Ephrin-A1 Facilitates Mammary Tumor Metastasis through an Angiogenesis-Dependent Mechanism Mediated by EphA Receptor and Vascular Endothelial Growth Factor in Mice

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Abstract

Ephrin-A1, the prototypic ligand for EphA receptor tyrosine kinases, is overexpressed in vascularized tumors relative to normal tissue. Moreover, ephrin-A1-Fc fusion proteins induce endothelial cell sprouting, migration, and assembly in vitro, and s.c. vascular remodeling in vivo. Based on these data, we hypothesized that native, membrane-bound ephrin-A1 regulates tumor angiogenesis and progression. We tested this hypothesis using a transplantable mouse mammary tumor model. Small interfering RNA–mediated ephrin-A1 knockdown in metastatic mammary tumor cells significantly diminishes lung metastasis without affecting tumor volume, invasion, intravasation, or lung colonization upon i.v. injection in vivo. Ephrin-A1 knockdown reduced tumor-induced endothelial cell migration in vitro and microvascular density in vivo. Conversely, overexpression of ephrin-A1 in non-metastatic mammary tumor cells elevated microvascular density and vascular recruitment. Overexpression of ephrin-A1 elevated wild-type but not EphA2-deficient endothelial cell migration toward tumor cells, suggesting that activation of EphA2 on endothelial cells is one mechanism by which ephrin-A1 regulates angiogenesis. Furthermore, ephrin-A1 knockdown diminished, whereas overexpression of ephrin-A1 elevated, vascular endothelial growth factor (VEGF) levels in tumor cell–conditioned medium, suggesting that ephrin-A1–mediated modulation of the VEGF pathway is another mechanism by which membrane-tethered ephrin-A1 regulates angiogenic responses from initially distant host endothelium. These data suggest that ephrin-A1 is a proangiogenic signal, regulating VEGF expression and facilitating angiogenesis-dependent metastatic spread. (Cancer Res 2006; 66(21): 10315-24)

Introduction

Vascular recruitment by tumors is a critical step in malignant progression. Microvascular density is one prognostic indicator for the probability of metastasis, disease recurrence, and survival for many tumor types, including breast (1–4). Although tumors exploit a variety of mechanisms to acquire blood vessels, the best-characterized mechanism is angiogenesis, a process involving sprouting of new branches from preexisting host vessels (reviewed in refs. 5, 6). Through up-regulation of proangiogenic molecules and down-regulation of angiostatic factors, tumors elicit remodel-

ing of host blood vessels by activating endothelial cell invasion through basement membrane and sprouting, proliferation, migration, and tube formation/supporting cell recruitment to form new functional vessels (reviewed in refs. 5–7). These new vessels supply oxygen, nutrients, and growth/survival factors to the tumor, creating a permissive environment for malignant progression. In addition, invasive tumor cells can use these vessels to enter circulation for metastatic spread (6, 8). Therefore, understanding molecular mechanisms that facilitate tumor angiogenesis is crucial for development of more effective anticancer therapies.

Eph receptor tyrosine kinases and their ligands, the ephrins, have emerged as important mediators of vascular remodeling during embryonic development and in disease. This family consists of at least 16 receptors and nine ligands identified in multiple species (reviewed in refs. 9–12). One unusual feature of this family is that Eph receptors bind to membrane-anchored ligands rather than to soluble factors. The family is subdivided into two classes based on homology and binding to two distinct classes of ephrins (13). EphA-class receptors in general bind to class A ephrins, which are bound to the cell membrane by a glycosyl-phosphatidylinositol linkage. EphB class receptors generally bind to class B ephrins, which are anchored to the cell membrane by a transmembrane-spanning domain. As ephrins are membrane tethered, cellular contact is required to initiate signaling upon binding to Eph receptors on adjacent cells, which initiates intracellular signaling cascades leading to changes in cell shape, cellular adhesion, and chemotaxis through repulsion, depending on cell type (reviewed in refs. 9–12).

In addition to roles in embryonic patterning and axonal guidance, Eph receptors regulate angiogenesis in embryonic development and in adult tissues (reviewed in refs. 11, 12). Several B-class receptors and ligands promote remodeling of embryonic vessels, as well as marking boundaries between arterial and venous domains in the developing circulatory system, and B-class receptors and ligands have recently been linked to tumor neovascularization (reviewed in refs. 11, 14). Although genetic studies have not yet revealed a function for A-class receptors and ligands in embryonic vascular remodeling, recent evidence has linked EphA2 and ephrin-A1 to postnatal angiogenesis regulation and tumor angiogenesis. Expression analysis of several mouse xenograft models and human tumors revealed that both ephrin-A1 and EphA2 were widely expressed in tumor parenchyma and tumor endothelium (15). Studies using soluble receptors as inhibitors revealed that A-class receptors are necessary for vascular remodeling in response to soluble ephrin-A1-Fc ligand and in tumor neovascularization (16–18). Ephrin-A1-Fc induces angiogenic responses in cultured endothelial cells in vitro, as well as angiogenic remodeling of s.c. and corneal vessels in vivo (reviewed in refs. 9, 19), and EphA2-deficient mice display diminished...
vascular remodeling in response to ephrin-A1-Fc, as well as impaired tumor angiogenesis in response to ephrin-A1–expressing tumor cells in vivo (19, 20). Although expression has been observed in vascularized tumors (reviewed in refs. 11, 14), a causal role for ephrin-A1 in tumor vessel recruitment remains unclear. Previous studies, along with the observation that ephrin-A1 mRNA is up-regulated in metastatic mammary tumor cell lines relative to less malignant cells (21), lead us to hypothesize that that native, membrane-bound ephrin-A1 regulates tumor angiogenesis and progression. Using both small-interfering RNA (siRNA)–mediated knockdown and overexpression approaches, we present data that suggest ephrin-A1 expression by tumor cells promotes angiogenesis and facilitates metastasis in vivo.

Materials and Methods

Cell culture. Primary mouse mammary epithelial cells (PMEC) were isolated from BALB/c female mice and maintained as previously described (22). Mouse mammary adenocarcinoma cell lines 67NR, 168FARN, 4T07, and 4T1 were generously provided by Dr. Fred Miller (Karmanos Cancer Institute, Wayne State University, Detroit, MI; ref. 23). Bovine pulmonary microvascular endothelial cells (BPMEC) were purchased from VEC Technologies (Rensselar, NY) and maintained in endothelial basal medium-2 (Cambrex Bioscience, Walkersville, MD) supplemented with Clonetics EGM-2 Singlequots (Cambrex Bioscience) plus 2 mmol/L L-glutamine and 5 units/mL penicillin-streptomycin. Murine pulmonary microvascular endothelial cells (MMPMC) were prepared as described previously (20, 24) and maintained in EGM-2. Cells were grown on tissue culture plates coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) diluted in PBS.

Reagents. siRNA sequences for mouse ephrin-A1 or irrelevant control were generated by computer algorithm (Ambion, Austin, TX): 5′-GGAGTATGTGCGATGCAGATT-3′ ephrin-A1 siRNA 1; 5′-GGTGGTGATATTAGG- GTTTTT-3′ ephrin-A1 siRNA 2; 5′-UUGAAGGCGGCAUCAGU-3′ control siRNA (25). PAGE-purified oligonucleotides encoding siRNA sequences or irrelevant control sequence (Integrated DNA Technologies, Coralville, IA) were annealed, phosphorylated, and cloned into siRNA pRetroSuper vector (generously provided by Dr. Reuven Agami, The Netherlands Cancer Institute, Amsterdam, Netherlands; ref. 26) to produce retroviruses for delivery to target cells as described previously (26, 27). Pools of 4T1 cells stably expressing siRNAs were selected and maintained by selection with 4 μg/mL puromycin (Sigma-Aldrich). Human ephrin-A1 cDNA (generously provided by Dr. Doug Cerretti, Amgen, Seattle, WA) was cloned into pAdEasy adenoviral vector (Qbiogene, Irvine, CA; ref. 28) for production of recombinant adenovirus as per instructions of the manufacturer.

Antibodies include anti-von Willebrand factor (vWF; DakoCytomation, Glostrup, Denmark), anti-ephrin-A1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-tubulin (Sigma-Aldrich), anti-CD31 (BD Biosciences, San Diego, CA), anti–vascular endothelial growth factor (VEGF) antibody for immunohistochemistry (Neomarkers, Lab Vision Corporation, Fremont, CA), neutralizing antibody for VEGF (R&D Systems, Minneapolis, MN), and anti-actin (Santa Cruz Biotechnology). Additional rabbit polyclonal antibodies raised against human ephrin-A1 and ephrin-A5 were provided by Dr. Doug Cerretti. CellTracker dyes 5-chloromethylfluorescein diacetate and 5- and 6-((1-chloromethyl)-benzoylamino)tetramethyl-rhodamine were purchased from Molecular Probes (Eugene, OR). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Sigma-Aldrich.

In vivo analysis of tumor progression. BALB/c female animals were obtained from Harlan Sprague-Dawley and housed under pathogen-free conditions. All experiments were done in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. Ten-week-old BALB/c female mice were injected with 5 × 10^6 4T1 or 67NR cells in the left inguinal mammary gland (19, 21). 67NR cells were transduced with 10^8 plaque-forming unit (pfu) adenoviruses harboring control β-galactosidase (20) or human ephrin-A1 before transplantation. Tumors were harvested after 14 days (67NR) or 21 days (4T1) and measured by caliper. Volume was quantified using the following formula: volume = length × width^2 × 0.52 (29). For 4T1 tumors studies, a total of 15 mice per condition were analyzed from three independent experiments with five mice per condition in each experiment. For 67NR tumor studies, a total of 9 to 10 mice per condition were analyzed from three independent experiments with three to four mice per condition in each experiment.

For lung colonization experiments, 10,000 tumor cells were injected into 10-week-old BALB/c female mice i.v. (30). Lungs were collected 12 days after transplantation. Data are a representation of two independent experiments with four mice per condition for each experiment, eight mice per condition total. For quantification of total metastatic lesions, lungs were stained with hematoxylin as previously described (19). Lungs were photographed using an Olympus SZX12 dissecting microscope with digital camera and lung lesions were enumerated.

Histologic analyses. For quantification of microvascular density, tumor sections were probed with primary antibodies against VWF (DakoCytomation; 1:250) as described previously (19) and counterstained with DAPI (1.5 μg/mL) to visualize nuclei. Photographs were captured using a BX60 microscope plus digital camera. Microvascular density was quantified in three random ×10 fields by scoring pixel area of Cy3 (vWF) fluorescence using NIH Image 1.63 software. Ephrin-A1 staining in tumor sections was done as described previously (16) using anti-ephrin-A1 (P1, 1:200; Amgen) or equivalent rabbit IgG control. Staining for β-galactosidase was done as previously described (31) using anti-β-gal antibody (1:100; 5-Prime 3-Prime, Boulder, CO). Staining for VEGF was done as previously described (16) using anti-VEGF antibody (10 μg/mL; Neomarkers). CD31 staining was done by the Vanderbilt Immunohistochemistry Core Laboratory and quantified by counting the number of CD31-positive vessels per ×20 field in four random fields per section. Analysis of tumor cell proliferation and apoptosis from sections by staining for nuclear proliferating cell nuclear antigen and terminal deoxynucleotid transferase-mediated nick-end labeling assay, respectively, was done as described previously (16).

Tumor cell/endothelial cell coculture migration assays and invasion assays. Coculture assays were done as described previously (16). Migrating cells were photographed using a BX60 microscope plus digital camera and invasion scored by quantification of fluorescent pixel area using NIH Image I.63 software. For gain-of-function experiments, 67NR cells were transduced with 10^6 pfu adenoviruses harboring control β-galactosidase or human ephrin-A1 before migration assay. For all coculture experiments, data are a representation of nine independent samples per condition from three independent experiments.

Three-dimensional invasion assays were done according to Debnath et al. (32). Cultures were photographed using a CK40 inverted microscope plus digital camera. Data are a representation of eight independent cultures per clone from two independent experiments.

Analysis of VEGF expression and function. VEGF protein levels were assessed in tumor cell conditioned medium by immunassay using mouse VEGF Immunoassay Quantikine kit (R&D Systems; ref. 33). Tumor cells (100,000) were plated in six-well dishes and incubated with Opti-MEM supplemented with 5% FCS for 48 hours. Before incubation, 67NR cells were transduced with 10^6 pfu adenoviruses harboring control β-galactosidase or human ephrin-A1 before migration assay. For all coculture experiments, data are a representation of nine independent samples per condition from three independent experiments.

For inhibition studies, coculture migration assays using Ad-ephrin-A1 transduced 67NR cells or controls were done in the presence of 5 μg/mL anti-VEGF antibody or control goat IgG (Santa Cruz Biotechnology). Efficacy of the neutralizing antibodies was validated by the ability to inhibit VEGF-induced (20 ng/mL) endothelial coalescence in two-dimensional assembly assays (data not shown). 67NR transduced with Ad-β-gal or Ad-ephrin-A1 (100,000) were plated in 24-well plates and incubated in OptiMEM overnight. Neutralizing antibodies were added to the well containing cells and conditioned medium and incubated for 30 minutes before the migration assay. MMPMCEs (20) were added to the upper chamber (100,000)
of Matrigel-coated transwells and incubated for 5 hours. Migration was scored as described previously (20) by fixing endothelial cells on transwells, staining with crystal violet, and counting cells on the lower surface that migrated in response to conditioned medium. Coculture assays in which 67NR cells were plated on the lower surface of transwells were done as described above. Neutralizing antibodies or control IgGs were added to the well containing conditioned medium for 30 minutes before the migration assay and migration of wild-type or EphA2-deficient MPMEC was scored as described above. Data are a representation of nine independent samples per condition from three independent experiments.

Results

Ephrin-A1 expression correlates with malignancy and vascular density in mammary adenocarcinoma cells. To assess ephrin-A1 expression in relation to degree of malignancy, we chose a mouse mammary tumor model that consists of four distinct tumor cell lines, 67NR, 168FARN, 4T07, and 4T1. These cell lines were derived from a spontaneous BALB/c mouse mammary tumor and are capable of reproducing each stage of metastatic disease progression when transplanted into syngeneic animals, including development of primary tumors only (67NR), local invasion to the lymph node (168FARN), entry into circulation and lung micrometastases (4T07), and visible lung nodules (4T1; Fig. 1A; refs. 21, 23). Consistent with previously reported microarray data (21), ephrin-A1 protein expression was elevated in metastatic mammary adenocarcinoma cell lines 168FARN, 4T07, and 4T1 relative to nonmetastatic 67NR and PMECs (Fig. 1B). Moreover, microvascular density was significantly higher in mammary tumors produced by 4T1 cells relative to 67NR when transplanted in vivo (Fig. 1C). Tumor volume was also significantly higher for 4T1 tumors compared with 67NR tumors (data not shown; refs. 21, 23).

Down-regulation of ephrin-A1 reduces tumor vascular density and metastasis in vivo. To determine if ephrin-A1 affects malignant progression, we inhibited ephrin-A1 expression in 4T1 tumor cells. We transduced 4T1 cells with retroviruses expressing two independent siRNA constructs targeting mouse ephrin-A1 and generated stable pools by selection with puromycin. We observed a 60% to 80% reduction in ephrin-A1 expression in 4T1 cells stably transduced with ephrin-A1 siRNA 1 or 2 relative to control siRNA (Fig. 2A). Knockdown was specific for ephrin-A1, as ephrin-A5 expression levels remained unchanged in these cells (Fig. 2A). Moreover, siRNA-mediated knockdown was maintained when tumor cells were transplanted in BALB/c mice (Fig. 2B).

We transplanted parental 4T1, control siRNA clones, or siRNA clones 1 or 2 orthotopically into the mammary fat pads of BALB/c mice and collected primary tumors and lungs 3 weeks post-transplantation for analysis of tumor progression. Although ephrin-A1 knockdown had no effect on primary tumor volume, tumor cell survival, or tumor cell apoptosis (Fig. 3A; data not shown), expression of the endothelial-specific marker CD31 revealed a significant decrease in microvascular density upon ephrin-A1 siRNA knockdown (Fig. 3B). These results were confirmed by vWF staining (data not shown). In addition, the number of lung metastases was also significantly decreased in mice transplanted with ephrin-A1 siRNA clones relative to controls (Fig. 2C).

Down-regulation of ephrin-A1 impairs endothelial cell recruitment by tumor cells. The data from orthotopic tumor transplantation studies suggest a role for ephrin-A1 in recruitment of host vascular endothelial cells, which express its receptor EphA2 (16, 19). To test this hypothesis, we did modified transwell assays to assess the ability of ephrin-A1 knockdown tumor cells or controls to initiate migration of microvascular endothelial cells (Fig. 4A, diagram). Control cells or ephrin-A1 siRNA clones were plated on the lower surface of transwell chambers and labeled with CellTracker Green Dye. Primary microvascular endothelial cells from bovine lung (BPMEC) were labeled with CellTracker Orange Dye and added to the upper chamber of the transwell to assess endothelial migration and intercalation into the tumor cell layer below. After 5 hours, the cells remaining on the upper transwell membrane surface were removed with a cotton swab, and the cells on the lower surface were photographed under fluorescence to measure endothelial cell (red) migration and intercalation into the tumor cell (green) layer on the lower surface. Endothelial cell migration in response to ephrin-A1 siRNA clones was significantly impaired relative to parental 4T1 or control siRNA clones (Fig. 4A), suggesting a proangiogenic function for ephrin-A1 in vitro. To assess any effects on tumor cell migration in response to endothelial cells, we did an in vitro intravasation assay in which we assessed migration of tumor cells in the upper chamber of the transwell in response to endothelial cells coated on the lower surface of the transwell (Fig. 4B, diagram). No difference was observed in tumor cell migration induced by endothelial cells (Fig. 4B).

Although our data provide evidence that ephrin-A1 induces angiogenesis, these results do not exclude the possibility that down-regulation of ephrin-A1 affects intrinsic malignancy. To address this possibility, we examined tumor cell invasiveness in three-dimensional Matrigel culture assays (32). Invasiveness, as assessed by morphology and protrusion of cells from individual colonies, was unaltered by ephrin-A1 siRNA-mediated knockdown relative to controls (Fig. 4C). To elucidate any changes in malignancy separate from vascular recruitment defects in vivo, we tested the ability of these cells to colonize the lung upon i.v. injection. A slight, but not statistically significant, decrease in the number of lung colonies was observed comparing ephrin-A1 siRNA clones versus parental or control siRNA clones (Fig. 4D), suggesting that ephrin-A1 is not required for colonization of secondary organs by circulating tumor cells.

Up-regulation of ephrin-A1 elevates tumor-induced endothelial cell migration in vitro and microvascular density in vivo. To examine the proangiogenic role of ephrin-A1 in tumor progression, we also did gain-of-function studies in which we overexpressed ephrin-A1 in poorly malignant 67NR cells by adenoviral transduction (Fig. 5A). Relative to untransduced or control β-galactosidase–transduced cells, 67NR transduced with ephrin-A1 displayed a significant increase in tumor cell–induced endothelial migration in the modified transwell assay (Fig. 5A). Consistent with our observations in the 4T1 model, 67NR overexpressing ephrin-A1 did not show any difference in intrinsic malignancy compared with controls in three-dimensional Matrigel cultures (data not shown). To determine if ephrin-A1 overexpression affects tumor neovascularization in vivo, we transplanted control or ephrin-A1–overexpressing 67NR cells orthotopically into the mammary glands of BALB/c female mice. Expression of adenoviral gene products in 67NR tumors was validated by immunohistochemistry (data not shown). Although there was no significant increase in tumor volume (Fig. 5C), tumors collected from mice transplanted with ephrin-A1–overexpressing 67NR cells displayed a significantly higher microvascular density than tumors collected from control animals (Fig. 5B).

Ephrin-A1 regulation of VEGF in vascular recruitment. Although previous studies provide evidence for the proangiogenic activity of soluble ephrin-A1-Fc (16, 34), the mechanism through...
which membrane-tethered ephrin-A1 expressed by tumor cells facilitates recruitment of initially distant host endothelium remains unclear. It is possible that ephrin-A1 cooperates with other proangiogenic factors that are soluble and thus can function over a greater distance. Therefore, we assessed expression levels of VEGF, a potent soluble factor that has been linked to tumor angiogenesis in several models of cancer, including 4T1 (35, 36). Moreover, coexpression of ephrin-A1 and VEGF by 4T1 tumor cells along with reports that soluble EphA-Fc receptors can inhibit VEGF-mediated angiogenic responses in cultured endothelial cells in vitro and corneal angiogenesis in vivo provide a rationale for cooperation between these two pathways (16, 18, 34). Immunohistochemical analysis suggested that VEGF is down-regulated in tumors derived from ephrin-A1 siRNA 1 or siRNA 2 4T1 clones relative to parental or control siRNA tumors (Fig. 6A). To quantify this decrease, we measured VEGF protein levels in conditioned medium collected from tumor cells. We observed a significant decrease in VEGF protein levels for siRNA clones versus controls by ELISA (Fig. 6B). Moreover, we detected elevated VEGF protein levels in conditioned medium from ephrin-A1–overexpressing 67NR relative to controls.

To determine if up-regulated VEGF levels produced by ephrin-A1–overexpressing cells is required for tumor-induced endothelial cell migration, we assessed endothelial migration in response to conditioned medium generated by 67NR cells (Fig. 6C, diagram). Cells were transduced with Ad-β-gal or Ad-ephrin-A1 and plated. After 24-hour incubation with serum-free medium, control IgGs or anti-VEGF–neutralizing antibodies were added to the conditioned medium, and endothelial cells were added to the upper chamber of transwells above the cells/conditioned medium. We observed significantly higher numbers of migrating endothelial cells in response to Ad-ephrin-A1–expressing 67NR medium containing control IgG relative to Ad-β-gal–expressing 67NR medium (Fig. 6C). Neutralizing anti-VEGF antibodies abrogated endothelial cell migration induced by Ad-ephrin-A1–expressing 67NR conditioned medium (Fig. 6C), suggesting a functional link between ephrin-A1 and VEGF in angiogenesis regulation.

Although up-regulated VEGF levels produced by ephrin-A1–overexpressing cells contributes to endothelial cell migration, previous studies reported that ephrin-A1 by itself is capable of regulating endothelial cell migration, and migration induced by ephrin-A1 requires EphA2 receptor function in responding endothelial cells (16, 34). To distinguish between the independent angiogenic function of ephrin-A1 relative to VEGF–dependent function, we did modified coculture migration assays in which control or ephrin-A1–overexpressing 67NR cells were plated on the lower surface of transwells and conditioned medium generated for 24 hours. Control IgG or anti-VEGF–neutralizing antibodies were then added to conditioned medium, and endothelial cell migration in response to tumor cells/conditioned medium was scored (Fig. 6D, diagram). Interestingly, we did not observe any significant differences in endothelial migration between control IgG and anti-VEGF neutralizing antibody–treated samples, although ephrin-A1 overexpression still elevated endothelial cell migration relative to
control β-gal-expressing cells (Fig. 6D). These data suggest that ephrin-A1 itself is required for migration of endothelial cells when expressed on tumor cells adjacent to endothelial cells. We did observe significantly lower levels of migration induced by ephrin-A1–overexpressing 67NR for EphA2-deficient endothelial cells derived from mouse lung (MPMEC) relative to wild-type endothelial cells (Fig. 6D). As ephrin-A1 is the principal ligand for EphA2, these data provide further support for angiogenic functions of ephrin-A1 that are independent of VEGF. Taken together, these data suggest that ephrin-A1 facilitates vascular recruitment both independently and through regulation of VEGF.

Discussion

Although initiation of tumorigenesis involves tumor cell intrinsic activation of oncogenes and/or down-regulation of tumor-suppressor gene function, tumor progression requires complex interactions between tumor tissue and host tissue (37–39). In particular, interaction between tumor cells and microvascular endothelial cells within the microenvironment plays a key role in tumor survival, growth, and metastatic spread. In this study, we provide direct evidence for native, membrane-bound ephrin-A1 ligand as a proangiogenic factor in tumor progression. Diminished ephrin-A1 expression in malignant mammary adenocarcinoma cells by siRNA knockdown reduces tumor microvascular density and lung metastasis in vivo. At a cellular level, ephrin-A1 knockdown impairs tumor cell–induced endothelial cell migration in coculture assays. Consistent with these data, gain-of-function studies in which ephrin-A1 was overexpressed in nonmetastatic cells resulted in elevated tumor cell–induced endothelial cell migration in vitro and elevated tumor microvascular density in vivo. These data provide first functional evidence that membrane-bound ephrin-A1 expressed on tumor cells, which is up-regulated in a wide variety of vascularized cancers relative to normal tissue (reviewed in refs. 11, 14), facilitates tumor angiogenesis and metastatic spread.

Metastatic spread of tumor cells is a complex process involving many steps, including (a) local invasion of cells from the primary tumor mass into surrounding tissue and acquisition of tumor blood vessels, (b) intravasation into tumor blood vessels/entry into circulation, (c) survival in circulation, (d) extravasation from blood vessels/exit from circulation, and (e) establishment of a new tumor mass in secondary organs (reviewed in refs. 40, 41). Although microvascular density was diminished by ephrin-A1 siRNA knockdown, primary tumor volume was not affected, nor was invasiveness in three-dimensional cultures, suggesting that ephrin-A1 is dispensable for tumor cell growth/survival and local invasion. As no differences in the ability of tumor cells to migrate in response to microvascular endothelial cells were detected between control and siRNA knockdown cells, our data do not support a function for ephrin-A1 in intravasation. In addition, both control and siRNA knockdown cells were able to form secondary lung lesions when injected i.v., suggesting that ephrin-A1 is not required for survival in circulation, extravasation, or formation of lung colonies. Given the modulation of microvascular density in vivo upon up- or down-regulation of ephrin-A1 expression levels, as well as the effects on tumor cell–induced endothelial cell migration in vivo, the effect of ephrin-A1 knockdown on metastasis seems to be angiogenesis dependent.

Mechanistically, our data support a model in which ephrin-A1 ligand serves as a proangiogenic factor through at least two distinct mechanisms: (a) activating EphA2 receptor on host blood vessel endothelial cells, and (b) up-regulating VEGF expression by tumor cells and subsequent activation of endothelial cells. To support the first hypothesis, we and others have previously shown that EphA2 receptor tyrosine kinase modulates ephrin-A1-Fc–induced angiogenic remodeling in vitro and in vivo (15–17, 20, 34). More specifically, EphA2-deficient mouse display defects in s.c. or mammary gland vessel remodeling in response to soluble ephrin-A1-Fc– or

Figure 2. Diminished ephrin-A1 expression in 4T1 tumor cells expressing ephrin-A1 siRNAs. A, 4T1 mammary adenocarcinoma cells were transduced with retroviruses expressing control or two independent ephrin-A1 siRNA constructs and pools were selected by puromycin treatment. Relative to parental and control siRNA clones, cells expressing ephrin-A1 siRNAs (siRNA#1 or siRNA#2) displayed significantly lower levels of ephrin-A1 expression. Knockdown was specific, as ephrin-A5 levels were unaltered. B, ephrin-A1 knockdown persists in vivo up to 3 weeks posttransplantation. Sections from tumors collected 3 weeks posttransplantation were stained for expression of ephrin-A1. Significantly less ephrin-A1 expression was observed in tumors expressing ephrin-A1 siRNA 1 or 2 than in parental or control siRNA tumors. Bar, 50 μm.
ephrin-A1–expressing 4T1 mammary tumors, respectively (19, 20). Endothelial cells isolated from these animals also display defective assembly and migration in response to ephrin-A1-Fc or 4T1 tumor cells in vitro (19, 20), suggesting that ephrin-A1–mediated angiogenic responses require EphA2 receptor function. In this report, we observed that overexpression of ephrin-A1 in nonmetastatic cells elevated wild-type but not EphA2-deficient endothelial cell migration toward tumor cells, providing further support for EphA2 receptor in ephrin-A1–induced tumor angiogenesis.

We previously reported that VEGF stimulation of endothelial cells leads to up-regulated expression of ephrin-A1 within the endothelium (17, 34), and blockade of EphA-class receptor activation can inhibit several VEGF-mediated angiogenic processes, including migration, assembly, sprouting, survival, and corneal/retinal angiogenesis (34, 42). These data suggest that autocrine ephrin-A1 plays an important role in angiogenic responses of endothelial cells to proangiogenic signals. In addition, recent analysis of a murine model of skin hypoxia showed that VEGF, EphA2, and ephrin-A1 are all up-regulated in response to hypoxia and therefore could be subject to the same regulation and cooperate in facilitating angiogenesis in vivo (43). Our current study now suggests that ephrin-A1 expression by tumor cells can regulate expression of VEGF, presumably through interaction with EphA receptors on tumor cells. Up-regulated VEGF in tumor cells

**Figure 3.** Diminished tumor microvascular density and lung metastasis in 4T1 tumor cells expressing ephrin-A1 siRNAs. Tumor cell lines were orthotopically transplanted into the mammary fat pad of BALB/c female mice and lysates from tumors collected 3 weeks after transplantation were analyzed for tumor volume, microvascular density, and lung metastasis. A, no change in tumor volume was detected between parental and control siRNA tumors versus ephrin-A1 siRNAs 1 and 2. B, microvascular density was assessed by staining sections for CD31 expression. Arrows, CD31+ tumor blood vessels. A significant decrease in microvascular density was observed in siRNA tumors relative to parental 4T1 or 4T1 expressing control siRNAs. *, P < 0.05, Bar, 100 μm; n = 15 independent tumors per condition. C, metastases were enumerated in cleared, hematoxylin-stained lungs. Arrowheads, surface lung lesions. A significant decrease in the number of lung lesions was observed for mice receiving siRNA tumors relative to mice receiving parental or control siRNA tumors: **, P < 0.05; n = 10 independent lungs per condition.
by an ephrin-A1–mediated mechanism may explain how ephrin-A1, a membrane-tethered factor, is able to regulate angiogenic responses in initially distant host endothelium. Ephrin-A1–induced VEGF may initiate proliferation and sprouting of new branches from distant host vessels and thus promote angiogenesis indirectly. Upon recruitment and direct contact with tumor cells, as mimicked by in transwell coculture assays in which tumor cells and endothelial cells are separated only by a thin, 8 μm porous membrane, endothelial cells may be stimulated by ephrin-A1 directly to guide endothelial migration within the tumor. This model is supported by our observation that inhibition of VEGF function only affects migration of endothelial cells in response to tumor cells that are far removed (e.g., at the bottom of the well) rather than in close proximity (e.g., on the lower surface of the transwell). Presumably, tumor cells that are in close proximity to endothelial cells can initiate signaling cascades through direct

Figure 4. Diminished tumor-induced endothelial cell migration, but not intrinsic malignancy, in 4T1 tumor cells expressing ephrin-A1 siRNAs. A, endothelial cell migration in response to 4T1 tumor cells was assessed in modified transwell coculture assays as depicted in the diagram. Tumor cells were seeded on the lower surface of transwells and labeled with a green fluorescent marker. Endothelial cells that migrated and intercalated into the tumor cell layer after 5 hours were photographed for quantification of tumor-induced endothelial cell migration. A significant decrease in endothelial cell migration was detected in ephrin-A1 siRNA cocultures relative to parental or control siRNA cocultures. *, \(P < 0.05\). Arrowheads, endothelial cells (red) that migrated in response to tumor cells (green). Bar, 50 μm. B, tumor cell migration in response to endothelial cells in modified transwell intravasation assays as depicted in the diagram. Tumor cell (green) migration and intercalation in response to endothelial cells (red) seeded on the lower surface of transwells was scored as described above. No significant change in migration was detected between ephrin-A1 siRNA-expressing tumor cells and parental or control siRNA-expressing tumor cells. Arrows, tumor cells (green) that migrated in response to endothelial cells (red); \(n = 9\) independent cocultures from three experiments. C, parental, control siRNA, or ephrin-A1 siRNA–expressing 4T1 clones were plated at low density on Matrigel to induce three-dimensional spheroid formation. No changes in morphology or malignancy, as assessed by protrusion from the primary cell mass, were detected between control and ephrin-A1 siRNA cultures 10 days after plating; \(n = 8\) independent cultures from two experiments. Bar, 50 μm. D, parental, control siRNA, or ephrin-A1–expressing 4T1 clones were injected i.v. into BALB/c female mice and lung lesions were enumerated 12 days postinjection. No significant difference in the number of lung lesions was observed between control and ephrin-A1 siRNA–expressing cells; *, \(P = 0.85\); \(n = 9\) independent mice per condition from two experiments.
interaction of membrane-tethered ephrin-A1 with EphA receptors expressed on the surface of endothelial cells. The lack of migratory response to elevated ephrin-A1 levels by EphA2-deficient endothelial cells supports this model.

It is interesting to note that siRNA-mediated ephrin-A1 knockdown in malignant tumor cells inhibited tumor angiogenesis without affecting primary tumor volume. These results are distinct from studies resulted from soluble EphA-Fc receptor treatment or host-EphA2 deficiency (16, 18, 19). In these studies, primary tumor volume, growth, and/or survival were also significantly diminished by blockade of EphA2 receptor activation in tumors/host tissue by soluble receptor (16–19). This likely reflects the importance of the tumor cell intrinsic function of EphA2 in promoting malignancy, which contributes to cellular transformation and metastasis in a kinase-dependent manner (30, 44, 45).

Soluble receptor could block EphA receptor tyrosine kinase function both in vascular endothelial cells and within tumor cells, affecting both primary tumor growth as well as tumor metastasis. In the case of host-EphA2 deficiency, loss of EphA2 receptor in other components of the tumor stroma may contribute to more severe phenotype (19). Although we observed no change in tumor size upon overexpression or underexpression of ephrin-A1, we still observed a significant effect on tumor-induced endothelial cell migration. A significant increase in endothelial cell migration was detected in Ad-ephrin-A1 cocultures relative to untransduced or control Ad-β-gal cocultures. *, P < 0.05. Arrows, endothelial cells (red) that migrated in response to tumor cells (green). Bar, 50 μm. n = 9 independent cocultures from three experiments.

B, 67NR cells were transduced with adenoviruses in culture and orthotopically transplanted into the mammary fat pad of BALB/c female mice and tumors collected 2 weeks after transplantation were analyzed for microvascular density by staining tumor sections for vWF expression. Arrows, vWF+ tumor blood vessels (red). Sections were counterstained with DAPI (blue) to visualize nuclei. A significant increase in microvascular density was observed in Ad-ephrin-A1 tumors relative to parental 67NR or 67NR-expressing control Ad-β-gal. *, P < 0.05. Bar, 100 μm. C, no significant change in tumor volume was detected between parental and Ad-β-gal-expressing 67NR tumors or Ad-ephrin-A1-overexpressing 67NR tumors. n = 9 to 10 independent tumors per condition.
Our data argue for caution in the use of ephrin-A1-Fc proteins as therapeutic agents, as has been suggested by at least two recent studies (46, 47). These studies report that ligation of tumor cell–expressed EphA2 receptor by soluble ligands can target EphA2 for increased turnover and proteosomal degradation, thus inhibiting tumor growth and invasion mediated by EphA2. However, our data support a positive role for membrane-bound ephrin-A1 in vascular recruitment, similar to previously reported proangiogenic

Figure 6. Modulation of ephrin-A1 affects VEGF expression, which is required for tumor-induced endothelial cell migration in vitro. A, sections from tumors collected 3 weeks posttransplantation were stained for expression of VEGF (brown) and counterstained hematoxylin (blue) to visualize nuclei. Significantly less VEGF expression was observed in tumors expressing eprhin-A1 siRNAs 1 and 2 than in parental or control siRNA tumors. Bar, 100 μm. B, expression of VEGF by cultured tumor cells was quantified by ELISA assay. Tumor cells were plated in six-well dishes and conditioned medium was collected after 48-hour incubation. Levels of VEGF present in conditioned medium relative to cell number upon collection were scored. A significant reduction in VEGF levels was observed in siRNA 1 and siRNA 2 clones relative to parental or control siRNA 4T1 cells. *, P < 0.05. A significant increase in VEGF levels was observed in 67NR cells expressing Ad-ephrin-A1 relative to control cells expressing Ad-β-gal. **, P < 0.05. Data are consolidated from three independent experiments. C, neutralizing anti-VEGF antibodies, but not control IgG, can inhibit migration of endothelial cells induced by Ad-ephrin-A1–overexpressing 67NR conditioned medium. Coculture migration assays were done in which 67NR tumor cells were plated in 24-well dishes and incubated 24 hours to generate conditioned medium. Migration of endothelial cells through a transwell membrane in response to this medium was assessed as depicted in the diagram. Relative to control Ad-β-gal–expressing 67NR, Ad-ephrin-A1–expressing 67NR medium containing control IgG induced a significantly elevated endothelial cell migration response. ***, P < 0.05. This response was inhibited in Ad-ephrin-A1–expressing 67NR medium containing anti-VEGF neutralizing antibodies. ****, P < 0.05. D, coculture migration assays were done in which 67NR tumor cells were plated on the lower surface of transwells and incubated for 24 hours to generate conditioned medium. Migration of endothelial cells through the transwell membrane in response to cells/medium was assessed as depicted in the diagram. Neutralizing VEGF antibodies did not affect migration in response to Ad-ephrin-A1–expressing 67NR medium containing anti-VEGF neutralizing antibodies. ***, P < 0.05. E, migration of MPMECs isolated from EphA2-deficient mice (EphA2−/− MPMEC) was significantly diminished relative to wild-type endothelial cells in response to Ad-ephrin-A1–overexpressing 67NR. ***, P < 0.05. Representative of three independent experiments.
functions of ephrin-A1-Fc (reviewed in refs. 9, 11). This suggests that ephrin-A1-Fc, in addition to induction of EphA2 degradation within tumor cells, may also elicit an angiogenic response from the surrounding host tissue and thereby facilitate tumor progression. Further investigation into the role of ephrin-A1 in angiogenesis versus EphA2 receptor turnover in tumor cells is required before clinical development.

In summary, our results support a role for ephrin-A1 in promoting tumor angiogenesis in mammary adenocarcinoma, thus providing a permissive environment for tumor growth/survival and progression toward metastasis. Ephrin-A1 likely promotes angiogenesis through activation of EphA2 receptor tyrosine kinase on host endothelium to facilitate tumor-induced endothelial cell migration. In addition, ephrin-A1 also regulates VEGF production by tumor cells, providing an additional mechanism to promote vascular recruitment. These data suggest

that targeting the ephrin-A1/EphA2 pathway, which likely promotes tumor progression through both host- and tumor-dependent mechanisms simultaneously, could provide a new avenue for treatment of breast cancer.

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