Review

Eph receptors in breast cancer: roles in tumor promotion and tumor suppression

David Vaught[2], Dana M Brantley-Sieders[1] and Jin Chen[1-4]

[1]Department of Medicine, Division of Rheumatology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
[2]Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
[3]Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
[4]Department of Cell & Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

Corresponding author: Jin Chen, jin.chen@vanderbilt.edu

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Abstract

Eph receptor tyrosine kinase signaling regulates cancer initiation and metastatic progression through multiple mechanisms. Studies of tumor-cell-autonomous effects of Eph receptors demonstrate their dual roles in tumor suppression and tumor promotion. In addition, Eph molecules function in the tumor microenvironment, such as in vascular endothelial cells, influencing the ability of these molecules to promote carcinoma progression and metastasis. The complex nature of Eph receptor signaling and crosstalk with other receptor tyrosine kinases presents a unique challenge and an opportunity to develop therapeutic intervention strategies for targeting breast cancer.

Introduction

The Eph receptors comprise the largest family of receptor tyrosine kinases. The family is subdivided into class A and class B, based on sequence homology and binding affinity for two distinct types of membrane-anchored ephrin ligands. Ephrins and Eph receptors are key regulators of physiological and pathological processes in development and disease (for reviews, see [1-6]). Expression of many of the Eph receptors is often elevated in a wide variety of tumors, including breast cancer, yet their precise roles in cancer are not well understood. Data from recent studies demonstrated that Eph receptors and ephrins function in both tumor cells and the tumor microenvironment, with dual roles in tumor suppression and tumor promotion. In this review we highlight key results in the area of Eph receptor expression, tumor biology, and therapeutics in breast cancer, with an emphasis on EphA2 and EphB4 receptors.

Eph receptor in mammary gland development

Mammary epithelial morphogenesis is a complex developmental process during which an extensive network of branched ducts forms from a rudimentary epithelial bud. This process, termed 'branching morphogenesis', is regulated by endocrine hormones and local paracrine interaction between the developing epithelial ducts and their adjacent mesenchymal stroma. Expression of multiple Eph family receptors and their ligands has been reported in the mammary gland. Ephrin-B2 is expressed on the luminal cells, and its receptor, EphB4, is expressed complementarily on myoepithelial cells in mice. The expression of EphB4 and ephrin-B2 is dependent on estrogen and is regulated during the estrus cycle [7]. Over-expression of EphB4 under the control of the mouse mammary tumor virus (MMTV) promoter/enhancer induced delayed development of the mammary epithelium at puberty and during pregnancy, with unscheduled epithelial apoptotic cell death during pregnancy and abnormal epithelial DNA synthesis at early postlactational involution, indicating an abnormal response to proliferative/apoptotic signals [8].

In addition to EphB4, developmentally controlled expression of EphA2 in the mammary epithelium has also been reported [9,10]. Loss of EphA2 receptor resulted in decreased penetration of mammary epithelium into fat pad, reduced epithelial proliferation, and inhibition of epithelial branching, suggesting a positive role for EphA2 during normal mammary gland development (Vaught and coworkers, unpublished data). EphA2 is also expressed in human mammary epithelial cells [11-14]. Fournier and coworkers analyzed gene expression in two nonmalignant human mammary epithelial cell lines in three-dimensional cultures. When these cells underwent growth arrest and differentiated into polarized acini, EphA2 levels were significantly decreased [15], consistent with the observation that EphA2 is expressed at a low level in normal mammary gland epithelium, whereas expression increases in breast cancer [3]. Indeed, analysis of

EGFR = epidermal growth factor receptor; MMTV = mouse mammary tumor virus; PI3K = phosphoinositide-3 kinase; siRNA = small interfering RNA; VEGF = vascular endothelial growth factor.

a set of 19 genes that were downregulated in differentiated acini of human mammary epithelial cells in three-dimensional cultures against two independent breast cancer microarray datasets revealed that increased EphA2 levels are associated with poor patient prognosis [13,15]. Taken together, these data suggest that EphA2 is required for mammary gland morphogenesis, and increased EphA2 expression in human breast cancer is associated with tumor cell malignancy and poor patient survival.

Role of Eph receptors in breast cancer promotion

In screens for new receptor tyrosine kinases in cancer, many Eph receptors were found to be over-expressed in multiple types of human tumors [1,3]. Of Eph receptors expressed in breast cancer [11,12,16,17], EphA2 and EphB4 are the two that have been most extensively studied. EphA2 is expressed at low levels in normal human breast epithelium [9,18] and over-expressed in 60% to 80% of breast cancers [11,12,19] (Brantley-Sieders and Chen, unpublished data). Experimentally induced over-expression of EphA2 resulted in malignant transformation of nontransformed MCF10A breast cells and enhanced malignancy of pancreatic carcinoma cells [12,20]. Conversely, small interfering RNA (siRNA)-mediated inhibition of EphA2 expression impaired malignant progression of pancreatic, ovarian, and mesothelioma tumor cell lines, and over-expression of dominant-negative EphA2 constructs suppressed growth and metastasis of 4T1 metastatic mouse mammary adenocarcinoma cells *in vivo* [20-23]. To determine whether EphA2 plays a causative role in breast cancer initiation and metastatic progression, EphA2 knockout mice were crossed to MMTV-Neu transgenic animals that express a rat homolog of the ErbB2 receptor tyrosine kinase. Loss of EphA2 impairs both tumor initiation and lung metastasis in MMTV-Neu mice [24]. Similarly, EphB4 levels are also elevated in human breast cancer [17]. EphB4 knockdown inhibited breast cancer survival, migration, and invasion *in vitro* and tumor growth in a xenograft model *in vivo* [25]. Furthermore, over-expression of EphB4 in the mammary epithelium accelerates tumor onset and lung metastasis in MMTV-Neu animals [8]. Taken together, these data indicate a role for Eph receptor in tumor promotion.

The mechanisms regulating the oncogenic effects of Eph receptors are not entirely clear. In many instances, Eph receptors in tumor cells are under-phosphorylated in spite of over-expression. This could be due to increased activity of phosphotyrosine phosphatase or loss of E-cadherin in tumor cells. E-cadherin regulates cell surface localization of EphA2 and/or loss of cell-cell contacts prevent interaction with endogenous ephrin ligands [26-28]. Because both Eph receptors and ligands are membrane anchored and reside in separate microdomains on the cell surface [29,30], loss of cell-cell adhesion in tumor cells impairs activation of Eph receptor by ephrins on adjacent cells. Thus, the oncogenic activity of Eph receptors appears to be ligand independent. High levels of EphA2 receptor have been shown to interact physically with both the epidermal growth factor receptor (EGFR) and ErbB2, promoting Erk and RhoA GTPase activity [24,31]. These data suggest that crosstalk between Eph receptor and other oncogenic pathways promotes tumor cell malignancy, possibly in an ephrin-independent manner. Furthermore, a high level of EphA2 was found to upregulate matrix metalloproteinase 2 [20,32] and extracellular matrix protein fibronectin [33]. Modulation of tumor cell interaction with the microenvironment may also contribute to Eph receptor function in tumor promotion.

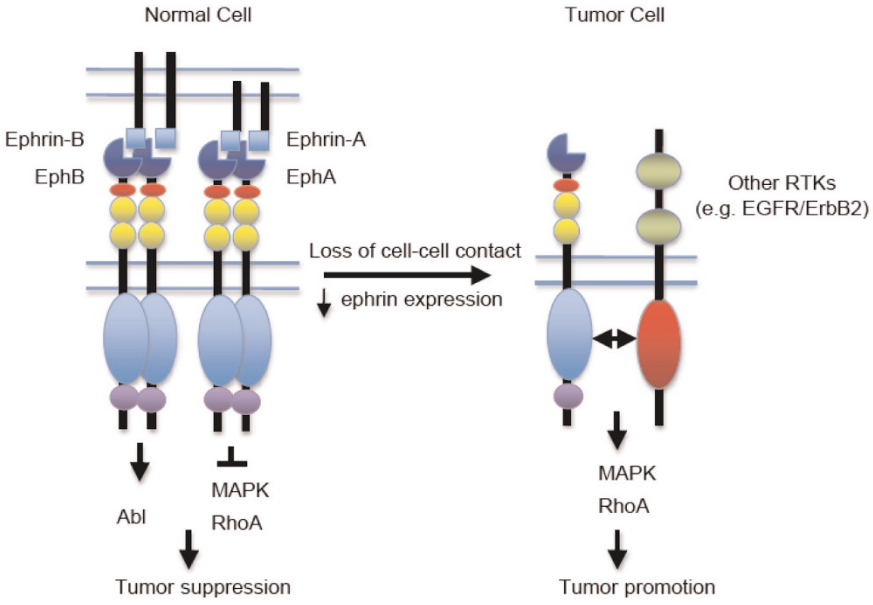
Role of Eph receptors in tumor suppression

Many studies have demonstrated a role for Eph receptor in tumor suppression. 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 [34,35]. Macrae and coworkers [35] also reported that treatment of human breast cancer cell lines with ephrin-A1-Fc attenuated epidermal growth factor mediated phosphorylation of Erk and inhibited transformation of NIH3T3 cells expressing v-erbB2. In addition, EphA2-deficient gene-trap mice displayed increased susceptibility to chemical carcinogen-induced skin cancer, accompanied by increased tumor cell proliferation and phosphorylation of Erk [36]. These data suggest that ephrin-A-induced EphA2 receptor forward signaling inhibits tumor malignancy.

In addition to EphA2, EphB4 forward signaling also appears to inhibit tumor progression. Systemic delivery of ephrin-B2-Fc inhibits the growth of MDA-MB-435 tumor xenografts [37]. EphB4 forward signaling apparently activates the Abl/Crk pathway to inhibit tumor cell growth and motility in breast cancer cells [37]. Furthermore, EphB receptor signaling is also able to suppress tumor expansion in colon cancer. Over-expression of a dominant negative EphB2 cytoplasmic truncation mutant or knockout of EphB3 or ephrin-B1 in the intestinal epithelium significantly increases tumor numbers and tumor invasiveness in the $APC^{min/+}$ model [38,39]. EphB receptors have been proposed to compartmentalize the expansion of colon cancer cells through a mechanism dependent on E-cadherin-mediated adhesion [39].

In summary, ephrin-induced Eph receptor forward signaling in nontransformed mammary epithelial cells appears to transduce an inhibitory signal that may keep cells quiescent and noninvasive [34,35,37]. Upon tumor initiation, Eph receptor expression is upregulated by oncogenic signaling pathways such as the Ras/mitogen-activated protein kinase pathway in breast cancer or the Wnt-β-catenin pathway in colon cancer, whereas their ephrin ligands are often down-regulated [35,40] or unable to bind to receptor because of loss of cell-cell adhesion [28]. Crosstalk between elevated Eph receptors and other oncogenes, such as the ErbB family of receptor tyrosine kinases [24,31], leads to enhanced cell

Figure 1



Working model for Eph receptor function in tumor promotion and tumor suppression. In normal cells, engagement of Eph receptors with ephrins on adjacent cells *in trans* induces receptor forward signaling, leading to inhibition of Ras/mitogen-activated protein kinase (MAPK) activity, or suppression of Crk activation via Abl kinase activity, and tumor suppression. In tumor cells, disruption of cell-cell junctions inhibits Eph receptor interaction with endogenous ephrins *in trans*. In addition, Eph receptors are often upregulated whereas ephrins are downregulated. Crosstalk between Eph receptors and other receptor tyrosine kinases such as ErbB2 and epidermal growth factor receptor (EGFR) results in increased activity of the Ras-MAPK pathway and the RhoA GTPase, and enhanced tumor malignancy.

proliferation and tumorigenesis, presumably independently of ephrin stimulation (Figure 1).

Eph receptors and ephrins in tumor angiogenesis

Tumor angiogenesis is critical for growth, survival, and malignant progression of tumors. Tumor vessels not only supply the nutrients and oxygen necessary for tumor cell growth and survival, but they also actively promote malignant progression by providing an entry point into the circulation for the dissemination of metastatic cells [41]. In addition to regulating developmental angiogenesis, Eph receptors and ephrins have also emerged as critical regulators of tumor angiogenesis. The first ligand discovered for the Eph receptors, ephrin-A1, is a tumor necrosis factor-α inducible gene in endothelial cells [42]. Early studies demonstrated that ephrin-A1 promotes angiogenic responses *in vitro* and corneal neovascularization *in vivo* [42]. Ephrin-A1 is expressed in developing embryonic and tumor vasculature [11,18,43]. More importantly, ephrin-A1 is further induced by hypoxia in tumors that are resistant to anti-vascular endothelial growth factor (VEGF) therapy [44]. Ephrin-A1 knockout mice were recently generated. Interestingly, ephrin-A1-deficient mice survive to adulthood with only minor heart valve defects (Frieden and Chen, unpublished data), suggesting that loss of ephrin-A1 can be functionally compensated for in vascular development. It remains to be determined whether tumor angiogenesis is affected in these mice.

EphA2, a major receptor for ephrin-A1 in vascular endothelial cells, plays a significant role in promoting tumor angiogenesis. Implantation of tumor cells into the mammary gland of EphA2-deficient host mice results in reduced tumor volume, microvascular density, and lung metastasis. These findings suggest that loss of EphA2 in the tumor microenvironment impairs tumor angiogenesis and metastatic progression [24,45]. Indeed, EphA2-deficient vascular endothelial cells fail to migrate and assemble in response to angiogenic cues *in vitro* [46] and are unable to incorporate into tumor blood vessels when they are co-transplanted with tumor cells *in vivo* [45,47], indicating a critical function for EphA2 in tumor angiogenesis.

In contrast to the complex effects of Eph signaling in tumor cells, Eph receptor signaling in vascular endothelial cells promotes tumor angiogenesis. Brantley-Sieders and co-workers [46] showed that EphA2 receptor forward signaling regulates endothelial cell migration and assembly through phosphoinositide-3 kinase (PI3K)-mediated Rac1 GTPase activation. A yeast two-hybrid screen for EphA2 interacting proteins revealed that Vav2 and Vav3 guanine nucleotide

exchange factors are recruited to activated EphA2 receptor and subsequently elevate Rac1-GTP levels [48]. Loss of Vav2 and Vav3 inhibits Rac1 activity and ephrin-A1-induced angiogenic responses both *in vitro* and *in vivo* [48]. Furthermore, Fang and coworkers [47] mapped phosphorylated tyrosine residues of EphA2 in vascular endothelial cells. Ephrin-A1-induced phosphorylation of Y587 and Y593 in the EphA2 receptor recruits Vav2 and Vav3 exchange factors, whereas phosphorylation of Y734 provides a docking site for the p85 regulatory subunit of PI3K. EphA2-null endothelial cells reconstituted with EphA2 mutants lacking these binding sites fail to activate Rac1 GTPase, are defective in cell migration and assembly *in vitro*, and are unable to incorporate into tumor vasculature *in vivo*. These findings suggest a critical role for these tyrosine phosphorylation sites in transducing EphA2 forward signaling in vascular endothelial cells and validated the involvement of PI3K-dependent activation of Vav exchange factors and Rac1 GTPase in ephrin-A1-induced angiogenesis.

Gene targeting studies have established ephrin-B2 and EphB4 as key regulators of embryonic vascular development [49,50]. Ephrin-B2 expression has also been observed in tumor vasculature in a variety of tumor types, suggesting that this ligand may regulate tumor neovascularization [51-53]. In support of this hypothesis, A375 melanomas form smaller, less vascularized tumors in the presence of the soluble, monomeric EphB4 extracellular domain *in vivo* [54]. Soluble EphB4 may act, at least in part, by preventing binding of tumor cell EphB receptors to ephrin-B2-positive endothelium, thus disrupting tumor angiogenesis. Further support for this hypothesis is provided by studies in which over-expression of a truncated cytoplasmic deletion EphB4 receptor construct produced increased tumor growth and vascularity in mammary tumors, probably through ephrin-B2 mediated reverse signaling in host endothelium [53]. Upregulation of ephrin-B1 expression has been reported in hepatocellular carcinoma, and over-expression of ephrin-B1 enhances tumor neovascularization *in vivo* [55]. Although proliferation of ephrin-B1 over-expressing cells was not affected in culture, soluble ephrin-B1-Fc enhanced endothelial cell proliferation and migration *in vitro*, suggesting that at least one function of ephrin-B1 in tumor progression involves facilitation of tumor angiogenesis [55,56]. Taken together, these studies reveal a critical role for B class receptors and ligands in tumor progression and vascular recruitment for multiple types of human cancer.

Eph receptors as targets for breast cancer therapeutics

Because Eph receptors are often over-expressed in malignant cancer, and reduction in Eph receptor levels was found to be efficacious in tumor inhibition in animal models, a wide range of therapeutic strategies targeting Eph receptors has been recently developed for cancer treatment. These approaches include activating monoclonal antibodies against Eph receptors, ligand- or activating antibody-cytotoxin conjugates, siRNA, antagonistic peptides, small molecular inhibitors, and immunotherapy (Table 1).

Carles-Kinch and coworkers [57] first reported that activating monoclonal antibodies against EphA2 inhibited tumor growth in soft agar and prevented tubular network formation on Matrigel. Coffman and colleagues [58] subsequently showed that similar anti-EphA2 agonistic antibodies selectively bind epitopes on malignant cells and decrease tumor growth in xenograft tumor models. The mechanism of action of these antibodies appears to mimic ephrin ligands, inducing receptor phosphorylation and subsequent internalization and degradation [57,58]. However, it is not clear whether agonistic antibody-induced EphA2 receptor forward signaling also conveys an inhibitory signal to promote tumor suppression. Regardless, the ability of ephrins and anti-EphA2 antibodies to distinguish malignant from nonmalignant cells prompted the development of ligand- or agonistic antibody-toxin conjugates. Wykosky and coworkers [59] reported a novel cytotoxin composed of the ephrin-A1 ligand conjugated to a genetically modified bacterial toxin, namely *Pseudomonas* exotoxin A. Ephrin-A1-conjugates exhibit potent and dose-dependent killing of cancer cells that express high levels of the EphA2 receptor, including glioblastoma multiforme cells, as well as breast and prostate cancer cells. An anti-EphA2 antibody conjugated with the microtubule polymerization inhibitor monomethylauristatin phenylalanine (MMAF) has also been developed by MedImmune Inc. (Gaithersburg, MD, USA). The conjugated antibody significantly inhibits tumor cell growth both *in vitro* and *in vivo* without any observable adverse effects [60]. These findings make ephrinA1- or anti-EphA2-based cytotoxins a potentially attractive therapeutic strategy in the treatment of breast cancer.

siRNAs that specifically inhibit gene expression have rapidly become a powerful tool in both mechanistic studies and targeted therapeutics. It was previously reported that siRNAs directed against EphA2 resulted in decreased protein expression and decreased tumor growth in a pancreatic cancer xenograft model [20]. More recently, siRNAs against EphA2 were incorporated into packaging liposomes composed of the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) for efficient *in vivo* delivery. Neutral liposome-coupled EphA2 siRNA reduced tumor growth in an orthotopic mouse model of ovarian cancer both in the presence and absence of paclitaxel [21,61], suggesting the feasibility of siRNA as a clinically applicable therapeutic approach.

Eph receptors and ephrins have emerged as critical regulators of tumor angiogenesis, making them attractive targets for inhibition of neovascularization [1,5]. More importantly, Eph/ephrin signaling provides a possible mechanism responsible for resistance to anti-VEGF therapy [44]. Soluble Eph receptors have been used to inhibit endogenous Eph

Table 1

Therapeutic strategies targeting Eph receptors

Treatment	Target	Tumor	Institution	References
Activating antibodies	EphA2	Breast cancer	MedImmune/AstraZeneca	[24,57,58]
Antibody-conjugates	EphA2	Prostate cancer, glioma	MedImmune/AstraZeneca	[60]
Ephrin-conjugates	EphA2	Glioblastoma	Wake Forest University	[59]
siRNAs	EphA2	Ovarian cancer	MD Anderson	[21]
		Pancreatic cancer	Harvard	[20]
Soluble receptors	EphA	Breast cancer	Immunex/Amgen	[18,62,63]
		Pancreatic cancer	Cephalon	
	EphB	Melanoma	University of Frieburg	[54]
Inhibitory peptides	EphB4	Angiogenesis	Burnham Institute	[64,67,73]
	EphB2			[65]
	EphA2			[74]
Small molecule inhibitors	EphA2	Angiogenesis	Burnham Institute	[66]
	EphA4			
Immunotherapy	EphA2	Colon cancer	Osaka University	[68]
		Glioblastoma	University of Pittsburgh	[69]

receptor signaling in vascular endothelium and tumor angiogenesis *in vivo* [18,54,62,63]. More recently, the Pasquale laboratory has developed a peptide, TNYL-RAW, which competes with ephrin-B2 for binding to EphB4 receptor [64,65]. In addition, two isomeric small molecule compounds have been identified that selectively inhibit ephrin binding to EphA4 and EphA2 [66]. Both the EphB4 blocking peptide and EphA2/EphA4 antagonistic compounds inhibit Eph receptor phosphorylation and capillary-like tube formation in human umbilical vein endothelial cells [66,67]. This suggests that they can potentially serve as starting points from which to develop antiangiogenic therapies in cancer treatment.

In addition to being direct targets for therapeutic intervention, EphA2-derived peptides have been used in a dendritic cell-based vaccine for immunotherapy in glioblastoma multiforme and colon cancer [68-70]. Early studies showed that, in renal cell carcinoma, EphA2-derived peptides induced specific, tumor-reactive $CD8^+$ and $CD4^+$ T-cell responses. The reactivity of $CD8^+$ T cells to EphA2 peptides was stronger in T cells isolated from postsurgery disease-free patients than in those from patients with active disease, suggesting that the immune system of cancer patients actively monitors EphA2-derived epitopes [70]. More recently, vaccination using dendritic cells pulsed with EphA2 peptides in a murine colon cancer model revealed that immunization inhibited the growth of MC38 tumors expressing EphA2, but did not have an effect on BL6 tumors that do not express EphA2 [68]. Furthermore, Hatano and coworkers [69] reported that stimulation of peripheral blood mononuclear cells from glioma patients and control healthy donors with dendritic cells loaded with EphA2 peptide elicited an antigen-specific cytotoxic T cell response. These preliminary results demonstrate that EphA2-derived epitopes may represent important candidate vaccines to be tested in clinical trials for the treatment of malignant cancers.

Conclusions

Eph receptor expression is often increased in human cancer, including breast cancer. The available evidence suggests that ligand-induced Eph receptor signaling in tumor cells plays a role in tumor suppression, whereas ligand-independent Eph receptor signaling functions in tumor promotion. Further investigation into molecular pathways that may be differentially regulated by EphA2 activation in response to endogenous ephrin ligands versus activation by association with other receptor tyrosine kinases, such as ErbB2, could provide insight into the differential effects of EphA2 signaling in breast cancer. Moreover, analysis of EphA2 and ErbB2/EGFR co-expression in human breast cancer samples, coupled with genetic evidence suggesting that EphA2 co-operates with ErbB2 to promote tumor progression in mice [24], may provide a rationale for combining EphA2-targeted therapies with inhibitors of ErbB2/EGFR signaling in patients that express both receptors.

For EphB4, expression patterns and function of B class Eph receptors in intestinal epithelial patterning and tumorigenesis

provide a model for elucidating the roles played by this and other EphB receptors in breast cancer. Expression and functional studies suggest that EphB/ephrin-B family members coordinate positional patterning of specific cell types in intestinal epithelium to prevent intermingling, and expression of several B class molecules are regulated by the Wnt/β-catenin signaling pathway in the gut [40,71]. Complementary expression of Ephrin-B2 on luminal cells and EphB4 on myoepithelial cells in the mouse mammary gland [7] could function to maintain a boundary between these cell types, similar to what is observed in the intestinal models. Moreover, because several members of the Wnt signaling pathway have been implicated in mammary gland development and breast cancer (for review see [72]), similar upregulation of EphB receptors by Wnt/β-catenin could contribute to breast cancer progression. Further research is required to test these hypotheses.

In addition to a tumor cell autonomous role, Eph receptor also regulates important tumor-host interactions, notably in stimulation of tumor angiogenesis during tumor progression. As such, targeting Eph receptors holds both promises and challenges for therapeutic intervention in breast cancer. The effects of Eph receptors on both tumor cells and tumor microenvironment provide a unique therapeutic opportunity to block multiple steps in tumor progression. At the same time, the nature of bidirectional signaling of the Eph/ephrin system, and contribution of cell-type and context-dependent factors to the multi-faceted role of Eph receptors in cancer cells may complicate the development of effective therapeutic agents. Further research in dissecting bidirectional signaling and the context-dependent role of Eph receptors in cancer is essential for developing successful therapeutic strategies.

Competing interests

We acknowledge that we have received grant funding from MedImmune, Inc. within the past 3 years, and have submitted a provisional patent application with MedImmune concerning therapeutic targeting of EphA2.

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