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Masaru Nakamoto, *Valparaiso University*
Andrew D Bergeman, *Mount Sinai School of Medicine*

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MASARU NAKAMOTO[1] AND ANDREW D. BERGEMANN[2]*
[1]*Department of Neurosciences/NC30, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195*
[2]*Department of Pathology, Mount Sinai School of Medicine, New York, New York 10029*

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ABSTRACT The Eph family of receptor tyrosine kinases and their cell-presented ligands, the ephrins, are frequently overexpressed in a wide variety of cancers, including breast, small-cell lung and gastrointestinal cancers, melanomas, and neuroblastomas. In particular, one Eph family member, EphA2, is overexpressed in many cancers, including 40% of breast cancers. EphA2 can also transform breast epithelial cells in vitro to display properties commonly associated with the development of metastasis. Remarkably, the oncogenic properties of EphA2 contravene traditional dogma with regard to the oncogenic properties of a growth factor and its receptor tyrosine kinase: while stimulation of EphA2 by its ligand (ephrin-A1) results in EphA2 autophosphorylation, the stimulation reverses the oncogenic transformation. As will be discussed in this review, the apparent dependence of oncogenicity on the dephosphorylated state of EphA2 most probably reflects the unique nature of Eph signaling. In particular, oncogenecity may depend on the capacity of unactivated EphA2 to interact with a variety of signaling molecules. As well as acting in oncogenic transformation, a growing body of evidence supports the importance of the concerted actions of ephrins and Eph molecules in tumor angiogenesis. Genetic studies, using targeted mutagenesis in mice, reveal that ephrin-B1, ephrin-B2, and EphB4 are essential for the normal morphogenesis of the embryonic vasculature into a sophisticated network of arteries, veins, and capillaries. Initial studies indicate that these molecules are also angiogenic in tumors, and as such represent important new targets for the development of chemotherapeutic treatments. *Microsc. Res. Tech. 59: 58–67, 2002.*

INTRODUCTION

As a group, the receptor tyrosine kinases (RTKS) have well-established roles in both normal physiology and oncogenesis. In oncogenesis, numerous examples abound of the dysregulated or excessive activity of a tyrosine kinase, allowing it to function as a classical oncogene (Cantley et al., 1991; Lamorte and Park, 2001). However, the role in oncogenesis of the largest family of receptor tyrosine kinases, the Eph family, is apparently complex and remains ill-defined. Nevertheless, Eph molecules are overexpressed in a wide range of cancers and additional evidence indicates that overexpression is an important component of the carcinogenic process in breast cancers (Zantek et al., 1999; Zelinski et al., 2001). In this review we provide an overview of the evidence to date as it supports roles for the Eph family in cellular transformation, metastasis, and the tumor-driven induction of angiogenesis.

The first family member identified, originally named eph but since renamed EphA1, was isolated from a human hepatocellular carcinoma cell line (Hirai et al., 1987). Since then the family has grown to include 14 members, characterized by shared features in both the extracellular and intracellular domains (Dodelet and Pasquale, 2000; Flanagan and Vanderhaeghen, 1998; Tuzi and Gullick, 1994). The extracellular domain is composed of a globular, amino-terminal domain, a cysteine-rich region, and two fibronectin type III repeats (Himanen et al., 1998; Tuzi and Gullick, 1994). The globular, amino-terminal domain is both necessary and sufficient for ligand-binding (Himanen et al., 1998). The intracellular domain includes a tyrosine kinase domain and a carboxy-terminal SAM (sterile alpha motif) domain, which is a low-affinity oligomerization domain (Smalla et al., 1999). The carboxy-terminal portion of the extracellular domain can also act as an oligomerization domain (Lackmann et al., 1998). Long before any ligands were identified for these receptors or, indeed, before any function could be ascribed to them, it was recognized that many Eph family members display remarkable expression patterns during vertebrate development. In particular, it was recognized that many display segment-specific expression patterns in the segments of the rhombomeres of the developing hindbrain (Becker et al., 1994; Nieto et al., 1992).

An understanding of the functions of these receptors has been considerably enhanced over the last 7 years by the discovery of a family of ligands (Beckmann et al., 1994; Brambilla et al., 1995; Flanagan and Vanderhaeghen, 1998; Gale et al., 1996). The ligand family has

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*Correspondence to: Andrew D. Bergemann, Department of Pathology, Box 1194, Mount Sinai School of Medicine, New York, NY 10029.
E-mail: andrew.bergemann@mssm.edu

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now grown to encompass nine members, which can be divided into two groups—the GPI (glycosylphosphatidylinositol) anchored A-class of ligands and the transmembrane B-class of ligands (Menzel et al., 2001; Eph Nomenclature Committee, 1997). The Eph receptors can also be divided into two groups based on sequence homologies and ligand-binding characteristics, the A-class and the B-class. Many of the ephrins display a remarkable degree of promiscuity in their interactions with their receptors (Bergemann et al., 1995; Gale et al., 1996). However, as a general rule members of the A-class of ligands interact with the A-class of receptors and members of the B-class of ligands interact with the B-class of receptors (Brambilla et al., 1995; Gale et al., 1996). One important exception to this rule is the interaction of EphA4 with ephrin-B2, which has well-established biological importance in hindbrain segmentation (Mellitzer et al., 1999; Xu et al., 1995). A distinguishing feature of the Eph-ephrin interaction, as compared to the interactions of other RTKs with their ligands, is that soluble derivatives of the ephrins (in monomeric form), as a general rule, can bind to the receptors but fail to induce their autophosphorylation. However, clustering of the ligands, through ectopic means, such as antibody-crosslinking, enables the ligands to activate the receptors (Davis et al., 1994).

The discovery of the ligands has led to an explosion of functions being defined for the Eph family in normal embryonic development. In particular, it is now very well established that the interactions of Eph molecules with the ephrins act to guide a diverse array of migrating cells and extending axons to their targets. In the classical model, Eph receptors on the surface of an extending growth cone (or the leading filipodia and lamelipodia of a migrating cell) sense ephrins, acting as guidance cues, in the surrounding milieu (Cheng et al., 1995; Drescher et al., 1995; Krull et al., 1997; Nakamoto et al., 1996; Tessier-Lavigne, 1995; Wang and Anderson, 1997). These interactions of Eph molecules and ephrins have classically been considered to be repulsive in nature, the axon or migrating cell stopping, or retracting, as the Eph receptors detect a rising concentration of ligand. However, it is now clear that their interactions can also mediate cell adhesion. It would appear the these cell-adhesive effects may be due to kinase dead Eph forms, such as alternate splice forms which lack the kinase domain (Holmberg et al., 2000). The kinase dead forms may act as dominant-negative inhibitors of normal Eph signaling from full-length receptors, allowing cell adhesion to occur.

SIGNAL TRANSDUCTION EVENTS INDUCED BY THE EPH-EPHRIN INTERACTION

Signaling Through Eph Receptors

Upon ephrin binding, the Eph receptors are phosphorylated at specific tyrosine residues in the cytoplasmic region, which then serve as docking sites for various signaling molecules. Many SH2 domain-containing proteins have been found to interact with phosphotyrosines of activated Eph receptors, including cytoplasmic tyrosine kinases of the Src family (Fyn, Src) (Ellis et al., 1996; Zisch et al., 1998), adaptor proteins (Grb2, Grb10, Crk, SLAP, and SHEP-1) (Dodelet et al., 1999; Hock et al., 1998; Pandey et al., 1995; Stein et al., 1996, 1998a), p85 subunit of phosphatidylinositol 3-kinase (Pandey et al., 1994), a cytoplasmic phosphotyrosine phosphatase LMW-PTP (low molecular weight phosphotyrosine phosphatase) (Stein et al., 1998b), and Ras-GAP (Ras GTPase-activating protein) (Hock et al., 1998; Holland et al., 1997).

In early studies, two tyrosine residues in a highly conserved juxtamembrane motif were identified to be the major autophosphorylation sites. These phosphotyrosines act as a binding site for Fyn, Src, RasGAP, Crk, Nck, and SLAP. Grb10 and LMW-PTP are thought to bind to conserved tyrosine residues in the tyrosine kinase domain and the SAM domain, respectively. In addition, a recent in vivo study has shown that 10 or more tyrosine residues are phosphorylated in activated Eph receptors (Kalo and Pasquale, 1999) and those phosphotyrosines may also provide sites for interaction with specific signaling molecules. Interestingly, EphB receptors are also extensively phosphorylated on serine/threonine residues (Kalo and Pasquale, 1999), suggesting that novel, phosphotyrosine-independent pathways are involved in the signaling mechanisms through Eph receptors.

The carboxyl terminus of the Eph receptors contains a PDZ (postsynaptic density protein, disc large, zona occuludence) domain-binding motif. Several PDZ domain-containing proteins, such as AF6/afadin, Pick1, Syntenin, and Grip, have been shown to associate with the carboxyl terminal motif of Eph receptors (Buchert et al., 1999; Hock et al., 1998; Torres et al., 1998).

Many of the signaling proteins that interact with Eph receptors have been implicated in cell adhesion, cell motility, and cytoskeleton rearrangement, which is consistent with previously identified functions of Eph receptors, such as axon guidance and cell migration. However, the mechanisms by which individual molecules and signaling pathways exert specific functions still remains to be elucidated. In addition, it should be noted that many of the above-mentioned signaling proteins were tested for molecular interactions with only a subset of the Eph receptors and may not bind to all members of the family. Indeed, several molecules have been reported to show specificity in association with Eph receptors. Therefore, despite a striking sequence similarity between the cytoplasmic domains, individual Eph receptors may interact with distinct sets of intracellular signaling molecules and exert specific functions.

While many of the signaling pathways activated by Eph receptors are shared with many other RTKs, recently, signaling pathways have been reported for Eph receptors that may prove to be unique to this group of RTKs. Gu and Park (2001) have reported that EphA8 can signal in a kinase-independent fashion, in which sequences within the juxtamembrane domain of EphA8 interact with, and activate, phosphatidylinositol 3-kinaseγ. Miao et al. (2001) have observed that EphA2 can also signal in a kinase-independent fashion, through activation of the focal adhesion kinase (FAK). Shamah et al. (2001) reported that EphA4 can activate RhoA, and in turn inhibit Cdc42 and Rac1, through an interaction with ephexin, a guanine nucleotide exchange factor. The interaction of EphA4 with ephexin also occurs independent of receptor autophosphorylation. However, the most remarkable feature of ephrin-Eph signaling remains the ability of Eph molecules to induce signal transduction from the ephrins, activating

signaling pathways in the ephrin-presenting cell, as we described below.

Ephrins as Receptors

The high level of conservation of the carboxy-terminal intracellular tail of B-class ephrins led investigators to postulate a possible signaling role for these molecules initiating signaling cascades within the cells that present them. The first biochemical evidence that these molecules do indeed signal resulted from studies indicating that the highly conserved carboxy-terminal tail became tyrosine phosphorylated upon interaction of the ephrin with its receptor (Bruckner et al., 1997; Holland et al., 1996). Genetic evidence supporting a receptor-like function for ephrins (which is now often referred to as reverse signaling, while signaling from the Eph receptor is referred to as forward signaling) was provided by targeted disruption of the EphB2 gene. The EphB2$^{-/-}$ mouse displays defects in the formation of the anterior commissures, with axons from the temporal cortices failing to reach the midline (Henkemeyer et al., 1996). While this initially would appear to be another example of an Eph molecule acting as a guidance cue receptor, the absence of any expression of EphB2 in the cortical neurons precludes such a simple model. However, an EphB2 ligand, ephrin-B1, is expressed in the cortical neurons, raising the possibility that the "ligand" acts as a receptor, guiding the cortical axons by sensing EphB2. Consistent with this hypothesis, EphB2 is expressed in the tissues ventral to the anterior commissures, in a distribution appropriate for acting as a chemorepellant for the cortical axons (Henkemeyer et al., 1996). Stronger evidence for EphB2 acting through ephrin-B1-mediated reverse-signaling is provided by mice in which the EphB2 gene is not a complete null, but instead the endogenous gene is replaced by an EphB2lacZ gene (a fusion of the EphB2 extracellular and transmembrane domains to lacZ). In these mice the cortical axons project normally, despite the inability of the EphB2lacZ to transduce a signal (Henkemeyer et al., 1996). More recently, genetic studies have supported reverse signaling in axon mapping within the developing vertebrate retina (Birgbauer et al., 2000), in the guidance of migrating cells in developing *Caenorhabditis elegans* (George et al., 1998), and most importantly, from the aspect of tumorigenesis, in vertebrate angiogenesis (Adams et al., 2001) (however, it should be noted that at least in some of these studies the phenotypic change is potentially due to kinase-independent forward signaling, similar to that described by Gu and Park [2001]).

An important clue as to mechanisms by which ephrins may transduce signals was provided by the discovery that the carboxy-terminal domains of ephrins (like those of Eph molecules) interact with a variety of PDZ domain proteins (Bruckner et al., 1999; Torres et al., 1998). Further, Bruckner et al. discovered that ephrin-B1 molecules are localized within sphingo-lipid/cholesterol-enriched raft microdomains through a mechanism that is dependent on their carboxy-termini. Furthermore, they can draw the PDZ domain proteins GRIP and ABP/GRIP2 into these domains (Bruckner et al., 1999). Cross-linking of ephrin-B1 with EphB2, or with cross-linking antibodies, results in the fusion of rafts into large patches and an association of a serine/threonine kinase activity with the PDZ domain proteins. The end result is presumably the formation of an extensive signaling complex.

In a very recent study, Flanagan and co-workers (Lu et al., 2001) established a novel signaling pathway for ephrins that antagonizes signaling from heterotrimeric G-protein-coupled receptors. This activity is mediated by the binding of a novel PDZ domain protein, PDZ-RGS3 (RGS: regulator of heterotrimeric G protein signaling), to the carboxy-terminus of B-class ephrins. In the developing hindbrain this activity functions to inhibit premature migration of cerebellar granule cells, which would otherwise migrate under the influence of the chemokine SDF-1, acting through its G-protein-coupled receptor, CXCR-4. The inhibition of heterotrimeric G-protein signaling is mediated by PDZ-RGS3 exhibiting a GAP (GTPase-activating protein) activity specific for heterotrimeric G-proteins. While the inhibition of granule cells is dependent on the presence of an EphB molecule, the association of PDZ-RGS3 with ephrin-B1 is not.

It is now apparent that A-class ephrins can also mediate signaling (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001; Knoll et al., 2001). Both ephrin-A2 and ephrin-A5 act to regulate integrin function. These are not the first GPI-anchored molecules to be shown to act as receptors, with previous examples including the family of GDNF (glial cell-line-derived neurotrophic factor) receptors called GFRs. While some of these GPI-anchored molecules plainly can signal through interactions with transmembrane proteins, such as in the case of GFRα signaling through an interaction with c-RET (Saarma, 2000), in other cases transmembrane cofactors are not apparent. Instead, signaling is dependent on nonreceptor type tyrosine kinases of the src family. In these cases, the ligand-dependent cross-linking of GPI-anchored molecules, which are concentrated in the outer leaflets of lipid microdomains (also known as rafts, or glycospingolipid enriched membranes), may lead to the coalescence of the lipid microdomains. The acyl moieties of src family kinases are concentrated in the inner leaflets and the coalescence of the microdomains may lead to the clustering and activation of these kinases (Ilangumaran et al., 2000; Simons and Ikonen, 1997). Consistent with this signaling mechanism, the signaling by ephrin-A2 and ephrin-A5 is at least partially dependent on Fyn (Davy et al., 1999).

MULTIPLE ROLES FOR EPHRINS AND THEIR RECEPTORS IN CANCER

Disregulation of cellular tyrosine-kinase activity has a widely recognized role in the establishment of carcinogenesis. Further, excessive activation of receptor tyrosine kinases occurs frequently in human cancers and inhibition of the disregulated activity can frequently lead to remission (Kumar et al., 2000). However, there have been only a relatively limited number of occurrences in which investigators have demonstrated that excessive activation of Eph receptor tyrosine kinases can lead to cell proliferation or transformation. This no doubt relates to their in vivo functions primarily being the guidance of migrating cells and growing axons and the establishment of segment boundaries. Therefore, it may well be that the major role in carcinogenesis of this group of RTKs lies in angiogenesis or metastasis, not as a primary oncogene (Dodelet and Pasquale,

2000). In the following sections we review the possibilities for ephrins in each of the roles.

EphA2, EphB4, and Ephrin-B2 in Normal Breast Physiology

Two Eph molecules, EphA2 and EphB4, display remarkable expression patterns during murine breast development and tumorigenesis (Andres et al., 1994; Zelinski et al., 2001). Both are induced at puberty and undergo cyclical changes in expression during the estrous cycle. Their expression peaks during the estrogen phase of the cycle, during which time mammary epithelial cells are entering S-phase.

Further, pregnancy-induced differentiation of the mammary epithelium downregulated both. Nikolova et al. (1998) furthered this study by demonstrating that while EphB4 is restricted to the myoepithelial cells surrounding the ducts and alveoli of adult mouse mammary glands, its ligand, ephrin-B2, was restricted to the luminal epithelial cells. Ovariectomy resulted in loss of expression of both receptor and ligand, but could be reversed by the administration of estrogen.

Eph and Ephrin Molecules as Classical Transforming Oncogenes

There are many reports of ephrin or Eph upregulation in cancer cells relative to untransformed cell lines and in tumors relative to surrounding tissue (Dodelet and Pasquale, 2000) (see Table 1 for an extensive list). It is noteworthy that many of the reports of ephrin or Eph upregulation focus on breast cancers, gastrointestinal cancers, and melanoma and small-cell lung carcinoma (SCLC).

However, the evidence for most of the ephrins and their receptors acting as classical oncogenic growth factors and their receptors is relatively scarce. While, as outlined in Table 1, there are many reports of their expression being upregulated in cancers and cancer cell lines, in very few instances have these molecules been demonstrated to have transforming capacity in vitro. Further, mutations in human cancer have not been tightly linked to Eph or ephrin genes and mice transgenically overexpressing these genes have not, as yet, been reported to be prone to tumor development. In fact, it has been reported that tumor expression of EphB6, ephrin-B2, and ephrin-B3 in neuroblastoma patients correlates with a good prognosis and that ectopic expression of EphB6 can suppress the malignant phenotype of neuroblastoma cell lines (Tang et al., 2000a). Furthermore, under certain conditions activation of Eph receptors antagonizes the activation of mitogen-activated protein kinase (MAPK) by other growth factors (Miao et al., 2001). However, there are a few instances in which good evidence exists for Eph molecules to act as transforming oncogenes. In particular, the EphA1 gene has a well-established potential to act as a classical oncogene. Overexpression of EphA1 in NIH3T3 cells confers upon these cells the ability to both form colonies in soft agar and to create tumors in nude mice (Maru et al., 1990).

Activation of EphA2 can induce cell proliferation (Easty et al., 1995), although this effect may be cell-type-specific (Miao et al., 2001). EphA2 is expressed in the human colonic adenocarcinoma cell line, Caco-2. Caco-2 also express an EphA2 ligand, ephrin-A1, raising the possibility of an autocrine loop. Currently, it is not clear whether the predominant interactions are autocrine or paracrine when ephrins and Eph molecules are coexpressed (an interaction of an ephrin with an Eph molecule on the same cell has yet to be demonstrated). Addition of exogenous ephrin-A1 to Caco-2 cells represses their expression of differentiation markers (Rosenberg et al., 1997). Similar results, suggesting that EphA2 and ephrin-A1 may initiate an autocrine signal that stimulates proliferation, have been described for a number of metastatic melanoma cell lines (Easty et al., 1995b). However, as outlined in the next section, the relationship of ephrin-A1 and EphA2 in carcinogenesis may differ fundamentally from the classical dogma of a growth factor activating its RTK to initiate, through its tyrosine kinase domain, oncogenic signals.

EphA2 Can Induce Metastatic Phenotypes in Breast Epithelial Cells Through Mechanisms Independent of Its Autophosphorylation

Transgenic expression in mice of either the c-myc or Ha-ras genes from the whey acidic protein promotor (WAP) results in a predisposition to mammary tumors (Andres et al., 1991; Schoenenberger et al., 1988). EphA2 (as well as EphB4) has been found to become overexpressed in the undifferentiated, invasive tumors generated in mice by transgenic expression of Ha-ras, but not in the relatively differentiated and nonmetastatic tumors induced by c-myc (Andres et al., 1994).

Zantek et al. (1999) found that EphA2 was upregulated 2–5-fold higher in human breast cancer cell lines than in nontransformed epithelial-derived cell lines. In the nontransformed cell lines the EphA2 was phosphorylated and localized to points of cell–cell contact. However, in breast cancer cell lines that lack E-cadherin, EphA2 was relatively unphosphorylated and localized to membrane ruffles. Expression of E-cadherin in such cell lines led to a restoration of EphA2 phosphorylation and a redistribution of the EphA2 to the cell–cell contact points. Direct activation of EphA2 by cross-linking antibodies decreased both cell proliferation and extracellular matrix adhesion by breast cancer lines. Furthering this work, Zelinski et al. (2001) found that EphA2 could act in vitro to transform mammary epithelial cells. Introduction of EphA2 into the breast epithelial cell line MCF-10A (a spontaneously immortalized, nearly diploid line) caused cellular transformation, including anchorage-independent growth, weakened cell–cell contacts, increased affinity for extracellular matrix (ECM) components, and increased invasiveness in Matrigel. All of these phenotypes are common, and probably essential, features of breast cancer cells that have attained metastatic potential (Even-Ram et al., 2001; Kinch et al., 1995; Ruoslahti, 1994; Sommers et al., 1994; Weaver et al., 1995). Once again, Zelinski et al. (2001) found that the EphA2 in the transformed cells was relatively unphosphorylated and, quite remarkably, that activation of the EphA2 by exogenous soluble ligand reversed the capacity of EphA2 to induce transformation. EphA2-transformed breast epithelial cells also displayed a marked increase in tumorigenicity when subcutaneously injected into nude mice. The resulting tumors were histologically invasive. These results are noteworthy, in light of the fact that such tumorigenicity cannot be conveyed upon MCF-10A by TC21, Ras, or ErbB2/Neu

TABLE 1. Reported occurrences of Eph RTKs and ephrins in cancer

Type of cancer or cancer cell line. (with references)	Eph family members										Ephrins						
	A1	A2	A3	A4	A5	B1	B2	B3	B4	B6	A1	A3	A4	A5	B1	B2	B3
Breast cancers and breast cancer-derived cell-lines (1,2,3,4,5,6,7,8,12,37,45,46)	U R	* U						P R	P R		* U			P R			
Liver cancers and derived cell lines (4,6,8,46)	U R								P R								
Gastrointestinal cancers and derived cell lines (4,6,7,8,9,11,29,32,35,36,45,46)	U R	U R				U R	U R		P R		P					P	
Neuroblastomas, neural cancers, and cell lines (10,13,24,31,33,34,38,42,43)		P	P		P		P			P R		P R			P R	P R	P R
Leukemias and lymphomas and derived cell lines (6,14,16,28,39,40,41,44)			P						P R	P		P R	P R		P R	P R	
Prostate cancer and derived cell lines (17,18,19)	P	* U		P R				U R	P R								
Pancreatic cancers (4,46)	P R																
Lung cancers, including SCLC, and derived cell lines (4,6,7,8,10,15,34,46)	U	P	U			P R	P	P R	P R	P	P				P R	P R	P R
Melanoma and derived cell lines (6,10,20,21,22,23,26,27,30)		* U	* U	P					P R		* U					* U R	
Ovarian cancer, endometrial tumors, choriocarcinoma and teratocarcinoma (9,25,34)							P R		* U							* U	
HeLa cells (6,42)		P R							P R								
Thyroid cancers (4,46)	P R																
Sarcomas (9,10)			* U				P R										
Renal carcinomas (9,10)			U				P R										
Epidermoid cancer cell lines (5,42)		P R						P									

"P" (for present) indicates reported expression of the relevant Eph or ephrin gene in a tumor (or tumor-derived cell line). "U" (for upregulated) indicates increased expression of the gene in a tumor relative to appropriate surrounding tissue (or in the tumor-derived cell line relative to an appropriate control cell line). An asterisk in the upper right-hand corner of a box indicates seven or more tumors or cell lines were screened, and of these more than 30% displayed increased expression. "R" in the bottom right-hand corner of a box indicates results based on presence of the gene's mRNA, as determined by Northern analysis, RT-PCR, or cloning. Absence of "R" indicates the results were obtained through analysis of protein levels, most often by immunohistochemical or immunoblotting analyses. It should be noted that the intended use of this table is as a reference resource, rather than to depict trends. References: (1) Berclaz et al., 1996; (2) Zantek et al., 1999; (3) Zelinski et al., 2001; (4) Maru et al., 1990; (5) Bohme et al., 1993; (6) Bennett et al., 1994; (7) Ogawa et al., 2000; (8) Hirai et al., 1987; (9) Kiyokawa et al., 1994; (10) Chiari et al., 2000; (11) Oba et al., 2001; (12) Andres et al., 1994; (13) Bruce et al., 1999; (14) Steube et al., 1999; (15) Tang et al., 1999; (16) Wicks et al., 1992; (17) Walker-Daniels et al., 1999; (18) Chaib et al., 2001; (19) Robinson et al., 1996; (20) Easty et al., 1997; (21) Easty and Bennett, 2000; (22) Vogt et al., 1998; (23) Hess et al., 2001; (24) Tang et al., 2000a; (25) Takai et al., 2001; (26) Easty et al., 1999; (27) Easty et al., 1995a; (28) Shimoyama et al., 2000; (29) Rosenberg et al., 1997; (30) Easty et al., 1995b; (31) Miescher et al., 1997; (32) Sakano et al., 1996; (33) Tang et al., 2000b; (34) Ikegaki et al., 1995; (35) Iwase et al., 1993; (36); Chen et al., 1999 (37) Winslow et al., 1995; (38) Davis et al., 1994 (39) Boyd et al., 1992; (40) Dottori et al., 1999; (41) Kozlosky et al., 1995; (42) Lindberg and Hunter, 1990; (43) Tang et al., 1998; (44) Munthe et al., 2000; (45) Bartley et al., 1994; (46) Maru et al., 1988.

(Clark et al., 1996; Giunciuglio et al., 1995), suggesting a special role for EphA2, either in neoplastic breast transformation (Zelinski et al., 2001) or in the development of metastatic potential.

These observations indicate that EphA2 may function in the late stages of carcinogenesis, particularly during metastasis, and that it does so in a fashion that is independent of, as well as inhibited by, autophos-

phorylation, implying very different mechanisms of oncogenesis than those traditionally associated with receptor tyrosine kinases and their growth factor ligands. The traditional notion that abnormally high levels of tyrosine kinase activity is the major determinant of the oncogenic potential of an RTK, as is the case for ErbB2/Neu (Segatto et al., 1988), plainly does not apply for EphA2. What is the mechanism of EphA2-mediated transformation? Three possibilities exist that may explain an autophosphorylation-independent oncogenic capacity of EphA2.

The first of these possibilities is that autophosphorylation-independent transformation could result from a kinase-independent, ligand-dependent, forward-signaling mechanism, similar to that described for EphA8. As mentioned previously, EphA8 has already been observed to induce extracellular matrix adhesion through a PI3 kinase γ-mediated regulation of integrin activity, independent of its own kinase (Gu and Park, 2001). If a similar activity exists for EphA2, then kinase-independent forward signaling may well account for some or all of the changes occurring during EphA2-mediated transformation. Another plausible mechanism is that the transforming activity of EphA2 is due to an interaction with endogenous A-class ephrins, resulting in reverse signaling. As noted previously, coexpression of EphA2 and one of its ligands, ephrin-A1, has been detected in certain cancer cell lines (Easty et al., 1995b). A major disadvantage of both kinase-independent forward signaling (at least if it occurs by a mechanism similar to that reported for EphA8) and reverse signaling is that they are both dependent on ephrin–Eph interactions, and hence cannot easily explain the observed unphosphorylated state of EphA2 in breast cancer cell lines. Nor can they easily explain the decrease in transformed phenotypes as EphA2 phosphorylation increases. Therefore, a third possibility must be considered, namely, that the EphA2 may be initiating signaling through a mechanism that is not dependent on either ligand binding or its kinase activity. The possibility of such a signaling mechanism is raised by experiments showing that unactivated EphA molecules (or unphosphorylated fragments of EphA molecules) can associate with a variety of signaling factors, including focal adhesion kinase (FAK) (Miao et al., 2000), ephexin (Shamah et al., 2001), AF6/afadin, Pick1, Syntenin, and Grip1 (Bruckner et al., 1999; Torres et al., 1998). As the overexpressed EphA2 in transformed cell lines is displaced from points of cell–cell contact (Zantek et al., 1999; Zelinski et al., 2001), it is likely that this overexpression can in turn mislocalize one or more of these molecules. Such a mislocalization could generate aberrant pro-oncogenic signaling. In fact, it has previously been shown that activation of FAK is essential for contact-independent growth of breast cancer lines (Xu et al., 2000) and that localization of FAK to the cell membrane is sufficient to constitutively activate it (Frisch et al., 1996; Shen and Schaller, 1999). Ligand-induced autophosphorylation of EphA2 inhibits its association with FAK and in turn reduces both FAK phosphorylation and FAK activity (Miao et al., 2000) and, therefore, would counteract any oncogenic activity due to the disregulated FAK activity.

Miao et al. (2000) also report that EphA2 activation (through FAK inhibition) can inhibit integrin-mediated cell adhesion. The concentration of exogenous ligand necessary for this EphA2-mediated inhibition of adhesion corresponds closely with that necessary to achieve significant phosphorylation of the EphA2. Further, they show that EphA2 activation can also repress other activities normally associated with transformation, including repressing the MAP kinase pathway, cell proliferation, and cell migration (Miao et al., 2000, 2001). Taken together, these results appear to indicate that kinase-dependent forward signaling may be antagonistic to an oncogenic activity mediated by kinase-independent signaling and that the best candidate mechanism for this antagonism is the kinase-mediated inhibition of FAK association, which ties EphA2 directly to a control of extracellular matrix adhesion and cell migration (and plausibly through FAKs interaction with src, control of other aspects of transformation). An FAK-associated mechanism of oncogenesis has the considerable advantage that not only is it phosphorylation-independent, but is also ligand-inhibited, and therefore can most readily account for the unphosphorylated state of EphA2 in cancer cell lines. Also, by being ligand-independent, this mechanism allows for the continued function of EphA2 in a migrating metastatic cancer cell in vivo, where at times it is likely to be isolated from ligand-presenting cells.

Ephrins as Angiogenic Factors in Cancer and Normal Development

Inhibition of tumor angiogenesis is now recognized as having great potential as a chemotherapeutic approach. As we outline in this section, targeted mutagenesis studies in mice have clearly established several ephrins and Eph molecules as critical factors in the refinement of the plexus into a sophisticated network of veins, capillaries, and arteries. Recent results indicate they are also upregulated as blood vessels invade tumors. As a result, they must be considered as viable targets for the development of drugs antagonizing tumor angiogenesis.

Several B-class ephrins and their receptors are expressed in the murine embryonic vasculature, including EphB2, EphB3, EphB4, and ephrin-B1 and ephrin-B2. Ephrin-B2 is expressed in most arteries but is absent from veins (Adams et al., 1999; Wang et al., 1998) (although very weak expression can be detected in certain veins of the adult [Gale et al., 2001; Shin et al., 2001]). EphB4 expression in the murine embryo is restricted to endothelial cells, with much higher expression in arteries than veins (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). The expression of both of these molecules in endothelial cells extends into adulthood (Shin et al., 2001). This distribution provides the strongest evidence to date for a cellular difference between venous and arterial endothelial cells. It is well established in the embryonic hindbrain that ephrins and Eph molecules interact to establish boundaries between tissue segments. These results have been elegantly recapitulated by in vitro studies in which cells expressing ephrin-B2 do not intermingle with those expressing EphB2 or EphA4, both of which are ephrin-B2 receptors (Mellitzer et al., 1999). In light of these roles in establishing neural segment boundaries, these vascular expression patterns implicate B-class ephrins and their receptors in establishing the boundary between veins and arteries, and hence the location of capillary beds.

Ephrin-B2 is also expressed in the smooth muscles and pericytes of arteries, although this expression appears considerably later in development than does expression in the endothelial cells (Gale et al., 2001; Shin et al., 2001). This pattern of expression raises the possibility that ephrin-B2 expression in the endothelial cells imposes an appropriate arterial nature upon the surrounding support cells.

Targeted mutagenesis of ephrin-B2 in mice results in a dramatic failure of normal vascular development during embryogenesis (Wang et al., 1998; Adams et al., 1999), as well as mild defects in branchial arch development and neural crest migration (Adams et al., 2001). Vascular development can be divided into two phases: vasculogenesis (angioblastic growth) and angiogenesis (angiotrophic growth). Vasculogenesis is defined as the de novo differentiation of mesodermal cells into endothelial cells resulting in a crude vascular network, called the primary capillary plexus. Vasculogenesis is also responsible for the formation of the primordia of the heart and the large trunk vessels. Angiogenesis is the refinement of this vascular network into a mature vasculature, including recognizable capillary beds, through processes that include endothelial cell proliferation, sprouting, and migration. While some defects in vasculogenesis are observable in the ephrin-B2$^{-/-}$ mouse, the major defects appear to be in angiogenesis. As a result of the failed formation of the vascular system, ephrin-B2$^{-/-}$ mice die in midgestation.

When mice were created which were not null for ephrin-B2, but instead expressed a C-terminal deletion of ephrin-B2 lacking most of the intracellular domain, neural crest migration was normal but vascular development remained severely dysfunctional (Adams et al., 2001). This indicated that the reverse signaling of ephrin-B2 may be essential for vascular development, but that its ligand (i.e., Eph activating) function alone may be sufficient for normal neural crest migration. Although alternative explanations, such as a requirement of the intracellular domain for clustering of the ephrin-B2, and hence greater Eph activation, could explain this observation, the ability of EphB-Fc (soluble molecules resulting from the fusion of the Eph extracellular domain to an immunoglobulin domain) to stimulate endothelial sprouting in vitro strongly supports this hypothesis (Adams et al., 2001).

Targeted disruption of EphB4 also results in midgestation embryonic lethality, most likely due to failed cardiovascular development (Gerety et al., 1999). The expression of EphB4 is restricted to the cardiovascular system in the E10 mouse and is preferentially expressed in veins as opposed to arteries. Expression on arteries is very weak and patchy. A prominent feature of the EphB4$^{-/-}$ mouse was that its anterior cardinal vein was split into multiple branches, a feature which closely duplicated the ephrin-B2$^{-/-}$ mouse. In combination with defects in arteries, which normally express EphB4 weakly, these results indicate that aspects of the EphB4$^{-/-}$ phenotype may be due to a failure to activate the receptor-like activity of ephrin-B2.

While genetics now provides strong evidence for a role for reverse signaling in angiogenesis, there is also clear evidence for forward signaling in this process. Soluble molecules, formed from the fusion of the extracellular domains of B-class ephrins to Fc domains, can induce endothelial cells in vitro to replicate various aspects of angiogenesis, including vessel formation and sprouting (Adams et al., 1999; Stein et al., 1998b). It should be noted that at least for some of these processes the ephrin-Fc fusions need to be ectopically clustered into oligomers.

While ephrin-B2 is generally artery-specific and EphB4 is generally vein-specific, the expression pattern of other Eph and ephrin molecules in the mouse embryonic vasculature is more complex. A general summary of results would be that arteries coexpress ephrin-B1 and ephrin-B2, the aortic arch coexpresses ephrin-B1, ephrin-B2, and EphB3, and veins coexpress ephrin-B1, EphB3, and EphB4 (Adams et al., 1999). The coexpression of ephrins and Eph molecules indicates that as well as acting in venous-arterial boundary formation, they may also act in other aspects of angiogenesis such as sprouting and proliferation. Further, EphB2 and ephrin-B2 are often expressed in mesenchyme surrounding vessels, indicating a role in mesenchymal–endothelial interactions.

Ectopic expression of dominant-negative forms of EphB4 or any B-class ephrin in *Xenopus* embryos resulted in defects in the vasculature, particularly the intersomitic veins (Helbling et al., 2000). EphB4 is expressed in the intersomitic veins, while the B-class ephrins are expressed in the surrounding somites. The treated embryos displayed intersomitic veins inappropriately entering the somites, strongly suggesting a role for these molecules in the modeling of the vasculature by endothelial–mesodermal interactions.

Mice lacking both EphB2 and EphB3 display, at low penetrance, similar defects to those of the ephrin-B2$^{-/-}$ mouse (Adams et al., 1999). Included in these defects are a failure to form the intersomitic vessels (Adams et al., 2001). Ephrin-B2 is also expressed in the vessels of tumors, arguing against the traditional view that tumor vessels arise exclusively from postcapillary venules (Shin et al., 2001). Ephrin-B2-expressing (and, presumably therefore, arterial) endothelial cells can be observed to invade tumors introduced into nude mice (Shin et al., 2001). It will be of considerable interest when future studies determine the degree to which disruption of ephrin-B2, or its receptors, confer upon mice resistance to tumorigenesis. If these molecules are essential for tumor angiogenesis, then tumors in mice deficient in them may fail to progress beyond diminutive size. These studies have been hampered so far through the lethal nature to the mouse of these deficiencies. However, the advent of hypomorphic mutants likely ensures these studies will be completed soon.

Soluble ephrin-A1 can act as an angiogenic factor in vitro and is essential for TNF-alpha-induced angiogenesis. Further, its expression during early murine development is restricted to regions of vasculogenesis or angiogenesis, including the endocardial cells and the intersomitic vessels (McBride and Ruiz, 1998). A number of its receptors are also expressed in discrete regions of the embryonic vasculature, raising the possibilities of ephrin-A1 functioning through either autocrine or paracrine systems in normal angiogenesis (Bovenkamp and Greer, 2001). Its expression in certain tumors, including melanomas, may therefore correlate with tumors attracting invasion by endothelial cells (Easty et al., 1999). In mice models, in which human breast-derived tumors or Karposi-sarcoma-derived tu-

mors were used as xenografts, the vasculature invading the tumors strongly expressed ephrin-A1 and one of its receptors, EphA2 (Ogawa et al., 2000). Further, ephrin-A1 and EphA2 can be detected on the surface of endothelial cells in a variety of human tumors (Ogawa et al., 2000).

CONCLUSIONS

At this point in time, considerable evidence is accumulating for roles for the Eph RTKs in both metastasis and tumor angiogenesis. This is particularly true for certain tumor types, including breast. As such, the Eph family and its ligands represent targets for chemotherapeutic drug development. As well as traditional small molecule antagonist approaches, drugs antagonizing Eph molecule-mediated carcinogenesis may include appropriate derivatives of either Eph or ephrin molecules. However, before drug development proceeds, significant gaps in the basic research need to be filled. Among the most pressing of these is a need to understand why, depending on the circumstances, expression of Eph and ephrin family members can correlate with a better or a worse prognosis, and why phosphorylation of the Eph molecules can sometimes correlate with reduced metastatic potential. The recent results in breast epithelial cells and breast tumors, which we have detailed in this review, provide new insights into the fundamental biological processes which may underlie these paradoxes.

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