05
Parental
Control siRNA
siRNA#1
siRNA#2

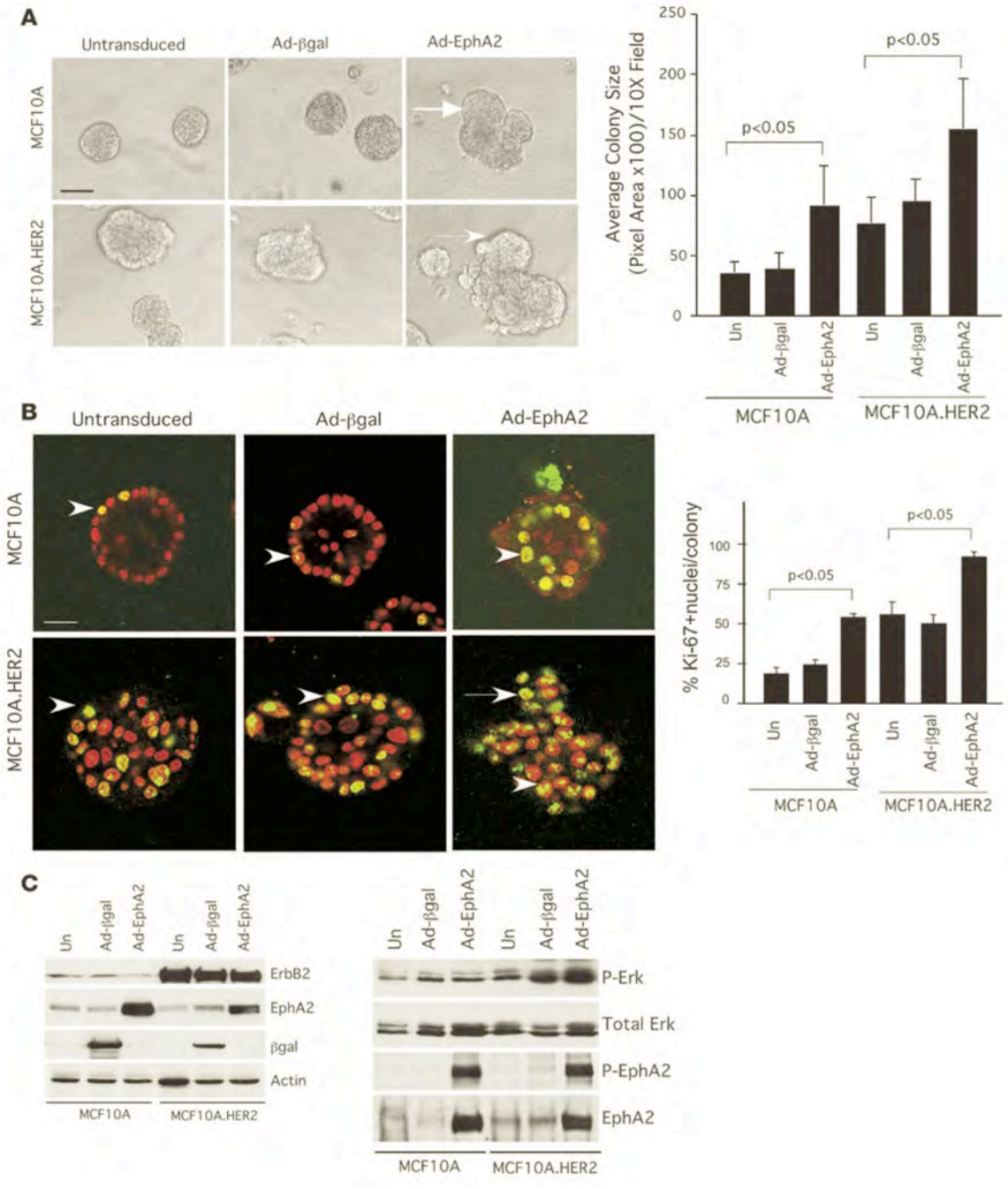
expression by siRNA correlated with diminished levels of p-Erk, a known regulator of proliferation in the MMTV-Neu model (reviewed in ref. [227]), in EphA2 siRNA clones (Figure 3.3A). Parental MMTV-Neu cells and cells transduced with the control siRNA formed large, multiacinar structures and failed to form lumens in 3-dimensional Matrigel culture, consistent with previous descriptions of the effects of ErbB2 activity on 3-dimensional cultures of human MCF10A cells [228]. In contrast, diminished EphA2 expression impaired the ErbB2/Neu-driven multiacinar phenotype of the MMTV-Neu cells in 3-dimensional culture. Instead, these cells primarily formed small, organized acini composed of epithelial cells surrounding a single central lumen (Figure 3.3B and C). Furthermore, the size of individual 3-dimensional colonies formed by control cells was 3- to 4-fold greater than cells with decreased EphA2 expression (Figure 3.3B). While MMTV-Neu parental cells or cells expressing control siRNAs formed tumors when orthotopically transplanted in the cleared fat pads of FVB recipient female mice, MMTV-Neu cells with diminished EphA2 expression failed to establish tumors or formed very small, nonpalpable tumors in a small percentage of animals (Figure 3.3D). These data suggest that EphA2 activity is required for tumor cell–intrinsic growth and invasiveness in the context of the ErbB2/Neu oncogene.

Elevated EphA2 expression augments growth and invasiveness of MCF10A cells overexpressing human ErbB2

To determine whether EphA2 enhances ErbB2-mediated growth and invasiveness in human cells, we overexpressed EphA2 in both nontransformed MCF10A human mammary epithelial cells and in MCF10A cells that stably express the human homolog of

Figure 3.4. Elevated EphA2 expression in MCF10A.HER2 cells enhances cell proliferation and invasiveness in vitro.

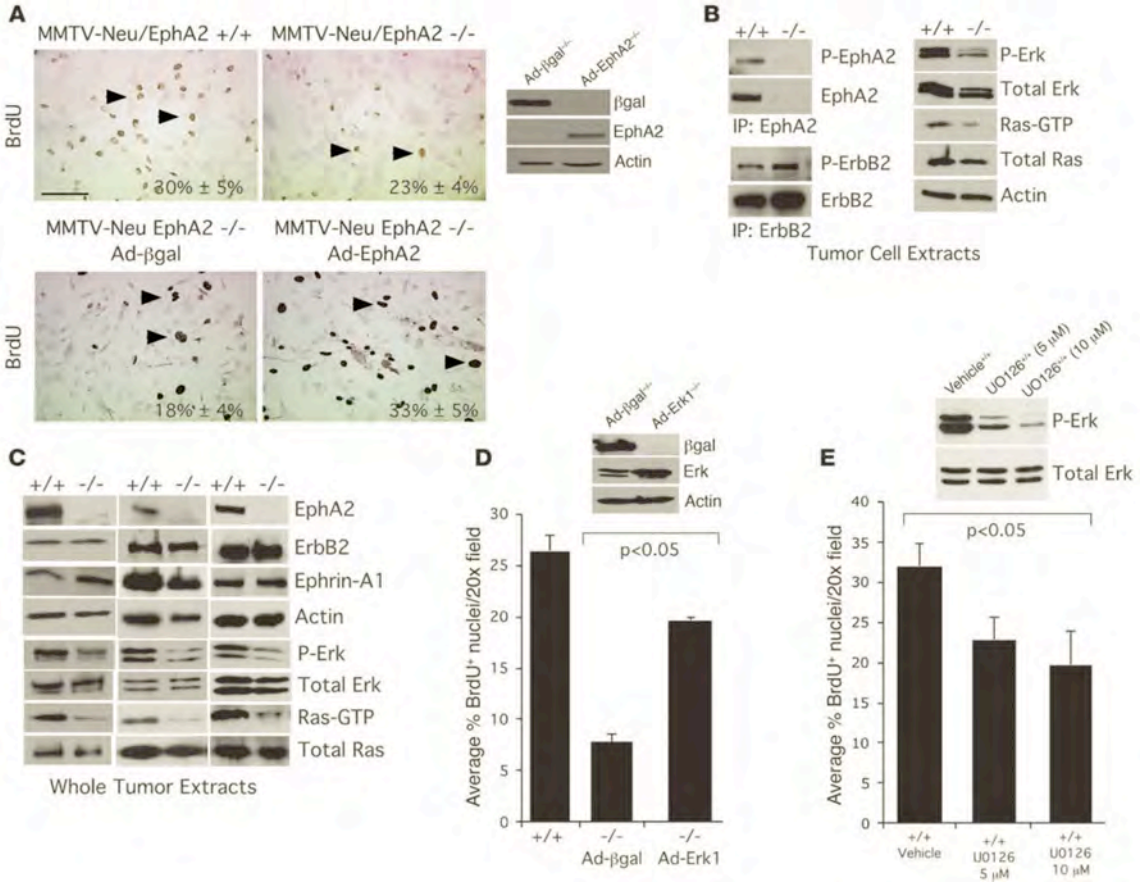
A. Parental MCF10A human breast cells and MCF10A.HER2 cells were transduced with adenoviruses (Ad) expressing EphA2 or control β-gal and plated on growth factor–reduced Matrigel to generate 3-dimensional spheroid cultures. After 10 days in culture, parental MCF10A cells and cells expressing Ad–β-gal formed small, round acinar structures, while MCF10A.HER2 cells formed larger colonies with irregular, invasive protrusions (arrows). Expression of Ad-EphA2 in MCF10A cells resulted in larger, irregular colonies, an effect that was amplified in MCF10A.HER2 cells ($P < 0.05$; single-factor ANOVA). Scale bar: 25 μm. **B.** Cultures were stained with TO-PRO-3 iodide nuclear stain (red) and anti-Ki67 (green) and imaged by confocal microscopy. Confocal analysis revealed that parental and Ad–β-gal–transduced MCF10A formed uniform acinar structures composed of a single layer of epithelial cells surrounding a central lumen, while MCF10A.HER2 cells formed multiacinar structures with invasive protrusions (arrows) and a poorly defined lumen containing several cells. MCF10A cells transduced with Ad-EphA2 also formed multiacinar structures with a poorly defined lumen. Invasion and lumen filling were enhanced in MCF10A.HER2 cells overexpressing EphA2. Scale bar: 20 μm. EphA2 overexpression significantly enhanced proliferation ($Ki67^+$ nuclei, arrows) within acinar structures formed by MCF10A and MCF10A.HER2 cells ($P < 0.05$; single-factor ANOVA). **C.** Expression of adenoviral gene products and overexpression of ErbB2/HER2 in MCF10A.HER2 cells was confirmed by immunoblot, and uniform loading was verified by immunoblot for actin. Expression of p-Erk, total Erk, p-EphA2, and total EphA2 was also assessed by immunoblot.


A
Untransduced
Ad-βgal
Ad-EphA2
MCF10A
MCF10A.HER2
Average Colony Size (Pixel Area x100)/10X Field
250
200
150
100
50
0
p<0.05
p<0.05
Un
Ad-βgal
Ad-EphA2
Un
Ad-βgal
Ad-EphA2
MCF10A
MCF10A.HER2
B
Untransduced
Ad-βgal
Ad-EphA2
MCF10A
MCF10A.HER2
% Ki-67+nuclei/colony
100
75
50
25
0
p<0.05
p<0.05
Un
Ad-βgal
Ad-EphA2
Un
Ad-βgal
Ad-EphA2
MCF10A
MCF10A.HER2
C
Un
Ad-βgal
Ad-EphA2
Un
Ad-βgal
Ad-EphA2
ErbB2
EphA2
βgal
Actin
MCF10A
MCF10A.HER2
Un
Ad-βgal
Ad-EphA2
Un
Ad-βgal
Ad-EphA2
P-Erk
Total Erk
P-EphA2
EphA2
MCF10A
MCF10A.HER2

ErbB2 (HER2; ref. [226]) by adenoviral transduction. Consistent with previous studies [74], overexpression of EphA2 enhanced growth, as we observed increased colony size in3-dimensional Matrigel culture (Figure 3.4A). Relative to parental MCF10A, HER2-overexpressing (MCF10A.HER2) cells formed larger, multiacinar structures that failed to form lumens in 3-dimensional Matrigel culture (Figure 3.4A), consistent with previous reports [226, 228]. Overexpression of EphA2 by adenoviral transduction in MCF10A.HER2 cells led to a 2-fold increase in the size of individual colonies relative to untransduced controls or cells transduced with adenovirus expressing β-gal (Figure 3.4A). In addition, there was an increase in lumen filling and invasive protrusions in acinar structures formed by MCF10A and MCF10A.HER2 cells upon overexpression of EphA2, as assessed by confocal microscopy (Figure 3.4B). Quantification of nuclear Ki67 revealed that overexpression of EphA2 in MCF10A and MCF10A.HER2 cells increases proliferation nearly 3-fold compared with levels observed in control cells (Figure 3.4B). Overexpression of HER2 in MCF10A.HER2 cells, as well as expression of adenoviral gene products, was confirmed by immunoblot (Figure 3.4). Increased p-Erk levels in cells overexpressing HER2, and to a greater extent cells overexpressing both HER2 and EphA2, correlated with increased levels of proliferation in culture (Figure 3.4C). These data suggest that EphA2 enhances mammary epithelial proliferation and invasion and augments growth and invasive properties induced by ErbB2/HER2 in human breast epithelial cells.

Figure 3.5. EphA2 is required for Ras/Erk activation and proliferation in the context of Neu/ErbB2-mediated neoplasia.

A. Proliferation of PMTCs isolated from EphA2$^{-/-}$ animals, as assessed by nuclear incorporation of BrdU (arrowheads), was reduced relative to EphA2$^{+/+}$ cells. For rescue experiments, PMTCs were transduced with adenoviruses expressing EphA2 or β-gal 48 hours prior to BrdU incorporation assay. Overexpression of EphA2 significantly elevated serum-induced proliferation relative to control ($P < 0.05$; 2-tailed, paired Student's *t* test). Scale bar: 20 μm. Expression of adenoviral transgenes was confirmed by immunoblot. **B.** Ras activity in unstimulated cells, as measured by effector pulldown assay of GTP-bound Ras by GST-Raf Ras-binding domain, was reduced in EphA2$^{-/-}$ PMTCs relative to control, as was Erk phosphorylation. Uniform loading was confirmed by immunoblotting for total Ras/Erk and actin. EphA2 deficiency and uniform expression of Neu/ErbB2 was confirmed by effector pulldown assay and immunoblotting for EphA2 and ErbB2. EphA2 was phosphorylated in unstimulated EphA2$^{+/+}$ tumor cells, and no changes in ErbB2 phosphorylation were detected in EphA2$^{+/+}$ versus EphA2$^{-/-}$ PMTCs. **C.** Diminished Ras and Erk activity were confirmed in whole tumor extracts isolated from 3 independent EphA2$^{+/+}$ or EphA2$^{-/-}$ tumors. **D.** For rescue experiments EphA2$^{-/-}$ PMTCs were transduced with adenoviruses expressing Erk-1 or control βgal. Overexpression of Erk-1 in EphA2$^{-/-}$ PMTCs significantly elevated serum-induced proliferation relative to control ($P < 0.05$, EphA2$^{-/-}$ Ad–β-gal versus EphA2$^{+/+}$ or EphA2$^{-/-}$ Ad-Erk-1; single-factor ANOVA). Expression of adenoviral transgenes was confirmed by immunoblot. **E.** Treatment of EphA2$^{+/+}$ PMTCs with the MEK inhibitor U0126 for 12 hours significantly inhibited serum-induced proliferation relative to vehicle control ($P < 0.05$, 5- and 10-μM U0126 versus vehicle). Inhibition of Erk phosphorylation by U0126 was confirmed by immunoblot.


A
MMTV-Neu/EphA2 +/+
MMTV-Neu/EphA2 -/-
BrdU
30% ± 5%
23% ± 4%
MMTV-Neu EphA2 -/-
Ad-βgal
MMTV-Neu EphA2 -/-
Ad-EphA2
18% ± 4%
33% ± 5%
Ad-βgal-/-
Ad-EphA2-/-
βgal
EphA2
Actin
B
+/+ -/-
P-EphA2
EphA2
IP: EphA2
P-ErbB2
ErbB2
IP: ErbB2
+/+ -/-
P-Erk
Total Erk
Ras-GTP
Total Ras
Actin
Tumor Cell Extracts
C
+/+ -/- +/+ -/- +/+ -/-
EphA2
ErbB2
Ephrin-A1
Actin
P-Erk
Total Erk
Ras-GTP
Total Ras
Whole Tumor Extracts
D
Ad-βgal-/-
Ad-Erk1-/-
βgal
Erk
Actin
Average % BrdU+ nuclei/20x field
p<0.05
+/+
-/- Ad-βgal
-/- Ad-Erk1
E
Vehicle-/-
UO126-/- (5 μM)
UO126-/- (10 μM)
P-Erk
Total Erk
Average % BrdU+ nuclei/20x field
p<0.05
+/+ Vehicle
+/+ U0126 5 μM
+/+ U0126 10 μM

EphA2 promotes activation of Ras/MAPK and tumor cell proliferation

To examine the specific EphA2 signaling events intrinsic to the breast epithelial cells that regulate proliferation, we purified MMTV-Neu hyperplastic PMECs and primary mammary tumor cells (PMTCs) from EphA2–/– and EphA2+/+ mice. EphA2–/– tumor cells expressed no detectable levels of EphA2, but ErbB2 expression or phosphorylation was not affected in these cells (Figure 3.5B), suggesting that EphA2 did not regulate ErbB2 expression or activity. Both PMECs and PMTCs that were EphA2–/– exhibited a decrease in proliferation relative to that in EphA2+/+ cells (Figure 3.5A and Figure 3.1D), and the proliferation defect was rescued by restoring EphA2 expression (Figure 3.5A). While there were no significant changes in levels of p-src, p-stat5, p–cyclin-D1, or p-PLCγ (data not shown), levels of p-Erk and active GTP-bound Ras were significantly diminished in EphA2–/– relative to EphA2+/+ cells (Figure 3.5B). Similarly, there was a substantial reduction in Erk and Ras activity in whole tumor lysates from EphA2–/– animals compared with tumors from EphA2+/+ MMTV-Neu mice (Figure 3.5C). Overexpression of exogenous Erk-1 rescued proliferation defects in EphA2–/– PMTCs relative to cells expressing control β-gal (Figure 3.5D), suggesting that modulation of Ras/Erk signaling is a primary mechanism through which EphA2 affects Neu-mediated tumor growth. Treatment of EphA2+/+ PMTCs with the MEK inhibitor U0126 significantly impaired proliferation. As MEK activates Erk in response to activation of Ras, these data confirm that Ras/MEK/Erk pathway activation regulates growth in these cells (Figure 3.5E).

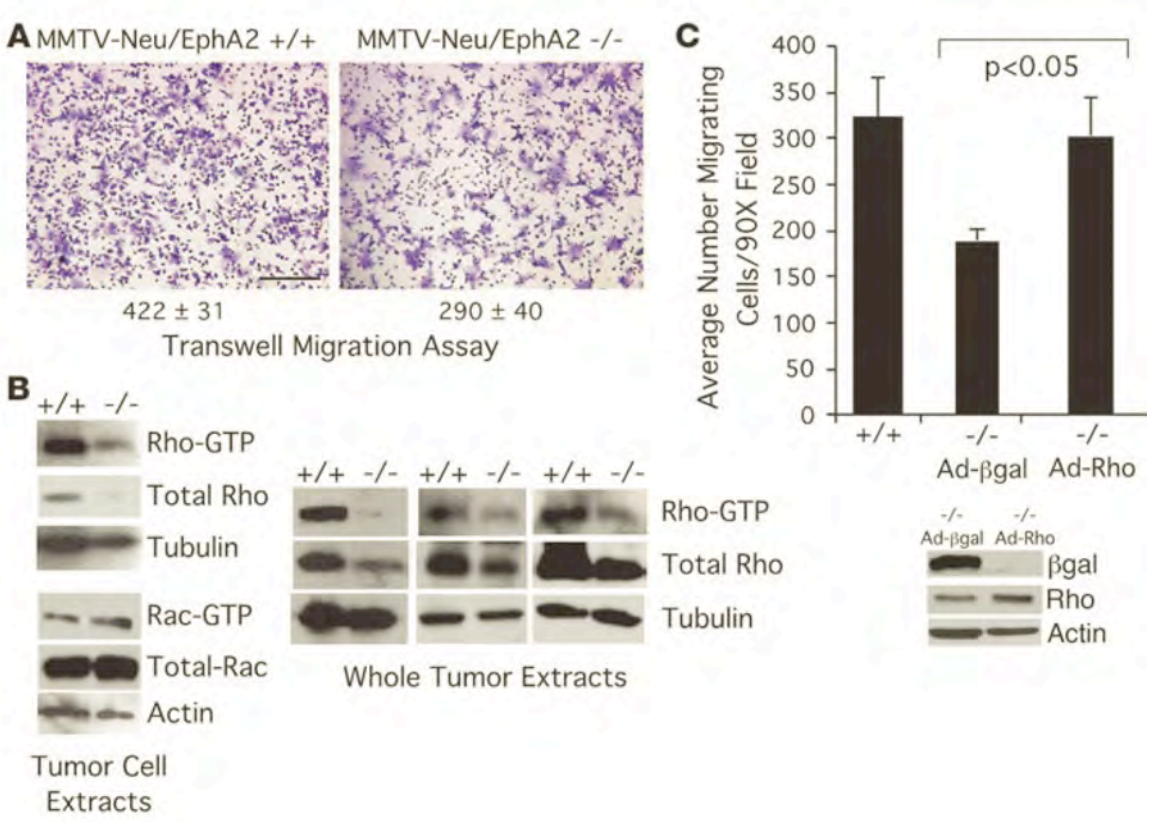


Figure 3.6. EphA2 is required for RhoA activation and tumor cell migration in the context of Neu/ErbB2-mediated malignancy.

A. EphA2$^{-/-}$ PMTCs displayed significantly reduced migration in response to growth media supplemented with 10% serum compared with EphA2$^{+/+}$ PMTCs in transwell migration assays ($P < 0.05$; 2-tailed, paired Student's *t* test). **B.** RhoA activity, as measured by effector pulldown assay of GTP-bound RhoA in tumor cell lysates and in whole tumor extracts by GST-Rhotekin Rho-binding domain, was reduced in EphA2$^{-/-}$ PMTCs and intact tumors relative to EphA2$^{+/+}$ cells and tumors. We also observed a decrease in total RhoA protein levels in EphA2$^{-/-}$ MMTV-Neu tumor cells and in whole tumor extracts relative to EphA2$^{+/+}$ controls. We observed no change in GTP-bound, activated Rac, or total Rac protein levels in tumor cell lysates from EphA2$^{-/-}$ or EphA2$^{+/+}$ PMTCs. **C.** For rescue experiments, EphA2$^{-/-}$ MMTV-Neu primary tumor cells were transduced with adenoviruses expressing constitutively active RhoA (Q63L) or control β-gal 48 hours prior to migration assay. Expression of constitutively active RhoA restored serum-induced migration of EphA2$^{-/-}$ tumor cells to levels comparable to those observed in tumor cells derived from EphA2$^{+/+}$ animals, while control β-gal had no effect ($P < 0.05$, EphA2$^{-/-}$ Ad–β-gal versus EphA2$^{+/+}$ and EphA2$^{-/-}$ Ad-Rho; single-factor ANOVA). Expression of adenoviral transgenes was confirmed by immunoblot assays.

EphA2 promotes tumor cell migration through activation of RhoA GTPase

To dissect the mechanisms by which EphA2 promotes tumor metastasis, we analyzed motility of MMTV-Neu tumor cells in the context of EphA2 deficiency using a transwell migration assay. EphA2–/– MMTV-Neu tumor cells displayed a 1.5-fold decrease in serum-stimulated migration relative to EphA2+/+ cells (Figure 3.6A). Because expression and activity of Rho family small GTPases are integral components of signaling pathways that regulate cell migration, we sought to determine whether EphA2 regulates tumor cell motility through a Rho-dependent mechanism. Diminished levels of active GTP-bound RhoA were present in both EphA2–/– tumors and in purified EphA2–/– PMTCs relative to EphA2+/+ controls (Figure 3.6B). EphA2–/– tumor cells also displayed a decrease in total RhoA protein expression. In contrast, there were no detectable changes in levels of activated Rac1 under our experimental conditions. To determine whether activation of RhoA mediates EphA2-dependent cell migration, we expressed a constitutively active RhoA in EphA2–/– MMTV-Neu tumor cells. While expression from a control adenovirus expressing β-gal had no effect on migration in EphA2–/– PMTCs, expression of exogenous activated RhoA restored migration to levels similar to those of EphA2+/+ control cells (Figure 3.6C). These findings suggest that RhoA activation contributes to EphA2-mediated tumor cell migration.

While Rho family GTPases, including RhoA, have also been shown to regulate cell cycle progression [229, 230], expression of constitutively active RhoA did not rescue proliferation in EphA2–/– PMTCs to the levels observed in control cells (data not shown), suggesting that RhoA activation specifically contributes to EphA2-mediated tumor cell

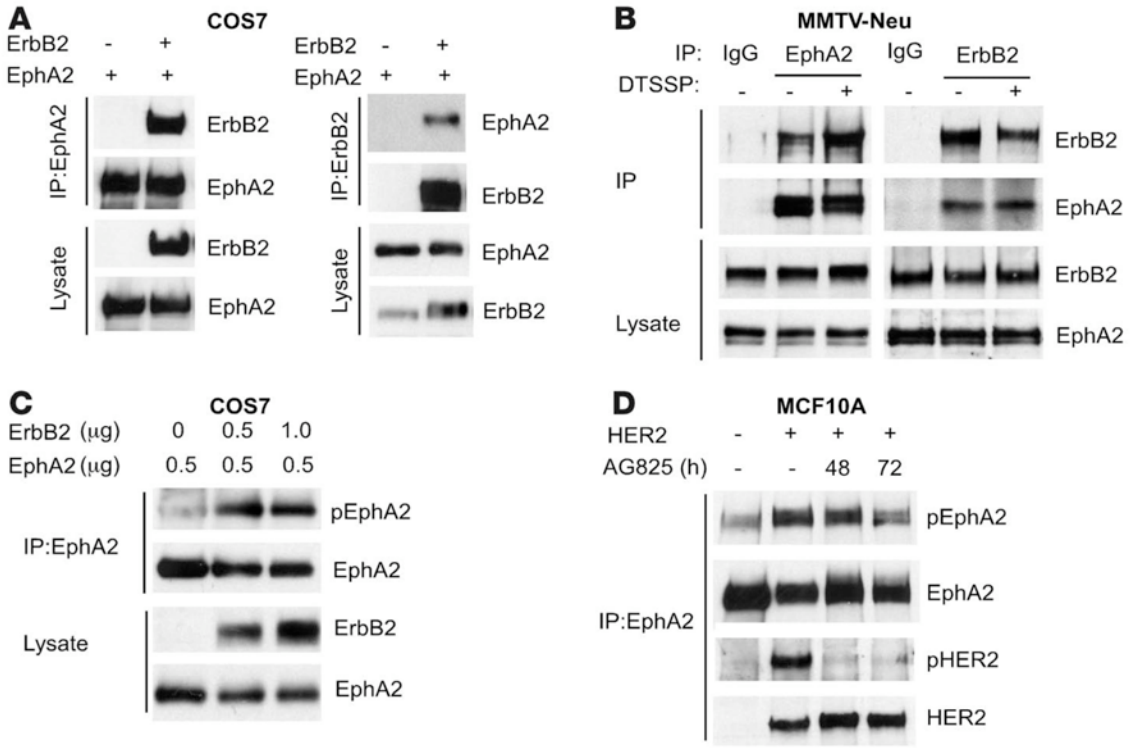


Figure 3.7. EphA2 physically and functionally interacts with ErbB2.

A. COS7 cells were transfected with plasmids for expression of EphA2 or/and ErbB2. EphA2 or ErbB2 was immunoprecipitated from cell lysates, and products were analyzed for ErbB2 or/and EphA2. Coexpression of EphA2 and ErbB2 was sufficient to permit coimmunoprecipitation. **B.** Endogenous ErbB2 and EphA2 were coimmunoprecipitated with anti-EphA2 or anti-ErbB2 antibodies, respectively, in EphA2$^{+/+}$ MMTV-Neu tumor cells that were untreated or treated with the chemical crosslinker DTSSP. The interaction detected was specific: EphA2 and ErbB2 were not immunoprecipitated by control IgG. Uniform input was validated by probing lysates for expression of EphA2 and ErbB2. **C.** COS7 cells were transfected with plasmids for expression of EphA2 or ErbB2. EphA2 was immunoprecipitated from cell lysates, and products were analyzed for EphA2 expression and tyrosine phosphorylation. Coexpression of ErbB2 and EphA2 was sufficient to induce phosphorylation of EphA2 in COS7 cells in the absence of ephrin ligand stimulation. **D.** Interaction between EphA2 and HER2 in MCF10A cells overexpressing HER2 was observed, as EphA2 and HER2 were coimmunoprecipitated with anti-EphA2 antibodies in HER2-overexpressing cells, but not in parental MCF10A cells. Elevated EphA2 phosphorylation was observed in MCF10A cells overexpressing HER2 relative to parental MCF10A cells, and treatment with the ErbB2 kinase inhibitor AG825 reduced EphA2 phosphorylation as well as ErbB2 phosphorylation in MCF10A cells overexpressing HER2.

migration rather than growth. Conversely, we did not observe any change in migration of EphA2–/– PMTCs upon overexpression of Erk-1 (data not shown). These data suggest that proliferation and motility are regulated separately by Erk-1 and Rho, respectively, in the context of ErbB2/EphA2-mediated tumor progression.

EphA2 physically and functionally interacts with ErbB2

To investigate the molecular mechanism(s) by which EphA2 modulates Neu/ErbB2-mediated proliferation and invasiveness, biochemical studies were performed to assess physical interaction between EphA2 and ErbB2 in COS7 cells overexpressing both proteins and between endogenous proteins in MMTV-Neu–derived PMTCs. We detected the presence of ErbB2 in EphA2 immunoprecipitates, and EphA2 in ErbB2 immunoprecipitates, in lysates from COS7 cells overexpressing the human isoforms of EphA2 and ErbB2 (Figure 3.7A). Coimmunoprecipitation analysis of endogenous proteins from PMTCs also confirmed that ErbB2 formed a complex with EphA2 (Figure 3.7B). In both PMTCs and COS7 cells, EphA2 and ErbB2 were expressed at high levels, and the EphA2/ErbB2 interaction occurred constitutively in the absence of ligand stimulation (Figure 3.7C). Strikingly, coexpression of ErbB2 and EphA2 in COS7 cells was sufficient to induce tyrosine phosphorylation of EphA2 in the absence of ligand or serum stimulation (Figure 3.7C). Likewise, elevated EphA2 phosphorylation was observed in MCF10A.HER2 cells overexpressing ErbB2 relative to parental MCF10A cells (Figure 3.7D). Consistent with coexpression data in COS7 cells, treatment with an ErbB2 kinase inhibitor diminished EphA2 phosphorylation as well as HER2 phosphorylation in MCF10A.HER2 cells (Figure 3.7D). Given evidence for physical

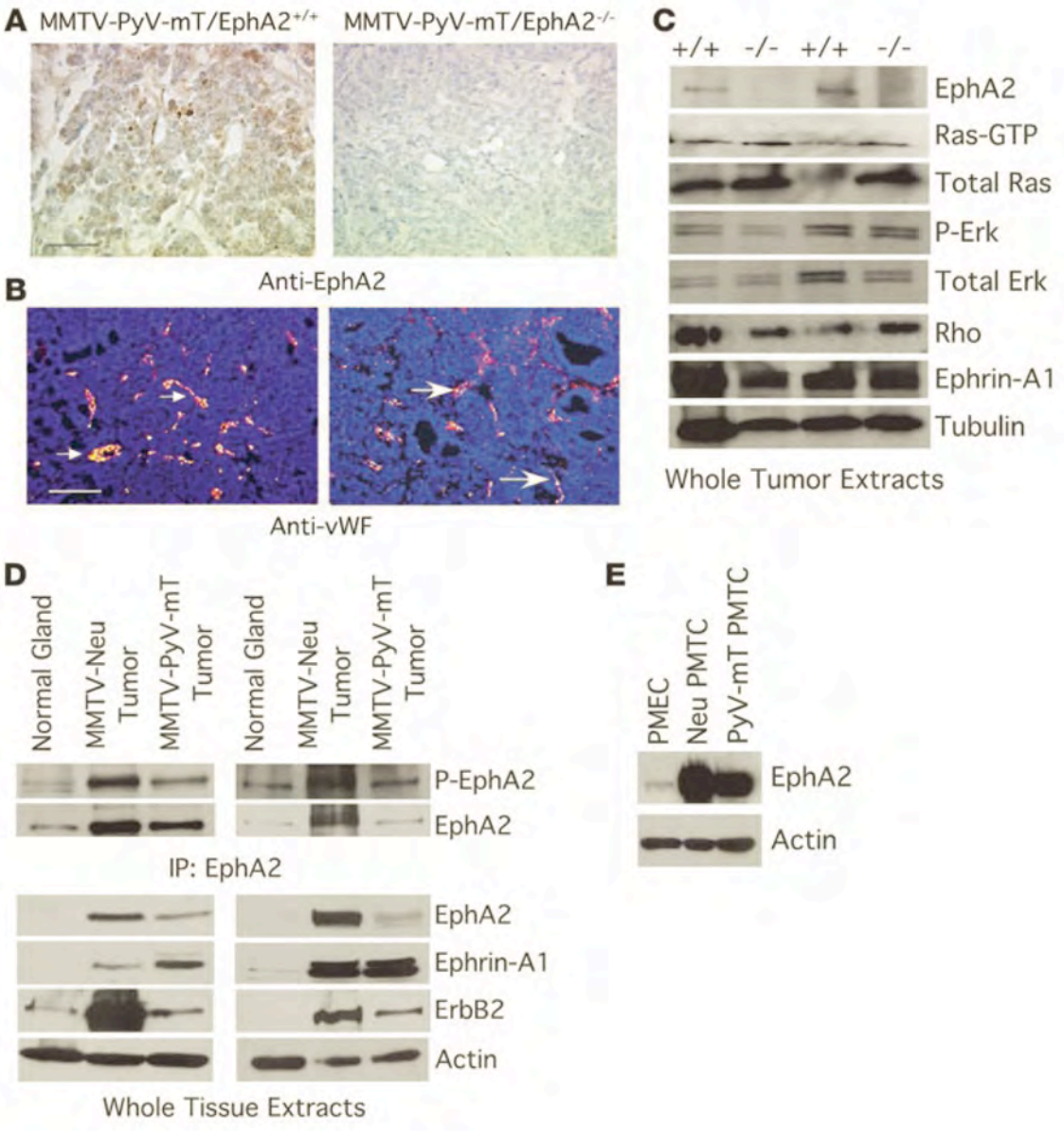
interaction between ErbB2 and EphA2 and the functional requirement of EphA2 expression for maximal activation of signaling pathways downstream of ErbB2, these data suggest that that EphA2 participates in ErbB2 signaling.

EphA2 deficiency has no impact on tumor progression, angiogenesis, or metastasis in MMTV–PyV-mT transgenic animals

To assess EphA2 function in an independent endogenous model of mammary tumorigenesis that is also dependent upon the Ras/MAPK pathway, we crossed MMTV–PyV-mT mice with EphA2–/– mice. These animals were used to generate MMTV–PyV-mT mice that were EphA2+/+, EphA2+/–, or EphA2–/–. Virgin female mice were monitored for tumor formation through 100 days. Despite confirmed loss of EphA2 deficiency in the MMTV–PyV-mT model (Figure 3.8A and C), EphA2 deficiency did not affect rate of tumor formation, tumor volume, number of surface lung lesions (data not shown), or microvascular density (Figure 3.8B). Additionally, there were no differences in levels of total Ras, active GTP-bound Ras, p-Erk, or total Rho in MMTV–PyV-mT tumors derived from EphA2+/+ versus EphA2–/– mice (Figure 3.8C). These findings are in striking contrast to the effects of EphA2 deficiency observed in the MMTV-Neu model. These data suggest that, in marked contrast to the MMTV-Neu model, EphA2 does not affect tumor initiation, metastasis, or vascular density in the MMTV–PyV-mT model, nor does loss of EphA2 affect the signaling pathways that contribute to these aspects of tumor progression in this model.

Figure 3.8. EphA2 deficiency does not affect tumorigenesis, microvascular density, or growth regulatory signaling pathways in MMTV–PyV-mT tumors.

A. Loss of EphA2 protein expression was confirmed by immunohistochemical staining. Scale bar: 50 μm. **B.** We detected no change in MMTV–PyV-mT tumor microvascular density based on vWF staining (arrows indicate vWF^+ blood vessels). Scale bar: 100 μm. **C.** We did not observe any change in levels of GTP-bound active Ras or p-Erk in $EphA2^{-/-}$ MMTV–PyV-mT whole tumor extracts relative to controls, nor did we observe any change in levels of RhoA. Uniform loading was confirmed by immunoblotting for total Ras, total Erk, and tubulin. **D.** We observed EphA2 overexpression and elevated phosphorylation in MMTV-Neu and MMTV–PyV-mT tumors relative to normal mammary tissue isolated from control FVB mice, with the highest levels observed in MMTV-Neu tumors. We also observed overexpression of ErbB2 and ephrin-A1 in both tumor types, with comparable ephrin-A1 expression in both tumor types and higher ErbB2 levels in MMTV-Neu tumors. Uniform loading was confirmed by immunoblot for actin. **E.** We confirmed EphA2 overexpression specifically in epithelium by comparing EphA2 levels in PMEC lysates versus PMTCs derived from MMTV-Neu and MMTV–PyV-mT mice.


A
MMTV-PyV-mT/EphA2+/+
MMTV-PyV-mT/EphA2-/-
Anti-EphA2
B
Anti-vWF
C
+/+ -/- +/+ -/-
EphA2
Ras-GTP
Total Ras
P-Erk
Total Erk
Rho
Ephrin-A1
Tubulin
Whole Tumor Extracts
D
Normal Gland
MMTV-Neu Tumor
MMTV-PyV-mT Tumor
P-EphA2
EphA2
IP: EphA2
EphA2
Ephrin-A1
ErbB2
Actin
Whole Tissue Extracts
E
PMEC
Neu PMTC
PyV-mT PMTC
EphA2
Actin

We next assessed expression and activation of EphA2 in normal mammary tissue isolated from FVB female mice, in MMTV-Neu and MMTV–PyV-mT tumor tissue, and in PMECs and PMTCs isolated from both MMTV-Neu and MMTV–PyV-mT animals. EphA2 was overexpressed and phosphorylated in tumor tissue derived from both MMTV-Neu and MMTV–PyV-mT models compared with normal mammary tissue.

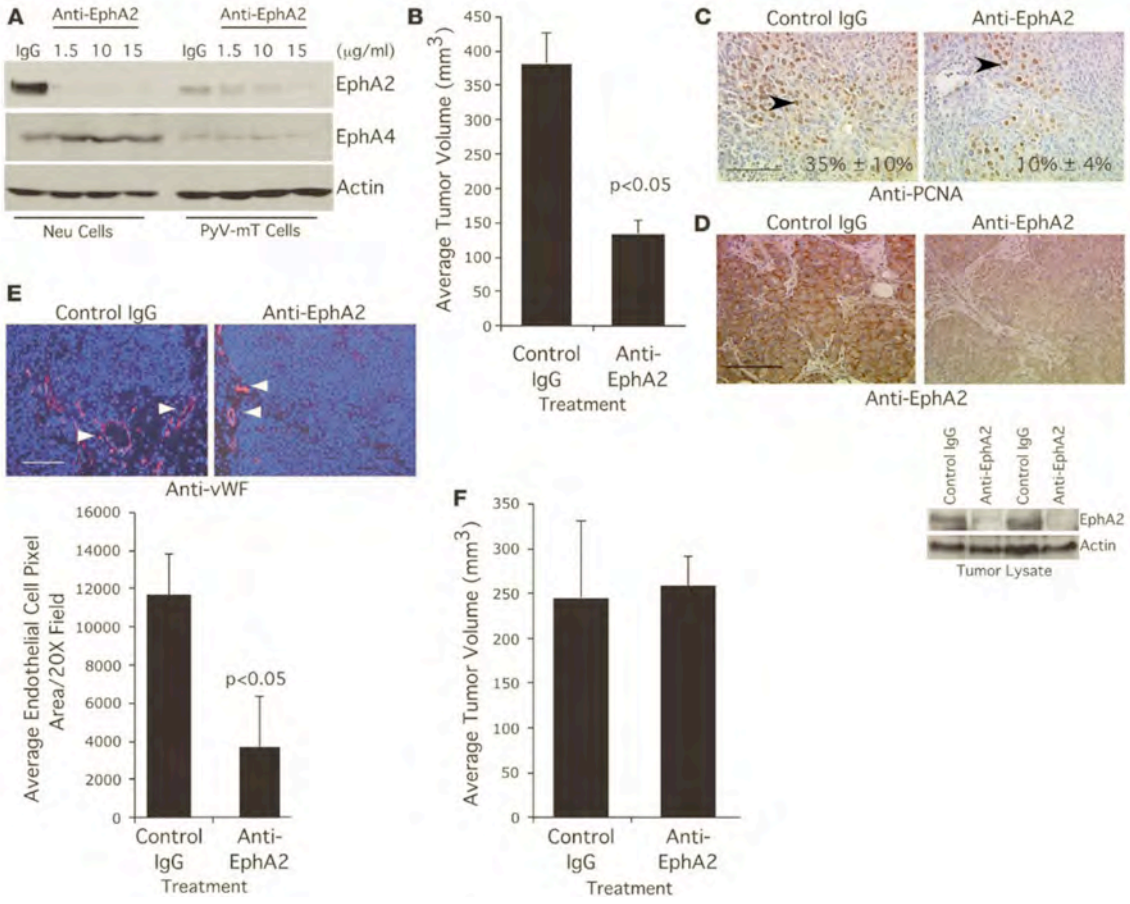
Furthermore, expression of ephrin-A1 ligand was elevated in tumor lysates from both models compared with normal mammary tissue (Figure 3.8C and D). Levels of ephrin-A1 were comparable in EphA2+/+ and EphA2–/– tumor lysates (Figure 3.8C and D). Notably, however, levels of both total EphA2 and p-EphA2 were higher in MMTV-Neu tumors compared with MMTV–PyV-mT tumors (Figure 3.8D). EphA2 overexpression was detected specifically in tumor cells and not in non-neoplastic epithelial cells (Figure 3.8E). While ErbB2 overexpression has been previously reported in MMTV–PyV-mT tumors [231] and was also observed in our tumor lysates, MMTV-Neu tumors displayed a much higher level of ErbB2 overexpression (Figure 3.8D). The evidence therefore suggests that EphA2 augments ErbB2/Neu-mediated signaling and that increased expression of EphA2 may be a mechanism by which ErbB2 signaling pathways are amplified in tumors.

Anti-EphA2 therapy shows efficacy in the MMTV-Neu tumor model

To determine whether MMTV-Neu tumors are responsive to targeted anti-EphA2 therapy in vivo, we transplanted wild-type MMTV-Neu tumor cells into the cleared fat pads of wild-type FVB recipient animals. At 2 weeks after transplantation, animals were injected

Figure 3.9. Treatment with an anti-EphA2 antibody inhibits tumor growth in MMTV-Neu but not MMTV–PyV-mT tumors.

A. Treatment with anti–murine EphA2 antibody diminished EphA2 protein expression in tumor cells derived from MMTV-Neu and MMTV–PyV-mT mice. Tumor cells were treated with control IgG (10 μg/ml) or increasing concentrations of anti-EphA2 antibody for 48 hours. Uniform loading was confirmed by immunoblot for actin. Blots were stripped and reprobed with anti-EphA4 antibodies as a control for antibody specificity. **B.** Cells derived from $EphA2^{+/+}$ MMTV-Neu mice were orthotopically transplanted into the cleared fat pads of female FVB recipient mice. At 2 weeks following transplantation, mice were injected intraperitoneally with anti-EphA2 antibody or control IgG (10 mg/kg) twice weekly for 3 weeks. We observed a significant reduction in tumor volume in anti-EphA2–treated animals relative to control IgG–treated mice ($P < 0.05$; 2-tailed, paired Student's *t* test). Data are mean ± SEM. **C.** Tumor cell proliferation was significantly impaired in anti-EphA2–treated animals relative to controls ($P < 0.05$; single-factor ANOVA; arrowheads indicate $PCNA^+$ nuclei). Scale bar: 50 μm. **D.** EphA2 expression was significantly diminished in anti-EphA2–treated tumors relative to IgG controls, as assessed by immunohistochemistry and immunoblot. Blots were stripped and reprobed for actin expression to verify uniform loading. Scale bar: 50 μm. **E.** We observed significantly reduced ($P < 0.05$; 2-tailed, paired Student's *t* test) microvascular density in tumors isolated from anti-EphA2–treated mice relative to controls (arrowheads indicate vWF^+ blood vessels). Scale bar: 100 μm. (**F**) Cells derived from MMTV–PyV-mT mice were orthotopically transplanted in the cleared fat pad of FVB female recipient mice and were treated with anti-EphA2 antibody or control IgG as described above. We observed no change in tumor volume between animals treated with anti-EphA2 antibody relative to control IgG-treated mice.


A
Anti-EphA2
Anti-EphA2
IgG 1.5 10 15
IgG 1.5 10 15
(μg/ml)
EphA2
EphA4
Actin
Neu Cells
PyV-mT Cells
B
Average Tumor Volume (mm3)
p<0.05
Control IgG
Anti-EphA2
Treatment
C
Control IgG
Anti-EphA2
35% ± 10%
10% ± 4%
Anti-PCNA
D
Control IgG
Anti-EphA2
Anti-EphA2
Control IgG
Anti-EphA2
Control IgG
Anti-EphA2
EphA2
Actin
Tumor Lysate
E
Control IgG
Anti-EphA2
Anti-vWF
Average Endothelial Cell Pixel Area/20X Field
p<0.05
Control IgG
Anti-EphA2
Treatment
F
Average Tumor Volume (mm3)
Control IgG
Anti-EphA2
Treatment

intraperitoneally twice weekly for 3 weeks with either control IgG or an anti-EphA2 antibody that targets murine EphA2 for degradation (Figure 3.9A). The anti-EphA2 antibody specifically targeted EphA2, as expression of the related receptor EphA4 was unaffected in antibody-treated tumor cells derived from MMTV-Neu and MMTV–PyV-mT animals (Figure 3.9A). MMTV-Neu tumors harvested from anti-EphA2–treated animals displayed a 3-fold reduction in tumor volume relative to tumors isolated from IgG-treated mice (Figure 3.9B). In addition, tumor cell proliferation was significantly decreased in anti-EphA2–treated animals relative to controls, as determined by quantifying nuclear PCNA staining (Figure 3.9C). As predicted, EphA2 protein levels were significantly reduced in anti-EphA2–treated tumors relative to control IgG–treated tumors, as assessed by immunohistochemistry and immunoblot (Figure 3.9D), although downregulation of EphA2 expression did not affect expression of ErbB2 in anti-EphA2–treated tumors, nor did control IgG treatment affect ErbB2 expression in tumors (data not shown). We also observed a significant reduction in microvascular density in tumors harvested from anti-EphA2–treated animals relative to those treated with control IgG (Figure 3.9E). In contrast to these results, anti-EphA2 treatment had no effect on tumor volume (Figure 3.9F) in animals transplanted with MMTV–PyV-mT tumors in spite of downregulated levels of EphA2 protein in anti-EphA2-treated tumors. These data suggest that the efficacy of anti-EphA2 therapy depends upon the oncogene context in which tumor progression occurs, as treatment of MMTV–PyV-mT tumor-bearing animals did not affect tumor progression as in MMTV-Neu tumor-bearing mice in spite of EphA2 overexpression in both tumor models.

Discussion

Role of EphA2 in ErbB2-driven mammary tumor initiation and metastatic progression

The role of EphA2 in breast tumor progression has remained controversial, based on discrepancies among individual studies. For example, recent screens of RTK expression in tumors revealed that EphA2 RTK is overexpressed in a variety of human epithelial cancers, including more than 80% of breast cancer clinical samples [232]. While these studies did not discriminate between stromal cell–intrinsic versus tumor cell–intrinsic EphA2 expression, they clearly demonstrated a correlation between EphA2 expression and breast cancer progression. In contrast, some initial studies suggested that EphA2 does not play a role in tumor initiation, including reports indicating that stimulation of tumor cells with soluble ephrins does not promote, and may even inhibit, MAPK activation and tumor cell proliferation [34, 113, 116]. In addition, the majority of previous in vivo studies regarding EphA2 function in tumor progression involved tumor xenograft models that do not recapitulate endogenous tumor initiation and progression [74, 76-78] [95, 98, 208, 233]. To provide a comprehensive examination of the role of EphA2 in the multiple stages of mammary tumorigenesis, we generated EphA2–/– MMTV-Neu mice. We chose the MMTV-Neu model because the mammary epithelium progresses from hyperplasia to carcinoma in situ and to invasive and metastatic carcinoma in a stepwise manner is similar to that seen in ErbB2-overexpressing human breast cancers [216]. EphA2–/– MMTV-Neu female mice exhibited a reduction in mammary epithelial hyperplasia and developed tumors with reduced frequency secondary to a significant reduction in epithelial cell proliferation (Figure 3.1), consistent with a role for EphA2 in the earliest

stages of breast cancer formation. This proliferation defect was, at least in part, intrinsic to epithelial cells, as BrdU incorporation was also reduced in primary mammary epithelial cells and tumor cells isolated from EphA2–/– animals relative to controls (Figures 3.1 and 3.5). Taken together, these results suggest that EphA2 is required for mammary tumor onset and growth.

At later stages of tumor progression, MMTV-Neu mammary tumors progress from hyperplasia to metastatic carcinoma through multiple steps, including increased tumor cell invasion and migration and sustained angiogenesis. Loss of EphA2 significantly inhibited MMTV-Neu tumor metastasis to the lung. While EphA2-dependent tumor cell proliferation is intrinsic to the epithelium, EphA2-dependent tumor metastasis can be attributed to dual roles for EphA2 in both tumor cells and host microenvironment. Ex vivo studies using EphA2–/– MMTV-Neu tumor cells revealed that loss of EphA2 impaired serum-induced cell migration (Figure 3.6), indicating a tumor cell–intrinsic role for EphA2-mediated malignant progression. Additionally, tumor angiogenesis was significantly diminished when MMTV-Neu cells were transplanted into EphA2–/– host environment (Figure 3.2). These studies underscore the complex nature of EphA2 signaling.

Oncogene interaction in mammary tumorigenesis and metastatic progression

A large body of work indicates that tumorigenesis is a multistep process, and different oncogenes often cooperate to promote different steps of tumor progression (reviewed in refs. [202-204]). Here we demonstrated a physical interaction between EphA2 and ErbB2

at the tumor cell surface, inducing phosphorylation of the EphA2 in the absence of ligand stimulation. This interaction between ErbB2 and EphA2 amplified Ras/Erk signaling and Rho GTPase activation (Figures 3.5 and 3.6), likely contributing to the increased proliferation and motility of EphA2-expressing tumor cells. This observation holds repercussions regarding how ErbB2-expressing breast cancers are treated, especially those that are refractory to anti-ErbB2 therapies. Our findings provide translational evidence that anti-EphA2 therapy may be effective against ErbB2-expressing tumors, alone or in combination with methods targeting ErbB2.

By contrast, EphA2 deficiency in MMTV–PyV-mT transgenic mice had no effect on tumor progression or in observed levels of activated Ras or p-Erk. Because Ras and Erk displayed high basal activity in PyV-mT–expressing tumors regardless of EphA2 expression, these data suggest that PyV-mT antigen activates Ras/MAPK pathway by alternate mechanisms, bypassing the requirement for EphA2 function. More importantly, this observation demonstrates that EphA2 function in tumor progression depends upon the context of other oncogenic/tumor suppressive determinants of malignancy, which may underlie some of the controversy regarding the role of EphA2 in tumor progression. While ErbB2 and PyV-mT activate many of the same signaling pathways that contribute to malignancy, such as those mediated by Ras, PI3K, src-family kinases, and Stat transcription factors (reviewed in refs. [234, 235]), our data indicate that modulation of EphA2 signaling represents an important molecular distinction between these pathways to malignancy.

EphA2 RTK: oncogene or tumor suppressor?

The contradictory observations that EphA2 may promote tumorigenesis under some circumstances (e.g., ErbB2-expressing breast tumors) and fail to influence tumor progression under others (e.g., PyV-mT–expressing tumors), or may even prevent tumor formation (e.g., carcinogen-induced skin cancers; ref. [113]), may be reconciled by the following model that we propose. Under physiologic conditions, epithelial cells form adherens junction, permitting ephrins to interact with EphA2s on adjacent cells. Ligand stimulation induces receptor endocytosis and degradation, keeping EphA2 levels low. Upon tumor initiation, EphA2 expression is upregulated. Elevated EphA2 can be phosphorylated by other RTKs, such as ErbB2, independent of ligand stimulation, leading to enhanced cell proliferation and tumorigenesis. Thus, ligand stimulation may play an antitumorigenic role by downregulation of EphA2, while in the absence of ligand, EphA2 crosstalk with other receptors may promote tumorigenesis. This model is supported by several lines of experimental evidence. First, ligand stimulation has been shown to downregulate EphA2 through endocytosis [40, 116, 166] as well as Cbl ubiquitin ligase–mediated proteasome degradation [40, 170, 171]. Second, adenoviral delivery of ephrin-A1 [40] and EphA2 activating antibodies have been shown to be effective in inhibition of malignant cell behavior in vitro and in treating malignant ovarian tumors in vivo [131, 134]. Third, we have shown that ErbB-2 interacted with EphA2 physically in MMTV-Neu tumor cells and that EphA2 was phosphorylated by activated ErbB2. Fourth, ablation of EphA2 by gene targeting inhibited ErbB2-induced mammary tumorigenesis. Thus, EphA2 remains an important therapeutic target, and

downregulation of EphA2 expression or inhibition of EphA2 signaling could lead to tumor inhibition.

EphA2 as a therapeutic target

Although EphA2 is overexpressed in a wide variety of tumors, including breast adenocarcinomas, our data suggest that overexpression in and of itself does not necessarily indicate an active role in tumorigenesis. Significant levels of EphA2 overexpression were documented in tumors arising in both MMTV-Neu and MMTV–PyV-mT models of mammary carcinogenesis in this study. However, while deletion of EphA2 significantly impaired tumor initiation and progression in MMTV-Neu animals, there was no effect of EphA2 deficiency on tumor progression in the MMTV–PyV-mT model, which expressed only moderate levels of ErbB2. Thus, the functional consequences of EphA2 overexpression depend upon the context of coexpressed oncogenes. Therefore, effective therapeutic targeting of EphA2 requires an understanding of how EphA2 cooperates with and functionally influences coexisting oncogenic signaling networks within specific tumor types. For example, while downregulation of EphA2 protein levels showed efficacy against human ovarian tumor xenografts [131], an independent, similarly designed antibody reagent had no effect on CT26 human colon cancer xenografts or human mammary adenocarcinoma xenografts [236]. Interestingly, like MMTV–PyV-mT tumor cells, CT26 cells do not overexpress ErbB2/HER2 [237], suggesting that EphA2 overexpression enhances malignant transformation and progression particularly in the context of ErbB2 overexpression and is therefore an appropriate target in such tumors.

While EphA2 overexpression has been reported in a variety of human epithelial cancers, including more than 80% of breast cancer clinical samples, HER2 overexpression is observed in only 30% of human breast cancers [238]. Moreover, no correlation was reported between EphA2 and HER2 expression in a recent screen of 134 human breast cancer specimens. Our data demonstrated that EphA2 interacted with ErbB2. Other EGFR family members, including EGFR/ErbB1 and an EGFR variant (EGFRvIII, a constitutively active deletion mutant implicated in carcinogenesis; refs. [239, 240]), have also been shown to physically and functionally interact EphA2 [42]. Overexpression of EGFR and EGFRvIII has been reported in a broader subset of human breast cancers, with as many as 48% of cases analyzed reported to be positive for EGFR expression [241-246]. Thus, EphA2 may act in concert with the EGFR family of RTKs in general, and not exclusively with ErbB2, to enhance proliferation and malignant progression. Functional interaction between EphA2 and EGFR as well as ErbB2 may be required for breast tumor growth and progression. Further investigation of the relationship between EphA2 and EGFR family members may provide a sound rationale for targeting EphA2 in breast cancers that display amplification and/or activation of 1 or more of this family of RTKs.

Conclusions

Our data suggest that the role of EphA2 RTK in cancer is context dependent, as EphA2 deficiency impairs tumor progression in MMTV-Neu, but not MMTV–PyV-mT, transgenic models of mammary epithelial adenocarcinoma. We provide evidence that EphA2 physically and functionally interacts with ErbB2 to amplify Ras/MAPK and RhoA signaling in tumor cells. Ras/MAPK contributes to cell proliferation, while

activated Rho GTPase is required for tumor cell motility. Together, these results indicate that EphA2 cooperates with ErbB2/Neu to promote tumor progression and may be a novel target for tumors that are dependent upon ErbB receptor signaling.

CHAPTER IV

ELEVATION OF RECEPTOR TYROSINE KINASE EPHA2 MEDIATES RESISTANCE TO TRASTUZUMAB THERAPY

The work presented in this chapter is published with the same title in *Cancer Research*, Jan 2010 [247].

Abstract

One arising challenge in the treatment of breast cancer is the development of therapeutic resistance to trastuzumab, an antibody targeting the human epidermal growth factor receptor-2 (HER2), which is frequently amplified in breast cancers. In this study, we provide evidence that elevated level of the receptor tyrosine kinase Eph receptor A2 (EphA2) is an important contributor to trastuzumab resistance. In a screen of a large cohort of human breast cancers, we found that EphA2 overexpression correlated with a decrease in disease-free and overall survival of HER2-overexpressing patients. Trastuzumab-resistant cell lines overexpressed EphA2, whereas inhibiting EphA2 restored sensitivity to trastuzumab treatment in vivo. Notably, trastuzumab treatment could promote EphA2 phosphorylation by activating Src kinase, leading in turn to an amplification of phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase signaling in resistant cells. Our findings offer mechanistic insights into the basis for trastuzumab resistance and rationalize strategies to target EphA2 as a tactic to reverse trastuzumab resistance.

Introduction

Recent advances in the development and application of molecularly targeted therapies for cancer have generated promising new treatments. One such treatment is the recombinant humanized monoclonal anti-HER2 antibody trastuzumab (Herceptin, Genentech). Trastuzumab targets the human epidermal growth factor receptor-2 (HER2/ErbB2) oncoprotein [248], a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTK). HER2 is overexpressed in 25% to 30% of human breast cancers and is associated with poor patient survival [249]. Despite the proven benefit of trastuzumab in treating breast cancer [250-252], not all patients with amplified HER2 respond to trastuzumab. Indeed, only one third of women with newly diagnosed HER2-positive breast cancer exhibit tumor regression with trastuzumab monotherapy [252]. In addition, the majority of patients who achieve an initial response develop trastuzumab resistance within 1 year [252, 253]. Therefore, identifying mechanisms that modulate trastuzumab response and resistance is vital to improving the therapeutic index of this agent.

Eph receptor A2 (EphA2), an Eph-family RTK, has been recently linked to breast tumor initiation and metastatic progression [25, 26, 254]. Experimentally induced overexpression of EphA2 resulted in malignant transformation of nontransformed MCF10A breast epithelial cells and enhanced malignancy of pancreatic carcinoma cells [74, 75]. Conversely, small interfering RNA (siRNA)–mediated inhibition of EphA2 expression impaired the malignant progression of pancreatic, ovarian, and mesothelioma human tumor cell lines, and overexpression of dominant-negative EphA2 constructs

suppressed the growth and metastasis of 4T1 mouse mammary adenocarcinoma cells in vivo [75-78]. EphA2-mediated oncogenesis seems to be ligand independent, and EphA2 often signals through cross talk with other cell surface receptors [27, 42]. We recently reported that loss of EphA2 receptor impaired tumor initiation and metastatic progression in mouse mammary tumor virus (MMTV)-Neu mice [201]. In human and murine breast carcinoma cells, EphA2 forms a complex with HER2, resulting in enhanced activation of Ras-mitogen-activated protein kinase (MAPK) and RhoA GTPase and increased cell proliferation and motility. These data indicate that EphA2 promotes breast tumor formation and metastatic progression by amplifying HER2 signaling.

In this report, we investigated the role of EphA2 in regulation of breast cancer sensitivity to trastuzumab. We found that high EphA2 levels enhanced both intrinsic and acquired trastuzumab resistance. Elevated EphA2 in resistant cells seems to be activated by trastuzumab treatment–induced Src kinase, and activated EphA2 amplifies signaling through the phosphoinositide 3-kinase (PI3K)/Akt and MAPK pathways in resistant cells. In addition, microarray analysis of a large cohort of human breast cancer specimens revealed that high levels of EphA2 expression in HER2-positive patients predict poor prognosis. Thus, these results provide new mechanistic insights into the molecular basis of anti-HER2 resistance, and targeting EphA2 could represent an appealing therapeutic strategy to increase the efficacy of HER2-based treatments in breast cancer.

Methods

Survival analysis

The van der Vijver database, with microarray profiles of 295 human breast tumors and associated clinical data, was obtained from Rosetta Inpharmatics.8 The first 25% patients that exhibit higher HER2 expression were defined as HER2 positive, as described [255-257]. The HER2-positive patients were further stratified into two groups based on the expression levels of EphA2. Kaplan-Meier analyses were computed using R survival package. Statistical differences were determined by log-rank tests.

Cell culture

The MMTV-Neu tumor–derived cell line [220], parental MCF10A cells, and MCF10A cells stably overexpressing HER2 were maintained as described previously [201]. Parental and trastuzumab-resistant SK-BR-3 and BT-474 cells were generously provided by Francisco Esteva (The University of Texas M.D. Anderson Cancer Center, Houston, TX; ref. [258]) and Carlos Arteaga (Vanderbilt University, Nashville, TN; ref. [259]), respectively. Three-dimensional spheroid cultures were established on Matrigel as described [224]. Cultures were maintained for 8 d before photodocumentation. Digital images were analyzed and the percentage of Ki67-positive cells was quantified using LSM Image Browser (Zeiss) software. Results were derived from 10 colonies in two independent experiments. Statistical differences among groups were determined by Student's t test.

Mice and in vivo tumor studies

Athymic nude female mice, 3 to 4 wk old, were implanted with 1.5-mg, 60-d-release 17β-estradiol pellets s.c. The next day, trastuzumab-resistant BT-474 cells (1.5×10^7; HR5) were resuspended in 100 μL PBS/100 μL growth factor–reduced Matrigel and injected into the number 4 inguinal mammary gland fat pad as previously described [259]. Tumor engraftment and growth was verified by palpation and tumor volume was measured by a caliper. Two weeks after transplantation, the mice were treated with control IgG (10 mg/kg; clone R347, MedImmune, LLC), anti-EphA2 antibody (10 mg/kg; clone 3F2-3M, MedImmune, LLC), trastuzumab (20 mg/kg), or the combination of anti-EphA2 antibody and trastuzumab by twice-weekly i.p. injections. Tumors were harvested 2 wk after treatment and data were derived from 10 independent animals per treatment group in two independent experiments.

Histologic analyses

Tumors were sectioned by the Vanderbilt University Immunohistochemistry Core Facility. Immunohistochemical staining for EphA2, proliferating cell nuclear antigen (PCNA), and CD31 was done as described previously [93]. Proliferation or apoptosis was quantified by calculating the average percentage of PCNA- or terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive nuclei relative to total nuclei (four random fields of at least four independent tumor samples).

Fluorescence resonance energy transfer analysis of Src biosensor

The MCF7 cells expressing HER2 were transfected with Src biosensor (generously provided by Yingxiao Wang, University of Illinois, Urbana-Champaign, IL) and serum starved for 48 h before being treated with trastuzumab (10 μg/mL). Imaging and fluorescence resonance energy transfer (FRET) analysis were done on an LSM 510 META confocal microscope (Zeiss) using a 40x/1.3 NAPlan-Neofluar objective lens and 458-nm laser excitation for cyan fluorescent protein (CFP) and FRET. Emission from CFP versus yellow fluorescent protein (YFP)/FRET was discriminated using appropriate bandpass emission filters (BP 475-525 for CFP and LP560 for YFP/FRET). The fluorescence intensities of CFP and YFP images were measured using the Zeiss Image Examiner software before being quantified and analyzed by Prism 5 (GraphPad). Quantification was based on 20 cells per time point in two independent experiments. Statistical differences were analyzed using Student's t test.

Results

Overexpression of EphA2 in HER2-positive patients predicts poor prognosis

Because our previous investigations in mouse models suggest that cooperation between HER2 and EphA2 may promote mammary tumor formation, we sought to determine if EphA2 could be an effective therapeutic target for HER2-positive breast cancer patients. To analyze the effect of EphA2 overexpression on the prognosis of HER2-positive breast cancer patients, we examined previously published microarray data for a panel of 295 breast cancer samples [260]. Seventy-four HER2-positive samples were examined for

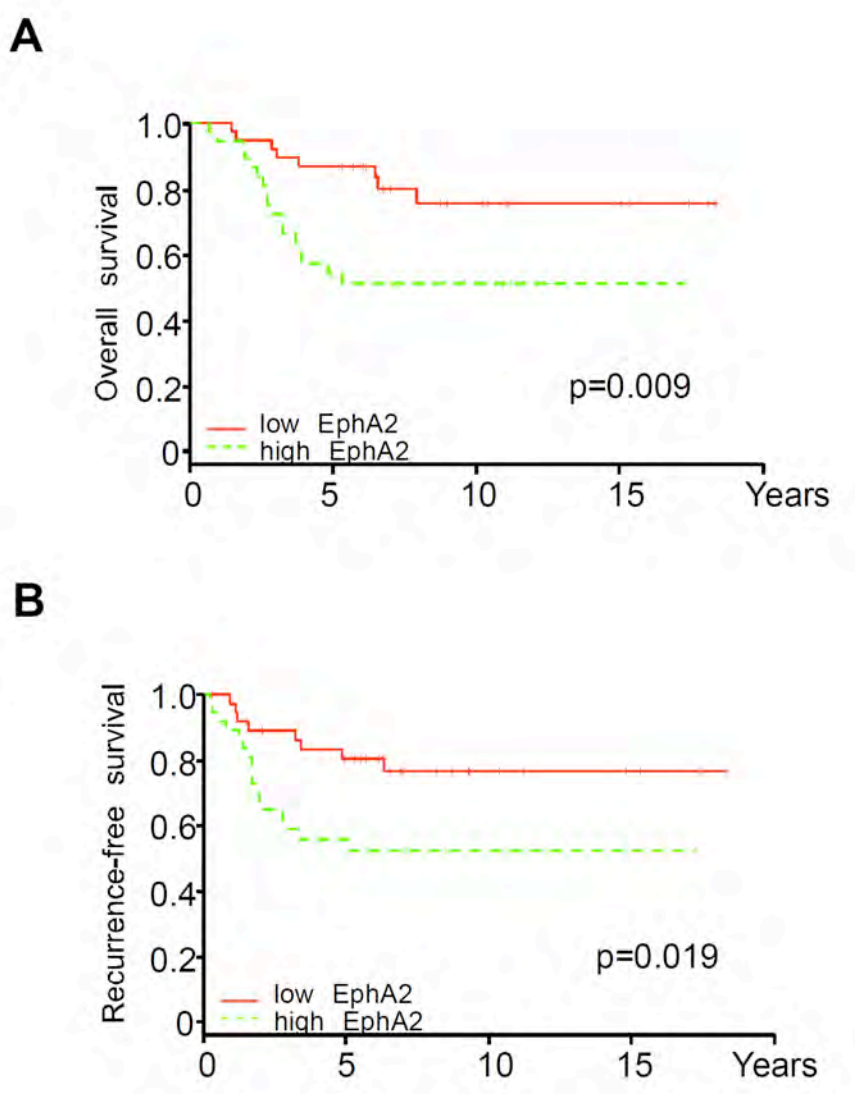


Figure 4.1. Overexpression of EphA2 in HER2-positive patients predicts poor prognosis.

A previously published microarray data set from the fresh-frozen tissue bank of the Netherlands Cancer Institute for a panel of 295 breast cancer samples was analyzed. The resulting Kaplan-Meier kinetic analyses of survival data revealed that high levels of EphA2 mRNA expression correlated with a decrease in overall survival (**A;** P = 0.009) and recurrence-free survival (**B;** P = 0.019).

EphA2 mRNA expression. The resulting Kaplan-Meier analysis of survival data revealed that high levels of EphA2 expression correlated with a decrease in overall (Fig. 4.1A) and recurrence-free survival (Fig. 4.1B) in HER2-positive breast cancer patients. These data indicate that EphA2 overexpression in HER2-positive patients may predict poor prognosis, and elevated EphA2 may enable breast cancer cells to resist anti-HER2 treatment.

EphA2 overexpression confers cellular intrinsic resistance to trastuzumab

To investigate whether EphA2 overexpression is sufficient to confer resistance to trastuzumab, we transduced a constitutively activated (CA-EphA2) or a kinase-dead (KD-EphA2) form of human EphA2 into MCF10A.HER2 cells [261]. MCF10A.HER2 cells formed large acinar-like structure with a filled lumen and were sensitive to trastuzumab treatment (Fig. 2A; ref. [228]). Introduction of CA-EphA2 into in MCF10A.HER2 cells further enhanced cell proliferation, but this increased cell growth in MCF10A.HER2 cells expressing CA-EphA2 was refractory to trastuzumab (Fig. 4.2A and B). In contrast, expression of catalytically inactive KD-EphA2 in MCF10A.HER2 cells decreased the basal rates of proliferation, which were further decreased on treatment with trastuzumab (Fig. 4.2A and B). These data are consistent with previous data showing cooperation between HER2 and EphA2 to drive cellular proliferation [201], and further suggest that EphA2 kinase activity is able to promote trastuzumab resistance in HER2-overexpressing breast cells.

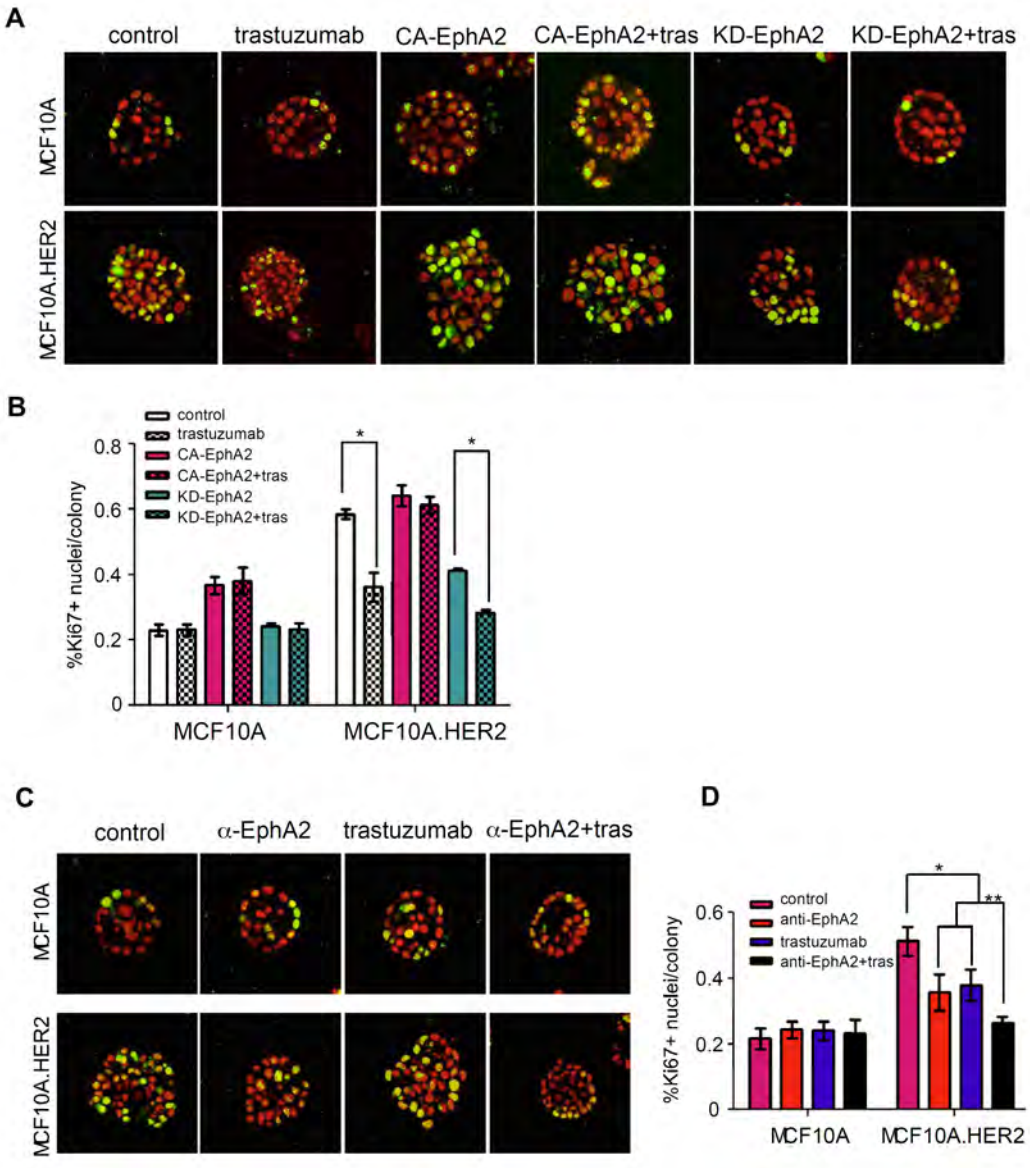


Figure 4.2. EphA2 overexpression confers cellular intrinsic resistance to trastuzumab.

A. constitutively activated (CA-EphA2) or kinase-dead (KD-EphA2) EphA2 receptor were introduced into MCF10A or MCF10A.HER2 cells by retroviral transduction. tras, trastuzumab. Pooled G418-resistant cell populations were cultured in three-dimensional Matrigel and stained for Ki67 (green) to assess proliferation and counterstained for To-Pro-3 (red) to visualize nuclei. Overexpression of CA-EphA2, but not KD-EphA2, desensitizes MCF10A.HER2 cells to trastuzumab. Cell proliferation was quantified in **B.** *, $P < 0.01$, Student's t test. **C.** MCF10A or MCF10A.HER2 cells were cultured in three-dimensional Matrigel and treated with antibodies as indicated. Anti-EphA2 antibody inhibited cell growth in MCF10A.HER2 cells. Cell proliferation in C is qualified in **D.** *, $P < 0.01$; **, $P < 0.05$, Student's t test.

Interestingly, MCF10A.HER2 cells express elevated levels of EphA2 protein relative to those in parental MCF10A cells (data not shown). To determine if inhibition of EphA2 increases innate sensitivity to trastuzumab, MCF10A.HER2 cells were treated with an antihuman EphA2 antibody, a ligand-mimetic activating antibody that specifically binds to EphA2 and induces receptor internalization and degradation. Whereas the anti-EphA2 antibody had no effect on nontransformed MCF10A cells that express low levels of EphA2, the antibody significantly inhibited cell growth in MCF10A.HER2 cells. More importantly, the combination of anti-EphA2 antibody and trastuzumab inhibited cell growth with greater potency than either antibody alone (Fig. 4.2C and D). Taken together, these data suggest that EphA2 overexpression is one mechanism of intrinsic resistance to trastuzumab.

As an independent approach to determine whether EphA2 expression levels correlate with trastuzumab resistance, we overexpressed HER2 in a panel of human breast cancer cell lines that express EphA2 protein at low or high levels (Fig. 4.3A). BT-474 and SK-BR-3 cells that express high levels of endogenous HER2 but low levels of EphA2 were growth inhibited in response to trastuzumab, and so were MCF7 and T47D that overexpress HER2 (Fig. 4.3B). In contrast, HBL100, MDA-468, MDA-231, and BT-549 expressed high levels of EphA2 and were resistant to the growth inhibitory effects of trastuzumab. These data are consistent with a correlation between EphA2 expression and trastuzumab response in HER2-overexpressing human breast cancer cells.

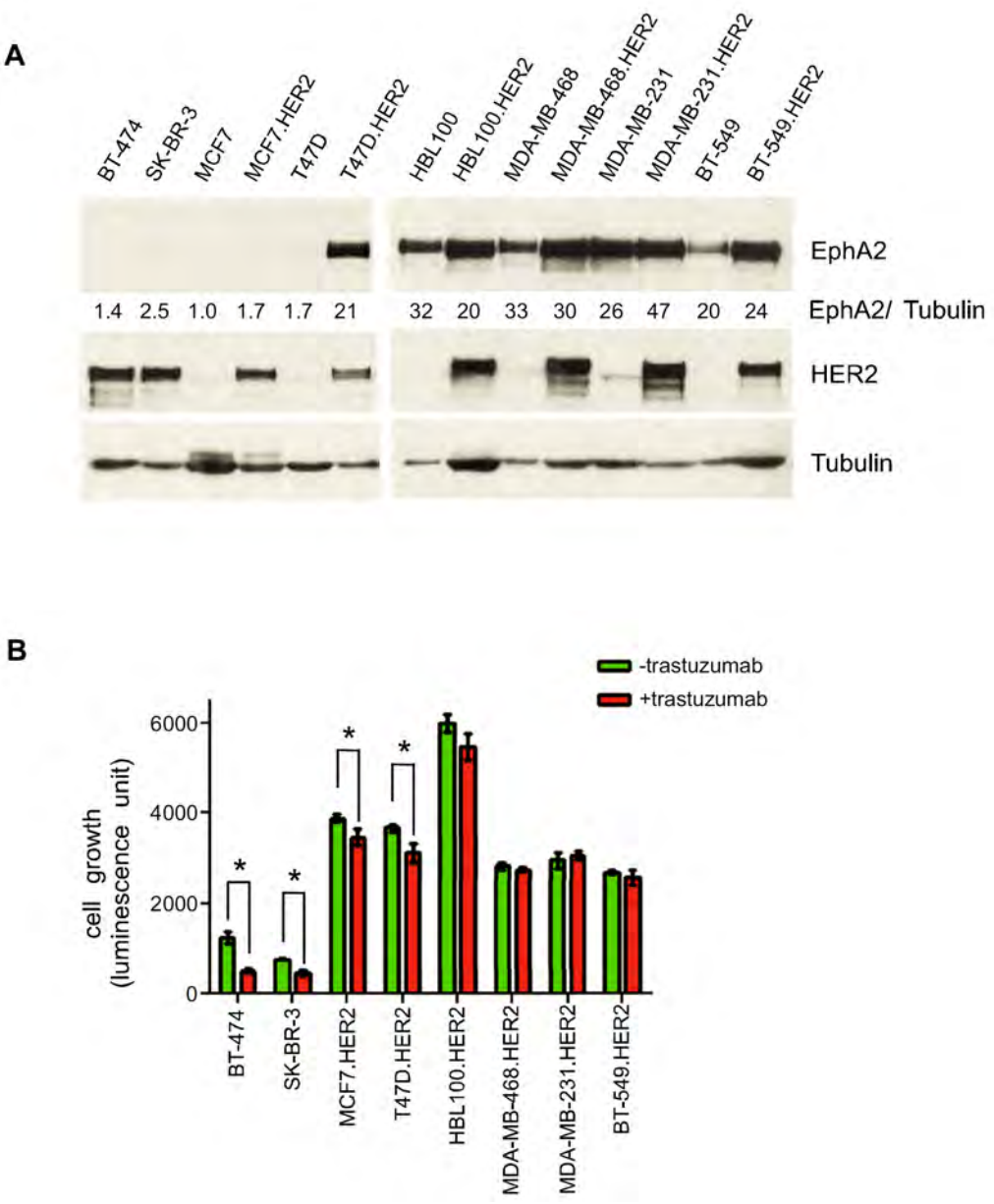


Figure 4.3. EphA2 expression levels correlate with trastuzumab resistance.

A. Human breast cancer cell lines expressing low or high levels of EphA2 were transduced with pBABE retrovirus expressing HER2. Expression of EphA2 and overexpression of HER2 in these cell lines were confirmed by western blot. **B.** The effect of trastuzumab on cell growth of above human breast cancer cell lines was determined by CellTiter-Glo luminescent cell viability assay. * $P<0.05$; Student's t test.

EphA2 elevation contributes to acquired trastuzumab resistance

Genome-wide profiling of gene expression showed that EphA2 and HER2 are not always coexpressed in human breast cancer. We reasoned that on prolonged trastuzumab treatment, a subset of HER2-positive tumors that initially express low levels of EphA2 and respond to trastuzumab may increase EphA2 expression, leading to a decrease in trastuzumab sensitivity. To test this possibility, we analyzed EphA2 expression in two independent trastuzumab-resistant human breast cancer cell lines, SK-BR-3 and BT-474, which were derived from in vitro or in vivo selection for acquired resistance to trastuzumab, respectively [258, 259]. As shown in Fig. 4.4A, EphA2 levels were considerably higher in two independently derived trastuzumab-resistant clones from each cell line relative to their trastuzumab-sensitive parental cells. To test whether this EphA2 overexpression is required to maintain trastuzumab resistance, we treated the parental and the trastuzumab-resistant cells with anti-EphA2 antibody in the presence or absence of trastuzumab. As expected, sensitive SK-BR-3 and BT-474 cells were growth inhibited by trastuzumab whereas resistant cells were not. Anti-EphA2 antibody alone did not significantly affect cell growth in SK-BR-3 or BT-474 cells. However, EphA2 inhibition restored cellular sensitivity to trastuzumab in each resistant cell line, as shown in both two-dimensional cell culture (Fig. 4.4B) and three-dimensional Matrigel culture (Fig. 4.4C). These data suggest that EphA2 is upregulated in treatment-induced, trastuzumab-resistant cells and that high levels of EphA2 in resistant cells contribute to acquired trastuzumab resistance.

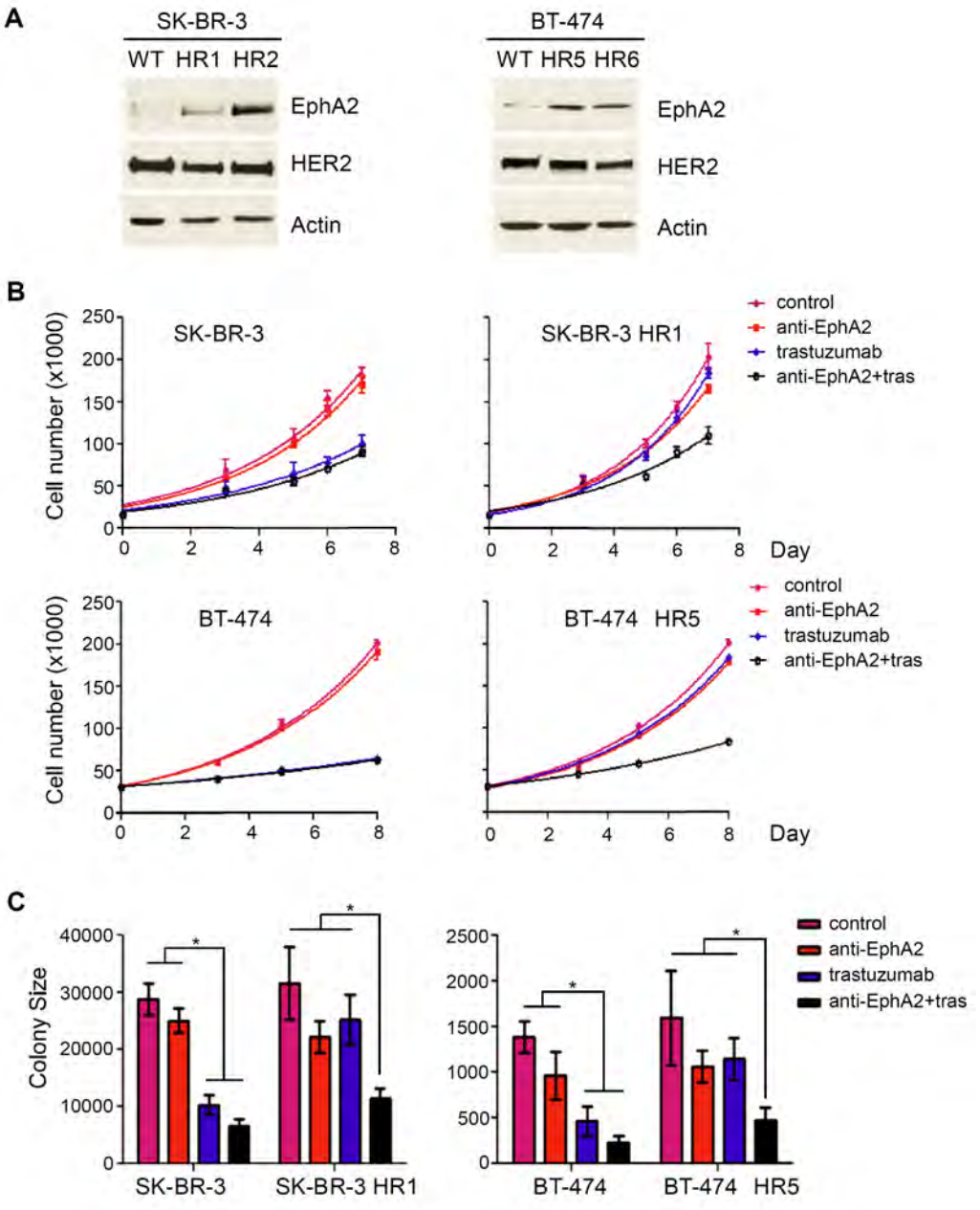


Figure 4.4. EphA2 elevation contributes to acquired trastuzumab resistance.

A. trastuzumab-sensitive (WT) or trastuzumab-resistant (HR) SK-BR-3 or BT-474 cells were subjected to Western blot analysis to assess EphA2 expression levels. **B.** sensitive or resistant SK-BR-3 or BT-474 cells were treated with IgG control, anti-EphA2, trastuzumab, or the combination of anti-EphA2 antibody and trastuzumab. Anti-EphA2 antibody restores cellular sensitivity to trastuzumab. **C.** sensitive or resistant SK-BR-3 or BT-474 cells were cultured in three-dimensional Matrigel. Colonies were photographed at day 7 and colony size was quantified. *, $P < 0.01$, Student's t test.

Targeting EphA2 inhibits trastuzumab-resistant tumor growth in vivo

Having shown the combinatorial activity of anti-EphA2 antibody and trastuzumab for growth inhibition of trastuzumab-resistant cells in vitro, we next investigated the therapeutic potential of an anti-EphA2 antibody for the treatment of trastuzumab-resistant tumor growth in vivo in an orthotopic xenograft model. Trastuzumab-resistant BT-474 cells were injected into the mammary fat pad of female athymic nude mice. Two weeks after transplantation, when tumor volume reached ~200 mm^3, mice were treated with either control IgG or antihuman EphA2 antibody (10 mg/kg) in the presence or absence of trastuzumab (20 mg/kg). Consistent with a prior report [259], resistant BT-474 tumors did not respond to trastuzumab treatment as compared with IgG-treated tumors. Anti-EphA2 antibody treatment moderately reduced tumor size relative to controls. In contrast, coadministration of anti-EphA2 antibody with trastuzumab markedly reduced tumor volume (Fig. 4.5A and B).

To examine cellular changes within treated tumors, we analyzed cell proliferation and apoptosis in tissue sections by staining for PCNA and by TUNEL assay, respectively. Quantitation of PCNA-positive nuclei revealed a nearly 2-fold decrease in PCNA staining in tumors treated with the combination of anti-EphA2 antibody versus tumors treated with control IgG ($P < 0.05$; Fig. 4.5C). In contrast, treatment with anti-EphA2 antibody alone or with trastuzumab alone did not significantly alter the proportion of PCNA-positive cells as compared with IgG-treated tumors. Similarly, apoptosis was increased ~6-fold in tumors treated with the combination of anti-EphA2 antibody and trastuzumab ($P < 0.01$; Fig. 4.5C) but was unaltered in tumors treated with either

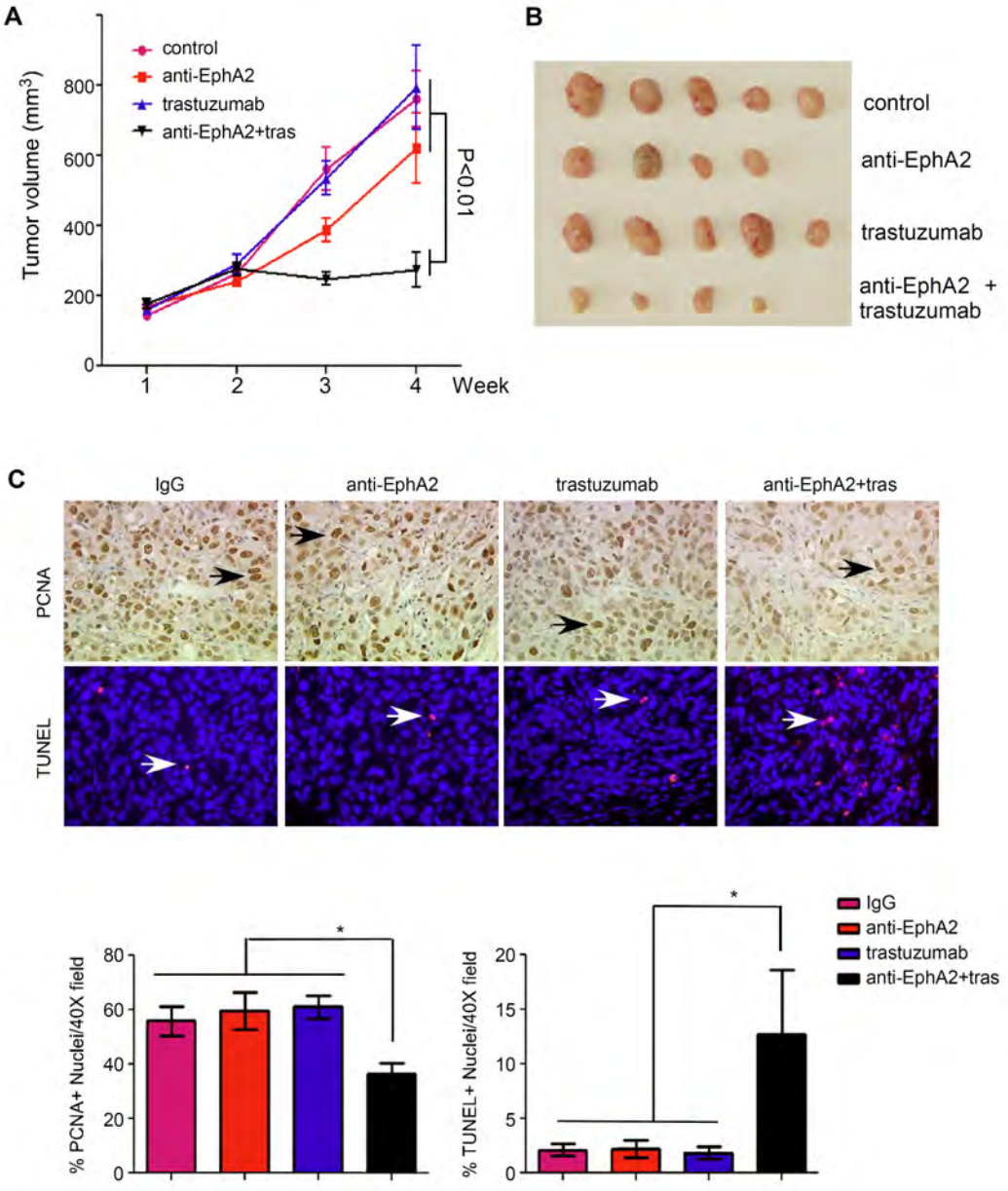


Figure 4.5. Targeting EphA2 inhibits trastuzumab-resistant tumor growth.

A. trastuzumab-resistant BT-474 cells were orthotopically transplanted into the mammary glands of nude female mice. Two weeks after transplantation, tumors were treated with control IgG, anti-EphA2 antibody (10 mg/kg), trastuzumab (20 mg/kg), or the combination of anti-EphA2 antibody and trastuzumab twice weekly via i.p. injection. Points, mean of 10 mice per treatment group from two independent experiments; bars, SEM. **B.** tumors were harvested and photographed. **C.** cell proliferation and apoptosis in tumor sections were evaluated by PCNA immunohistochemistry and TUNEL assay, respectively. *, $P < 0.01$. Arrowheads, PCNA- or TUNEL-positive nuclei.

antibody alone. Taken together, these data suggest that targeting EphA2 may be effective for the suppression of trastuzumab-resistant breast tumor growth.

EphA2 regulates breast cancer cell sensitivity to trastuzumab by modulation of Akt and MAPK activities

Breast cancer resistance to HER2 inhibitors could arise through multiple mechanisms, including activation of alternative growth factor receptors or enhancing downstream signaling pathways. We investigated potential mechanisms by which EphA2 contributes to trastuzumab resistance in HER2-overexpressing breast cancer. We found that elimination of EphA2 by siRNA knockdown or anti-EphA2 antibody reduced phospho-Akt and phospho-extracellular signal-regulated kinase (Erk) levels in trastuzumab-resistant cells (Fig. 4.6A and B), suggesting that EphA2 expression and activity are required to maintain signaling through the PI3K-Akt and MAPK signaling pathways.

To determine whether the PI3K-Akt and Ras-MAPK signaling pathways play a causal role in trastuzumab resistance, we treated SK-BR-3 cells with a PI3K inhibitor, LY294002 (Fig. 4.6C), or a mitogen-activated protein/Erk kinase (MEK) inhibitor, U0126 (Fig. 4.6D), and analyzed cell growth in the presence or absence of trastuzumab. In sensitive cells, cell growth is inhibited by trastuzumab, and addition of LY294002 or U0126 did not further affect cell growth significantly. However, although resistant cells do not respond to trastuzumab, they are exquisitely sensitive to the MEK inhibitor (Fig. 4.6D). In fact, resistant cells are more sensitive to U0126 than trastuzumab-sensitive cells, suggesting that trastuzumab-resistant cells are dependent on MAPK signaling. In addition,

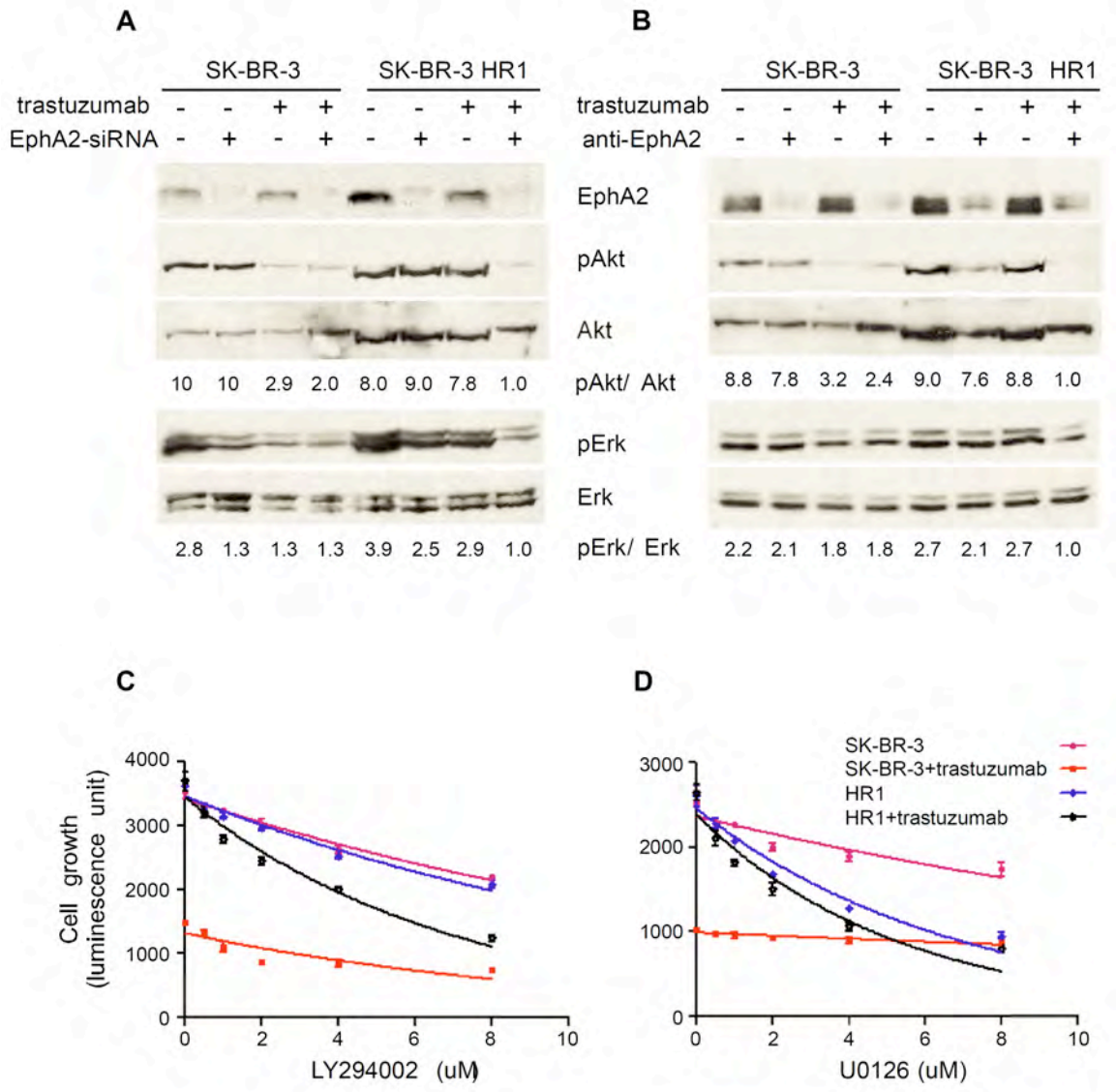


Figure 4.6. EphA2 regulates breast cancer sensitivity to trastuzumab by modulation of Akt and MAPK activity.

A. EphA2 was knocked down by siRNA in either parental or trastuzumab-resistant (HR1) SK-BR-3 cells. EphA2, phosphor-Akt, and phospho-Erk levels were assessed by Western blot analysis. The ratio of phospho-protein/total protein was determined by densitometry and expressed in arbitrary units. **B.** trastuzumab-sensitive or trastuzumab-resistant cells were treated with control, trastuzumab, anti-EphA2 antibody, or the combination of trastuzumab and anti-EphA2 antibody in the presence of 10% serum. Quantification of phospho-protein/total protein was determined as above. **C.** and **D.** trastuzumab-sensitive or trastuzumab-resistant (HR1) SK-BR-3 cells were treated with increasing dose of either PI3K inhibitor LY294002 or MEK inhibitor U0126 for 3 d and cell viability was determined.

either PI3K inhibitor or MEK inhibitor significantly restored trastuzumab sensitivity in resistant cells. Together, our data suggest that anti-EphA2 antibody therapy reverses trastuzumab resistance by inhibiting the activation of both Akt and MAPK.

Chronic trastuzumab treatment activates EphA2 through Src kinase

To investigate how EphA2 is activated in trastuzumab-resistant cells, we examined the involvement of Src kinase because prior studies showed that Src directly interacts with HER2 and is activated in HER2-overexpressing cancer cells [262, 263]. Coexpression of HER2 and EphA2 in COS7 cells was sufficient to induce tyrosine phosphorylation of EphA2, and this process was inhibited by a Src inhibitor, PP2. In addition, constitutively activated v-Src induced phosphorylation of EphA2 independently of HER2 (Fig. 4.7A), suggesting that HER2 may modulate EphA2 activity through Src. We next investigated whether Src can be activated by trastuzumab. A previous study suggested that short exposure to trastuzumab rapidly inhibits Src kinase activity [264]. However, we found that longer treatment of SK-BR-3 cells with trastuzumab increased Src phosphorylation at Y416, an indicator of Src activation (Fig. 4.7B). To further determine whether prolonged trastuzumab treatment can activate Src kinase, we used a Src biosensor that enables the visualization of Src activity in live cells with high spatiotemporal resolution by FRET technology [265, 266]. Trastuzumab induced a 15% to 25% reduction in Src activity within 1 hour in MCF7.HER2 cells transfected with the Src biosensor, but the decrease in Src activity gradually recovered with prolonged trastuzumab incubation (Fig. 4.8A). After 24 hours of treatment, Src activity increased by 35% in MCF7.HER2 relative to control cells (Fig. 4.8B), whereas EphA2 levels were not changed (data not

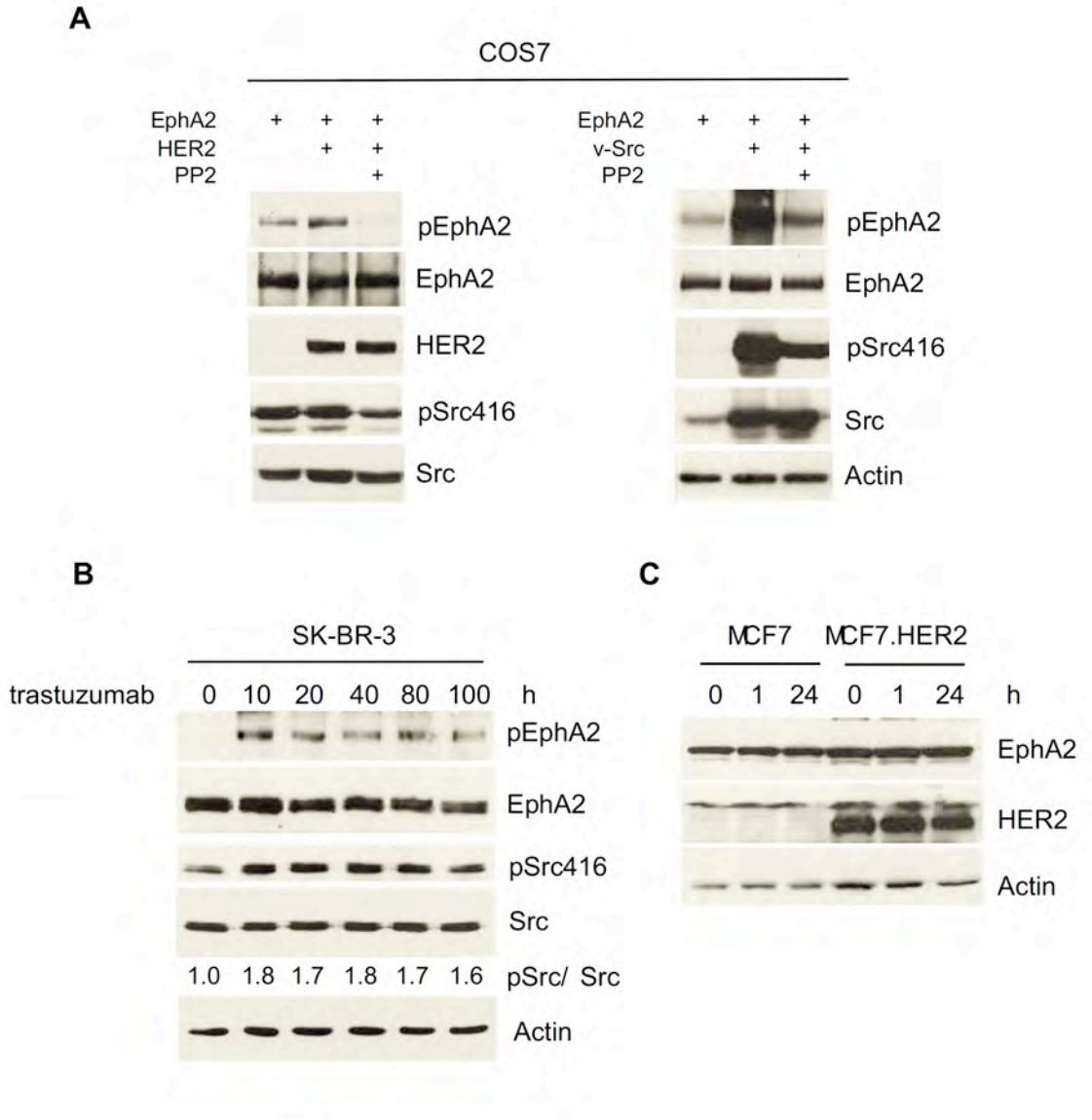


Figure 4.7. Chronic trastuzumab treatment activates EphA2 through Src kinase.

A. COS7 cells were transiently transfected with EphA2 alone or in combination with HER2 or v-Src. EphA2 immunoprecipitate was subjected to western blot analysis to assess EphA2 phosphorylation. Phosphorylation of EphA2 is increased in the presence of HER2 or v-Src. Src inhibitor, PP2, inhibited EphA2 phosphorylation induced by either HER2 or v-Src. **B.** Activation of Src and EphA2 in SK-BR-3 cells with long-term trastuzumab treatment was assessed by western blot analysis. **C.** EphA2 levels in MCF7 cells with trastuzumab treatment were assessed by western blot analysis.

shown). These data support the existence of a switch from trastuzumab-induced Src inhibition to activation, which could modulate EphA2 activity in resistant cells. Indeed, EphA2 and Src were highly phosphorylated in trastuzumab-resistant cells. Src inhibitors, PP2 (Fig. 4.8C) or dasatinib (data not shown), inhibited the activities of both Src and EphA2.

To determine whether Src kinase contributes to trastuzumab resistance, we treated SK-BR-3 cells with trastuzumab, dasatinib, or their combination and assessed cell viability. Dasatinib inhibited cell growth in both sensitive and resistant cells. Whereas resistant cells did not respond to trastuzumab, dasatinib partially restored trastuzumab sensitivity in resistant cells (Fig. 4.8D). Together, these results provide a clear link between activation of Src and EphA2 in trastuzumab resistance.

Discussion

In this report, we described a novel mechanism by which HER2-positive breast cancers acquire resistance to trastuzumab. The RTK EphA2 was found to correlate with a poor prognosis in patients with HER2-overexpressing breast cancers and had a greater negative impact on patient survival in HER2-overexpressing breast cancers as compared with other breast cancers. We found that overexpression of EphA2 in HER2-positive breast cancer cells was sufficient to confer innate resistance to trastuzumab. Furthermore, antibody-mediated EphA2 inhibition enhanced tumor response to trastuzumab both in cell culture and in vivo. These data suggest that therapeutic inhibition of EphA2 may represent a strategy for improving the clinical response of trastuzumab.

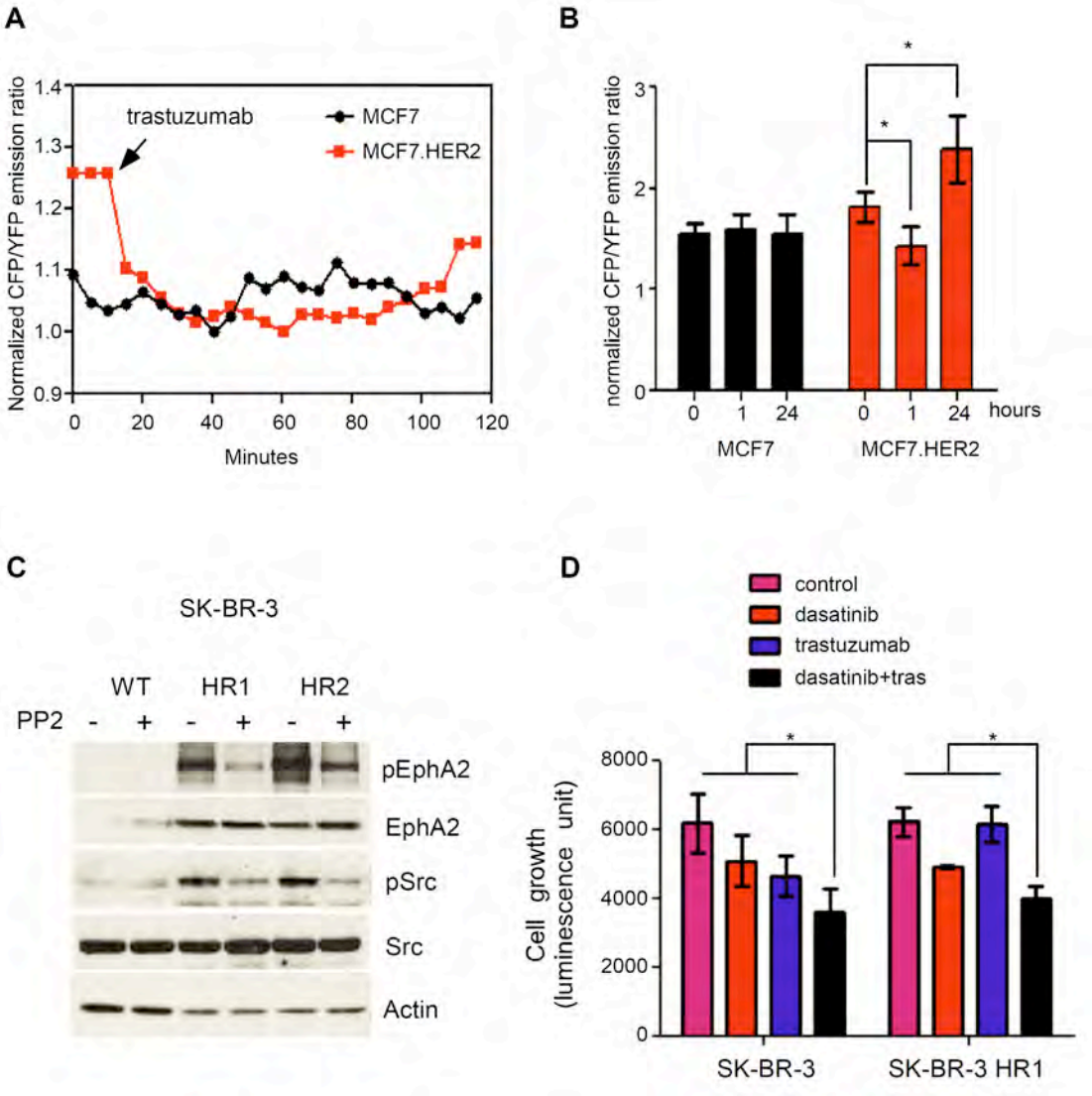


Figure 4.8. Trastuzumab treatment activates EphA2 through Src kinase.

A. MCF7.HER2 cells expressing Src reporter were treated with trastuzumab over a time course. The normalized CFP/YFP emission ratio of the Src biosensor over time in response to trastuzumab is shown. **B.** MCF7.HER2 cells expressing Src reporter were treated with trastuzumab for 0, 1, and 24 h. Changes in CFP/YFP emission ratio were quantified in 20 cells per experimental group at given time point. *, $P < 0.01$. **C.** sensitive (WT) or resistant (HR) SK-BR-3 cells were treated with Src inhibitor PP2 or vehicle control. Phospho-Src and phospho-EphA2 levels were assessed by immunoprecipitation and Western blot analysis. **D.** trastuzumab-sensitive or trastuzumab-resistant SK-BR-3 cells were treated with control, dasatinib, trastuzumab, or the combination of dasatinib and trastuzumab for 3 d. Cell growth was determined by luminescent cell viability assay. *, $P < 0.05$, Student's t test.

What is the mechanism by which elevated EphA2 confers tumor cell resistance to trastuzumab?

Resistance to anti-HER2/ErbB2 agents could arise through multiple mechanisms, including altered receptor-antibody interaction, activation of alternative growth factor receptor signaling pathways, and deregulation of downstream signaling pathways [267, 268]. The most common downstream signaling pathway that contributes to trastuzumab resistance is the PI3K-Akt pathway. Persistent activation of PI3K-Akt signaling in resistant cells could result from multiple mechanisms such as oncogenic mutations of PI3K [269], loss of PTEN [264], or upregulation of insulin-like growth factor-I receptor and EGFR activity [259, 270]. In this case, targeting EphA2 inhibited the PI3K-Akt pathway in trastuzumab resistant cells (Fig. 4.6). In addition to regulating Akt activity, we discovered that EphA2 also modulates phospho-Erk levels in resistant cells. Increased EphA2 expression in resistant cells enhanced phospho-Erk levels, and targeting EphA2 with siRNA or anti-EphA2 antibody inhibited Erk activity (Fig. 4.6). These data, together with reports from other laboratories [271, 272], suggest that the development of trastuzumab resistance may involve simultaneous activation of multiple parallel signaling cascades including the PI3K-Akt and MAPK pathways [273-275]. Indeed, a MEK inhibitor that suppresses phospho-Erk significantly decreased the viability of resistant cells (Fig. 4.6D). Suppression of MAPK activity by EphA2 antibody was also observed in MCF10A three-dimensional culture (data not shown), as well as in MMTV-Neu cells (Fig. 4.9A), where Erk phosphorylation recovered after prolonged treatment with gefitinib, a dual inhibitor of EGFR and ErbB2/Neu [276]. The combination of anti-EphA2 antibody and gefitinib completely abrogated MAPK activity and inhibited tumor

growth in vivo (Fig. 4.9B). Together, these data suggest that modulation of both Akt and MAPK signaling is a primary mechanism through which EphA2 contributes to trastuzumab resistance.

How is EphA2 receptor activated in trastuzumab-resistant cells?

We have previously shown that EphA2 forms a complex with HER2/ErbB2 and can be phosphorylated in the presence of HER2/ErbB2 [201]. However, we failed to detect direct EphA2 tyrosine phosphorylation by HER2 in an in vitro kinase assay (data not shown), indicating the possibility of involvement of another kinase. One candidate is the non-RTK Src because Src directly interacts with HER2 and is activated in HER2-overexpressing cancer cells [262, 263]. Indeed, Src is sufficient to activate EphA2 and is required for the phosphorylation of EphA2 by HER2 (Fig. 4.7A). Although trastuzumab reportedly inhibits Src activity within a short time frame [264], we observed increased Src activity in cells on prolonged exposure to trastuzumab (Fig. 4.7B). Using a FRET-based Src reporter to monitor Src activity in live cells, we found that short-term exposure to trastuzumab inhibits Src kinase activity, consistent with a previous report [264]. However, prolonged treatment resulted in increased Src activity (Fig. 4.8A and B). These results were supported by biochemical studies, in which Src phosphorylation at Y416 was increased with prolonged trastuzumab treatment. The mechanism of switch between trastuzumab-induced Src inhibition and activation is unclear. We speculate that continuous exposure to trastuzumab may cluster HER2 at the plasma membrane and recruit Src into the HER2/EphA2 complex, resulting in activation of Src and phosphorylation of EphA2 receptor.

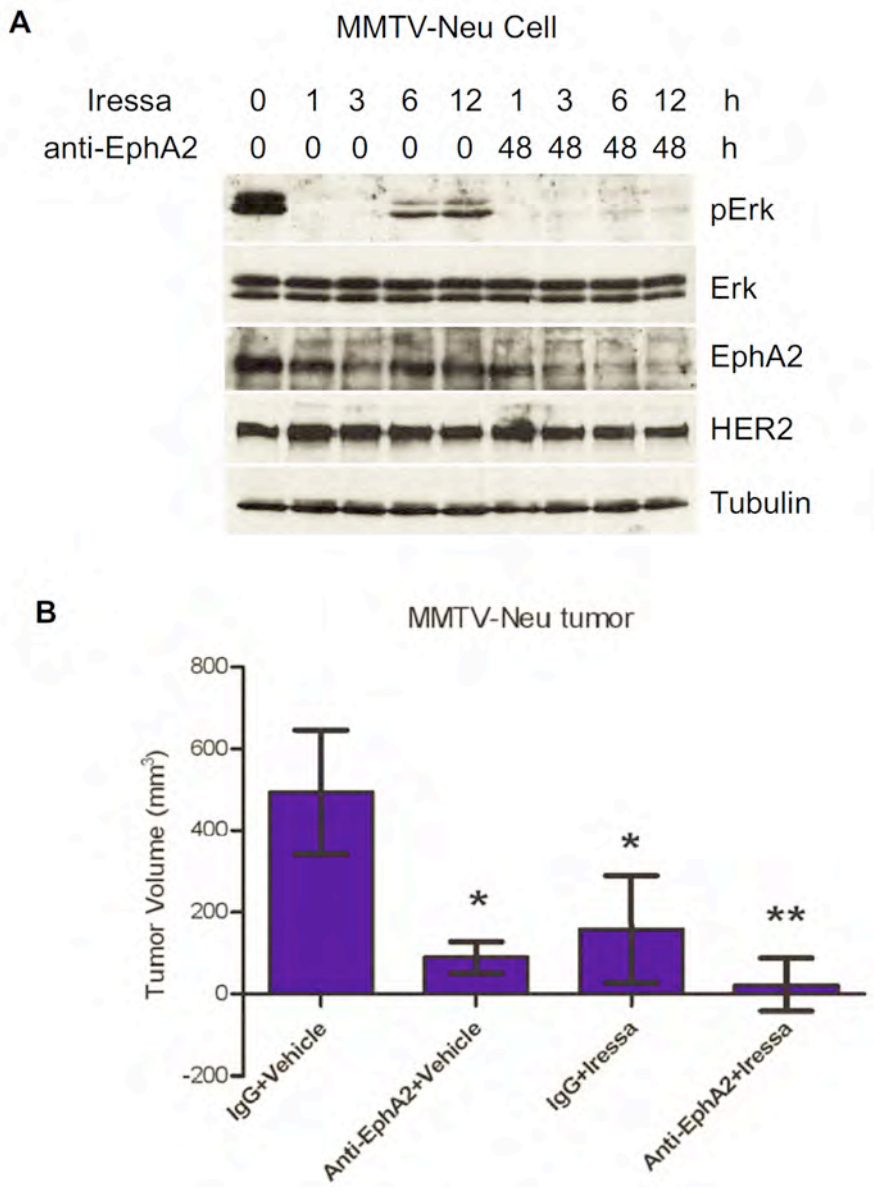


Figure 4.9. Suppression of MAPK activity by EphA2 antibody in MMTV-Neu cells.

A. MMTV-Neu cells were treated with 1μM gefitinib (Iressa) following a time course as indicated, in the presence or absence of anti-EphA2 (10μg/ ml). Phospho-Erk and phospho-Akt levels were determined by western blot analysis. pErk re-emerges after prolonged gefitinib treatment. The addition of anti-EphA2 completely abrogated MAPK activity. **B.** One million of MMTV-Neu cells were injected into the cleared mammary gland fat pad of recipient FVB female mice. Two weeks after transplantation, mice were treated with vehicle control (0.1% Tween 80) or gefitinib (50mg/ kg) by daily orogastric gavage, as well as with control IgG or anti-EphA2 (10mg/ kg) by twice weekly intraperitoneal injection. Tumors were harvested after two weeks treatment and tumor volume was calculated. * $P<0.05$, ** $P<0.01$; Student's t test.

Our findings that EphA2 coexpresses with HER2 and confers trastuzumab resistance in HER2-positive breast cancers could directly affect the clinical management of these patients. We propose that individuals with EphA2 and HER2-positive breast cancer might benefit from pharmacologic inhibition of EphA2 in combination with anti-HER2 therapies. EphA2 expression may also be used as a prognostic marker to predict trastuzumab resistance and treatment outcome. In patients who initially are negative for EphA2 but subsequently develop resistance to trastuzumab, elevated EphA2 could be one of the mechanisms that confer tumor resistance to HER2 inhibitors. Targeting EphA2 may represent a novel strategy to overcome trastuzumab resistance. In summary, our studies provide new mechanistic insights into the molecular basis of trastuzumab resistance. These studies provide a basis for rational design of combination therapies to overcome tumor resistance to trastuzumab.

CHAPTER V

GENOMIC AND MUTATION ANALYSES IDENTIFY EPHA3 RECEPTOR TYROSINE KINASE AS A TUMOR SUPPRESSOR IN NON-SMALL CELL LUNG CANCER

Abstract

Somatic mutations of Eph receptors in lung cancer have been recently identified in multiple systematic mutational screens. However, because Eph receptors can function in both tumor promotion and tumor suppression, the oncogenic potential of these mutations in lung cancer remains undefined. In this study, we analyzed copy numbers and expression levels of Eph receptors in large cohorts of both cell lines and primary lung cancer samples. We found that EphA3 is often deleted and/or under-expressed in human lung cancer. Further mutational analysis revealed that EphA3 somatic mutations identified in lung adenocarcinoma are loss-of-function mutation. Surprisingly, many EphA3 mutations can act dominant negatively to block wild-type EphA3 receptor tyrosine phosphorylation and kinase activity. Overexpression of wild-type, but not mutant, EphA3 inhibits tumor growth in vivo. EphA3 appears to promote cell apoptosis by negatively regulating mTOR complex 1 activity via activation of AMP-activated protein kinase. Expression of EphA3 in both tumor cell lines and lung cancer clinical specimens are negatively correlated with activities of S6K1 and S6, and higher EphA3 expression in tumor cell lines correlates with relative lower responsiveness to rapamycin. These findings identify EphA3 receptor tyrosine kinase as a tumor suppressor in non-small cell

lung cancer, and suggest that therapeutic intervention to diminish mTOR function may benefit patients with EphA3 deletion or mutation.

Introduction

Lung cancer is a leading cause of cancer-related deaths in the United States. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers and the overall five-year survival of NSCLC is approximately between 15-16% (NCI SEER Cancer Statistics Review, 2008). Although molecular targeted therapies such as EGFR kinase inhibitors have been developed, these agents often only target small percentage of patients, and the effectiveness is limited by the development of drug resistance. Recent gene sequencing studies in large cohorts of human lung cancer samples have identified a set of key mutations associated with NSCLC [122-125]. In one such sequencing effort, of 26 frequently mutated genes, Eph receptor tyrosine kinase (RTK) genes, notably EphA3, are mutated at significantly high frequencies and are predicted to be involved in tumor development [122]. However, due to the dichotomies of Eph receptor signaling in both tumor promotion and tumor suppression, the oncogenic potential of these mutations in lung cancer remains unknown.

EphA3 belongs to the largest family of RTKs, the Eph family, that mediate cell-cell communication. Since discovery in the 90s, Eph molecules have been increasingly recognized as key regulators both in development and disease [1, 25]. In cancer, the Eph receptors and their ligands, ephrins, regulate many processes that are essential for tumor initiation and progression, including proliferation, invasion/motility, angiogenesis and

metastasis [26, 27, 207]. Paradoxically, there is good evidence that Eph receptors could either promote or inhibit tumor, depending on ligand stimulation, signaling cross-talk, or other contextual factors. For example, EphA2 is overexpressed in a variety of human cancers and is associated with poor survival in breast, prostate, and lung cancer, as well as in glioblastoma multiform [78, 100, 101, 104, 247, 277-280]. In several studies, overexpression of EphA2 induces ligand-independent signaling, resulting in increased tumor cell malignancy in vitro and accelerated tumor growth and metastasis in vivo [74, 75]. Conversely, EphA2 knockdown or targeted gene deletion inhibited tumor initiation and metastatic progression [75, 76, 201]. However, ligand-dependent and kinase-dependent signaling of EphA2 in both breast cancer cells or glioblastoma lines inhibited tumor cell malignant behavior in vitro and tumor growth in vivo [35, 201]. Despite high frequency of EphA3 mutations discovered in a variety of tumor types, EphA3 is less well investigated in cancer. Early studies reported that ligand-dependent EphA3 signaling in melanoma cells induces cell de-adhesion through a CrkII and Rho-mediated mechanism [281]. However, ligand-induced EphA3 kinase-independent signaling in leukemia cells stimulated cell adhesion [118]. Together, these studies highlighted the complexity of Eph molecules in human cancer and the necessity of careful studies in each tumor type.

In this report, we analyzed copy numbers and expression levels of Eph receptors in NSCLC by a combination of SNP analysis and data mining of gene expression profiles in large cohorts of both cell lines and primary lung cancer samples. We found that EphA3 is often deleted and/or under-expressed in human lung cancer. Further mutational analysis revealed that EphA3 somatic mutations identified in lung adenocarcinoma are

loss-of-function mutation, but can act dominant negatively to block wild-type EphA3 receptor function. Overexpression of EphA3 suppressed tumor growth in vivo by regulating the mTOR signaling pathway. These findings uncovered a previously unknown mechanism by which EphA3 receptor inhibits lung tumor malignancy and demonstrated the power of an integrative approach to understand the complexities of Eph receptors in cancer.

Methods

Antibodies and reagents

Antibodies against the following proteins were used: EphA2 (Upstate Biotechnology); EphA3 (Santa Cruz Biotechnology for western, Sigma-Aldrich for IHC, and Millipore Corporation for IP); S6 ribosomal protein, phosphoserine-235/236 S6, p70 S6 kinase, phosphothreonine-389 p70 S6 kinase, Erk, phosphothreonine-202/serine-204 Erk, Akt, phosphoserine-473 Akt, AMPK, phosphothreonine-172 AMPK (Cell Signaling Technology); β-tubulin (Sigma-Aldrich); Actin, phosphotyrosine pY20 and pY99 (Santa Cruz Biotechnology); Ki67 (Zymed Laboratories); paxillin (BD Biosciences).

cDNA arrays containing paired normal lung tissues and NSCLC tumor samples or different stages of tumor specimens were purchased from Origene Inc. NSCLC cell lines and paired lung tissue and tumor sections were provided by Vanderbilt Lung SPORE program (Vanderbilt University, TN). Avidin peroxidase reagents were from Vector Laboratories, and liquid 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit

was from Zymed Laboratories. TO-PRO-3 iodide nuclear stain was purchased from Invitrogen. Rapamycin was purchased from Calbiochem. EphA3 TaqMan gene expression assay was from Applied Biosystems. EnzyLight ATP assay kit was purchased from Bioassay Systems.

Analysis of SNP array

The detailed descriptions of SNP arrays were published previously [282]. Affymetrix U133 microarray data (Gene Logic, Inc.) from NCI-60 cells were downloaded on DTP website (http://dtp.nci.nih.gov). SNP arrays were processed using the dChip software [283]. Both CentHind and CentXba SNP data were combined and filtered to reduce invariant SNPs. Filtered SNPs were subjected to unsupervised hierarchical clustering using the Pearson coefficient and average linkage method. Processed SNP data files were viewed in GenePattern software package [284]. SNP analyses of processed data from 84 human NSCLC lines and 371 clinical specimens were performed using published datasets [285, 286]. Analyses were based on the copy number thresholds: copy number 2.14 (amplifications) and 1.87 (deletions).

Cell viability and apoptosis assay

Cell viability was determined by CellTiter-Glo luminescent cell viability assay kit (Promega), based on quantitation of the amount of ATP in metabolically active cells. Two thousand and five hundred cells were seeded each well in 96-well plates in quadruplicates. Following a time course, cells were lysed and luminescence was measured by a luminometer according to manufacturer's instruction. For study of cellular

sensitivity to rapamycin, cells were plated at the density of 2,500/well in the presence or absence of 1μM rapamycin. Cells were harvested after 72h and viability determined as described above. Rapamycin resistance index was calculated as rapamycin-treated units/control units.

To determine the extent of apoptosis, histone-associated DNA fragments were quantified using the Cell Death Detection ELISA (Roche) according to the manufacturer's instructions. Briefly, cells (5,000) in 96-well plates were serum starved for 5 days or treated with GST-TRAIL (5ug/ml) for 24 hours. Free nucleosomes were detected by immobilized anti-histone antibody and anti-DNA peroxidase immunoreagents. The amount of peroxidase retained in the immunocomplex was determined with ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) as substrates. The absorbance was measured on a microplate reader (Bio-Tek) at a wavelength of 405/490 nm.

Immunoprecipitation and immunoblot analyses

Immunoprecipitation and immunoblot were performed as described previously [166]. Briefly, Cells were lysed in 1% Nonidet P-40 buffer with protease and phosphatase inhibitors. For co-immunoprecipitation HA-tagged wild-type and Myc-tagged mutant EphA3, receptor complexes were sequentially precipitated with anti-HA and anti-Myc agarose, resolved on SDS-PAGE and subjected to Western blot with desired antibodies. For kinase assay, precipitated protein complexes were resuspended in 25μl kinase buffer (20mM HEPES pH=7.6, 20mM β-glycerophosphate, 0.1mM sodium orthovanadate, 10mM MgCl2, 50mM NaCl, 1mM DTT, 200mM ATP, and 20μCi γ-32P ATP),

incubated at 30°C for 30 mins, resolved on a 8% SDS-polyacrylamide gel, and transferred to nylon membrane for autoradiography.

Tumor studies

NSCLC tumor cells (5×10^6) were resuspended in 100µl Matrigel (BD Biosciences) and subcutaneously injected into the nude mice. Tumors were harvested three weeks after injection and dimensions measured by digital caliper. Tumor volume was calculated using the following formula: volume = length x width^2 x 0.52. The tumor tissue was then fixed in 4% paraformaldehyde (Sigma-Aldrich) and processed for immunohistochemistry analyses of Ki-67 and cleaved-caspase 3 to determine proliferation and apoptosis in tumor tissue. Data were derived from 8-10 animals/group in two independent experiments.

ATP measurements

Cellular ATP levels were determined using a bioluminescence ATP assay kit according to the manufacturer's instructions (EnzyLight ATP assay kit; BioAssay Systems, Hayward, CA, USA). Briefly, cells were plated on 96-well in serum-free medium. After 24 hours, cells were stimulated with 10% FBS and lysed with 90µl assay buffer containing substrate D-luciferin and luciferase. The luminescence signal was measured within 20min by a luminometer.

Analysis of lung tissue microarray

Two tissue microarrays (TMA) containing 104 lung tumor samples and 26 normal lung tissues were provided by the Vanderbilt Lung SPORE program. Antigen retrival of TMA sections were performed by boiling in citrus butter. Two adjacent TMA sections were stained by anti-EphA3 (Sigma) or anti-phosphoS6 (abcam), and subsequently scanned and analyzed using the Ariol® SL-50 platform. The samples were scored as positive or negative for EphA3 and 0, 1, 2, 3 for pS6 according to the percentage and intensity of positive color (brown) in the sections, followed by visual verification, as shown in Supplemental Figure 6. To determine the statistical significance, Fisher's exact test and Chi-square test were performed in normal lung tissues and lung tumor samples, respectively.

Expression Array Analyses

Two independent EphA3-associated gene expression signatures were generated in a training dataset [122], and queried in a large testing dataset containing 444 gene expression profiles and associated clinical outcome data [287], using EXALT system as previously described [288]. EphA3 mutation signature (MutSig) was selected based on differentially expressed genes in patients carrying EphA3 mutation relative to those carrying the wild-type EphA3 gene. EphA3 co-expression signature (ExpSig) includes a cluster of genes with expression patterns correlated with EphA3 defined by Pearson correlation coefficient. Kaplan-Meier analyses were computed using R survival package. Statistical differences were determined by log-rank tests.

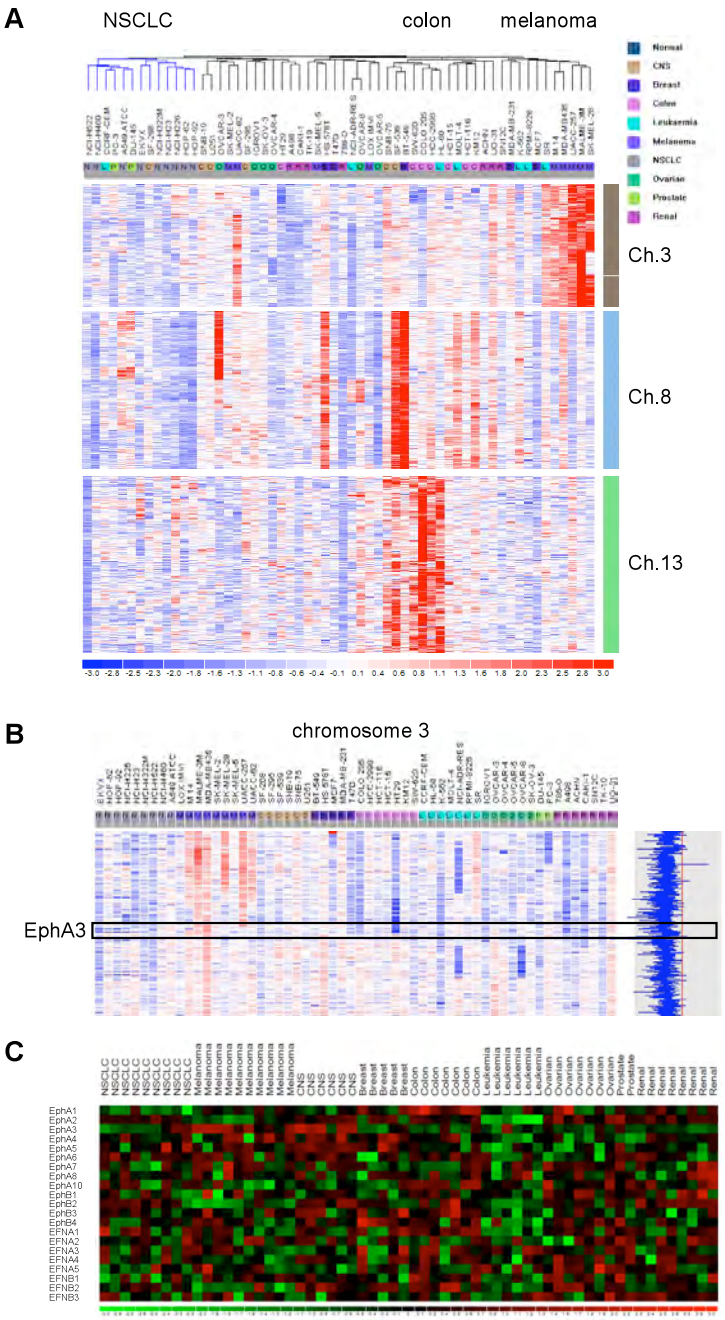


Figure 5.1. SNP array analysis of EphA3 gene copy number in NCI-60 tumor cell lines.

A. Hierarchical clustering of raw copy-number data from NCI60 cell lines showed cell line subclusters and chromosome-specific SNP clusters. Chromosome 3 is often deleted in NSCLC lines. Red indicates gain, blue indicates loss, and white indicates no change. **B.** Colorgrams of SNP copy number at chromosome 3p11.2. **C.** Microarray-based gene expression profiling showed loss of copy number of EphA3 and reduced EphA3 expression in 7 out of 9 NSCLC lines.

Results

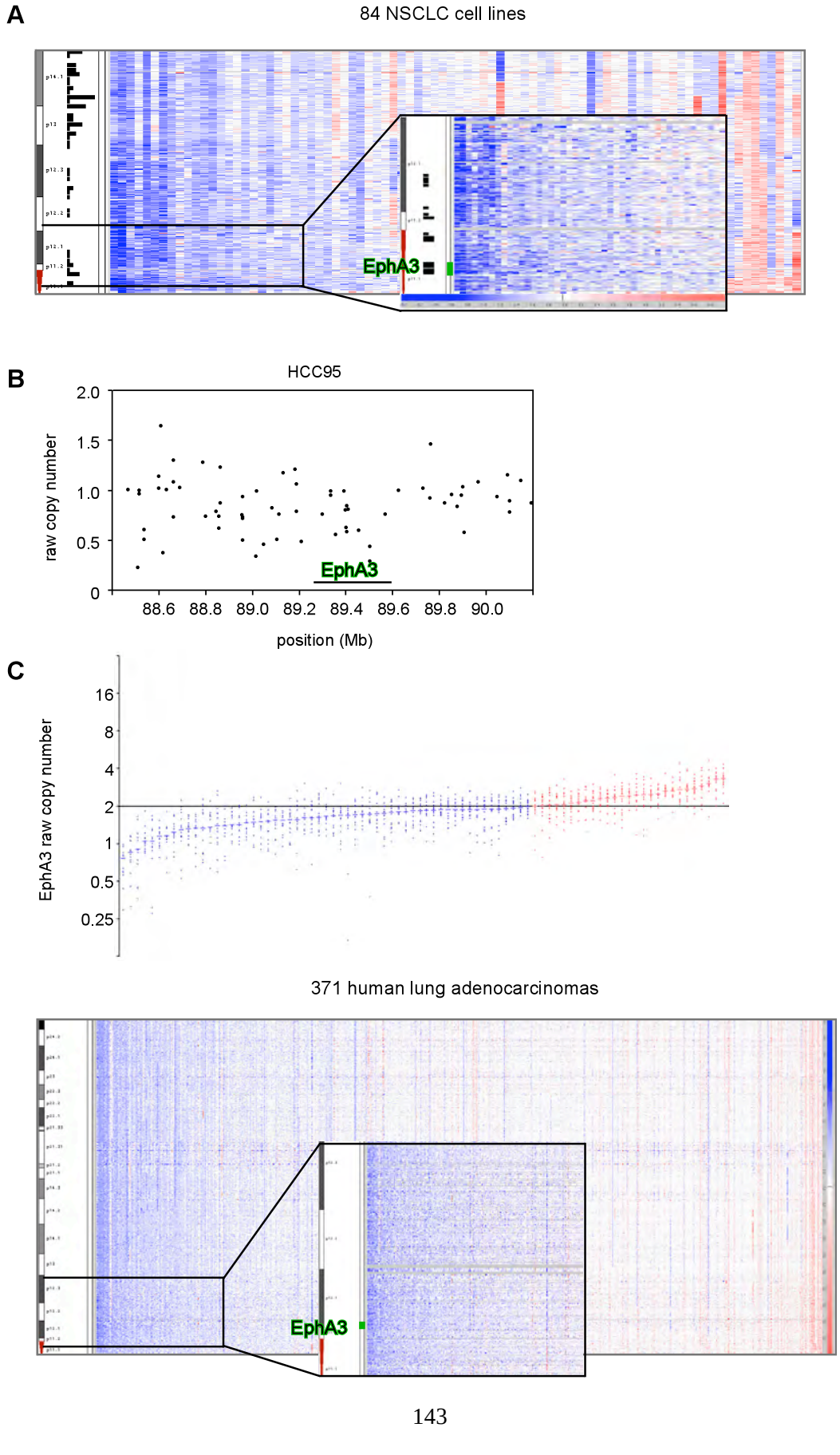
Loss of copy number and expression of EphA3 gene in human NSCLC

To analyze expression and function of Eph receptors in cancer systematically, we initially performed SNP analysis in NCI-60 human tumor cell lines [282]. Hierarchical clustering of copy number analysis revealed that regions in chromosome 3 are often deleted in NSCLC lines (Figure 5.1A). Further examination of Eph receptors in chromosome 3p11.2 showed loss of copy number of EphA3 (Figure 5.1B) and reduced EphA3 expression in 7 out of 9 NSCLC lines in the NCI-60 panel (Figure 5.1C). SNP analysis on a larger cohort of 84 NSCLC lines [286] supported the finding that EphA3 is frequently deleted (44 out of 84), with median copy number below 1.87 (Figure 5.2). To assess the relevance of EphA3 in human lung cancer, copy number of EphA3 gene on chromosome 3p were examined in a large dataset of 371 lung adenocarcinoma [285]. Consistent with the cell line data, EphA3 gene is often deleted in primary tumors (Figure 5.2D). Interestingly, genes encoding EphA3 ligands, ephrin-A1, -A3, and -A4, on chromosome 1q21-q22 are frequently amplified in these tumors (data not shown).

To determine whether EphA3 represents a target of 3p11 loss, we first asked whether EphA3 expression was downregulated in lung tumor samples relative to paired normal tissues. By quantitative RT-PCR, approximately 80% of samples (19 out of 24) showed 2- to 45-fold reduction of EphA3 mRNA in tumors relative to normal tissues (note of log2 scale on Y axis in Figure 5.3A). Next, we compared EphA3 expression in a panel of 85 NSCLC clinic specimens with different stages of tumor progression. EphA3 expression is significantly lower in all stages of tumors, compared to control normal

Figure 5.2. SNP array analysis of EphA3 gene copy number in 84 NSCLC lines and 371 lung tumor samples.

A. Chromosomal copy number of EphA3 was analyzed in 84 NSCLC lines and listed from lowest (blue) to highest (red). **B.** Raw copy number data (y axis) for HCC95 cell line are plotted according to chromosome 3 position (x axis). Genomic position of EphA3 is labeled along the x axis. **C.** Of 84 NSCLC lines, 44 lines showed copy number loss (below 1.87), compared to 20 lines showed copy number gain (above 2.14). **D.** Copy numbers of EphA3 on chromosome 3p are shown in 371 lung adenocarcinomas.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84 NSCLC cell lines
EphA3
B
HCC95
raw copy number
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1.5
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89.2
89.4
89.6
89.8
90.0
position (Mb)
C
EphA3 raw copy number
16
8
4
2
1
0.5
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D
371 human lung adenocarcinomas
EphA3

tissue ($p<0.001$, Figure 5.3B). Western blot analysis of 28 NSCLC lines revealed low or undetectable levels of EphA3, relative to high expression level of EphA2 receptor (Figure 5.3C). EphA3 protein expression was also analyzed in 9 paired NSCLC tumor samples and adjacent normal lung tissue by immunohistochemistry. While 8 normal tissues express EphA3, only 2 of the tumor samples express detectable levels of EphA3 ($p<0.05$, Figure 5.3D). Taken together, both copy number analysis and expression data indicate that EphA3 gene is frequently deleted and its expression is significantly downregulated in human lung cancer.

Overexpression of EphA3 in tumor cells promotes cell apoptosis

To determine the function of EphA3 in lung cancer cells, we overexpressed EphA3 in three lung cancer cell lines, A549, H1975, and H1299, via retroviral transduction (Figure 5.4A). To test the effects of EphA3 on cell growth, we initially measured clonal growth by colony formation assay. Tumor cells overexpressing EphA3 displayed 30%-50% reduction of numbers of colonies on tissue culture dish, although the size of colonies did not differ significantly between tumor cells overexpressing EphA3 and vector control cells (Figure 5.4B), indicating an equal growth rate but reduced plating efficiency. These results were confirmed by an MTT cell viability assay, in which tumor cells overexpressing EphA3 were less viable over a time course (Figure 5.4C). To determine whether reduced cell viability is due to decreased proliferation or increased apoptosis, we measured cellular incorporation of BrdU for proliferating cells and performed an ELISA-based apoptosis assay (see Methods). As shown in Figure 5.4D, there was no significant change in tumor cell proliferation between control and cells expressing EphA3. In

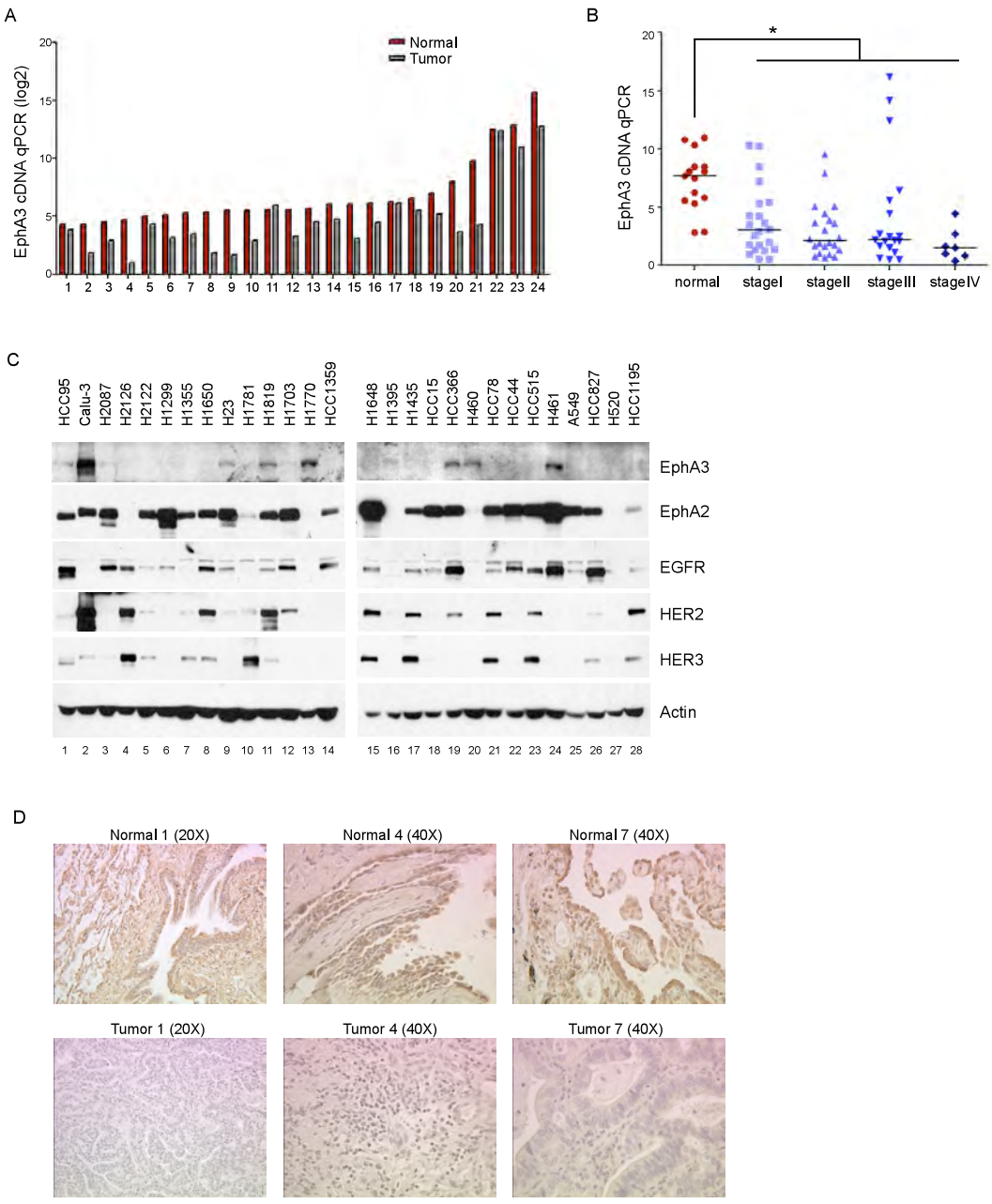


Figure 5.3. EphA3 expression is downregulated in NSCLC.

A. EphA3 mRNA levels were assessed by quantitative RT-PCR in paired normal tissues and lung tumor samples. 19 out of 24 tumors express lower levels of EphA3 compared to normal controls. **B.** EphA3 mRNA levels were quantified in different stages of NSCLC tumor samples. EphA3 is downregulated in all tumor stages relative to normal tissues (*, $P < 0.01$). **C.** Western blot analysis of 28 NSCLC lines revealed low or undetectable levels of EphA3 protein in tumor cell lines. **D.** Immunohistochemistry staining of 9 paired normal lung tissues and tumor samples (3 pairs were shown) revealed reduced EphA3 expression in tumor sections, compared to adjacent normal lung tissues.

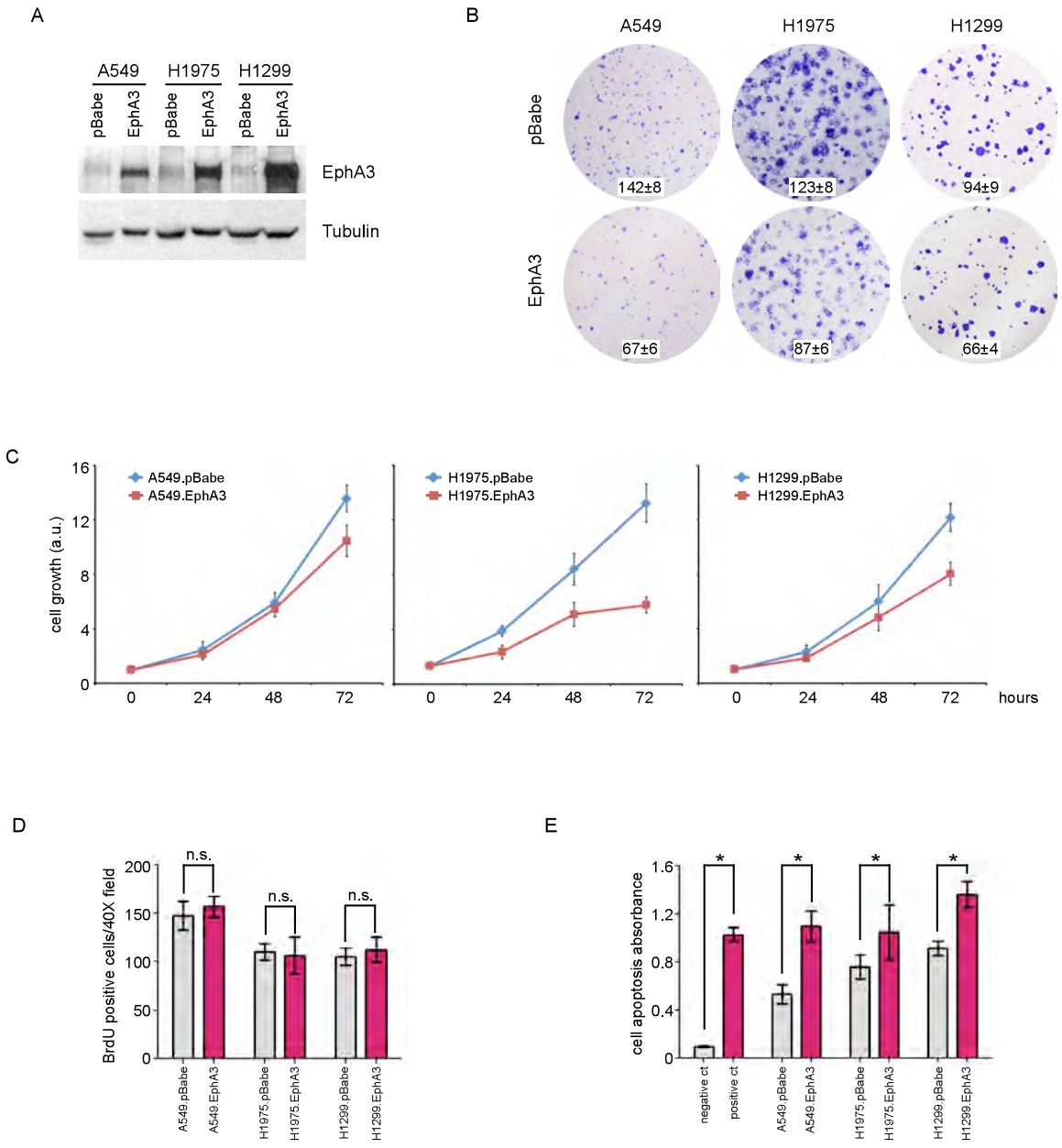


Figure 5.4. Overexpression of EphA3 in tumor cells promotes apoptosis.

A. Three NSCLC lines, A549, H1975, and H1299, were transduced with retroviruses carrying wild-type EphA3 or vector control. EphA3 overexpression in these lines was confirmed by Western blot analysis. **B.** Colony formation assay indicates that EphA3 overexpression decreases cell viability, which is confirmed by MTT assay shown in **C. D.** BrdU incorporation assay. **E.** Cells were serum starved for 5 days and subjected to apoptosis assay by Cell Death Detection ELISA kit. Overexpression of EphA3 increased apoptosis level. *, $P < 0.01$, Student's t test.

contrast, cells expressing of EphA3 displayed markedly enhanced cellular apoptosis (Figure 5.4E). Together, these results indicate that increased apoptosis, rather than decreased cell proliferation, was responsible for the reduction in colony numbers.

EphA3 mutations exhibit impaired ligand-binding or kinase activities and can function dominant negatively to suppress wild-type EphA3 receptor function

Somatic mutations in Eph receptors in lung cancer have been recently identified in multiple systematic mutational screens [122-125]. In one such gene sequencing effort, Eph mutations were discovered in 16% of primary lung adenocarcinoma, among which EphA3 was the most significantly mutated gene. Eleven mutations were identified in EphA3, with 8 mutations in extracellular domain and 3 in the kinase domain [123]. Additional EphA3 mutations were also identified by other studies (Table 5). These studies highlight the relevance of Eph family in lung cancer.

The majority of EphA3 mutations are clustered in the kinase domain and extracellular domain (Figure 5.5A). Multiple sequence alignments indicate that the mutated residues are highly conserved through species (data not shown). To determine whether the mutations in EphA3 affect ligand-binding and/or kinase activity, we generated 5 mutants in the kinase domain (T660K, D678E, R728L, K761N and G766E) and 6 mutants in the extracellular domain (T166N, G187R, N379K, T393K, A435S and S449F). Three mutations identified in colorectal cancer (D806N, T37K and N85S) were included for comparison. Both phospho-tyrosine blots and kinase assays showed that R728L, G766E, and D806N mutations in the kinase domain had impaired activity relative to wild type

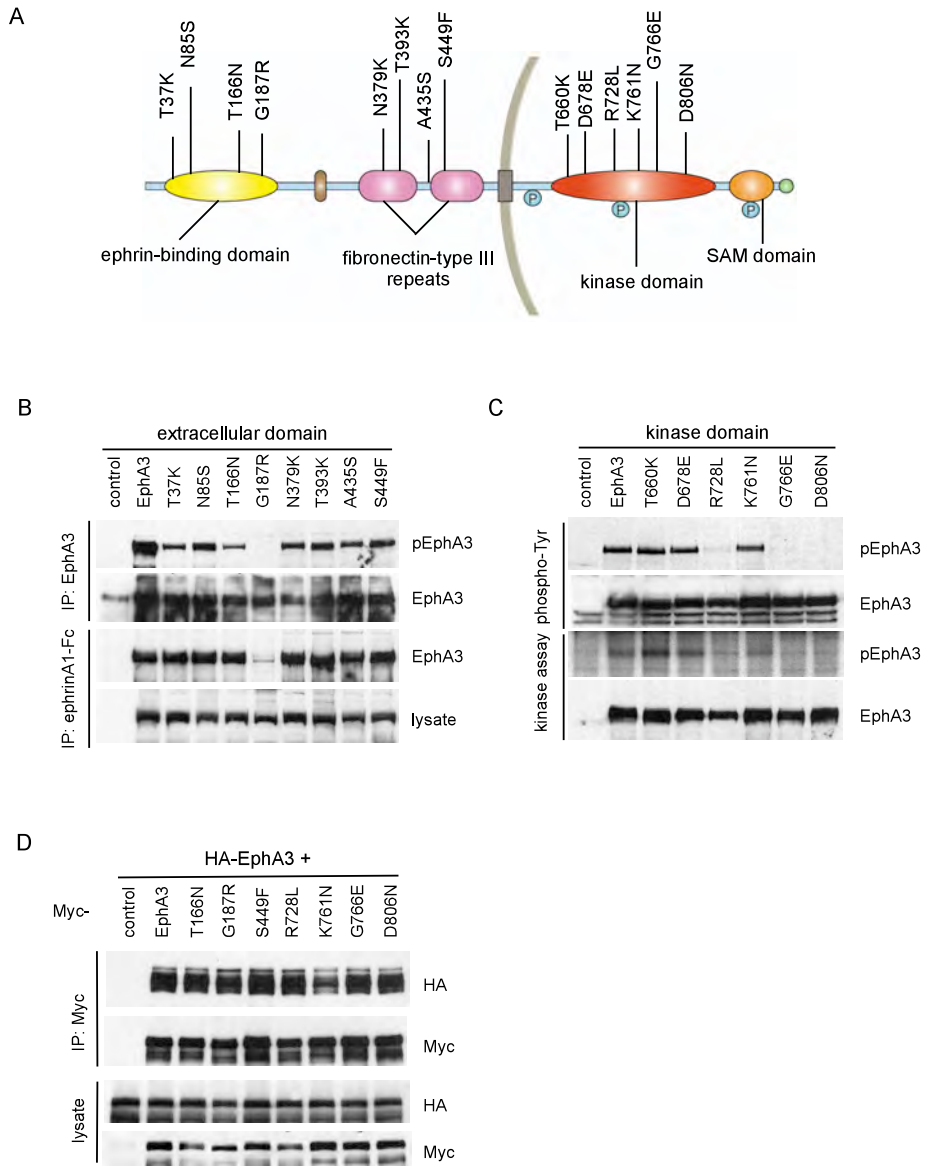


Figure 5.5. Ligand binding and kinase activity in EphA3 mutations.

A. Schematic diagram of EphA3 mutations, including 8 mutations in extracellular domain and 6 mutations in tyrosine kinase domain. **B.** Wild-type EphA3 or EphA3 bearing mutations in the extracellular domain was transfected into 293T cells. EphA3 proteins were immunoprecipitated with ephrinA1-Fc, and western blotted by anti-EphA3 and anti-pY to assess ligand-binding and tyrosine phosphorylation, respectively. **C.** Wild-type EphA3 or EphA3 bearing mutations in the kinase domain was precipitated as above, followed by phospho-tyrosine blot or kinase assay. **D.** HA-tagged wildtype EphA3 was co-transfected with Myc-tagged mutant EphA3 into 293T cells in equal amount (1:1). Transfected EphA3 were pulled down by Myc-agarose and western blotted by anti-HA. Wild-type EphA3 receptors are able to form complex with mutant EphA3 proteins.

Table 5. EphA3 mutations in lung and colon cancer

Sample	Tumor	Amino acid	Mutation type	Domain	%EphA3	%Eph	Reference
HCC15	lung	**T660K**	missense	PTK	2/23 (8.7%)	NA	(Wood et al., 2006)
HCC515	lung	T933M	missense	SAM			
H1770	lung	**S449F**	missense	FN3	3/33 (9.1%)	9/33 (27.3%)	(Davies et al., 2005)
H2126	lung	**G766E**	missense	PTK			
PD1362a	lung	S229Y	missense	CRR			
H1770	lung	**S449F**	missense	FN3	4/88 (4.5%)	13/88 (14.8%)	(Greenman et al., 2007)
H2126	lung	**G766E**	missense	PTK			
PD1362a	lung	S229Y	missense	CRR			
PD1369a	lung	G518L	missense	FN3			
17268	lung	**T166N**	missense	LBD	10/188 (5.3%)	25/188 (13.3%)	(Ding et al., 2008)
16863	lung	**G187R**	missense	LBD			
16802	lung	W250R	missense	CRR			
17290	lung	M269I	missense	CRR			
17210	lung	**N379K**	missense	FN3			
16835	lung	**T393K**	missense	FN3			
17735	lung	**A435S**	missense	FN3			
16949	lung	D446Y	missense	FN3			
17174	lung	**D678E**	missense	PTK			
16835	lung	**R728L**	missense	PTK			
16901	lung	**K761N**	missense	PTK			
NA	colon	S792P	missense	PTK	2/182 (1.1%)	4/182 (2.2%)	(Bardelli et al., 2003)
NA	colon	**D806N**	missense	PTK			
Mx41	colon	**T37K**	missense	LBD	4/37 (10.8%)	10/37 (27%)	(Sjoblom et al., 2006)
Co74	colon	**N85S**	missense	LBD			
Hx218	colon	I621L	missense	PTK			
Co84	colon	**D806N**	missense	PTK			

Mutation frequency was calculated based on the percentage of patients containing mutated EphA3 or Eph receptors. PTK, protein tyrosine kinase domain; SAM, sterile alpha motif; FN3, fibronectin-type III repeats; CRR, Cysteine-rich region; LBD, ligand-binding domain.

EphA3 (Figure 5.5C). Most of the mutants in extracellular domain also exhibited decreased levels of kinase activity as well (Figure 5.5B). To test whether mutations in the extracellular domain affects ligand binding, wild-type or mutant EphA3 proteins were precipitated by ephrin-A1-Fc beads and blotted by anti-EphA3 antibodies. While most mutants were able to bind to ephrin-A1, G187R, a mutation located in the conserved ligand-binding domain, was unable to bind to ephrin ligands effectively (Figure 5.5C). These data suggest that most EphA3 somatic mutations identified in human cancer are loss-of-function mutations. These results, together with the fact that EphA3 gene is frequently deleted and its expression downregulated in NSCLC, indicate that EphA3 functions as a tumor suppressor.

Because most EphA3 mutations have decreased activity, and because the mutations were not accompanied by frame-shift or nonsense mutations, or loss of heterozygosity (LOH) (data not shown), EphA3 mutants (MUT) may act dominant negatively to suppress wild-type EphA3 (WT) function in lung cancer. We hypothesize that the mutant EphA3 molecules form heterodimers with wild type EphA3, resulting in a catalytically inactive complex. To test this possibility directly, HA-tagged wild-type EphA3 was co-transfected with Myc-tagged mutants and MUT:WT EphA3 dimers/multipmers were isolated by sequential immunoprecipitation using anti-HA and anti-Myc monoclonal antibodies. As shown in Figure 5.5D, HA-tagged WT EphA3 was able to bind to Myc-tagged mutants. Co-expression of wild-type and mutant EphA3 significantly inhibited receptor tyrosine phosphorylation and kinase activity (data not shown), suggesting that EphA3 mutations function dominant negatively to suppress wild-type EphA3 function.

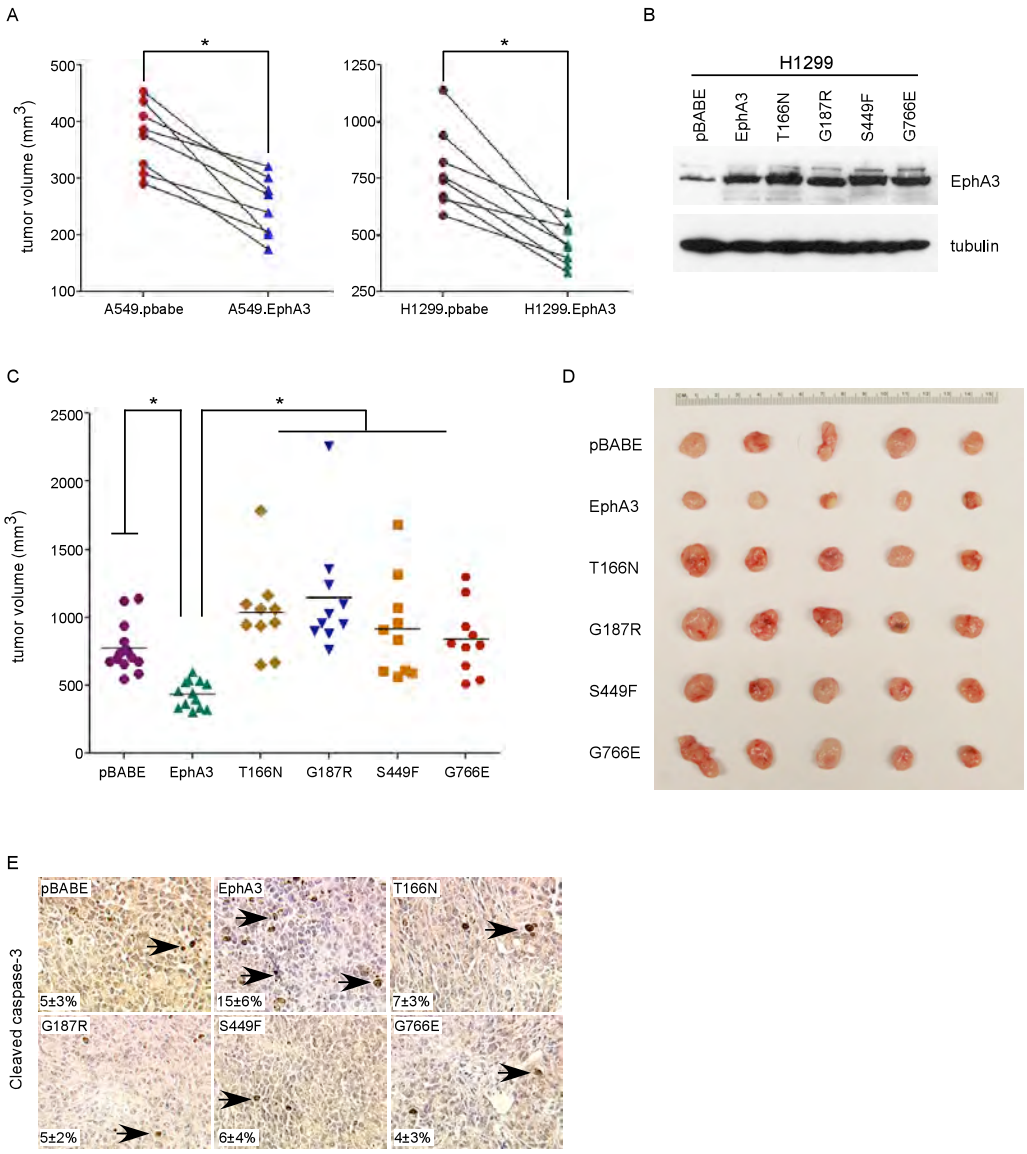


Figure 5.6. Overexpression of EphA3 inhibits tumor growth in vivo.

A. Cells overexpressing EphA3 were injected into nude mice subcutaneously. Cells carrying empty vector were injected contra-laterally in the same mouse as a control. Tumors were harvested three weeks after injection and tumor volume were measured. EphA3 overexpression in A549 or H1299 cells inhibits tumor growth. *, $P < 0.001$, paired t test. **B.** EphA3 mutants were introduced into H1299 cells and overexpression of the mutant proteins was confirmed by western blot analysis. **C.** H1299 cells expressing wild-type or mutated EphA3 were injected into the nude mice (n=10) subcutaneously. Tumors were harvested and measured three weeks after injection. EphA3 mutants did not inhibit tumor growth. *, $P < 0.001$, Student's t test. **D.** Tumors were harvested and photographed. **E.** Cell apoptosis in tumor sections were evaluated by cleaved-caspase3 immunohistochemistry. Apoptosis index was calculated as numbers of caspase-3 positive cell/total numbers of cell. Arrowheads indicate cleaved-caspase3 positive cells.

Overexpression of EphA3 inhibits tumor growth in vivo

Because most NSCLC cells express low to non-detectable levels of EphA3, we investigated the function of wild-type or mutant forms of EphA3 receptor by overexpression of these receptors in tumor cells. Expression of wild-type EphA3 in both A549 and H1299 cell lines significantly inhibited tumor growth in the xenograft animal model (Figure 5.6A). To determine whether EphA3 mutations are loss-of-function mutation in vivo, H1299 cells expressing wild-type or extracellular domain mutants G187R, T166N, S449, or kinase dead mutant G766E were injected into nude mice. Consistent with our in vitro data, EphA3 mutants did not affect tumor growth significantly *in vivo* (Figure 5.6B-D).

To examine cellular changes within tumors, we analyzed cell proliferation and apoptosis in tissue section by staining for Ki67 and cleaved caspase 3, respectively. Quantitation of Ki67-positive nuclei revealed that there were no significant changes of cell proliferation between tumors expressing wild-type and mutant EphA3 (not shown). In contrast, apoptosis was increased approximately 3-fold in tumor expressing wild-type, but not mutant, EphA3 receptor (Figure 5.6E), consistent with the apoptotic-promoting role of EphA3 *in vitro* (Figure 5.4).

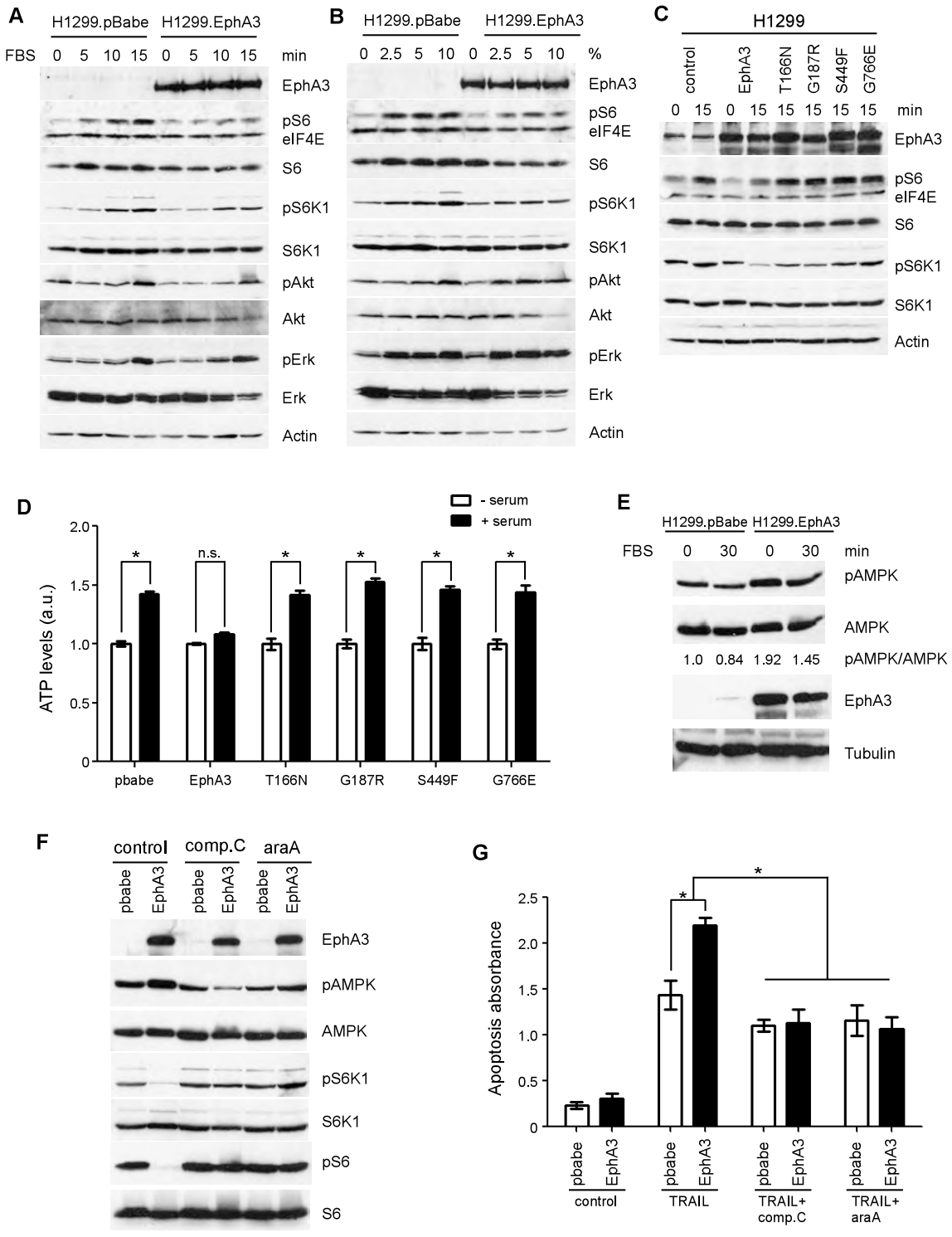
EphA3 inhibits mTORC1 activity via activation of AMP kinase

To gain mechanistic insight into EphA3's role in tumor suppression, we surveyed potential links between EphA3 and signaling molecules of known relevance to tumor growth and apoptosis. EphA3 overexpression had little or no effect on many key

molecules, including Akt, Erk, or Stat3. The one notable and consistent EphA3-induced alteration was inhibition of p70 ribosomal S6 kinase 1 (T389 phosphorylation) and S6 (S235/236 phosphorylation) activities (Figure 5.7A&B). S6 kinase 1 is a major substrate of mammalian target of rapamycin complex 1 (mTORC1) and can also be phosphorylated by PDK1 [289]. As overexpression of EphA3 does not affect activities of PDK1, Akt, and Erk, these results suggest that EphA3 inhibits mTOR complex 1 activity. The molecular link of EphA3 to mTORC1 activation was reinforced by the fact that mutant EphA3 did not affect S6K1 T389 phosphorylation level (Figure 5.7C). mTORC1 integrates three major signals to regulate many processes involved in cell growth, including growth factors, energy status, and amino acids [reviewed in [290-292]]. As EphA3 does not appear to affect mitogen-induced Akt or Erk activities (Figure 5.7A&B) or insulin or amino acid-induced S6K1 phosphorylation (data not shown), we investigated whether EphA3 regulates mTORC1 activity by energy status via AMP-activated protein kinase (AMPK), a master sensor of intracellular energy status. As a first step, we measured intracellular ATP level. Cells expressing wild-type EphA3 have significantly lower ATP level than those expressing mutant EphA3 or vector control (Figure 5.7D). Consistent with low ATP level, phosphorylation of Thr172 of AMPK is higher in cells expressing wild-type EphA3, relative to cells with vector control (Figure 5.7E). Inhibition of AMPK by either compound C or adenine 9-beta-d-arabinofuranoside (araA) abrogated EphA3-mediated suppression of activities of S6K1 and S6 (Figure 5.7F) and inhibited EphA3-mediated enhancement of apoptosis (Figure 5.7G). Taken together, these data suggest that EphA3 suppresses tumor growth by, at least in part, inhibition of mTORC1 via activation of AMPK.

Figure 5.7. EphA3 inhibits mTORC1 activity by activation of AMP kinase.

A and **B.** H1299 cells expressing EphA3 or vector control were serum starved and stimulated with either 10% of serum following a time course (A) or with increased percentage of serum (B). Phospho-S6, phospho-S6K1, phospho-Akt, and phospho-Erk levels were assessed by western blot analysis. Phospho-S6 and phospho-S6K1 were decreased in EphA3 expressing cells. **C.** EphA3 mutants fail to inhibit Phospho-S6 and phospho-S6K1 as determined by western blot anaysis. **D.** H1299 cells were stimulated with 10% FBS for 30 minutes and subjected to ATP measurements. Intracellular ATP concentration is lower in cells overexpressing wild-type, but not mutant, EphA3 in response to serum stimulation. *, $p < 0.01$, Student's t test. **E.** Phospho-AMPK levels were assessed by western blot analysis. The ratio of phospho-protein/total protein was determined by densitometry and expressed in arbitrary units. EphA3 expression resulted in higher phosphorylation of AMPK. **F** and **G.** Inhibition of AMPK by compound C (comp.C 10 μM) or adenine 9-beta-d-arabinofuranoside (araA, 1mM) abrogates EphA3-mediated suppression of activities of S6K1 and S6 (F) and Trail-induced apoptosis (G).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EphA3
T166N
G187R
S449F
G766E
0 15 0 15 15 15 15 15 min
EphA3
pS6
eIF4E
S6
pS6K1
S6K1
Actin
D
- serum
+ serum
ATP levels (a.u.)
2.0
1.5
1.0
0.5
0
*
n.s.
*
*
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*
pbabe
EphA3
T166N
G187R
S449F
G766E
E
H1299.pBabe H1299.EphA3
FBS 0 30 0 30 min
pAMPK
AMPK
1.0 0.84 1.92 1.45 pAMPK/AMPK
EphA3
Tubulin
F
control
comp.C
araA
pbabe
EphA3
EphA3
pAMPK
AMPK
pS6K1
S6K1
pS6
S6
G
Apoptosis absorbance
2.5
2.0
1.5
1.0
0.5
0
*
*
pbabe
EphA3
control
TRAIL
TRAIL+ comp.C
TRAIL+ araA

Higher EphA3 levels are associated with lower responsiveness to rapamycin

Although EphA3 expression is often reduced in NSCLC, some tumors do express various levels of EphA3 (Figure 5.3). To extrapolate our finding in cell lines to human lung cancer, we analyzed EphA3 and phospho-S6 expression in adjacent sections of two independent tissue microarray sets of human lung cancer specimens contains 104 tumor samples and 26 normal tissue controls. As shown in Figures 5.8A, normal tissue from 16 individuals (62%) were positive for EphA3, whereas majority of the tumor samples were largely negative for EphA3 (71, 68%). In contrast, phospho-S6 was low or undetectable in normal samples but dramatically increased in lung cancer specimens and the increased pS6K1 is correlated with decreased EphA3 levels in tumors ($p=0.03$) (Figure 5.8A, Table 6), suggesting that EphA3 also regulate mTORC1 activity in human lung cancer.

Because wild-type EphA3 inhibits mTORC1 activity and mTOR inhibitors have been developed for cancer therapy, we investigated whether EphA3 level is associated with tumor cell sensitivity to mTOR inhibitors. We have identified 18 NSCLC lines that do not express significant levels of EphA3 and 12 NSCLC lines that express various amount of EphA3. NSCLC lines that do not express appreciable amount of EphA3 are associated with consistently higher levels of phospho-S6K1 and phospho-S6 (Figure 5.8B). When these cells were treated with mTOR inhibitor rapamycin, cells expressing EphA3 (Figure5.8C, red bar) are more resistant to rapamycin than those lines that do not have significant amount of EphA3 (Figure 5.8C, grey bar) ($p=0.03$). Two EphA3 expressing cell lines, H1395 and H23, that are sensitive to rapamycin also carry LKB1 mutation, suggesting that LKB1 mutation upregulates mTORC1 activity, which render these cells

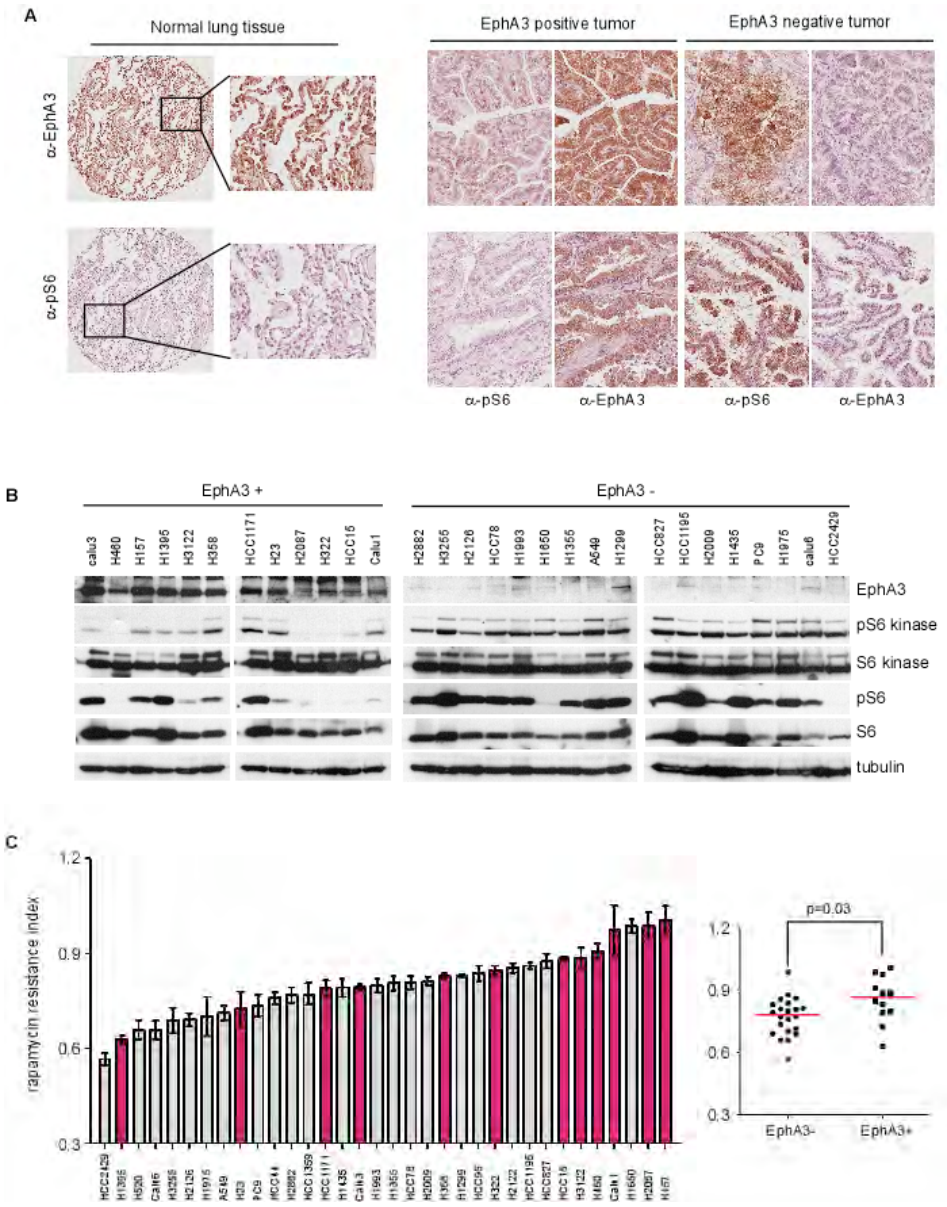


Figure 5.8. High EphA3 levels are associated with low pS6 and responsiveness to rapamycin in lung cancer.

A. EphA3 and phospho-S6 expression levels in lung tumors and control normal tissues were determined by staining adjacent sections of two lung tumor tissue microarrays. EphA3 levels in tumors are negatively correlated with pS6 expression level (quantification see supplemental table 2). **B.** EphA3, phospho-S6K1, and phospho-S6 levels were assessed by western blot analysis in a panel of 29 NSCLC lines. **C.** A panel of 29 lung cancer cell lines were treated with rapamycin (1μM) or vehicle control for 72 hours and subjected to cell viability assay. Rapamycin resistance index was defined by luminescence unit of treatment group/luminescence unit of control group. Cells expressing EphA3 (red bar) are more resistant to rapamycin (P=0.03, right panel).

Table 6. EphA3 and pS6 expression in lung tumor TMA

Normal Lung Tissue				NSCLC Tumors			
pS6 score	0&1	2&3	Total	pS6 score	0&1	2&3	Total
EphA3+	10	6	16	EphA3+	24	9	33
EphA3-	7	3	10	EphA3-	29	42	71
Total	17	9	26	Total	53	51	104

Immunohistochemical staining for EphA3 and pS6 was scanned and analyzed using the Ariol® SL-50 platform. The samples were scored as positive or negative according to the percentage of positive color (brown) in the section, followed by visual verification to ensure positive/negative staining in tumor cells. Example images for scoring criteria were shown in supplemental figure 6. EphA3 expression in tumors is negatively correlated with pS6 expression (p=0.003).

sensitive to rapamycin. Together, these data indicated that higher EphA3 levels are associated with tumor cell resistance to rapamycin, suggesting that therapeutic intervention to diminish mTOR function may benefits patients with EphA3 deletion or mutation.

EphA3-associated gene signatures predict clinical outcome

To determine the impact of EphA3 in lung cancer prognosis, we generated two independent EphA3-associated gene expression signatures in a training dataset [122], and queried EphA3 signatures in a large testing dataset containing 444 gene expression profiles and associated clinical outcome data [287]. EphA3 mutation signature (MutSig) was selected based on differentially expressed genes in patients carrying EphA3 mutations relative to those bearing the wild-type gene. EphA3 co-expression signature (ExpSig) includes a cluster of genes with expression patterns co-regulated with EphA3 defined by Pearson correlation coefficient. In an unsupervised hierarchical clustering analysis, both MutSig and ExpSig grouped lung tumors into two distinct clusters (Figure 5.9A&B, red and blue). The two clusters defined by MuSig overlapped significantly with those defined by ExpSig (417 of 444 samples). Kaplan-Meier analysis was performed using overall survival as an endpoint, and the two clusters were significantly different in clinical outcome. These data indicate that EphA3 signatures are associated with patient prognosis in lung adenocarcinoma, supporting the clinical relevance of EphA3 function in human lung cancer.

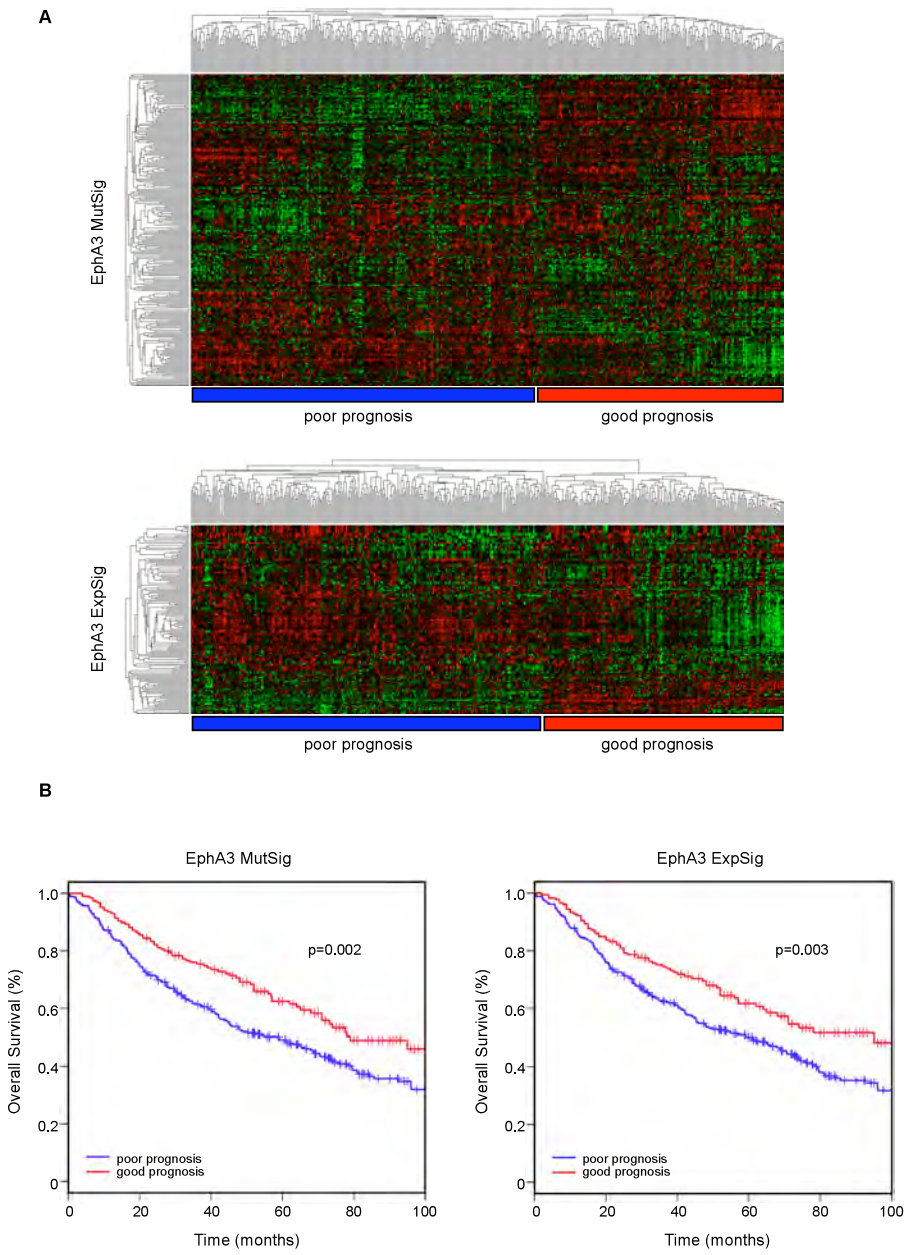


Figure 5.9. EphA3-associated gene signatures predict patient survival.

EphA3 mutation gene signature and EphA3 co-expression signatures were generated in a training dataset of Ding et al., and queried in a large testing dataset of Shedden et al. **A.** Hierarchical clustering of 444 clinically annotated human lung adenocarcinomas using two EphA3-associated gene signatures independently segregates tumors into two distinct clusters (blue, poor prognosis; red, good prognosis). **B.** Kaplan-Meier curves for the blue and red clusters of the hierarchical diagrams of A. The endpoint recorded for this dataset was death, measured in months.

Discussion

In this study, we used a systematic genome approach, combined with mutation analysis, to identify Eph receptors that play important roles in lung cancer. We have provided genetic, functional, and mechanistic evidence of a tumor suppressor role for EphA3 in non-small cell lung cancer and have translated these observations to human clinical material. Our results show that EphA3 functions as a tumor suppressor by regulating cellular apoptosis via AMPK- mTORC1 signaling pathway.

EphA3 receptor tyrosine kinase as a tumor suppressor

Malignant progression of solid tumors is a complex process that involves the activation of oncogenic signaling and downregulation of tumor suppressor pathways. Oncogenic conversion, amplification, or overexpression of proto-oncogenes, such as those encoding cell surface receptor tyrosine kinases (RTKs) like the EGF receptor family member ErbB2, are frequently observed in human cancers and contribute to malignancy. EphA3 is a member of the Eph receptor tyrosine kinase family. However, both tumor promoting and tumor suppression functions have been assigned to Eph receptors. As such, the discovery of EphA3 mutation at high frequency in human lung cancer indicates the clinical relevance but not the function of EphA3 receptor. Indeed, one mutation, K761N, is located in the kinase domain at a highly conserved position analogous to FGFR2 (K641) [293]. This mutation was predicted to be part of the "molecular brake" and functions as an activating mutation [122]. In addition, one recent report found EphA3 copy number gain in two NSCLC lines [286]. These studies implicate EphA3 as a possible "proto-oncogene". Our observation, however, argue against the above possibility. First, a

detailed SNP analysis using datasets from both NSCLC cell lines and human lung cancer samples revealed chromosomal region containing EphA3 is frequently deleted (Figure 5.1&5.2). Analysis of copy number of EphA3 in the same dataset as Sos et al revealed that EphA3 is deleted in 44 out of a total of 84 cell lines (below 1.87), compared to copy number gain (above 2.14) only in 20 cell lines (Figure 5.2C). Second, expression analyses by quantitative RT-PCR, western blot, and immunohistochemistry in both cell lines and tumor specimens showed reduced EphA3 expression in tumors, relative to normal tissues (Figure 5.3). Third, majority of EphA3 mutations lost kinase activity and exhibited reduced tyrosine phosphorylation status, whereas none of the mutations display increased activity (Figure 5.5). Finally, overexpression of wild-type EphA3, but not mutant, receptor in two NSCLC lines inhibited tumor growth in vivo (Figure 5.6). Taken together, these observations suggest that EphA3 receptor tyrosine kinase functions as a tumor suppressor in lung cancer.

Role of EphA3 mutations in lung cancer

Eph mutations that disrupt forward signaling by impairing ephrin binding or kinase activity have been discovered previously. For example, the EphA3 E53K mutation in the MeWo melanoma cell line abrogates ephrin binding [127], and the EphB2 G787R mutation found in colorectal cancer impairs kinase activity [128]. These data are consistent with our finding that majority of EphA3 mutations are loss-of-function mutations in lung cancer. It is interesting to note, however, that EphA3 mutations can form heterodimer/heteromultimer with wild-type receptor to inactivate wild-type EphA3 (Figure 5.5E and F). This finding has clinical relevance, since EphA3 mutations in human

lung adenocarcinoma were neither accompanied by loss of heterozygosity, nor frame-shift or nonsense mutations [data not shown, [122]], suggesting the possibility of EphA3 mutation playing an active role in tumor initiation and/or progression in human cancer.

In contrast to loss-of-function mutations in EphA3, gain-of-function mutation has been discovered in EphA2 receptor. Of particular interest is the R721Q activating mutation in EphA2 that is linked to cataract in human [294]. The R721 in EphA2 is corresponding to R728 in EphA3. However, R728L mutation in EphA3 resulted in loss of kinase activity (Figure 5.5C). Further investigation will be needed to determine whether the differences between glutamine and leucine in these mutations could account for the differences in kinase activity and phenotypic responses.

Regulation of mTOR signaling by EphA3

EphA3 receptor signaling was reported to mediate cell-cell repulsion in axonal guidance [295] and cell-matrix de-adhesion in tumor cells [281]. These biological responses appear to be mediated by EphA3 interacting adaptor Crk and RhoA GTPases [281]. The effect of EphA3 receptor on cell growth and survival has not been reported previously, although ephrin stimulation of EphA2 and EphA4 receptors inhibits cell growth via suppression of Erk1/2 activity in many normal and tumor cell types [34, 116]. Ligand-induced EphA2 signaling has also been shown to suppress cell motility by inhibiting Akt phosphorylation in glioma and prostate cancer cells [35]. However, overexpression of EphA3 in NSCLC lines did not appear to affect Erk1/2 or Akt activities (Figure 5.7). The mechanism by

which EphA3 inhibits cancer cell viability appears to involve regulation of mTORC1-S6K1 activity.

S6K1 activity in tumors is primarily regulated by mTORC1 positively in response to mitogen and amino acid, and negatively in the presence of energy stress. Signals that inhibit the TSC2 tumor suppressor, and thus activating mTORC1, include PI3K-Akt and MAPK pathways that are often activated in many types of cancer. Indeed, a recent report showed that ephrin-induced growth cone collapse is mediated by inhibition of Erk activity and reduced inhibition of TSC2 by Erk, resulting in enhanced activity of mTORC1 and S6K1 [296]. The PI3K-Akt and MAPK pathways, however, do not appear to be regulated by EphA3. Several observations support the notion that EphA3 regulates S6K1 activity through AMPK. First, intracellular ATP levels are significantly reduced in EphA3 expressing cells, suggesting EphA3 levels affect energy status in tumor cells (Figure 5.7D). Furthermore, phosphorylation of Thr172 of AMPK, an intracellular energy sensor, is higher in cells expressing wild-type EphA3, relative to cells with vector control (Figure 5.7E). Finally, inhibition of AMPK by compound C or araA abrogated EphA3-induced suppression of S6K1 and apoptosis, providing a functional between EphA3 and AMPK (Figure 5.7F&G). In addition to EphA3, AMPK can also be activated by LKB1, a serine/threonine kinase that is frequently mutated in NSCLC [297-299]. Although further work will be required to gain a complete understanding of whether EphA3 activates AMPK directly or indirectly through modulation of LKB1, the fact that EphA3 suppressed tumor growth in LKB1 mutation-bearing A549 cells suggest that EphA3 action on AMPK is independent of LKB1.

Due to the importance of mTOR signaling in tumor cells, inhibitors of mTOR have been developed as anti-cancer agents and early clinical trials showed effectiveness of these inhibitors in several types of tumors [300]. However, not all patients respond to mTOR inhibitors, thus molecular markers need to be developed for pre-screen patient for treatment. Towards this end, our data showed that higher level of EphA3 in lung cancer is correlated with reduced cellular sensitivity to rapamycin in 35 NSCLC cell lines and lower S6K1 activity in both NSCLC lines and in 104 tumor samples, suggesting that EphA3 may be included in a panel of markers for tumor sensitivity to mTOR inhibitors. Further, these studies suggest that therapeutic intervention to diminish mTOR function will improve the survival of patients with EphA3 deletion or mutation.

In summary, a combination of genomic and mutation analyses both in cell/animal model systems and in human tumor specimens revealed that EphA3 receptor tyrosine kinase functions as a tumor suppressor in lung cancer. Although our studies provide evidence that the kinase activity of EphA3 is required for its effect on cancer cell viability, future work is necessary to determine whether loss of other noncatalytic function will affect it tumor suppression function. Furthermore, because Eph receptor regulates communication between different cell types, the full impact of EphA3 mutations in vivo will require careful studies in inducible and tissue-specific transgenic tumor models. Finally, as another Eph family member, EphA2, appears to function in tumor promotion in NSCLC [101, 279], the discovery of EphA3 tumor suppressor function suggests that future therapeutic strategies targeting Eph receptors in cancer need to be directed at individual Eph molecule, rather than multiple or pan-Eph receptors. Such therapeutic intervention

efforts will require studies to dissect the mechanisms of opposing role between EphA2 and EphA3 in lung cancer.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

Conclusions

In the 20 years since the cloning of the first cDNA encoding an Eph receptor [301], EphA1, much progress has been made in characterizing the fundamental signaling mechanisms of Eph family, its biology and the pathological consequences of its deregulation [1, 25]. Notwithstanding these advances, a more complete understanding of Eph function and dysfunction in cancer is still to be achieved to make a significant impact on cancer therapy. These data in my thesis represent a step forward in exploring the remarkable multiplicity of Eph-ephrin signaling in tumor malignancy. We have now integrated genetically engineered mouse models with biochemical analyses in cell culture, and revealed that the role of one particular Eph receptor, EphA2, in breast tumor progression is dependent upon the oncogene/tumor suppressor context. More importantly, we showed the efficacy of a therapeutic antibody targeting EphA2 *in vivo,* suggesting that the mouse models are also useful for preclinical evaluation of new Eph-based therapies. In addition, EphA2 appears to serve as a predictor for prognosis and trastuzumab resistance in HER2 positive breast cancer, reflecting the intensive crosstalk between EphA2 and HER2. On the contrary, through an integrative approach by analyzing genome-wide profiling and examining biochemical properties of Eph mutations, we have identified another Eph receptor, EphA3, as a potent tumor suppressor in non-small cell lung cancer. Notably, EphA3 suppresses tumorigenicity in a kinase activity-dependent manner by regulating the mTOR signaling pathway. These seemingly paradoxical results

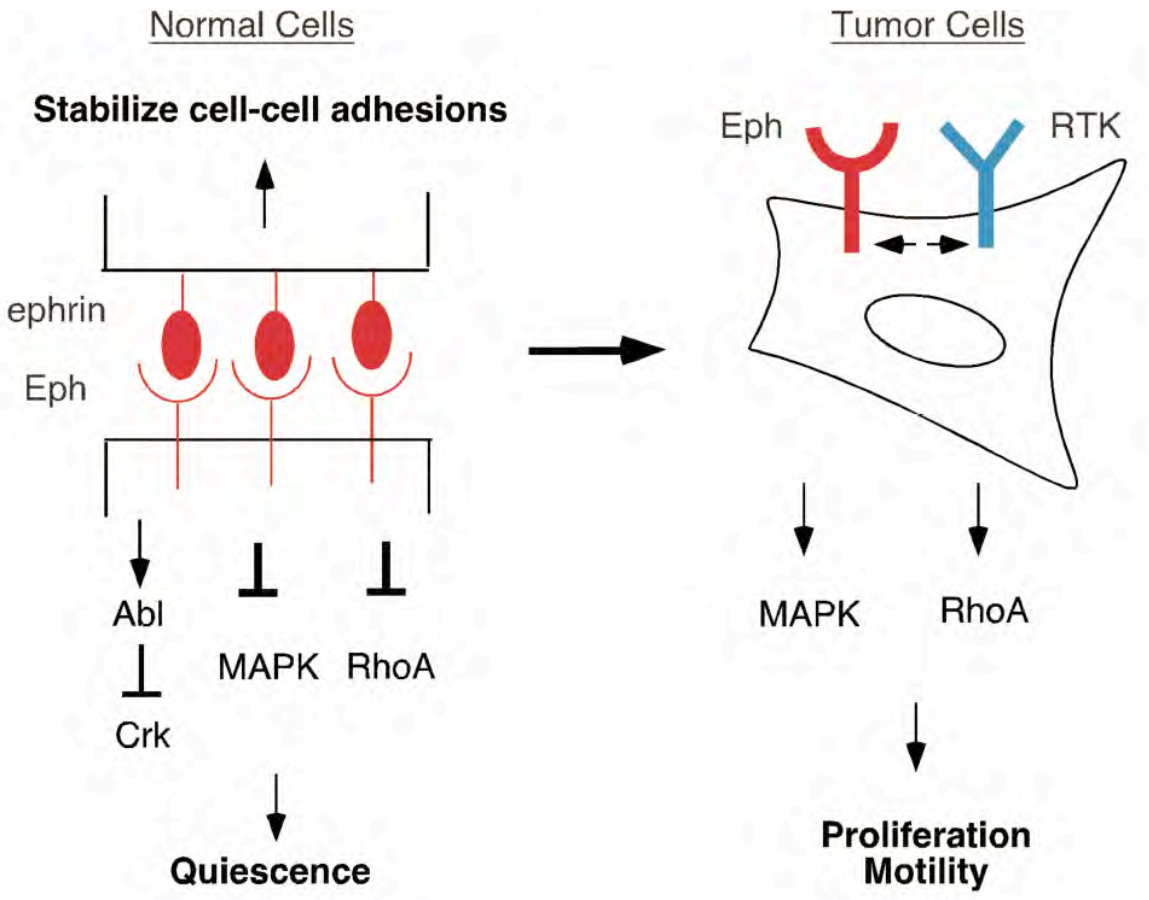


Figure 6.1. A working model of dual roles of Eph receptors.

In normal cells, engagement of Eph receptors with ephrins on adjacent cells induces forward signaling, leading to inhibition of Ras-MAPK, PI3K-Akt and Abl-Crk pathways. In tumor cells, disruption of cell-cell junctions inhibits Eph receptor interaction with ephrins. In addition, some tumor cells have low ephrin levels. Elevated Eph receptors crosstalk with other receptor tyrosine kinases, which results in increased activity of Ras-MAPK pathway and RhoA GTPase.

highlight the complexity of Eph functions and are consistent with the existing controversies in the field. Based on our studies and many others, we propose a working model in which the divergent roles reflect selective signaling pathways in Eph expressing cancer cells in different contexts (Figure 6.1). Generally, ligand-dependent Eph signaling is detrimental to cancer progression by inhibiting a variety of oncogenic cascades, such as Ras-MAPK, PI3K-Akt, Abl-Crk and mTOR-S6K pathways. On the other hand, non-canonical Eph receptor activity independent of ligand stimulation can promote tumor development through interaction with other oncogenes, suggestive of altered signaling networks in this situation. Intriguingly, cancer cells have developed a variety of mechanisms, for example EphA3 gene mutation and deletion in lung adenocarcinoma, to minimize ligand-dependent Eph signaling, and/or to hijack high levels of Eph receptors, in the case of EphA2 in breast cancer, to maximize oncogenic signaling pathways. Together, these findings have provided genetic, genomic, functional and mechanistic evidence and uncovered the complex roles for Eph signaling in tumorigenesis. We are confident that the continued translation of knowledge emerging from the field will ultimately facilitate cancer diagnostics, prognostics and therapeutics.

Future directions

Our work has raised as many questions as it has answered. The divergent roles of Eph receptors in human cancers are only beginning to be explored, and we have the privilege to speculate the new avenues of discovery in the future. Here is a partial list of unlimited possibilities.

How does receptor endocytosis regulate Eph signaling?

Previous studies have shown that EphB receptors are endocytosed after binding to ephrinB ligands [65, 66]. In this process, EphB receptors are initially activated, and induce Vav-Rac dependent cytoskeletal assembly that is required for internalization of the EphB–ephrinB complex [66, 169]. We have focused on EphA2 endocytosis, which has been explored as a means to reduce EphA2 levels and tumor malignancy. A negative regulatory loop via SHIP2 mediated Rac1 inhibition was identified to modulate EphA2 trafficking. Therefore, once the EphA2 internalization is triggered, Rac1 activity seems to be precisely controlled by EphA2 itself through both positive and negative regulation, suggestive of the importance of this accurately concerted process.

Several intriguing questions concern the role of receptor endocytosis in Eph signaling. Is it required for Eph signaling initiation, or just a way to terminate Eph signaling? Does Eph receptor continue to signal in cytoplasm, and if so, is the signaling different from that at the cell surface? The presence of phosphorylated EphB2 in intracellular vesicles suggests that EphB2 may continue to signal after endocytosis [65], as has also been found for other receptor tyrosine kinases such as the EGF receptor [302]. It remains to be determined whether EphA2 is also active in endocytic vesicles, and what the role of EphA2 endocytosis is in cancer.

What are the specific signaling activities of different Eph receptors?

There is a growing debate surrounding whether Eph receptors function as oncogene or tumor suppressor, as there is good evidence to support both roles. We proposed a

working model to reconcile the paradoxical effects of Eph signaling in malignancy. Under physiologic conditions, ephrin ligand interact with Eph receptors at cell-cell junctions, inhibiting the activation of Ras-Raf-MAPK, PI3K-Akt and Abl-Crk pathways, which is critical for development and tissue homeostasis, including the formation of tissue boundaries, assembly of intricate neuronal circuits and remodeling of blood vessels. However, tumor cells have developed a variety of mechanisms to prevent ligand-dependent Eph signaling, such as disruption of cell junctions and differential expression of Eph receptors and ephrin ligands. Meanwhile, other oncogenes including EGFR family of receptor tyrosine kinases are able to transduce downstream signals by crosstalk with Eph receptors, presumably independent upon ephrin stimulation. Thus, ligand-dependent Eph receptor signaling functions in tumor suppression, whereas ligand-independent Eph receptor activities appear to promote tumor progression.

Although our model is appropriate in most cases to decipher the complexities and paradoxes of Eph-ephrin signaling, there are several outliers which cannot be simply interpreted, suggestive of other potential mechanisms. For example, phosphorylation by Akt of a single serine (S897) in EphA2 appears to promote cancer cell migration and invasion, an effect that does not require, but is reversed by, ephrinA1 stimulation [35]. Thus, S897 of EphA2 serves as a binary switch to control cell motility, and it is conceivable that other Eph receptors may possess some types of molecular switch between agonistic and antagonistic. Interestingly, only EphA1 and EphA2 can be phosphorylated as substrates for Akt at this site, according to sequence conservation. Clearly, it is a distinct function in specific Eph members, and other Eph receptors may

have different mechanism for this switch. Further increasing complicacy, EphB2 signaling regulates the expression of PI3K catalytic subunits, which control cell positioning in intestinal epithelium independent of EphB2 kinase activity. On the contrary, EphB2 kinase is necessary to convey proliferative signals through an Abl-cyclin D1 pathway. Therefore, EphB2 receptor simultaneously promotes cell proliferation but suppress invasive growth of intestinal adenomas, and these two pathways could be dissociated [84, 303].

Particularly, we observed completely opposite expression patterns of EphA3 and EphA2 in non-small cell lung cancer. EphA3 is downregulated in cancer cells, while EphA2 is highly expressed which is consistent with previous reports. Our data indicate that EphA3 inhibits cell survival by suppressing mTOR signaling pathway, and the inhibition is dependent on EphA3 kinase activity. On the other hand, EphA3 decreased cell migration is kinase-independent, uncoupling two separate EphA3 signaling pathways. It is possible that EphA2 does not exhibit same effects as EphA3 considering the high levels of EphA3 in NSCLC. It would be interesting to find out the specific signaling partners of EphA3 that regulates cell motility and apoptosis, and to determine the reasons that account for the discrepancies between EphA2 and EphA3 in lung cancer.

Are EphA3 mutations in cancer drivers or passengers?

Cancer genomes carry two biological classes of somatic mutations [124]. “Driver” mutations confer growth advantage on the cell in which they occur, are causally implicated in cancer development and have therefore been positively selected.

Conversely, “passenger” mutations have not been subject to selection. They were present in the cell that was the progenitor of the final clonal expansion of the cancer, are biologically neutral and do not confer growth advantage. Most somatic mutations in cancer cells are likely to be passenger mutations, and only a subset of genes carry driver mutations and therefore function as cancer genes. Apparently, it is important to distinguish driver from passenger mutations in order to yield further insights into the development of human cancer, and to provide new opportunities for molecular diagnosis and therapeutics.

Our data suggest that most EphA3 mutations are loss-of-function mutations that impair EphA3 kinase activity and fail to inhibit tumor growth as wild-type EphA3 does. There are, however, mutants that are not associated with kinase inactivation, including mutations in the catalytic domain. These findings support the concept that acquired mutations in cancer may not contribute to malignant transformation and underscore the importance of functional studies to distinguish “driver” mutations underlying tumorigenesis from biologically neutral “passenger” alterations. More importantly, we found that EphA3 mutations can form heterodimers with wild-type receptor to inactivate wild-type EphA3, therefore obtain oncogenic properties. It remains to be determined whether mutated EphA3 could recruit specific effectors and activate alternative signaling pathways entirely different from wild-type EphA3.

We are also aware that appropriate mice models are essential to accurately elucidate the function of EphA3 mutations. Although easier to interpret, the results of *in vitro*

approaches might be sensitive to experimental conditions and methods, whereas experiments in animal models yield more physiologically relevant information especially valuable for recapitulating human cancer. This is particularly important for understanding Eph-ephrin system, which extensively mediates cell-cell communication in physiological contexts. Additionally, the genetically engineered mouse models carrying EphA3 deletion or mutation will enable us to answer many other open questions. Is EphA3 mutation sufficient to generate *de novo* lung adenocarcinoma? How does EphA3 affect lung tumor initiation, progression and metastasis? What are the common and specific signaling networks downstream of wild-type versus mutated EphA3? What is the effective therapeutic strategy for cancers harboring the EphA3 mutation? Perhaps by using a combination of *in vitro* experiments and genetic tools, it will be possible to unravel the significance of EphA3 signaling in lung cancer.

What is the role of ephrin reverse signaling in cancer cells?

One unique feature of Eph-ephrin complexes is their ability to generate bidirectional signals that affect both the receptor-expressing and ligand-expressing cells. Our studies presented here have mainly focused on understanding the role of Eph forward signaling in tumor development. Ephrin ligands are also present in tumor cells, suggesting that ephrin reverse signaling may in some cases contribute to tumorigenicity as well. Similar to Eph forward signaling, both pro- and anti-tumorigenic functions have been attributed to ephrin reverse signaling. In colon cancer cells, ephrinB1 tyrosine phosphorylation disrupts binding of the ephrin with the scaffolding protein Par6, promoting the formation of tight junctions between cells [304]. In addition, ephrinA5 reverse signaling displays

tumor suppressive effect in glioma by down-regulating EGFR levels [305]. Other examples imply ephrin ligands function as a tumor promoter. In fibroblasts, ephrinA5 reverse signaling activates the Fyn non-receptor tyrosine kinase, integrin-mediated cell adhesion and MAP kinases [29, 30]. Accordingly, ephrinA5 overexpression in murine fibroblasts can increase cell growth in soft agar, invasion and morphological transformation [306]. EphrinB reverse signaling has been reported to localize in lipid rafts, induce Rac1 activation and increase cancer cell migration and invasion [307-310].

It is not clear how ephrin reverse signaling is regulated in cancer cells. Both expression levels and selective signaling cascades could be involved in this regulation. Ephrin knockout mice and transgenic models, which abrogate reverse signaling but preserve forward signaling, will be instrumental for providing a detailed overview of the ephrin reverse signaling in cancer. Another interesting question is: how similar are ephrin reverse signaling and Eph forward signaling, and could they be functionally exchanged? An initial accomplishment has been achieved by applying a differential isotopic labeling technique to simultaneously and independently monitor signaling in two interacting populations of cells that express EphB2 and ephrinB1, respectively. Signaling networks were constructed, and the information processing by the two interacting cell types was modeled. This revealed that signaling between mixed EphB2- and ephrinB1-expressing cells is asymmetric and that the receptor forward and ligand reverse signaling use different tyrosine kinases and targets to process signals in most cases, but there are indeed shared modes of signal transduction. Unexpectedly, the intracellular tail of ephrinB1 also influences pTyr signaling in EphB2 positive cells, and soluble fusion

proteins containing the extracellular regions of ephrinB1 stimulate different signaling events compared with coculture [311]. Similar approaches should be applied into EphA-ephrinA system to intertwine quantitative measurements of cell behavior and signaling dynamics. The integrative information available from these studies could be of general utility in comprehensively studying the role of Eph bidirectional signaling network in cancer and other pathological processes.

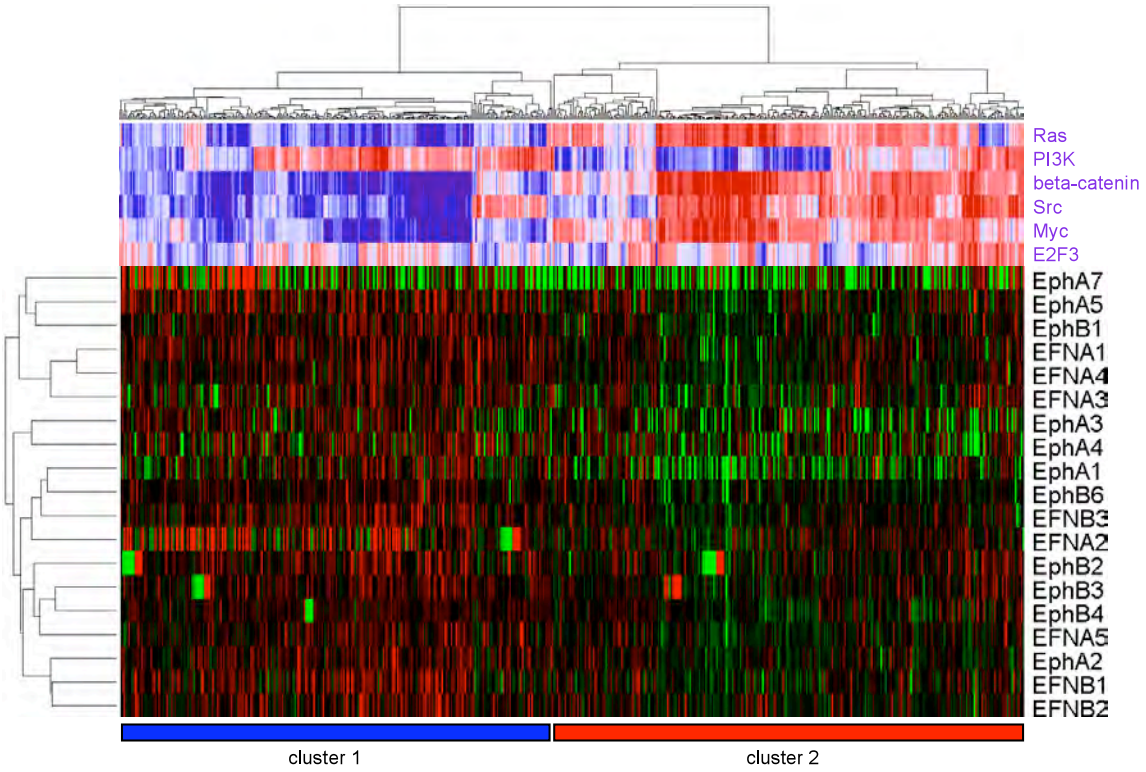
Is whole-genome analysis a feasible approach to dissect Eph-ephrin in cancer?

Cancer represents a special field of application for genomics, in which whole-genome analysis provides the opportunity for individualized diagnosis, prognosis and therapeutics. There are several reasons for this. First, the altered genome is the direct cause of cancer and precisely defines the tumor phenotype. Second, most cancer genomic alterations are somatic events, so that we can discern with confidence those changes specific to cancer relative to normal tissues. Third, genomic alterations are dynamic and progressive, related to disease stage, metastatic lesion and drug response. These connections have been largely uncovered with the rapid development of technologies. Therefore, the increasing sophistication of systems biology has made it possible that the complex interplay of events in cancer that activate and inactivate specific genes and pathways can now be deduced directly from deep genomic and transcriptomic analyses. Undoubtedly, these valuable information will make major contributions to the understanding of not only cancer in general, but also Eph-ephrin biology specifically.

Figure 6.2. Patterns of pathway deregulation and Eph-ephrin expression in human lung cancers.

A. Hierarchical clustering of predictions of pathway deregulation in samples of human lung tumours. Prediction of Ras, PI3K, Myc, E2F3, β-catenin and Src pathway status for each tumour sample was independently determined using supervised binary regression analysis, as described in text. Red indicates high probability of pathway activation, with blue indicating a low probability. Patterns in the tumour pathway predictions were identified by hierarchical clustering, and separate clusters are indicated below. The expression of Eph receptors and ephrin ligands was plotted based on the clustering of signaling pathways. **B.** Kaplan-Meier survival analysis for lung cancer patients based on pathway clusters. Patient clusters with correlative pathway deregulation correspond to clusters comprising each independent survival curve. Overall survival was defined as death due to any cause. P=0.03, log-rand test.

A


Ras
PI3K
beta-catenin
Src
Myc
E2F3
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EphA4
EphA1
EphB6
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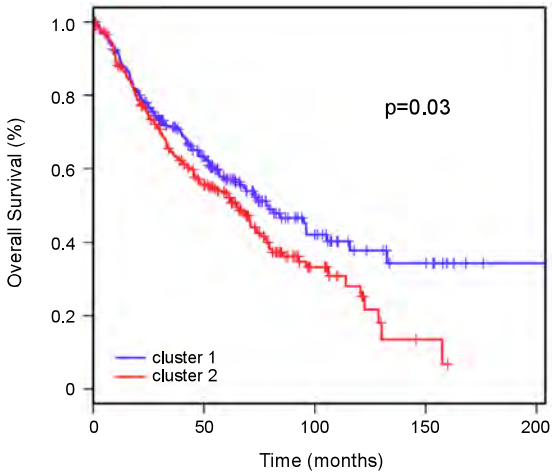
B


1.0
0.8
0.6
0.4
0.2
0
Overall Survival (%)
p=0.03
cluster 1
cluster 2
0
50
100
150
200
Time (months)

Bissell and colleagues used a three-dimensional culture model of non-malignant human mammary epithelial cells and developed a 22-gene signature, which accurately predicts breast cancer outcome across multiple datasets [100, 312]. Importantly, most individual genes in the 22-gene signature are significant predictors of patient survival, suggesting that these genes may be "master genes" with high predictive ability. The result supports the hypothesis that the 3D signature genes play important biological roles in breast cancer, and hence are potential targets for development of novel therapeutics. Interestingly, EphA2 is in the signature identified by this unbiased approach, which is consistent with our findings that EphA2 promotes tumorigenesis in breast cancer, at least under some circumstances. Another study combined nonsense-mediated RNA decay microarrays and array-based comparative genomic hybridization for the genome-wide identification of genes with biallelic inactivation involving nonsense mutations and loss of the wild-type allele. This approach, which identified previously unknown mutations in the EphB2 receptor, again demonstrated the power of an integrated strategy for the genome-wide screening of cancer-related genes to explore the fundamental and discriminating properties of Eph receptors [313].

In our studies, by analyzing SNP array-based genetic maps with gene expression signatures, we applied an integrative approach to identify EphA3 receptor, which is often deleted and under-expressed, as a potent tumor suppressor in non-small cell lung cancer. Additionally, we generated two EphA3 gene signatures, one related to EphA3 mutation in lung adenocarcinoma, and the other one correlated with EphA3 expression. In an unsupervised analysis using an independent large cohort validation dataset, both

signatures were able to group tumors into two distinct main clusters, which are associated with patient overall survival. This genome-wide transcriptional analysis supports our hypothesis that EphA3 signaling pathway plays a major role in lung cancer progression.

We sought to explore other possibilities for genomic analysis to be utilized in Eph-ephrin studies. Previous reports have shown that gene expression signatures can be identified which reflect the activation status of central oncogenic pathways [314-316]. These so-called oncogenic pathway signatures can predict pathway deregulation in human tumors, and cluster tumors based on patterns of pathway signatures. This approach is very appealing since the categorization of patients could define prognosis in respective patient subsets, and also pathway deregulation provides an opportunity to guide the use of targeted therapeutics. We applied this technique in 572 lung adenocarcinomas to predict the activity of key pathways including Ras, PI3K, β-catenin, Myc, Src, and E2F3. Unsupervised hierarchical clustering was performed to identify patterns of pathway deregulation (Figure 6.2). This analysis stratified tumors into two groups, one of which exhibited worse survival than the other. It is also evident that the tumors predicted as exhibiting relatively high Ras activity are generally predicted at higher levels of β-catenin, Myc and Src activity. Conversely, the tumors with relatively elevated Ras activity showed relatively lower levels of PI3K pathway. When expression levels of Eph receptors and ephrin ligands were projected on the dendogram, they are associated with patterns of pathway deregulation as well as patient outcome. Specifically, lower Eph-ephrin expression correlates with activated Ras, β-catenin, Myc and Src pathways, and poor prognosis. Notably, EphA3 is highly downregulated in the population of patients

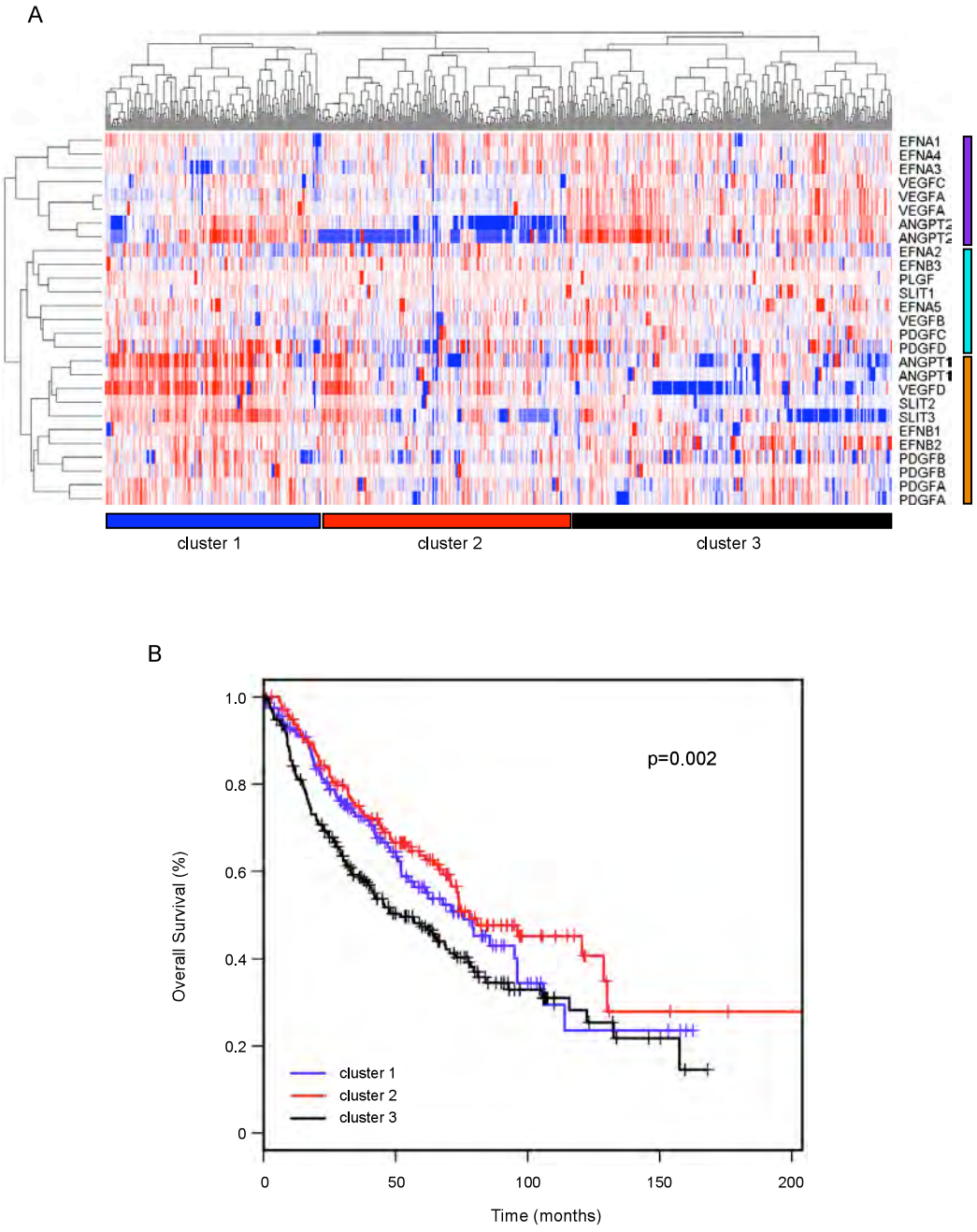


Figure 6.2. Patterns of the expression of angiogenic factors in human lung cancers.

A. Hierarchical clustering of angiogenic factors in samples of human lung tumours. Red indicates high expression, with blue indicating a low expression. Separate clusters on two dimensions are indicated in color bars. **B.** Kaplan-Meier survival analysis for lung cancer patients based on clusters. Overall survival was defined as death due to any cause. P=0.002, log-rand test.

with poor survival, supporting our findings that EphA3 functions as a tumor suppressor in lung cancer.

Such large, multi-site and well-performed gene expression studies can also help us to characterize the role of Eph-ephrin in tumor angiogenesis. We examined major classes of angiogenic factors expressed in tumor cells, including ephrins, VEGFs, PDGFs, angiopoietin, and Slits. Interestingly, these angiogenic modulators, which are grouped into three subsets, divided lung adenocarcinomas into three main clusters based on two-dimensional hierarchical clustering. Cluster 1 and cluster 3 are distinguished by higher PDGFs, ephrinB, Slits, angiopoietin 1, and VEGFs, ephrinA, angiopoietin 2, respectively, while cluster 2 express lower levels of these angiogenic factors in general. These data suggest to us that lung tumors have distinctive patterns of angiogenic growth, and angiogenesis is related to cancer prognosis. Accordingly, ephrin ligands are not identical in regulating tumor angiogenesis, and may have interplays with other families of activators such as VEGFs and Slits. These information should help in the design of better anti-angiogenic therapies and to overcome drug resistance frequently observed in clinic.

Concluding remarks

The results we have reported herein make a significant step forward in understanding the role of Eph signaling in the regulation of cancer malignancy. Through integrating genetic, genomic, functional and mechanistic studies, we have been able to show that the distinctive characteristics of Eph signaling enable it to exhibit both pro- and anti-tumorigenic effects. Canonical Eph signaling pathway, which is dependent on ephrin

stimulation, could suppress tumor progression by inhibiting crucial oncogenic events such as mTOR activity. On the other hand, non-canonical Eph signaling pathway, which is independent on ephrin stimulation, could promote tumorigenesis by coordinating with other oncogenes including HER2. We anticipate that important new insights into how tumors convert an inhibitory signal to a promoting one will emerge in the Eph field in the coming years. Such discoveries will become especially important when attempting to evaluate the effects of Eph-based therapies and establishing effective criteria for patient selection.

It is noteworthy that our model dramatically simplifies the diversities of Eph receptors and ephrin ligands, and in addition, it does not consider the function of Eph-ephrin in tumor microenvironment. Many questions remain to be answered. What are the specific signaling activities of different Eph receptors? What is the role of ephrin reverse signaling in cancer cells? Why are some Eph receptors mutated more frequently than the others? How does cancer-related Eph mutation contribute to tumor biology? Another area of great interest is whether the Eph system has impact on tumor infiltrating immune cells, as well as cancer stem cells. With the emerging new technologies and accumulating knowledge of Eph-ephrin, it will be possible to better understand the complexities of the Eph family in cancer.

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