Host Deficiency in Vav2/3 Guanine Nucleotide Exchange Factors Impairs Tumor Growth, Survival, and Angiogenesis In vivo

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Abstract
Vav guanine nucleotide exchange factors modulate changes in cytoskeletal organization through activation of Rho, Rac, and Cdc42 small GTPases. Although Vav1 expression is restricted to the immune system, Vav2 and Vav3 are expressed in several tissues, including highly vascularized organs. Here, we provide the first evidence that Vav2 and Vav3 function within the tumor microenvironment to promote tumor growth, survival, and neovascularization. Host Vav2/3 deficiency reduced microvascular density, as well as tumor growth and/or survival, in transplanted B16 melanoma and Lewis lung carcinoma models in vivo. These defects were due in part to Vav2/3 deficiency in endothelial cells. Vav2/3-deficient endothelial cells displayed reduced migration in response to tumor cells in coculture migration assays, and failed to incorporate into tumor vessels and enhance tumor volume in tumor-endothelial cotransplantation experiments. These data suggest that Vav2/3 guanine nucleotide exchange factors play a critical role in host-mediated tumor progression and angiogenesis, particularly in tumor endothelium. (Mol Cancer Res 2009;7(5):615–23)

Introduction
Recruitment of new blood vessels by tumors, primarily through angiogenesis, is crucial for sustained growth, survival, and metastatic dissemination (reviewed in refs. 1–3). Tumor neovascularization facilitates tumor survival, growth, and malignant progression through delivery of oxygen and host nutrients. The process of angiogenesis, or sprouting of new blood vessel branches from preexisting vasculature, is a complex, multistep process that includes (a) endothelial cell activation by factors secreted by tumor cells; (b) degradation of the basement membrane and extracellular matrix by proteases; (c) proliferation, invasion, and migration of the polarized endothelial cells; (d) coalescence and lumen formation; and (e) recruitment of perivascular support cells and production of extracellular matrix for stability of the new vessel (reviewed in refs. 3, 4). High-level expression of proangiogenic factors and/or elevated microvascular density have been correlated with malignant progression and a poor prognosis in patients suffering from several types of cancer, including lung cancer (5-10) and melanoma (11-14). Thus, understanding the molecular mechanisms that regulate tumor angiogenesis will enhance our efforts to target this process in the treatment of cancer.

Endothelial cell migration in response to tumor-derived signals is a key component of angiogenesis. Rho family GTPases, including Rho, Rac, and Cdc42, are critical mediators of cellular migration, and have been shown to regulate migration and morphogenesis of cultured endothelial cells (reviewed in refs. 15, 16). Rac, along with Cdc42, regulates endothelial morphogenesis and assembly (17, 18), whereas Rac alone was shown to regulate endothelial assembly and lumen formation in endothelial cells stimulated with vascular endothelial growth factor (VEGF; refs. 19, 20). In addition to VEGF, ephrin-A1 regulates Rac-mediated endothelial cell migration. Ephrins, membrane-tethered ligands for the Eph family of receptor tyrosine kinases, regulate angiogenesis during embryonic development, as well as in normal and diseases adult tissues (reviewed in refs. 21-24). In particular, ephrin-A1 and EphA2 have been linked with postnatal angiogenic remodeling and tumor angiogenesis (reviewed in refs. 21-24). We recently showed that EphA2-deficient mice display significantly reduced angiogenic remodeling upon stimulation with ephrin-A1 (25), as well as reduced microvascular density upon transplantation of ephrin-A1–expressing tumor cells (26, 27). In addition, modulating ephrin-A1 expression in tumor cells affects tumor microvascular density in vivo. Overexpression of ephrin-A1 in mammary adenocarcinoma cells enhanced, whereas siRNA-mediated down-regulation of ephrin-A1 diminished, microvascular density in vivo and tumor-induced endothelial cell migration in vitro (28). Ephrin-A1 stimulates Rac and Cdc42 activation in cultured endothelial cells in an EphA2 receptor–dependent manner (25). Moreover, inhibition of Rac function reduces ephrin-A1–induced endothelial cell migration in vitro (25). Taken together, these data suggest that ephrin-A1/EphA2 activation of Rac is a major mechanism by which this ligand/receptor pair mediates vascular remodeling.

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Rho family GTPases are molecular switches that cycle between an inactive, GDP-bound state and an active, GTP-bound conformation. In response to extracellular stimuli, guanine nucleotide exchange factors (GEF) catalyze the exchange of GDP for GTP to activate the GTPase. GTPases may be inactivated by guanine nucleotide dissociation inhibitors that likely block spontaneous activation, or by GTPase activating proteins that stimulate GTPase activity (reviewed in refs. 29, 30). GEFs serve as links between extracellular stimulation, particularly by receptor tyrosine kinases, and Rho family GTPase activity (reviewed in ref. 31). The Vav family of GEFs, including Vav1, Vav2, and Vav3, are known regulators of GDP-GTP exchange for Rho family GTPases. We recently showed that Vav2 and Vav3 physically associate with EphA2 receptor tyrosine kinase, linking EphA2 with Rac1 activation and endothelial cell assembly and migration *in vitro* (32). Moreover, Vav2/3-deficient mice display a greatly diminished angiogenic remodeling response to ephrin-A1 stimulation *in vivo* (32). The role of Vav2 and Vav3 GEFs in tumor angiogenesis, however, remains unclear. Here, we show that host Vav2/3 deficiency reduces microvascular density and tumor cell growth or survival in two independent mouse tumor models. These defects are, at least in part, intrinsic to loss of Vav2/3 in microvascular endothelium, as primary microvascular endothelial cells isolated from Vav2/3-deficient animals displayed defective tumor cell–induced migration in coculture assays *in vitro*. In addition, Vav2/3-deficient endothelial cells failed to incorporate into tumor vessels when cotransplanted with tumor cells *in vivo*. Taken together, these data suggest that Vav2 and Vav3 GEFs are required for tumor neovascularization, providing insight into the molecular mechanisms that regulate tumor angiogenesis that may be applied to the development of new antiangiogenic therapies in cancer treatment.

**Results**

**Host Vav2/3 Deficiency Impairs Tumor Growth and/or Survival and Microvascular Density In vivo**

To determine the effect of host Vav2/3 deficiency on tumor progression *in vivo*, we orthotopically transplanted Lewis lung carcinoma (LLC) cells into Vav2/3-deficient and wild-type control animals. Ten days after transplantation, we observed that average lung weights, as a measure of tumor burden, were significantly lower in tissue isolated from Vav2/3-deficient hosts versus wild-type controls (Fig. 1A), whereas there was no difference in normal lung weights in nontumor-bearing Vav2/3-deficient mice versus wild-type controls (data not shown). H&E-stained sections confirmed engraftment of orthotopically transplanted tumor cells into the lung tissue of recipient mice (Fig. 1A). Although we detected no significant change in tumor cell proliferation (Fig. 1B), apoptosis was significantly elevated in lung tumors derived from Vav2/3-deficient versus wild-type animals (Fig. 1C). In addition, there was a significant decrease in microvascular density in tumors from Vav2/3-deficient mice versus wild-type controls (Fig. 1D). These results are consistent with other studies involving antiangiogenic therapy in orthotopic LLC tumors, in which increased apoptosis, but not proliferation, was observed (33), suggesting that these conditions favor apoptotic rather than growth defects.

To confirm the effect of host Vav2/3 deficiency on tumor progression, we also assessed tumor volume, growth, survival, and vascularity in an independent allograft model. We injected B16 melanoma cells s.c. into Vav2/3-deficient and wild-type control animals. Two weeks after transplantation, we observed that tumor volume was significantly lower in tumors isolated from Vav2/3-deficient hosts relative to wild-type controls (Fig. 2A). In this model, there was a significant decrease in peripheral tumor cell proliferation (Fig. 2B) but no change in tumor cell apoptosis (Fig. 2C) in tumors derived from Vav2/3-deficient animals versus controls. Consistent with data from LLC tumors, we also observed a significant decrease in microvascular density in tumors isolated from Vav2/3-deficient mice relative to wild-type animals (Fig. 2D). These data suggest that host expression of Vav2/3 promotes tumor growth and/or survival, as well as neovascularization, *in vivo*.

**Vav2/3 Function Is Required for Endothelial Cell Migration in Response to Tumor Cells**

We observed reduced microvascular density in both LLC and B16 tumors in Vav2/3-deficient hosts, suggesting that Vav2/3 deficiency in host endothelium might affect tumor angiogenesis. We did coculture migration assays (Schematic; Fig. 3A) to assess tumor cell–induced migration of primary endothelial cells derived from mouse lung (25, 27) for Vav2/3-deficient (32) versus control wild-type C57BL/6 animals. Lung microvascular endothelial cells isolated from wild-type or Vav2/3-deficient animals exhibited cobblestone-like morphology (Fig. 4A) and over 95% of cells were CD31 positive (data not shown), confirming the endothelial character of these cells. Vav2 and Vav3 protein deficiency in these animals was confirmed by immunoblot of whole spleen extracts harvested at the time of endothelial cell isolation (Fig. 3A). Vav2/3-deficient endothelial cells displayed significantly reduced migration in response to LLC and B16 melanoma tumor cells (Fig. 3B), suggesting that the defects in tumor neovascularization are, at least in part, due to Vav2/3 deficiency in endothelium. We have previously shown that Vav2 mediates EphA2 receptor signaling in vascular endothelial cells (32). Consistent with our prior studies, both LLC and B16 tumor cells express ephrin-A1 ligand, and EphA2-deficient endothelial cells are defective in migration toward B16 melanoma cells (Fig. 3C; ref. 34). These observations indicate that Vav2/3 GEFs also likely act downstream of EphA2 receptor in response to tumor cell–derived signals.

**Vav2/3-Deficient Endothelial Cells Fail to Incorporate into Tumor Vasculature and Support Tumor Progression In vivo**

To determine if the defects in microvascular density and tumor progression in Vav2/3-deficient hosts are intrinsic to endothelial cells, we did tumor cell/endothelial cell cotransplantation experiments using primary microvascular endothelial cells derived from Vav2/3-deficient or wild-type control animals. B16 melanoma cells were mixed with wild-type or Vav2/3-deficient primary endothelial cells, resuspended in growth factor–reduced Matrigel, and injected into the subcutaneous dorsal flank of nude female mice as described previously (27). To distinguish donor versus host endothelial cells, donor endothelial cells were transduced with adenoviruses harboring β-galactosidase before cotransplantation. Within the time frame of adenovirus
transduction, over 95% of Vav2/3-deficient or wild-type control endothelial cells stained blue by X-gal, suggesting that transduction and expression of LacZ gene are equivalent in both cell populations (Fig. 4A). We observed that cotransplantation with wild-type endothelial cells significantly enhanced tumor volume relative to transplantation of B16 melanoma cells alone after 10 days (Fig. 4B). By contrast, cotransplantation with Vav2/3-deficient endothelial cells produced smaller tumor with significantly lower average tumor volume relative to cotransplantation with wild-type control cells (Fig. 4B). We observed fewer exogenous, LacZ+ endothelial cells in sections prepared from tumors harboring Vav2/3-deficient host animals relative to wild-type hosts (arrowheads, TUNEL+ nuclei; P < 0.05). Microvascular density, as scored by manual counting vWF+ vessels in tumor sections, was reduced in tumors isolated from Vav2/3-deficient host animals relative to controls (arrows, vWF+ vessels; P < 0.05). Right, reduced microvascular density in tumors isolated from Vav2/3-deficient host animals was confirmed by quantification of vWF+ pixel area in tumor sections.

**Discussion**

Investigation of signal transduction pathways through which Eph receptor tyrosine kinase and ephrins mediate such diverse
biological processes as embryonic patterning, axon guidance, embryonic vascular remodeling, tumor neovascularization, and malignant transformation increasingly center on Rho family GTPases. (reviewed in refs. 24, 35-38). More specifically, interactions between Eph receptor and GEFs such as ephexin (39), Tiam1 (40), kalirin (41), and vascular smooth muscle Rho-GEF (42) are critical for proper neuronal patterning and vascular remodeling. We and others previously reported a novel interaction between Eph receptor tyrosine kinases and Vav GEFs (32, 43). Here, we show for the first time that Vav2/3 GEFs are required in host tissue, specifically vascular endothelium, for maximal tumor neovascularization in vivo. Host Vav2/3 deficiency resulted in decreased microvascular density in two independent tumor allograft models in vivo, which correlated with decreased tumor cell survival and/or growth relative to allografts from wild-type control animals (Figs. 1 and 2). Primary microvascular endothelial cells derived from Vav2/3-deficient animals displayed impaired migration in response to LLC and B16 melanoma tumor cells in coculture assays (Fig. 3). Moreover, Vav2/3-deficient endothelial cells did not enhance tumor volume as robustly or incorporate into tumor vessels as efficiently as wild-type control cells when cotransplanted with B16 melanoma cells in vivo (Fig. 4). These data suggest that Vav2 and Vav3 GEFs are key mediators of tumor neovascularization in vivo.

Our previous studies showed that EphA2 receptor tyrosine kinase function in host endothelium is also necessary for tumor-induced endothelial cell migration in vitro and tumor angiogenesis in vivo (27). Moreover, ephrin-A1 expression in tumor cells facilitates tumor angiogenesis in vivo (28). Ephrin-A1 promotes migration and assembly of cultured endothelial cells through activation of Rac1 GTPase (25), and activation of Rac1 by ephrin-A1 was dependent on EphA2 receptor tyrosine kinase expression, as well as on phosphatidylinositol 3-kinase activity (25). More recently, we observed that Vav2 and Vav3 GEFs physically interact with the intracellular domain of EphA2 receptor (32). Rac1 activation in response to ephrin-A1 was impaired significantly in Vav2/3-deficient primary endothelial cells, as was ephrin-A1 induced assembly into interconnected vascular networks in vitro. These data, coupled with the observation that ephrin-A1 failed to stimulate a subcutaneous angiogenic remodeling response in Vav2/3-deficient mice, suggested that Vav2 and Vav3 GEFs are key mediators of ephrin-A1–induced angiogenesis. As both LLC and B16 melanoma cells express high levels of ephrin-A1, data presented in this study provide a correlation between Vav2/3 GEFs to ephrin-mediated tumor neovascularization.

As ephrins are membrane tethered, however, the mechanism(s) by which these tumor-expressed ligands could communicate...
with EphA2 receptor on initially distant host endothelium remained unclear. This may be explained by evidence suggesting cooperation between ephrin-A1/EphA2 receptor signaling and the VEGF pathway. We previously reported that EphA2 deficiency inhibits endothelial cell migration and assembly not only in response to ephrin-A1 but also upon stimulation with VEGF (44). Moreover, inhibition of class A Eph receptor signaling blocks corneal neovascularization in response to both ephrin-A1 and VEGF (45). More recently, we reported that down-regulation of ephrin-A1 ligand expression in mouse mammary adenocarcinoma cells also reduced expression of VEGF, and that neutralizing anti-VEGF antibodies inhibited migration of endothelial cells in response to tumor cells over-expressing ephrin-A1 (28). Interestingly, VEGF-induced Rac activation is also dependent on Vav2 function (46, 47). Moreover, down-regulation of Vav2 expression in endothelial cells by siRNA inhibited VEGF-induced migration in vitro (47). Taken together, these data suggest that Vav GEFs may represent a point of convergence between ephrin and VEGF pathways in promoting angiogenic remodeling. We assessed migration and assembly of EphA2-deficient endothelial cells reconstituted with a mutant receptor (Y587/593EE) that displays impaired binding to Vav2 and Vav3 proteins (48). Although VEGF-mediated migration and assembly were impaired in EphA2-deficient control endothelial cells, consistent with previous observations (44), EphA2.Y587/593EE-expressing cells displayed levels of migration and assembly comparable with levels observed in wild-type endothelial cells (data not shown). Moreover, expression of Vav2 in EphA2-deficient endothelial cells did not rescue migration and assembly in response to VEGF (data not shown). These data suggest that VEGF does not directly stimulate EphA2 receptor signaling.

FIGURE 3. Vav2/3-deficient endothelial cells display reduced migration in response to LLC and B16 tumor cells. A. Schematic description of coculture migration assay. Tumor cells harboring a green fluorescent dye are seeded on the lower surface of a transwell membrane and allowed to attach. Primary endothelial cells harboring a red fluorescent dye are placed in the top chamber and migration in response to tumor cells on the lower surface is measured after 5 h. Loss of Vav2/3 protein expression in Vav2/3-deficient (KO) donor animals versus control (WT) mice was confirmed by immunoblot analysis of whole spleen extracts harvested at the time of primary endothelial cell isolation. B. Primary lung microvascular endothelial cells isolated from Vav2/3-deficient mice displayed significantly reduced migration in response to LLC and B16 melanoma tumor cells relative to endothelial cells isolated from wild-type control mice (arrowheads, endothelial cells that migrated and intercalated into tumor cell monolayer on the lower surface of the chamber). C. We confirmed ephrin-A1 expression in B16 and LLC tumor cells by immunoblot analysis. Specificity of the antibody was confirmed by probing lysates from ephrin-A1–deficient cardiac tissue (ephrin-A1 KO). In coculture migration assays, EphA2-deficient endothelial cells (EphA2 KO) significantly reduced migration in response to B16 melanoma cells plated on the lower surface of transwells relative to wild-type control endothelial cells, suggesting that ephrin-A1 actively promotes EphA2-dependent chemotaxis in this tumor cell model.
Given that VEGF treatment up-regulates ephrin-A1 expression in endothelial cells (45), we hypothesize that cooperative signaling between VEGF and ephrins in parallel mediates angiogenic remodeling that is dependent on Vav GEF function. Further investigation is required to investigate this hypothesis in the context of tumor angiogenesis, particularly as many tumors co-express ephrin-A1 and VEGF. Other investigators have reported that inhibition of EphA class signaling by soluble EphA2-Fc impairs angiogenic remodeling in response to basic fibroblast growth factor (ref. 49). Thus, Eph/ephrin signaling may modulate chemotaxis in response to other cytokines, such as fibroblast growth factor 2, in tumor angiogenesis.

In summary, we provide the first evidence that Vav2/3 GEFs are required for maximal tumor neovascularization in vivo. Our study provides insight into the molecular mechanisms that regulate tumor angiogenesis that may be applied to the development of new antiangiogenic therapies in cancer treatment.

Materials and Methods

Reagents

Antibodies used include anti-Vav2 and anti-Vav3 (rabbit polyclonal produced in collaboration with Zymed Laboratories), anti-ephrin-A1 (Zymed Laboratories), anti-EphA2 (Santa Cruz Biotechnology), anti-actin (Santa Cruz Biotechnology), anti--von Willebrand factor (vWF; Dako), biotinylated anti--proliferating cell nuclear antigen (PCNA; BD Biosciences), anti--β-galactosidase (Millipore/Chemicon), anti-Rac1 antibody (BD Biosciences), and anti-CD31 (BD Biosciences). Growth factor--reduced Matrigel was purchased from BD Biosciences. Apoptag Red In Situ
Apoptosis detection kit was purchased from Millipore/Chemicon. Avidin-peroxidase reagents were from Vector Laboratories, and 3,3′-diaminobenzidine tetrahydrochloride substrate kit was from Zymed Laboratories. We purchased CellTracker Green CMFDA and Orange CMTMR dyes from Invitrogen/Molecular Probes. Costar transwells (8 μm polycarbonate membrane, 6.5 mm inserts) were obtained from Corning, Inc. Adenoviruses harboring control β-galactosidase were described previously (50). X-gal was purchased from Research Products International Corporation. We purchased ephrin-A1-Fc from R&D Systems.

Animal Studies
All animals were housed under pathogen-free conditions, and 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. Vav2/3-deficient animals were generously provided by Dr. Wojciech Swat (Washington University School of Medicine, St. Louis, MO; ref. 51). Athymic nude-Foxn1 mice (ages 3–4 wk) were purchased from Harlan Sprague-Dawley, Inc.

Cell Culture
B16 melanoma, LLC, and 4T1 mouse mammary adenocarcinoma cells were purchased from the American Type Culture Collection and maintained in DMEM (Mediatech) supplemented with 10% fetal bovine serum (Hyclone), 2 mmol/L L-glutamine, and 5 U/mL penicillin-streptomycin (Mediatech). Primary lung microvascular endothelial cells were isolated from wild-type C57BL/6J mice or syngeneic Vav2/3-deficient mice (51) as described previously (27, 32) and maintained in EGM-2 medium (Lonza) on tissue culture plates coated with 0.1% gelatin (Sigma-Aldrich) solution in PBS. We confirmed that ~98% of cells expressed CD31 by immunofluorescence staining, indicating that the majority of the cells that we isolated are endothelial cells (data not shown). We confirmed loss of Vav2 and Vav3 protein expression in Vav2/3-deficient animals from which primary endothelial cells were derived, as well as intact expression of Vav2/3 in wild-type animals, by probing whole spleen lysates for expression of Vav2 and Vav3.

Tumor Allografts
LLCs (2.5 × 10^5) were resuspended in 100 μL growth factor-reduced Matrigel. The Matrigel mixture was injected orthotopically into the left lateral thorax of syngeneic Vav2/3-deficient or wild-type control animals at the lateral dorsal axillary line to establish lung tumors as described by Wu et al. (52). Lungs were harvested 10 d postinjection and weighed as a measure of tumor burden. Data were derived from analysis of seven to eight independent allografts/condition from two independent experiments. B16 melanoma cells (5 × 10^5) were resuspended in 100 μL PBS, and injected s.c. into the dorsal flank of Vav2/3-deficient or wild-type control animals. Tumors were harvested 2 wk postinjection and dimensions measured by a digital caliper. Tumor volume was calculated using the following formula: volume = length × width^2 × 0.52 (53). Data were derived from analysis of 10 independent allografts/condition from 2 independent experiments.

Tumor Cell–Endothelial Cell Cotransplantation Assays
Primary lung microvascular endothelial cells isolated from Vav2/3-deficient and wild-type control mice were transduced with 10^5 plaque-forming unit/mL adenovirus β-galactosidase 48 h before cotransplantation. Cotransplantation experiments were done as previously described (27). Briefly, endothelial cells (5 × 10^5) plus B16 melanoma cells (1 × 10^6) were suspended in 250 μL growth factor-reduced Matrigel and injected into the subcutaneous dorsal flanks of 5-wk-old athymic nude-Foxn1 mice. Tumors were collected 10 d posttransplantation and tumor volume assessed as described above. Data are a representation of 10 independent tumors/condition from 2 independent experiments.

Histology
LLC and B16 tumors were fixed in 10% neutral buffered formalin (Fisher Scientific) overnight at 4°C and processed for paraffin embedding. For analysis of microvascular density, sections were stained with anti-vWF antibodies followed by anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories) secondary as described previously (27, 28). The sections were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich) to visualize nuclei, and microvascular density was measured in 40× fields photographed using an Olympus BX60 microscope plus digital camera. Microvascular density was quantified by measuring fluorescent pixel area using NIH Image J software as described previously (27, 28), as well as by manual counting of vWF+ vessels. For analysis of proliferation, sections were treated with biotinylated anti-PCNA antibody followed by avidinperoxidase and 3,3′-diaminobenzidine tetrahydrochloride substrate. The sections were counterstained with hematoxylin (Fisher Scientific) to visualize nuclei, and proliferation was assessed by calculating the average percent PCNA+ nuclei relative to the total number of nuclei in 40× fields as described previously (26). For analysis of apoptosis, sections were processed for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay using the Apoptag Red In Situ Apoptosis detection kit as per manufacturer’s instructions. Sections were counterstained with 4,6-diamidino-2-phenylindole to visualize nuclei, and apoptosis was assessed by calculating the average percent TUNEL+ nuclei relative to the total number of nuclei in 40× fields as described previously (26). For all quantifications, 4 to 6 random 40× fields were photographed in 3 to 5 independent tumor sections/group.

Tumors harboring exogenous endothelial cells were isolated from nude mice and processed for histology as previously described (25, 27). Cryosections (10 μm) were processed for Xgal staining (25, 27). Sections were counterstained with eosin. LacZ-positive, exogenous endothelial cells were counted in four independent 20× fields/section in 5 independent tumors/condition to quantify incorporation of exogenous endothelial cells into the tumor mass. Tumor sections were also costained with X-gal and an anti-CD31 antibody (BD Biosciences) or anti-vWF antibody (Dako) as described previously (25, 27).

Endothelial Coculture Migration
Coculture migration assays were done as described previously (28, 54). Migrating cells were photographed and migration scored by counting the number of tumor cells that migrated
and intercalated into the tumor cell monolayer on the lower surface of the transwell after 5 h of incubation. Data are a representation of nine independent samples per condition from three independent experiments.

Disclosure of Potential Conflicts of Interest

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