Subject Review

The EphA2 Receptor and EphrinA1 Ligand in Solid Tumors: Function and Therapeutic Targeting

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Abstract

The Eph receptor tyrosine kinases and ephrin ligands have been studied extensively for their roles in developmental processes. In recent years, Eph receptors and ephrins have been found to be integral players in cancer formation and progression. Among these are EphA2 and ephrinA1, which are involved in the development and maintenance of many different types of solid tumors. The function of EphA2 and ephrinA1 in tumorigenesis and tumor progression is complex and seems to be dependent on cell type and microenvironment. These variables affect the expression of the EphA2 and ephrinA1 proteins, the pathways through which they induce signaling, and the functional consequences of that signaling on the behavior of tumor cells and tumor-associated cells. This review will specifically focus on the roles that EphA2 and ephrinA1 play in the different cell types that contribute to the malignancy of solid tumors, with emphasis on the opportunities for therapeutic targeting.
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Introduction

The Eph receptor tyrosine kinases (RTK) and their ephrin ligands have been intensely studied for the roles they play during embryonic development, particularly within the central nervous system. Some of these proteins, particularly EphA2 and ephrinA1, are of increasing interest in recent years due to their documented or suspected involvement in mediating processes leading to the formation and progression of malignancy. The EphA2 receptor was first identified in 1990 as a result of screening an epithelial cell cDNA library with degenerate oligonucleotides designed to hybridize to highly conserved regions of protein tyrosine kinases (1). The same year, ephrinA1 was discovered as a novel tumor necrosis factor-α–inducible gene product in human umbilical vein endothelial cells (2) but only later identified as an EphA2 ligand (3). Since, both proteins have emerged as integral players in the pathogenesis of cancer, albeit in an extremely complex manner that is still not fully understood. This review will provide an overview of Ephs and ephrins in normal physiology and cancer and specifically outline advances in the field with respect to the expression and function of EphA2 and ephrinA1 in malignancy. A further emphasis will be on the pleiotropic effects of the receptor and ligand in the different cell types that contribute to tumor formation, maintenance, and progression. In addition, the specific ways in which this receptor/ligand system can be targeted for the development of anticancer therapeutics and diagnostics will be discussed.

Eph Receptors and Ephrin Ligands: Structure and Function in Physiology and Cancer

The Eph receptors comprise the largest family of tyrosine kinase receptors, with 16 known members across many species, 14 of which are found in mammals (4). The first receptor of this family, EphA1, was cloned from an erythropoietin-producing hepatocellular carcinoma cell line in 1987 during a screen for RTKs with homology to the viral oncogene *v-fps* (5). Initially, there were no known ligands for the Eph receptors, which were thus regarded as orphan RTKs. It was not until several years later that the family of ephrins, or Eph receptor-interacting proteins, was identified as Eph ligands (6, 7).

Ephs are divided into two distinct A and B classes based on sequence homology of the extracellular domain, which, in part, determines the ephrin ligands with which the receptors interact (Fig. 1A). This NH_2-terminal ephrin-binding domain is followed by a cysteine-rich domain and two fibronectin-type III repeats. Intracellularly, the juxtamembrane domain contains two tyrosines that undergo autophosphorylation and is followed by a tyrosine kinase domain. The COOH-terminal end of Eph receptors serves as a docking site for interacting proteins that may mediate downstream signal transduction processes and includes a sterile α motif and a PSD-95 postsynaptic density protein, Discs large, Zona occludens tight junction protein (PDZ) domain-binding motif (Fig. 1A).

The eight members of the ephrin family are also divided into distinct A and B subclasses, indicating the manner in which they are anchored to the membrane: either by a glycosylphosphatidylinositol (GPI) linkage (ephrinA1-A5) or by a

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transmembrane domain (ephrinB1-B3; Fig. 1B). A unique property of ephrins that is a result of membrane localization is their ability to transduce "reverse" signals into the cells on which they are expressed in addition to eliciting "forward" signaling into Eph receptor-expressing cells (8). EphrinB ligands possess a cytoplasmic tail involved in several intracellular signaling processes (Fig. 1B; ref. 9). Whereas less is known about reverse signaling through ephrinA ligands, it may involve the localization of proteins such as the Src family kinase Fyn to the GPI anchor, likely within lipid raft microdomains (10). Another unique aspect of ephrins that sets them apart from other RTK ligands is that their membrane-bound nature is thought to be required to elicit full Eph receptor activation. Plasma membrane–bound ephrins, as well as soluble ephrins that are artificially clustered or dimerized by the addition of antibodies against COOH-terminal epitope tags or fusion to the Fc portion of IgG, respectively, efficiently induce receptor phosphorylation (6). Soluble ephrinA1 and ephrinB1, for example, were shown to induce EphA5 and EphB1 receptor phosphorylation, respectively, only when membrane associated or clustered (6). This apparent requirement for ephrin membrane localization or clustering has been attributed to the possibility that Eph receptors themselves must undergo clustering to be activated (11-13). There is evidence, however, for the existence and function of soluble, unclustered, monomeric ephrinA1, which will be discussed later in this review. Furthermore, the functional consequences of Eph receptor activation have been shown to depend on the specific oligomeric state of the ephrin ligand, which could be one of the variety of ways in which the function of the system is regulated (14).

Structural Requirements for Eph-Ephrin Interactions

In general, ephrinA and ephrinB ligands interact with EphA and EphB receptors, respectively, but ephrins display promiscuous binding to Eph receptors within a class (4). There are some exceptions, however, in instances where ephrinA5 functionally interacts with EphB2 (15) and EphA4 binds to both ephrinA and ephrinB family members (8, 16). Many ephrins display preferences for binding to specific Eph receptors, which is manifested in variable binding affinity interactions (4). Structurally, the highly conserved 15-amino acid loop that joins the ephrin G and H β-strands (the G-H loop) is thought to be responsible for the initial dimerization of ephrin and Eph (17). Analysis of the specific residues within the G-H loop of ephrinA versus ephrinB as well as within the dimerization domains of EphA versus EphB has revealed a basis for binding preferences (11, 18). At position 109, B-ephrins typically have a bulky polar residue, such as glutamine or leucine, which participates in hydrogen bonding with a conserved threonine at position 38 of EphB. In contrast, position 109 of A-ephrins is most often occupied by a serine or alanine, which interacts with a bulky residue, such as glutamine or methionine, at position 38 of EphA. In addition, Thr^{114} of ephrinB participates in van der Waals interactions with valine at position 54 of EphB. EphrinA ligands, however, have a serine with a small side chain at position 114, and position 54 of EphA receptors is occupied by an amino acid with a bulky hydrophobic side chain, such as isoleucine or methionine. These studies offer an explanation for the ability of EphA4 to interact with both ephrinA and ephrinB ligands, being that this is the only EphA receptor with a valine at position 54. In addition, the basis for the ligand specificity of EphB4 for ephrinB2 and not other ephrin ligands was found to be due in part to the presence of leucine at position 95, which is occupied by a conserved arginine in all other Ephs (19). Other Eph/ephrin interfaces are responsible for mediating the higher-order clustering of Eph-ephrin dimeric complexes thought to occur during receptor activation and downstream signaling, and could potentially contribute to binding preferences.

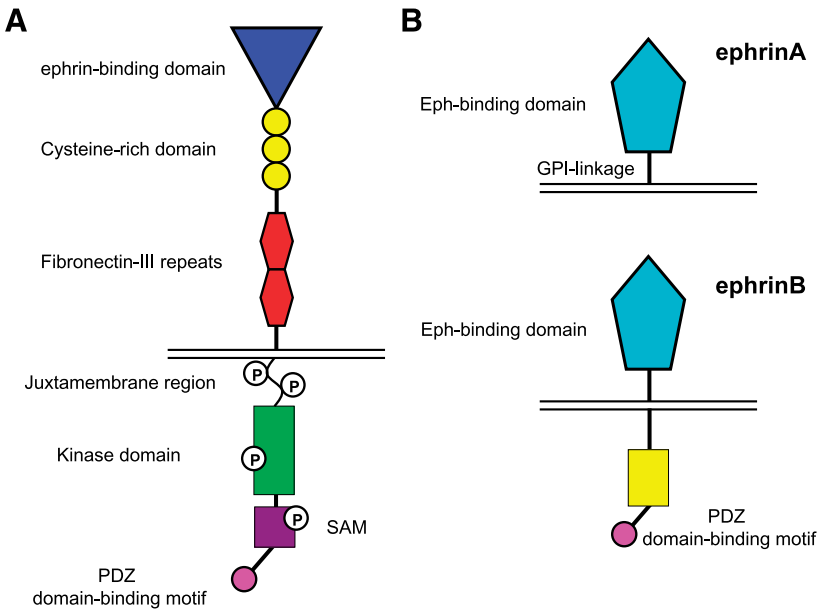

FIGURE 1. Eph receptors and ephrin ligands. Schematic drawing of the localization, structural, and signaling components of (**A**) Eph receptors and (**B**) ephrinA and ephrinB ligands (141). P, tyrosine phosphorylation site. =, plasma membrane. Each color represents a structurally or functionally distinct domain of the protein, as labeled. Yellow in ephrinB ligands represents the intracellular, cytoplasmic tail of the protein. Adapted from Cytokine Growth Factor Rev, Vol. 13, N. Cheng, D.M. Brantley, J. Chen, The ephrins and Eph receptors in angiogenesis, pp. 75-85, Copyright (2002), with permission from Elsevier.

Eph/Ephrin Signaling Pathways: Lessons from Development

Signaling cascades downstream of Eph receptors involve the interaction of the cytoplasmic domain with proteins such as p120RasGAP, a Ras family GTPase-activating protein (20, 21), guanine-nucleotide exchange factors such as Tiam1 (22), Vav1 and Vav2 (23, 24), and ephexin (25, 26), focal adhesion kinase (FAK; refs. 27, 28), PDZ domain-containing proteins such as the Ras-binding protein AF-6 (29, 30), and SH2/SH3 domain-containing proteins Nck (31), SLAP (32), Src family cytoplasmic tyrosine kinases (33, 34), and the p85 subunit of phosphatidylinositol 3-kinase (PI3K; ref. 35). These interactions are highly dependent on the specific Eph receptor involved, the ligand-activated status of the receptor, and the cell type in which the receptor is expressed. Functionally, the important role that Eph-ephrin signaling plays during development has been well characterized and is reviewed in detail elsewhere (4, 8, 36, 37). Most of these processes are cell-cell contact dependent, which would be expected considering the assigned plasma membrane localization of both receptor and ligand. Juxtacrine signaling between an Eph-expressing and an ephrin-expressing cell typically results in either adhesion or repulsion and governs processes such as axon guidance, boundary formation, and topographic mapping within the developing central nervous system. In addition, some Ephs and ephrins, particularly those in the EphB/ephrinB subclass, play an important role in the organization of the vasculature, both within the context of the central nervous system and in peripheral organs.

In general, Ephs and ephrins are expressed at the highest level during development and are found at low levels in normal adult tissue (38-40). Recent work highlights the role of Eph signaling in the formation, maintenance, and physiologic function of synapses. Several EphB receptors, EphA4, and ephrinA3 are involved in the regulation of dendritic spine morphology and synaptic plasticity in the developing and adult brain (39, 41-44). Specifically, ephrinA3 expressed on astrocytes in the adult mouse hippocampus interacts with EphA4 on dendritic spines of hippocampal neurons, causing shortening of spines and spine collapse reminiscent of neuronal growth cone collapse mediated by the ephrinA/EphA interaction during development (41). Overall, the function of Eph/ephrin proteins in the adult brain lies primarily in mediating processes, which involve remodeling and the formation of new neuronal connections, and gene expression seems to be mostly confined to the specific cell populations in the brain involved in these processes (39).

Ephs and Ephrins in Human Malignancy

Being that the first Eph receptor was cloned from a hepatocellular carcinoma cell line and was found to be at least 10-fold overexpressed in these cells compared with nonmalignant tissue (5), it is not surprising that most Eph proteins have since been found to be associated with a plethora of malignancies. The Eph-interacting adaptor proteins mentioned above intracellularly converge on downstream signaling pathways that are without a doubt integral to controlling processes such as cell growth, proliferation, migration, and invasion, all of which are inherent to cancer development and progression. For example, Eph receptors have been shown to affect signaling through pathways such as Janus-activated kinase/signal transducers and activators of transcription (45), Ras-mitogen-activated protein kinase (MAPK; refs. 46-53), PI3K/AKT (35, 52, 54, 55), and integrins/FAK/paxillin/Rho (24, 27, 28, 42, 50, 53, 55-58).

Specifically, EphB2 is overexpressed in gastrointestinal and liver tumors and plays a functional role in stimulating the invasion of human glioblastoma multiforme (GBM) cells by eliciting signaling through R-Ras and affecting integrin activity (51, 53). Interestingly, however, a tumor-suppressing role for EphB2 has been suggested in colorectal cancer (59-62) and in prostate cancer (63, 64). The dichotomy of EphB2 as an oncoprotein or tumor suppressor depending on cell type and microenvironment is just one example of the complex pleiotropic effects of Eph receptors in cancer. EphB4 has also been detected in several types of human cancers, including mammary (65), colon (66), and ovarian (67-69) carcinoma. Interestingly, EphB4 promotes ovarian tumor growth (68) and melanoma invasiveness (70), but signaling through this receptor has also been shown to suppress mammary carcinogenesis through an Abl-Crk pathway (71), again highlighting complex and seemingly contradictory roles for Eph receptors (72). Reverse EphB4 signaling through ephrinB2 also significantly affects tumor angiogenesis, evident in the finding that overexpression of signaling defective or truncated EphB4 still enhanced tumor growth (73). The important function of EphB4 and ephrinB2 in the formation and organization of the vasculature also points to a role for these proteins in tumor angiogenesis and metastasis. Among the EphA/ephrinA proteins, EphA2 and ephrinA1 are the most widely studied with respect to tumorigenesis, angiogenesis, and metastasis and represent sought-after therapeutic targets because of their expression and diverse functions in several different types of cancer.

EphA2 and EphrinA1 Expression in Human Tumors

The EphA2 receptor is a 130-kDa, 976-amino acid transmembrane glycoprotein and is abundantly overexpressed in several solid tumors (Table 1; refs. 56, 74-92). Overexpression has been shown both at the mRNA and protein level in established cell lines and human tumor tissue specimens (Table 1). Expression of *ephA2*, located on chromosome 1p36, is limited in normal tissues to those having a high proportion of dividing epithelial cells, such as the skin, lung, and small intestine (40), but to date, a systematic analysis of EphA2 protein expression in normal tissue has not been done. By immunohistochemistry, EphA2 is strongly overexpressed in 61% of GBM patient tumors (82), 76% of ovarian cancers (76), and 85% of prostate adenocarcinomas (79). Importantly, this protein is highly overexpressed with regard to percentage of patient tumors and percentage of cells within a tumor, and is a plasma membrane–localized receptor that can internalize on ligand binding (93). Moreover, expression of EphA2 is associated with poor prognosis, increased metastasis, and decreased survival (83, 85, 89, 94). Thus, due to its expression pattern, localization, and functional importance in the outcome

Table 1. Summary of EphA2 Expression Analyses in Human Solid Tumors

Cancer type	Publications	Cell lines	Tissue	Notes
Breast	Macrae et al. (47)	Y	N	EphA2 is overexpressed in 8 of 28 breast cancer cell lines and is a direct transcriptional target of the Ras-Raf-MAPK pathway.
	Zelinski et al. (75)	Y	Y	EphA2 overexpression sufficient to transform mammary epithelial cells.
	Fox and Kandpal (74)	Y	N	Up-regulation of *ephA2* and down-regulation of *ephrinA1* gene expression in more invasive and tumorigenic breast cancer cells.
Ovary	Thaker et al. (76)	Y	Y	75.9% of tumors overexpressed EphA2. EphA2 overexpression was significantly associated with higher grade, advanced disease stage, and shorter survival.
	Lin et al. (77)	Y	Y	EphA2 overexpressed in 76% of tumors and associated with advanced-stage disease. EphA2 expressed in tumor and endothelial cells was associated with factors involved in invasion and angiogenesis.
Prostate	Walker-Daniels et al. (78)	Y	Y	EphA2 overexpressed more in metastatic cells than noninvasive epithelial cells.
	Zeng et al. (79)	N	Y	EphA2 levels increase as prostatic epithelial cells move toward a more aggressive phenotype.
Pancreas	Mudali et al. (80)	Y	Y	EphA2 overexpressed in 95% of clinical specimens and associated with metastatic disease.
	Duxbury et al. (56)	Y	N	EphA2 expression associated with increased cellular invasiveness.
Glioblastoma	Wykosky et al. (81)	Y	Y	EphA2 is overexpressed but not tyrosine phosphorylated in GBM cells or tumors.
	Wykosky et al. (82)	Y	Y	EphA2 strongly overexpressed in 61% of GBM tumors and is significantly associated with astrocytoma grade.
	Liu et al. (83)	Y	Y	EphA2 expression is associated with poor patient survival.
	Wang et al. (84)	N	Y	EphA2 strongly overexpressed in 60% of primary and recurrent GBM tumors. Increased expression significantly associated with adverse patient outcome.
Renal	Herrem et al. (92)	N	Y	Higher levels of EphA2 correlated with tumors that were of higher grade, larger, and more highly vascularized. EphA2 was predictive of shorter survival.
Lung	Kinch et al. (85)	Y	Y	High levels of EphA2 predict shorter survival and brain metastases.
Melanoma	Hess et al. (86)	Y	Y	EphA2 is expressed and phosphorylated specifically in aggressive metastatic melanoma cells and functions in vasculogenic mimicry.
Bladder	Abraham et al. (87)	Y	Y	EphA2 staining intensity associated with advancing stages of urothelial carcinoma.
Gastric	Nakamura et al. (88)	Y	Y	Analysis of EphA2 gene and protein expression. EphA2 expression highest in macroscopic type 3 and 4 tumors.
Esophageal	Miyazaki et al. (89)	Y	Y	EphA2 overexpressed in 50% of patient tumors and correlated with lymph node metastasis and poor survival rates.
Colorectal	Kataoka et al. (90)	Y	Y	Overexpression of EphA2 more in early-stage tumors than late stage and more in smaller tumors than large tumors. Microvessel count correlated with overexpression of EphA2.
Cervical	Wu et al. (91)	Y	Y	High level of EphA2 predictive of shorter overall survival.

of cancer patients, EphA2 is a very attractive target for novel anticancer therapies.

EphrinA1 is a 205-amino acid, tumor necrosis factor-α early-inducible gene product located within chromosomal region 1q21-q22 and having a predicted molecular mass of 22 kDa. Interestingly, ephrinA1 was originally identified as a soluble protein but with a sequence of hydrophobic amino acids at the COOH-terminal end reminiscent of those found in proteins that undergo GPI linkage to the plasma membrane (3). Although this original study did not document the membrane attachment of ephrinA1, subsequent studies showed the GPI-anchored, plasma membrane localization of the protein. Treatment with phosphatidylinositol-specific phospholipase C, a bacterial enzyme that specifically cleaves GPI anchors, efficiently released ephrinA1 into the medium (95).

The expression pattern of ephrinA1 in tumors does not seem to be the same as that which has been documented for EphA2. In fact, ephrinA1 is expressed at low levels in both GBM and breast cancer cells when EphA2 is overexpressed (47, 96). Conversely, breast cancer cells that express high levels of endogenous ephrinA1 exhibit low EphA2, a pattern of differential expression that is reversed on small interfering RNA (siRNA)–mediated knockdown of ephrinA1 (47). Moreover, EphA2-expressing GBM cells producing ectopic ephrinA1 respond with a profound down-regulation of the receptor (96). These findings point to the possible existence of a feedback loop mechanism whereby EphA2 acts to suppress expression of ephrinA1 and vice versa. Other analyses of ephrinA1 expression in tumors have revealed this protein to be expressed in some cases at moderate to high levels in both tumor cells and tumor-associated endothelium but typically lacking an association with tumor grade or stage (87, 88, 91).

EphA2 and EphrinA1 Function in Human Tumors

One of the initial questions surrounding EphA2 as a newly discovered RTK overexpressed in cancer was whether it played a functional role in contributing to the malignant phenotype. This question was directly answered first by Zelinski et al. (75), who showed that expression of EphA2 was sufficient to transform mammary epithelial cells. An intriguing finding in this and subsequent studies is that EphA2, despite its abundant overexpression, is present in some tumor cells in a non–tyrosine-phosphorylated state and is localized to membrane ruffles at the leading edge of invasive cancer cells (81, 97). In nonneoplastic epithelia, however, EphA2 was expressed at much lower levels, tyrosine phosphorylated, and localized to points of cell-cell contact (97). Early studies attributed the lack of EphA2 tyrosine phosphorylation in epithelial malignancies to a failure of the receptor to interact with its membrane-bound ephrinA1 ligand (75, 97, 98). Malignant cells often exhibit a decrease in cell adhesion mediated by loss of function of E-cadherin, which would result in neighboring cells expressing

receptor and ligand having less opportunity to interact (97). In fact, EphA2 phosphorylation and localization to points of cell-cell contact was found to depend on the expression and function of E-cadherin (97). Alternatively, simply a lack of or low ephrinA1 expression could result in decreased EphA2 phosphorylation. In either case, decreased ligand-induced receptor internalization and degradation may increase stability of the protein and contribute to the overexpression of EphA2 (99). In instances when ligand and receptor are both expressed such that EphA2 may potentially undergo ephrinA1-mediated phosphorylation, it is possible that protein tyrosine phosphatases, such as LMW-PTP, which associates with EphA2 intracellularly (20, 100), act rapidly and contribute to a lack of detectable receptor phosphorylation in tumor cells.

The overexpression of a RTK that plays a role in malignancy in a non–tyrosine-phosphorylated state is intriguing, as hyperphosphorylation and constitutive activation are the ways in which most RTKs are known to contribute to tumorigenesis. EphA2 seems to be unique among the Eph receptors in that its kinase activity may not be entirely dependent on ligand binding and phosphorylation of the conserved activation loop tyrosine, which controls the kinase activity of many RTKs, including other Ephs as well as epidermal growth factor receptor (EGFR; refs. 75, 97, 101). In fact, EphA2 seems to possess ligand-independent kinase activity in tumor cells *in vitro*, which may offer some explanation for the oncogenic effects of the nonphosphorylated receptor (97, 101). Interestingly, it has recently been shown that mutation of conserved juxtamembrane tyrosines 587 and 593 of EphA2 severely reduces kinase activity in endothelial cells, suggesting that phosphorylation of these residues is, in fact, important for high EphA2 kinase activity, potentially in a cell type–dependent manner (102).

The exact mechanisms that govern EphA2 overexpression and function in cancer have yet to be fully elucidated. It is known, however, that *ephA2* is a direct transcriptional target of the Ras-MAPK pathway (47, 103) and that EphA2 is overexpressed in Ras-transformed cells as well as in Ras-overexpressing transgenic mice (104). Interestingly, *ephrinA1* expression in tumor cells is inhibited by this same pathway, contributing to the differential expression of the receptor and ligand within the same cell (47). Thus, it is possible that *ephA2* is transcriptionally up-regulated during the process of malignant transformation and progression, potentially as a result of aberrant growth factor signaling originating through another RTK. A likely candidate is EGFR, as EphA2 at the gene and protein level is, in fact, up-regulated in response to wild-type EGFR, and the constitutively active mutant form of this receptor, EGFRvIII (105, 106). It has recently been shown that EphA2 cooperates with ErbB2 in promoting mammary tumor progression, highlighting the ability of EphA2 to cross-talk with other RTKs (50). In addition, the *ephA2* promoter has DNA damage-responsive p53-binding sites (107) and the receptor is up-regulated in response to UV irradiation in a p53-independent, MAPK-regulated manner (108), providing other possible mechanisms for EphA2 overexpression. *EphA2* is also tightly regulated by homeobox transcription factors, which are known to dictate the temporal expression of this gene during development (109, 110). Some of these transcription factors, including HoxA1 (111-113), are up-regulated during malignancy, and *ephA2* could potentially be a target gene during this process. Furthermore, *ephA2* gene transcription is repressed by a variety of stimuli that are often lost in the most advanced stages of aggressive cancers, such as estrogen receptor signaling and c-Myc (114). Yet another possible contributing factor to the abundance of EphA2 in malignancies is gene mutation or amplification. To date, there is little evidence that these mechanisms significantly contribute to EphA2 overexpression. No somatic mutations of *ephA2* have been identified, and one study identified *ephA2* amplification, but only in 1 of 33 pancreatic cancer specimens (80). Interestingly, *ephA2* is located in a chromosomal region that is deleted in some cancers of neuroectodermal origin (115). Further studies are needed for full elucidation of the mechanism of EphA2 overexpression in solid tumors, but the current data point to a complex relationship with other growth factor signaling pathways and protein stability as well as the regulation of *ephrinA1* expression, about which our knowledge is currently not sufficient.

The majority of studies investigating the function of ephrinA1, both in the context of development and in cancer, have focused on the protein as a membrane-anchored ligand for EphA receptors, which mediates juxtacrine signaling. Thus, due to the plasma membrane localization in addition to the previously mentioned study by Davis et al. (6) in which some ephrins, including ephrinA1, only elicited full Eph receptor activation when membrane bound or in an artificially clustered soluble form, it has been widely accepted that clustering or membrane localization is required for function. Interestingly, ephrins have since been identified as substrates for the cross-linking enzyme tissue transglutaminase, which is capable of inducing oligomerization and, as a result, function of soluble ephrins (116). Recent work from our laboratory has uncovered that ephrinA1 is cleaved from the plasma membrane of ectopic ephrinA1-expressing GBM cells and endogenous ephrinA1-expressing breast cancer cells (96). This soluble ephrinA1 functionally interacts with EphA2 and other EphA receptors in an unclustered, monomeric state at molar concentrations similar to that of recombinant dimeric ephrinA1. Together, these studies suggest that the function of ephrinA1 may not necessarily depend on juxtacrine interactions as previously thought, and that there may also be a paracrine role for some of these ligands that has yet to be explored. Interestingly, the ability of ephrinA1 mimetic peptides to functionally interact with EphA2 (117) is also suggestive of such a scenario.

EphA2 and EphrinA1 Signaling in Tumor Cells

It is evident from several studies that the mere presence of EphA2 elicits oncogenic effects. EphA2 overexpression is transforming and imparts metastatic characteristics and potential on previously nonmetastatic cells (57, 75). The ligand-independent effects of EphA2 are, thus, important in influencing processes that are critical for malignant progression. In addition to controlling the enzymatic activity of the receptor in some cell types, ephrinA1 binding and resultant EphA2 phosphorylation and down-regulation is thought to influence the function and localization of intracellular proteins that interact with the receptor in tumor cells. For example,

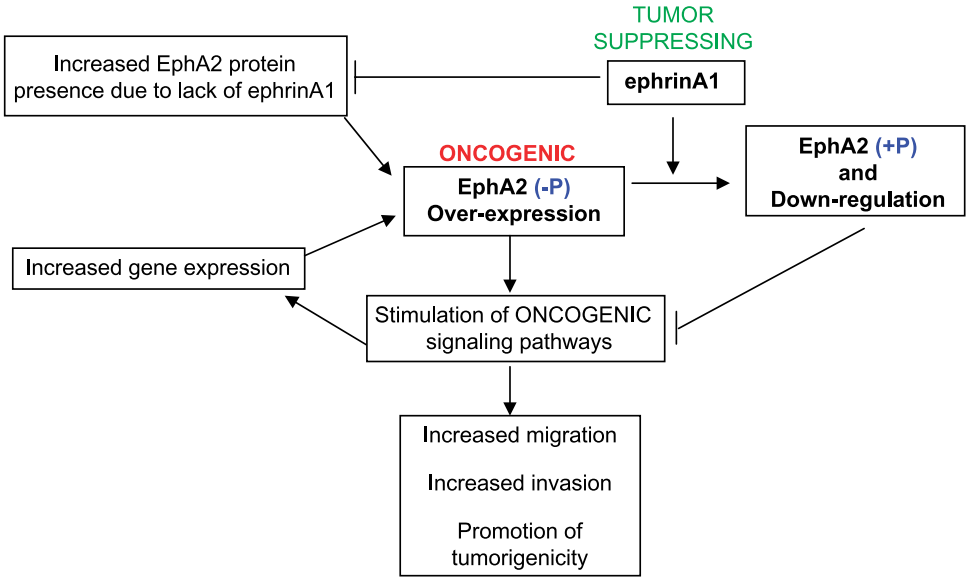

FIGURE 2. Hypothetical role of the EphA2/ephrinA1 system in solid tumor cells. EphA2 becomes overexpressed possibly due to increased gene expression or a lack of ephrinA1-induced receptor down-regulation. Overexpressed EphA2 is nonphosphorylated and stimulates oncogenic processes. EphrinA1 causes receptor phosphorylation and subsequent down-regulation, both of which likely contribute to the tumor-suppressing effects of the ligand in tumor cells. (−P), nonphosphorylated; (+P), phosphorylated.

overexpressed, nonphosphorylated EphA2 has been shown to associate with FAK in prostate and pancreatic cancer cells (27, 57). EphA2 overexpression induces and sustains phosphorylation and kinase activity of FAK, one consequence of which is to increase expression of matrix metalloproteinase-2 (57, 118, 119), an integral player in tumor cell invasion. Moreover, ephrin-dependent activation of EphA2 causes FAK dephosphorylation and dissociation, leading to integrin inactivation and likely affecting the invasive capacity of the tumor cells (27). It is further likely that adaptor proteins, such as those containing SH2 domains, associate with kinase-active EphA2 in tumor cells, sustaining signaling through downstream oncogenic pathways to directly affect neoplastic transformation, the evidence for which is discussed below.

It has been well documented that on stimulation of tumor cells with ephrinA1-Fc, a soluble recombinant ephrinA1 fused to the Fc portion of IgG, EphA2 is tyrosine phosphorylated and down-regulated (28, 57, 81, 93, 95). This process involves receptor degradation following ligand-induced internalization and association with the adaptor protein c-Cbl (93, 120). EphrinA1 stimulation has been observed in many cell types and systems to suppress the malignant character of EphA2-overexpressing tumor cells, both *in vitro* and *in vivo*, causing a profound change in cytoskeletal architecture involving the loss of processes and polarity (27), suppression of migration and invasion (57, 81), defects in colony formation in soft agar (75, 81), and decreased tumor growth *in vivo* (121). Mechanistically, ephrinA1-mediated tumor suppression could result from a dual process: direct signaling through EphA2 as well as from down-regulation of the receptor (Fig. 2). For example, on interaction of ephrinA1 and EphA2, the SHP-2 protein phosphatase is rapidly recruited to the receptor (27). SHP-2 causes dephosphorylation of FAK and disruption of the EphA2-FAK complex, which results in an immediate suppression of integrin function and decreased cell-extracellular matrix adhesion. It is likely, then, that subsequent down-regulation of EphA2 prevents further phosphorylation of FAK, resulting in a long-lasting suppression of tumor cell invasion. It is evident, therefore, that ephrinA1 induces tumor-suppressing signaling through EphA2, but that this is complemented by the suppressive effects of the subsequent EphA2 down-regulation. In addition, the ephrinA1-induced change in cell morphology seems to be a direct result of EphA2 phosphorylation and also involves FAK. In response to ephrinA1 stimulation, a kinase-active Src-FAK complex forms that leads to contraction of the cytoskeleton through the activity myosin II and the small GTPase RhoA (20), an event that could also contribute to the suppression of tumor cell migration and invasion. It is possible by extension that this event occurs following recruitment of SHP-2 and dissociation of the FAK-EphA2 complex. Furthermore, at short time points following stimulation of cells with ephrinA1 and before EphA2 down-regulation occurs, there is a marked decrease in phosphorylation of extracellular signal-regulated kinase (20, 46) and AKT.[4] Moreover, ephrinA1 treatment of EphA2-expressing breast cancer cells attenuates growth factor–induced activation of Ras (47). The suppression of the oncogenic/survival Ras-MAPK and PI3K signaling pathways and direct effect on cell architecture, integrin function, and invasion supports the function of ephrinA1-induced signaling in suppressing the malignant character of tumor cells.

Interestingly, however, EphA2 knockdown by siRNA-mediated or antisense oligonucleotide–mediated gene silencing

[4] J. Wykosky et al., unpublished results.

results in phenotypes that are similar, if not identical, to those described above on treatment with ephrinA1-Fc (83, 101). Taken together, the data suggest that the apparent tumor-suppressing properties of ephrinA1 may primarily be a result of the ability of the ligand to down-regulate EphA2. Separation of the effects of ephrinA1-induced signaling from EphA2 down-regulation offers a challenge. Thus, both of the processes seem to play an important role, but the relative contribution of each in the process of suppressing malignant progression has not been directly established. The above-mentioned studies support elements of tumor suppression that occur as a result of direct signaling through EphA2, but it is likely that this works in concert with the effects of subsequent EphA2 down-regulation. In summary, the data thus far suggest an oncogenic, ligand-independent role for EphA2 in tumor cells and a dual tumor-suppressing role for ephrinA1 that involves repression of oncogenic signaling as a result of both receptor phosphorylation as well as subsequent receptor down-regulation (Fig. 2). Thus, ephrinA1 may have a dual function that involves eliciting tumor-suppressing signaling through EphA2 and ridding cells of a protein, which, in and of itself in a non–ligand-activated state, is a powerful oncoprotein.

As mentioned previously, the Eph-ephrin interaction as characterized during development is known to cause either adhesion or repulsion of cells, and it is of interest as to how these seemingly opposite functions can arise from the same receptor-ligand interaction. Indeed, there are several reports of the mechanisms and consequences of EphA2-ephrinA1 signaling that are in accordance with this complex trend. One hypothesis is that the outcome is dictated by differential phosphorylation of residues within intracellular signaling proteins that directly associate with or are indirectly affected by EphA2. For example, the formation of the Src-FAK complex described above, which dictates retraction and repulsion of cell processes in response to ephrinA1, has been shown to involve phosphorylation of FAK on $Tyr^{576/77}$ (20). In contrast, EphA2 induces autophosphorylation of FAK Tyr^{397}, facilitating the interaction of FAK with other SH2 domain-containing proteins, which elicit downstream signaling to cause increased adhesion and cell spreading (28). In reality, the downstream effects of the EphA2-ephrinA1 interaction, or of EphA2 ligand-independent kinase activity, are likely due to a combination of complex factors, including cell type and microenvironment. For example, ephrinA1-mediated suppression of extracellular signal-regulated kinase phosphorylation has been documented (46, 47), as has the stimulation of extracellular signal-regulated kinase phosphorylation (122) using the same ephrinA1 ligand and some of the same breast and prostate cancer cell lines. These findings suggest that in addition to cell type, other factors that may be present in the microenvironment, perhaps manifested as varied artificial *in vitro* conditions, may dictate differences in function. It has been shown that in endothelial cells, Eph receptors discriminate specific ephrin oligomers to determine function (14). Experiments using ephrinA1-Fc, which the overwhelming majority of studies use, could result in varied homodimeric and/or oligomeric ephrinA1-Fc complexes and potentially contribute to the contrasting outcomes. Nonetheless, EphA2-ephrinA1 signaling in tumor cells is nothing aside from complex, but without a doubt involves those pathways that are most intimately involved in the pathogenesis of cancer. The function of EphA2 in contributing to this process seems to rely on its overexpression and kinase activity in the absence of ephrinA1, in which the receptor interacts with adaptor proteins to sustain signaling through these important pathways that dictate the behavior of cells and the progression to malignancy.

EphA2 and EphrinA1 in Tumor Neovascularization

As if EphA2 and ephrinA1 signaling and function is not complex enough in tumor cells, these proteins also play a very important role in angiogenesis and tumor neovascularization. EphrinA1 has long been known as tumor necrosis factor-α–inducible stimulator of endothelial cell migration (123). In recent years, however, several studies have directly addressed the importance of the EphA2/ephrinA1 interaction specifically in the context of tumor angiogenesis, both *in vitro* and *in vivo*. Ogawa et al. (124) was one of the first to report tumor vasculature–specific expression of EphA2 and ephrinA1 in blood vessels of breast carcinoma and Kaposi's sarcoma xenografts. An immunohistochemical analysis revealed detectable expression of both proteins in xenograft endothelial cells as well as tumor cells. Furthermore, EphA2 was reported to be present in a tyrosine-phosphorylated state, consistent with the observed expression of ephrinA1. This study, however, did not distinguish between the phosphorylation status of endothelial and tumor cell EphA2; EphA2 phosphorylation was observed using whole tumor lysates, which would be expected to contain all cell types present within the tumor as well as those in the microenvironment.

Subsequently, it was found that ephrinA1 possessed the capacity to stimulate EphA2-expressing endothelial cell migration and survival (125). Importantly, these processes were severely inhibited by administration of recombinant EphA2-Fc, unveiling the role of ephrinA-mediated EphA2 receptor activation (125). Further use of EphA2-Fc as a blocker of the ephrinA-EphA2 interaction revealed that disruption of this process *in vivo* inhibits angiogenesis in a RIP-Tag transgenic model of pancreatic carcinoma as well as in a 4T1 model of metastatic mammary adenocarcinoma (126). EphA2-Fc also inhibited microvessel formation in a rat aortic ring assay and the growth of xenograft and orthotopic human pancreatic tumors in mice (127). Strikingly, EphA2-Fc in the former study had no effect on tumor cells in culture but specifically inhibited the migration of endothelial cells in response to tumor cells. These findings suggest a possible scenario in which endothelial cell–specific EphA2 responds to an angiogenic cue from ephrinA1, which could be expressed in either tumor cells or endothelial cells.

The importance of EphA2 in angiogenesis and metastasis was even more evident in the finding that metastatic mammary carcinoma cells implanted into EphA2-deficient mice exhibited decreased tumor volume and metastasis (128). It is likely that endothelial cells within the tumor microenvironment, when devoid of EphA2, cannot respond to ephrinA1 and thus fail to induce the angiogenic processes required to support tumor growth and metastasis. Further studies using a transplantable

mouse mammary tumor model investigated the effects of native tumor cell–produced ephrinA1, whereas most previous studies had used exogenous recombinant, soluble homodimeric ephrinA1-Fc (129). Tumor cell ephrinA1 was proangiogenic through induction of EphA2-specific endothelial cell migration and sprouting and increasing vascular endothelial growth factor expression. Moreover, siRNA knockdown of ephrinA1 in mammary tumor cells inhibited angiogenesis and lung metastasis (129). Therefore, tumor angiogenesis, and thus metastasis and progression, depends in part on the EphA2-ephrinA1 interaction in endothelial cells, which is seemingly distinct from the role that this system plays in affecting the behavior of tumor cells.

Reconciling the Role of EphA2 and EphrinA1 in Tumorigenesis and Angiogenesis

One may perceive it contradictory that the function of EphA2 in affecting the malignancy of tumor cells may be ligand independent and the fact that EphA2-mediated endothelial cell migration, etc., depends on an interaction with the ephrinA1 ligand. Thus, one question of interest is whether the kinase activity of EphA2 is required for its function in each of these processes. To directly address this question in the context of tumor cells, Fang et al. (130) generated EphA2 variants lacking the cytoplasmic domain or carrying a D738N point mutation to inhibit kinase activity. When expressed in breast cancer cells and grown in mice, these mutants resulted in decreased tumor volume and metastasis. Notably, the decreased tumor growth was found not to be a result of defects in tumor angiogenesis. This finding supported previous findings that EphA2 expressed in endothelial cells, and not tumor cells, is responsible for supporting angiogenesis (125). It does, however, highlight the importance of EphA2 in mediating the malignant phenotype of tumor cells in a manner that requires the kinase activity of the receptor. Interestingly, the phenotype of mutants with a cytoplasmic deletion was more severe than those carrying the kinase-dead version of the receptor. Thus, phosphorylation-independent effects of EphA2, likely through association of signaling proteins, etc., with the cytoplasmic domain, seem to also be required for tumorigenesis.

This work also contributed further to the knowledge of tumor cell–specific signaling downstream of EphA2 in that cells harboring mutant EphA2 were defective in Rho GTPase activation and migration (130). Interestingly, EphA2-induced signaling in endothelial cells that is responsible for mediating angiogenesis also involves activation of Rho GTPases. In contrast to the signaling in tumor cells, however, EphA2-mediated angiogenesis seems to largely depend on PI3K and/or the binding of the guanine-nucleotide exchange factors Vav2 and Vav3 to phosphorylated EphA2 (24). In addition, it is known that the p85 subunit of PI3K binds to phosphorylated EphA2 in vascular smooth muscle cells (35). Specifically, it has recently been shown through mass spectrometry analysis and phosphopeptide mapping studies in endothelial cells that Y587 and Y593 of EphA2 bind to the Vav guanine-nucleotide exchange factors and Y734 binds to p85 of PI3K (102). Based on these findings, a possible scenario worthy of investigation is that the function of EphA2 in endothelial cells is more dependent on ephrinA1 binding than its counterpart in tumor cells, contributing to the complexity of the system but also potentially explaining how drastically differently this receptor functions depending on cell type and microenvironment.

Rather than inducing signaling to suppress processes such as growth and migration as has been shown for ephrinA1 and tumor cell–specific EphA2, the interaction of ephrinA1 with endothelial cell–specific EphA2 seems to stimulate these same effects. The previously mentioned differential expression of EphA2 and ephrinA1 in tumor cells could potentially explain how tumor cell–produced ephrinA1 acts through EphA2 on endothelial cells. When ephrinA1 is overexpressed by tumor cells, either endogenously or ectopically, these cells exhibit down-regulation of EphA2 (47, 96). Potentially as a result, ephrinA1 may interact with an Eph receptor on a different cell type in the microenvironment, such as endothelial cells. Interestingly, ephrinA1 is up-regulated at the gene and protein level by vascular endothelial growth factor (125), suggesting that overexpression of ephrinA1 in some tumor cells and potentially also in endothelial cells may be the result of early angiogenic events. Furthermore, because the function of ephrinA1 as an EphA2 ligand has mainly focused on its membrane-bound nature, it would be necessary for cells expressing ephrinA1 to come in contact with EphA2-expressing endothelial cells. However, our recent findings that a functional, soluble form of ephrinA1 is released from tumor cells suggest a possible paracrine role for ephrinA1 in stimulating angiogenesis that is not dependent on juxtacrine signaling (96). It is further possible that production and subsequent cleavage and release of ephrinA1 only occurs in cells that have been subjected to angiogenic stimuli to up-regulate ephrinA1 and/or the enzyme required to release the ligand as a paracrine factor. Down-regulation of EphA2 in response to ephrinA1 overexpression in tumor cells could be a way for these cells to circumvent quenching the released ephrinA1 before it has a chance to exert a paracrine function on the endothelial cells within the tumor microenvironment.

Being that both EphA2 and ephrinA1 play an integral role in tumor cells as well as endothelial cells, a tumor *in vivo* is likely a mixture of EphA2 overexpression in both cell types that depends on variables such as growth factors, angiogenic stimuli, and other factors within the microenvironment that influence overall tumor growth. It is not unreasonable to suggest that there is a complex, temporal, dynamic flux of EphA2 and ephrinA1 expression and down-regulation with respect to one another. Although EphA2 overexpression is by far more prevalent than ephrinA1 in cancer cell lines, it is much more common to see high levels of ephrinA1 expression in tumors (81, 124, 131). This observation supports the up-regulation of ephrinA1 by other factors in the microenvironment, possibly dependent on tumor grade or stage. Overall, EphA2 and ephrinA1 are crucial in mediating tumorigenesis, tumor neovascularization, and progression, which are crucial to overall tumor growth, metastasis, and patient survival.

Therapeutic Targeting of EphA2 in Solid Tumors

The unique dual function of the EphA2/ephrinA1 system in tumorigenesis and angiogenesis makes it a very attractive therapeutic target. In general, there are at least two ways in

which this receptor/ligand system can be targeted with respect to cancer therapy and diagnostics. The first involves taking advantage of the tumor-promoting function of EphA2 to modulate the behavior of cells and suppress tumor growth and malignant progression. The second involves using the EphA2 receptor as a way of delivering agents to tumor cells and the associated vasculature in the form of either exogenous drugs or endogenous immune cells.

With regard to function-based EphA2 targeting, one issue is whether to pursue ephrinA1-based therapeutics or take an approach that involves direct knockdown of EphA2 expression. Ligand-based approaches, such as delivery of ephrinA1 to tumors by adenoviral vector gene therapy (121), have the advantage of potentially imparting tumor-suppressing signaling through EphA2 in tumor cells, as well as further preventing the tumorigenic effects of EphA2 by down-regulating the receptor. However, one must take caution due to the function of ephrinA1 in inducing angiogenesis through endothelial cell–specific EphA2. For targeting tumor vasculature, the best approach may likely be to either interrupt the EphA2-ephrinA1 interaction through use of a blocker such as EphA2-Fc (127, 132), for example, or targeted knockdown of EphA2 expression in endothelial cells so that it cannot respond to angiogenic cues. Interestingly, the important and nonredundant role of EphA2 in angiogenesis, evident in the finding that vascular endothelial growth factor receptor, for example, cannot compensate for EphA2 loss (127), further suggests it as an attractive target for antiangiogenic therapy. Based on the complex functions of EphA2 within the entire tumor, however, the most rational approach may be direct knockdown of receptor expression. An approach such as this would potentially be effective in removing both a powerful oncoprotein from tumor cells as well as an important inducer of angiogenesis from endothelial cells. As mentioned earlier, however, the expression and function of EphA2 may vary in these cell types within a tumor depending on factors such as tumor grade or stage or other cues from the microenvironment, which is a variable that must be taken into consideration. Monoclonal anti-EphA2 antibodies have been developed that are highly specific to the extracellular domain of EphA2 and induce receptor phosphorylation and down-regulation (101). Interestingly, the tumor-suppressing characteristics of these agonistic antibodies are thought to specifically be a result of EphA2 down-regulation rather than phosphorylation. Targeted direct gene knockdown by siRNA delivery into cells represents another option for directly depleting both tumor and endothelial cells of EphA2 (133). An additional consideration is that the efficacy of EphA2 antibody-based therapy may depend on tumor type, which was evident in one study that although agonistic EphA2 antibodies decreased total EphA2 protein levels, there was no antitumor effect (134). More specifically, a response may be dictated by whether a particular tumor is dependent on EphA2-mediated signaling pathways. For example, a colorectal tumor model was used for the previous study in which no growth-suppressive effect was observed, and these tumors often harbor Ras-activating mutations, which may render EphA2-targeted therapy ineffective (134). In addition, it has recently been shown that mice harboring ErbB2 in mammary epithelium were sensitive to therapeutic inhibition of EphA2, whereas mice harboring polyomavirus middle T antigen were not, further supporting the notion that efficacy of EphA2-targeted therapies is dependent on the context of the tumor (50).

The other possible scenario for EphA2 targeting lies in the fact that the receptor is an internalized plasma membrane protein that is abundant throughout the two cell types that are most integral to tumor growth and progression. This expression pattern represents an opportunity to deliver agents to tumors. One approach that has been taken is to use ephrinA1 as a targeting ligand for mutated bacterial toxins that are devoid of their natural receptor-binding domain (135). EphrinA1-targeted cytotoxins potently and specifically kill EphA2-expressing cells through an irreversible inhibition of protein synthesis due to the action of the toxin. An advantage of these agents is that any function ephrinA1 may elicit on its interaction with EphA2 is likely irrelevant, as the targeted cell is nearly immediately destined to die. In addition to using ephrinA1 ligands, ephrinA1 mimetic peptides have been identified (117) and could also be used to deliver toxins, other anticancer molecules, or diagnostic imaging agents to EphA2-expressing cells. Yet, another opportunity for using EphA2 as a target for anticancer therapeutics lies in immunotherapy and the development of EphA2-specific vaccines. EphA2 has been identified as a T-cell recognizable antigen, and immune cells can, indeed, elicit an EphA2-targeted cytotoxic response (136-139). In addition, a recent effort to use highly specific EphA2 monoclonal antibodies has generated a bispecific single-chain antibody that simultaneously targets EphA2 expressed on malignant cells as well as the T-cell receptor/CD3 complex on T cells (140). This construct efficiently redirects unstimulated T cells to destroy EphA2-expressing tumor cells, both *in vitro* and *in vivo*. The efficacy of EphA2-targeted therapeutics in affecting tumor growth and progression is encouraging thus far, and the different ways in which this receptor/ligand system can be targeted represent many possibilities for the continued research and clinical development of these agents.

Conclusion

Overall, although EphA2 and ephrinA1 are no doubt of major importance in both development and cancer, the exact contribution of these proteins to the specific processes involved to tumor formation, maintenance, and progression is extremely complex and dependent on many factors. Future studies focusing on *in vivo* systems and using natural ephrin ligands rather than artificial recombinant proteins may shed more light on the specific roles that EphA2 and ephrinA1 coordinately and temporally play in tumorigenesis and angiogenesis. Moreover, further elucidation is needed with regard to the function of EphA2 and ephrinA1 in the context of other growth factor receptors and their ligands. Hopefully, all of this will lead to new knowledge about how this system affects the pathobiology of neoplasia, which can be used for the generation of improved, rationally designed therapeutics to target the EphA2/ephrinA1 system in solid tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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