

Cooperative Signaling between Slit2 and Ephrin-A1 Regulates a Balance between Angiogenesis and Angiostasis^{∇†}

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Received 9 June 2010/Returned for modification 29 July 2010/Accepted 20 November 2010

Slit proteins induce cytoskeletal remodeling through interaction with roundabout (Robo) receptors, regulating migration of neurons and nonneuronal cells, including leukocytes, tumor cells, and endothelium. The role of Slit2 in vascular remodeling, however, remains controversial, with reports of both pro- and antiangiogenic activity. We report here that cooperation between Slit2 and ephrin-A1 regulates a balance between the pro- and antiangiogenic functions of Slit2. While Slit2 promotes angiogenesis in culture and *in vivo* as a single agent, Slit2 potently inhibits angiogenic remodeling in the presence of ephrin-A1. Slit2 stimulates angiogenesis through mTORC2-dependent activation of Akt and Rac GTPase, the activities of which are inhibited in the presence of ephrin-A1. Activated Rac or Akt partially rescues vascular assembly and motility in costimulated endothelium. Taken together, these data suggest that Slit2 differentially regulates angiogenesis in the context of ephrin-A1, providing a plausible mechanism for the pro- versus antiangiogenic functions of Slit2. Our results suggest that the complex roles of Slit-Robo signaling in angiogenesis involve context-dependent mechanisms.

Angiogenesis, the process by which new blood vessels sprout from preexisting vessels, is critical for proper embryonic development and normal tissue homeostasis and contributes to the pathogenesis of many diseases, including cancer. Proper vessel morphogenesis requires a balance between angiogenic stimuli, which regulate endothelial cell invasion and migration, proliferation, and tubulogenesis, and angiostatic factors that terminate or inhibit these processes upon vessel maturation to promote vascular stability (reviewed in references 13, 14, 24, and 58). Members of the Slit/roundabout (Robo) gene family have recently emerged as key regulators of vascular remodeling and homeostasis, particularly with the discovery of an endothelial cell-specific Robo receptor, Robo4/Magic roundabout (reviewed in reference 43).

The three Slit proteins (Slit1-3) identified in vertebrates interact with receptors of the Robo family (Robo1-4), Robo1 and Robo4 being most highly expressed in endothelial cells (76). While Robo receptors lack intrinsic kinase activity, the intracellular portions of the receptors contain several conserved CC motifs that can interact with intracellular kinases, such as Abelson kinase (Abl) and its substrate enabled (Ena), as well as GTPase activating proteins (GAPs) that modulate the activities of Rho family GTPases. These interactions link

Slit-Robo signaling to cytoskeletal remodeling, which promotes chemotaxis or chemorepulsion downstream of Robo signaling, depending upon the cell type and physiologic context (reviewed in references 28 and 43). Identified originally in *Drosophila melanogaster* (reviewed in reference 20) and later in vertebrates (12, 47), the role of Slit proteins in regulation of angiogenesis is controversial, with reports of both proangiogenic (37, 38, 63, 69, 75) and antiangiogenic (26, 34, 35, 46, 55) activity. Relatively few studies have examined the role of Slit2 as a single agent in angiogenesis, though in the context of vascular endothelial growth factor (VEGF) (34, 35, 46, 55), recent investigations have clearly demonstrated that Slit2 inhibits VEGF-induced vascular remodeling. Thus, the mechanism that governs pro- versus antiangiogenic functions of Slit2 is not clear.

The Eph family of receptor tyrosine kinases (RTKs) and their cell surface membrane-bound ephrin ligands have emerged as critical regulators of angiogenic remodeling associated with both normal physiology and disease (reviewed in references 1, 5, 7, and 40). This family, comprised of class A receptors that generally bind to glycosylphosphatidylinositol (GPI)-linked ephrin-A ligands and class B receptors that normally bind to transmembrane-linked ephrin-B ligands, is the largest RTK family identified in the genome, including at least 14 receptors and 8 ligands in vertebrates (reviewed in references 2 and 56). EphA2 and its primary ligand, ephrin-A1, have become the targets of intensive investigation due to their functions in tumorigenesis and neovascularization.

In this study, we found that Slit2 potently stimulates angiogenesis as a single agent. In the presence of ephrin-A1, how-

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† Supplemental material for this article may be found at <http://mc.manuscriptcentral.com/mcb>.

∇ Published ahead of print on 6 December 2010.

ever, Slit2-mediated vascular remodeling is impaired. We provide the first evidence linking the proangiogenic effects of Slit2 to mTORC2-dependent activation of Akt and Rac-GTPase, which is inhibited by ephrin-A1 cotreatment. These data suggest that Slit2 differentially regulates angiogenesis in the context of ephrin-A1, providing a plausible mechanism for the pro- versus antiangiogenic functions of Slit2.

MATERIALS AND METHODS

Reagents. Antibodies against the following proteins were used: Akt, phosphoserine 473 Akt, phosphothreonine 308, src, phospho-src family (Tyr416), and myc rictor (Cell Signaling Technology, Boston, MA); actin and ephrin-A1 (normal rabbit IgG; Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-ephrin-A1 antibody (62); Rac (BD Biosciences, San Jose, CA); von Willebrand factor (vWF; Zymed Laboratories, South San Francisco, CA); β -galactosidase (Millipore, Billerica, MA); tubulin (Sigma-Aldrich, St. Louis, MO); and rictor (Bethyl Laboratories, Montgomery, TX). Pak-PBD agarose Rac assay reagent was purchased from Millipore. Recombinant mouse Ephrin-A1-Fc, recombinant mouse EphA2-Fc, recombinant rat Robo1-Fc, human IgG, and recombinant Slit2 were purchased from R&D Systems (Minneapolis, MN). Gel-foam absorbable gelatin sponges (Pharmacia) were obtained from the Vanderbilt University Hospital pharmacy. Tetramethyl rhodamine isothiocyanate (TRITC)-dextran and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich. Growth factor-reduced Matrigel was purchased from BD Biosciences. Soluble Rac inhibitor (Insolution RacI inhibitor NSC23776) and the mTOR inhibitor rapamycin were obtained from Calbiochem (EMD Chemicals Inc./Merck KGaA, Darmstadt, Germany). The Akt1/2 inhibitor 5J8/0360263-1 was produced by the Vanderbilt University Department of Chemistry as described previously (45). Tamoxifen was purchased from Sigma. Constructs encoding constitutively active Rac (RacV12 [59]) and Akt (myristoylated Akt [myr-Akt], generated from addition of a src myristoylation sequence to Akt Δ 4-129 pleckstrin homology [PH] domain deletion mutant [39, 73]) were described previously. The construct encoding dominant negative Akt (K179M [77]) was purchased from Addgene, Inc. (Cambridge, MA). Adenoviruses harboring ephrin-A1 (Ad-ephrin-A1) and β -galactosidase (Ad- β -galactosidase) were described previously (9). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from MP Biomedicals (Solon, OH).

Endothelial cell culture. Primary murine pulmonary microvascular endothelial cells (MPMEC) were isolated from mice as described previously (6), and bovine pulmonary microvascular endothelial cells (BPMEC) were purchased from VEC Technologies, Inc. (Rensselaer, NY). Cells were maintained in EGM-2 medium (Lonza, Walkersville, MD) supplemented with penicillin-streptomycin (Cellgro/Mediatech, Herndon, VA) and 10% fetal bovine serum (HyClone, Logan, UT). Immortalized MPMEC were isolated from 1- to 3-month-old mice derived from the *H-2K^b-tsA58* transgenic "Immorto-mouse" background (33, 41) as described previously (23). These cells were grown at 33°C in EGM-2 medium supplemented with gamma interferon (10 ng/ml; Millipore), a permissive condition that allows the expression of the temperature-sensitive simian virus 40 (SV40) T-antigen (TAg) transgene. The cells were incubated at 37°C for at least 3 days in the absence of gamma interferon to downregulate TAg expression and revert the cells to a nonimmortalized state. MPMEC were also isolated from mice harboring a floxed *rictor* allele (66) and expressing a tamoxifen-inducible cre transgene (70) and treated with 100 nM tamoxifen or vehicle control for 7 days to induce rictor deficiency.

Recombinant Slit2 production. The HEK293 cells that produce full-length Slit2 proteins tagged with c-myc have been described previously (44, 71). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS). Slit2 was partially purified from the supernatants as described previously. The supernatant from parental HEK cells was used as controls. Working concentrations of Slit2 from diluted supernatants were estimated to be between 100 and 250 ng/ml, based on silver staining of serial dilutions of supernatants following SDS-PAGE fractionation (data not shown).

In vitro angiogenesis assays. *In vitro* vascular assembly assays were performed as described previously (6, 23). Briefly, 12-well plates were coated with 100 μ l of growth factor-reduced Matrigel (BD Biosciences). After 24 h of starvation in Opti-MEM, 25,000 MPMEC were plated in wells in the presence or absence of Slit2 (100 ng/ml recombinant or HEK293 supernatant), control HEK293 supernatant, ephrin-A-Fc (0.5 μ g/ml), or control IgG (0.5 μ g/ml) and photographed after 9 to 24 h. Images were acquired on an Olympus CK40 inverted microscope through an Optronics DEI-750C charge-coupled-device (CCD) video camera

using Scion Image version 1.62c capture software. The degree of assembly was quantified by measuring branch length, the distance from the branching point to the tip of assembled cells. The branch length in assembled endothelial cell networks was expressed as arbitrary units per $\times 10$ field in four random fields from each well, with triplicate samples per condition, using Scion Image software. For inhibitor studies, cells were pretreated with 50 μ M LY294002 PI 3-kinase inhibitor or soluble Rac inhibitor, 50 nM rapamycin mTOR inhibitor, or 1 μ M 5J8/0360263-1 Akt1/2 inhibitor for 1 h prior to assembly assay as well as for the duration of the assay. For rescue experiments, plasmids encoding myc-tagged V12Rac, as well as myr-Akt, were expressed in BPMEC by transfection with Lipofectamine 2000 reagent (Invitrogen), as described previously (6), prior to assembly assays. For some experiments, cells were transduced with 1×10^8 PFU/ml Ad-ephrin-A1 or Ad- β -galactosidase 48 h prior to assay. For some experiments, adenovirus-transduced cells were pretreated with PI-PLC (0.5 U/ml) in phosphate-buffered saline (PBS) buffer for 1 h at 4°C prior to assay. We assessed reduction of cell surface ephrin-A1 following PI-PLC treatment by immunofluorescence using anti-ephrin-A1 (3E6, 1:100) overnight at 4°C followed by detection with anti-mouse IgG-Alexa594 (Molecular Probes) and DAPI counterstain.

For migration assays, endothelial cells were serum starved for 24 h in Opti-MEM. Transwells were coated with growth factor-reduced Matrigel (1:20 dilution with Opti-MEM) for 30 min and blocked with 1% bovine serum albumin solution for an additional 30 min. Cells (100,000) were plated in the upper chamber of the transwells, and 600 μ l of Opti-MEM containing Slit2 (100 ng/ml recombinant or HEK293 supernatant), control HEK293 supernatant, ephrin-A1-Fc (0.5 μ g/ml), or control IgG (0.5 μ g/ml) was added to the lower chamber. After 5 h, cells were fixed and stained with crystal violet to visualize endothelial cells. Cells that migrated to the lower surface of transwell filters were counted in four random fields from each well, with triplicate samples per condition, as described previously (6, 23).

In vivo sponge assays for angiogenesis. All animals were housed under pathogen-free conditions, and experiments were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. Sponge assays for angiogenesis were performed as described previously (6, 31). Briefly, gel foam sponges were cut into small pieces (2.5 to 3 mm wide by 5 mm long) and soaked with 100 μ l of phosphate-buffered saline containing 5 μ g of ephrin-A1-Fc or IgG, Slit2 (approximately 0.5 μ g), or control HEK293 supernatant. The sponges were then implanted into the subcutaneous dorsal flank of recipient mice. Each recipient received one proangiogenic factor-impregnated sponge and one relevant control factor-impregnated sponge implanted in the opposite flank. After 7 days, the mice were injected with a 2% tetramethyl rhodamine isothiocyanate (TRITC)-dextran-phosphate-buffered saline solution to label host blood vessels (6, 31), and the sponges were collected and analyzed. Whole-mount images were acquired on an Olympus CK40 inverted microscope through an Optronics DEI-750C charge-coupled-device video camera using Scion Image version 1.62c capture software. Density of blood vessels within the sponges was quantified by fluorescence intensity ($\times 10$ magnification) of TRITC-dextran using Scion Image software. Data are a representation of results from five independent sponges under each condition. Statistical significance was determined by two-tailed, paired Student's *t* test. Vessel identity was confirmed in paraffin sections prepared from sponges and counterstained with DAPI and/or costained with the endothelial cell marker vWF as described previously (8, 10, 11).

Immunoblot analyses. Endothelial cells were serum starved for 24 h in Opti-MEM plus 2% FCS. Rac activation in approximately 500 μ g endothelial cell lysate was assessed by Pak-PBD agarose Rac assay reagent as described previously (6, 23). For analysis of src and Akt phosphorylation and expression, approximately 50 μ g of endothelial cell lysates was collected and processed as per antibody supplier's protocol (Cell Signaling Technologies). For all experiments, cells were stimulated with ephrin-A1-Fc (0.5 μ g/ml), control IgG (0.5 μ g/ml), Slit2 (100 ng/ml recombinant or HEK293 supernatant), or control HEK293 supernatant for 5 to 10 min. For some experiments, cells were pretreated with a pharmacologic inhibitor of PI 3-kinase, mTOR, or Akt prior to stimulation. Experiments scoring soluble versus membrane-bound ephrin-A1 were performed according to methods described previously (72). Endothelial cells were transduced with control Ad- β -galactosidase or Ad-ephrin-A1 and incubated in a low volume (3 ml/10-cm plate) of growth medium for 48 h. Cell supernatants and lysates were harvested and subjected to immunoblotting for ephrin-A1. Parallel plates were washed and treated with PI-PLC (0.5 U/ml) in PBS buffer for 1 h at 4°C, followed by supernatant harvest. To enrich for soluble ephrin-A1, 1-ml aliquots of supernatant from untreated or PI-PLC-treated samples were subjected to immunoprecipitation using 0.5 mg EphA2-Fc followed by immunoblotting for ephrin-A1. Lysate and/or pulldown products were fractionated on 10 to

12% SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose membranes and probed with primary antisera. Specific immunoreaction was detected using anti-IgG antibodies conjugated to horseradish peroxidase (Promega, Madison, WI) and a Pierce ECL chemiluminescence detection kit (Thermo Scientific, Rockford, IL). The blots were stripped and reprobed with antiactin (Santa Cruz Biotechnology) or antitubulin (Sigma-Aldrich) antibody to confirm uniform loading. Densitometry was performed on scanned blots using NIH Image J software version 1.32 to quantify the average pixel density/band area after normalization to the pixel density of the corresponding loading control. Data are a representation of three to five independent experiments.

Analysis and manipulation of digital images. Images were captured from Olympus CK40 inverted and BX-60 conventional microscopes using an Optronics NTSC digital camera system and NIH Scion Image/Image J software. Images were processed minimally using Adobe Photoshop CS2 software to optimize brightness and contrast. Control images were subjected to the same manipulations as images from experimental groups, and manipulations were applied to all parts of the image.

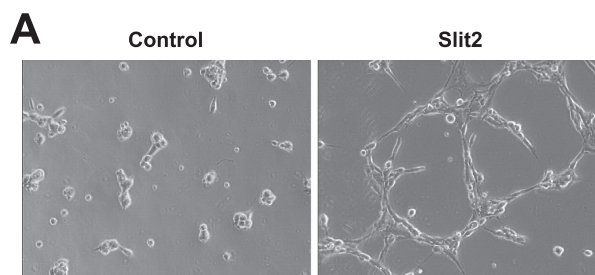
RESULTS

Slit2 promotes angiogenic remodeling in culture and *in vivo*.

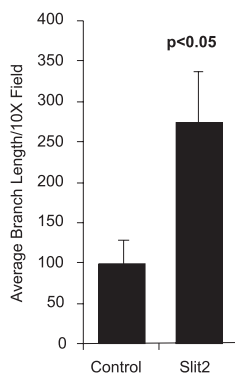
Though Slit2 was reported to inhibit angiogenesis (46, 55), particularly in response to VEGF (34, 35), the role of Slit signaling in angiogenesis remains controversial, as other studies reported that Slit proteins, including Slit2, can promote angiogenesis (37, 38, 63, 69). Therefore, we tested the effect of Slit2 on vascular remodeling as a single agent, in the absence of VEGF. We analyzed transiently immortalized endothelial cells isolated from *H-2K^b-tsA58* transgenic "Immorto-mice" (33), expressing a temperature-sensitive SV40 TAg cassette at a permissive temperature (33°C). Once cells are plated at physiologic temperature (37°C), protein levels of the thermolabile TAg are downregulated, and cells are restored to a nontransformed state over the course of several days (23, 33, 41).

We assessed microvascular endothelial cell assembly by plating cells on a thin layer of growth factor-reduced Matrigel in the presence of control IgG/control HEK293 supernatant versus recombinant Slit2/Slit2-containing HEK293 supernatant. Relative to cells cultured with control protein, endothelial cells treated with Slit2 displayed a significantly elevated remodeling response, assembling into interconnected structures resembling a primitive capillary plexus in cell lines from Immorto-mice as well as freshly isolated lung microvascular endothelial cells (Fig. 1A). Slit2 also stimulated endothelial cell migration in transwell assays (Fig. 1B).

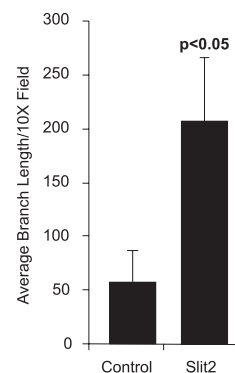
To determine if Slit2 affects vascular remodeling from intact vessels *in vivo*, we implanted sponges seeded with IgG/control HEK293 supernatant versus recombinant Slit2/Slit2-containing HEK293 supernatant subcutaneously into the dorsal flank of recipient mice. One week following implantation, we injected mice intravenously with TRITC-dextran in order to visualize blood vessels infiltrating the sponge. Sponges harboring Slit2 stimulated a robust angiogenic response, with a significant increase in TRITC-positive surface blood vessels relative to that for sponges containing IgG control (Fig. 2A and B). We also observed TRITC-positive blood vessels infiltrating sponges containing Slit2, but not control IgG, in tissue sections. The TRITC-positive structures in sponge sections costained with von Willebrand factor (vWF, green; Fig. 2C), a marker for vascular endothelium, confirming that the TRITC-positive structures observed were functional blood vessels. Taken together, these data support the proangiogenic function of Slit2.



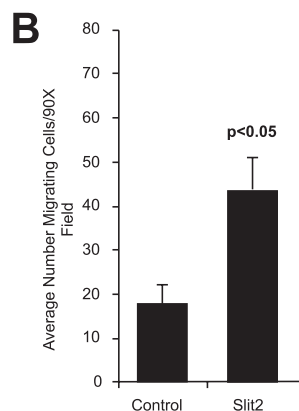
Immortalized Wild-type Endothelial Cell Assembly Assay



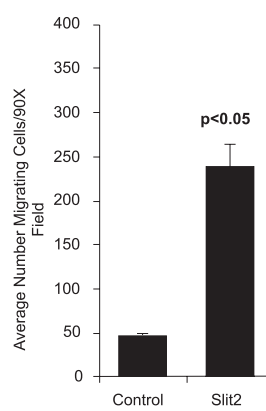
Assembly Immortalized Endothelial Cells



Assembly Primary Endothelial Cells



Transwell Migration Assay Immortalized Endothelial Cells



Transwell Migration Assay Primary Endothelial Cells

FIG. 1. Slit2 stimulates endothelial cell assembly and migration *in vitro*. (A) We plated immortalized or freshly isolated primary MPMEC on a thin layer of growth factor-reduced Matrigel and scored assembly into interconnected vascular networks in response to control medium/IgG versus Slit2-containing medium/recombinant Slit2. Slit2 stimulated assembly of both immortalized and primary MPMEC relative to control levels. (B) Slit2 also stimulated MPMEC migration in transwell assays. Data are a representation of the averages \pm standard deviations for three to five independent experiments, with replicate samples analyzed in each experiment.

Slit2 represses angiogenesis in the presence of ephrin-A1. Slit2 has also been reported to suppress angiogenesis, particularly in the presence of VEGF (34, 35, 46, 55). As VEGF regulates ephrin-A1 expression (18) and is dependent upon ephrin/EphA2 activity for vascular remodeling (4, 15, 17, 18, 21), we hypothesized that Slit2 could repress angiogenic re-

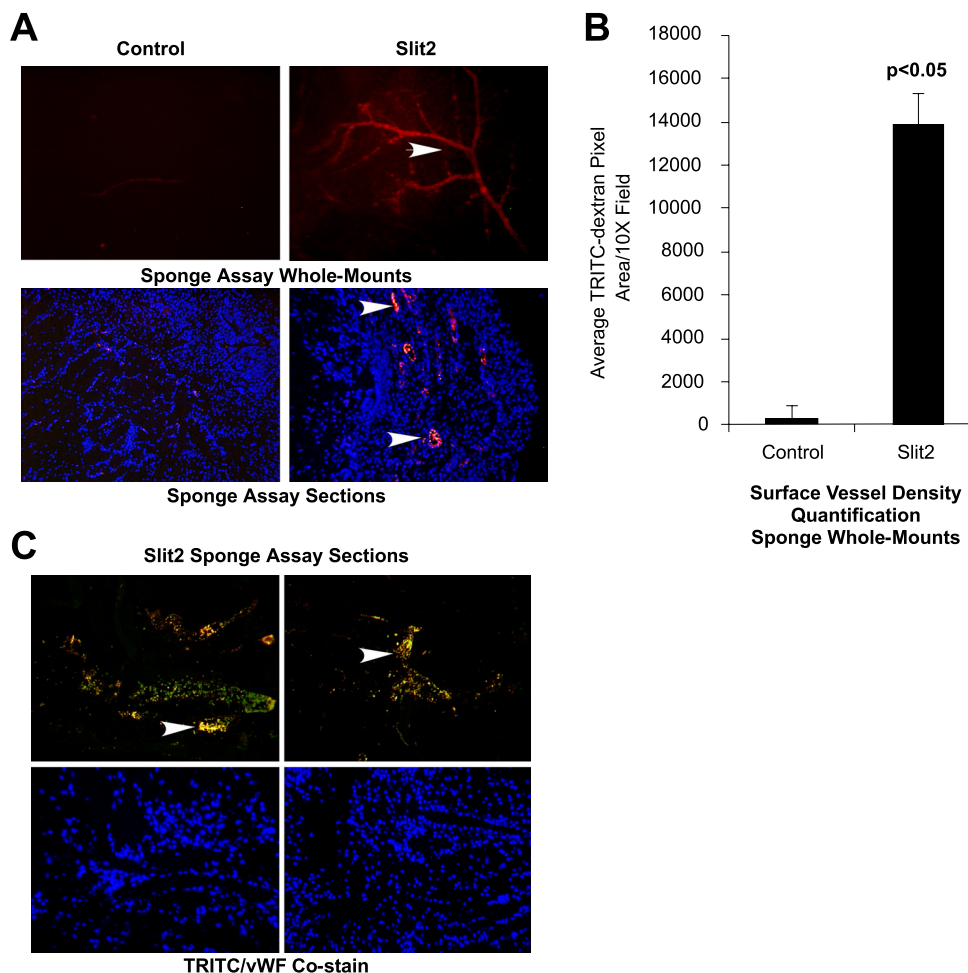


FIG. 2. Slit2 induces subcutaneous blood vessel remodeling *in vivo*. (A) We loaded Gelfoam sponges with control medium/IgG (negative control) versus Slit2-containing medium/recombinant Slit2 and subcutaneously implanted the sponges into the dorsal flank of recipient mice. After 7 days, the mice were injected intravenously with TRITC-dextran to label vasculature, and the sponges were excised for analysis. Sponges harboring Slit2 contained significantly more TRITC-positive blood vessels than did control sponges (arrows, whole mounts; arrowheads, sections). (B) We quantified surface vessel density in whole mounts based on TRITC-positive pixel area using NIH Image J software analysis. (C) We confirmed that TRITC-positive structures also expressed the endothelial cell marker von Willebrand factor (vWF, green staining; arrowheads indicate TRITC-positive/vWF-positive blood vessels). Data are a representation of the averages \pm standard deviations for 10 independent animals total/condition analyzed in 2 independent experiments (5 animals/condition/experiment).

modeling in the presence of ephrin-A1. To test this hypothesis, we assessed vascular assembly in cells stimulated with Slit2 in the presence of soluble ephrin-A1-Fc. Costimulation with Slit2 and ephrin-A1 abrogated assembly (Fig. 3A), whereas endothelial cells treated with ephrin-A1-Fc alone, as with single-agent Slit2 (Fig. 1A), displayed a significantly elevated remodeling response (Fig. 3A). Consistent with these observations, costimulation with Slit2 and ephrin-A1 resulted in significantly diminished endothelial cell migration relative to that for cells stimulated with only ephrin-A1-Fc (Fig. 3B) or Slit2 (Fig. 1B) as a single agent in transwell assays.

Endogenous ephrin ligands are membrane tethered. While ephrin-A1-Fc, which is presented to cells in dimeric form, mimics activation of EphA receptors induced by endogenous, clustered ligands, it is possible that this soluble form does not fully recapitulate signaling induced by native ligands (36, 67). To address this possibility, we overexpressed native ephrin-A1 in wild-type endothelial cells via adenoviral transduction. We

detected ephrin-A1 protein in cell lysates as well as culture medium, suggesting that both membrane-tethered ephrin-A1 and soluble ephrin-A1 are produced upon overexpression (see Fig. S1 in the supplemental material), consistent with detection of soluble ephrin-A1 expression in endothelial (29) and tumor (3, 72) cells. Adenovirus-mediated overexpression of native ephrin-A1 inhibited Slit2-induced vascular assembly (Fig. 3C), consistent with the effects we observed upon costimulation with ephrin-A1-Fc and Slit2. Interestingly, pretreatment with phosphatidylinositol-specific phospholipase C (PI-PLC), which liberates ephrin-A1 from the cell surface, impaired angiogenesis induced by both single agents, as well as the ability of Ad-ephrin-A1 expression to suppress Slit2-mediated angiogenesis (see Fig. S1 in the supplemental material). Taken together, these data suggest that Slit2 represses angiogenic remodeling in the presence of ephrin-A1 and that these effects are mediated by clustered ephrin-A1 rather than cleaved soluble, presumably monomeric, forms of the ligand.

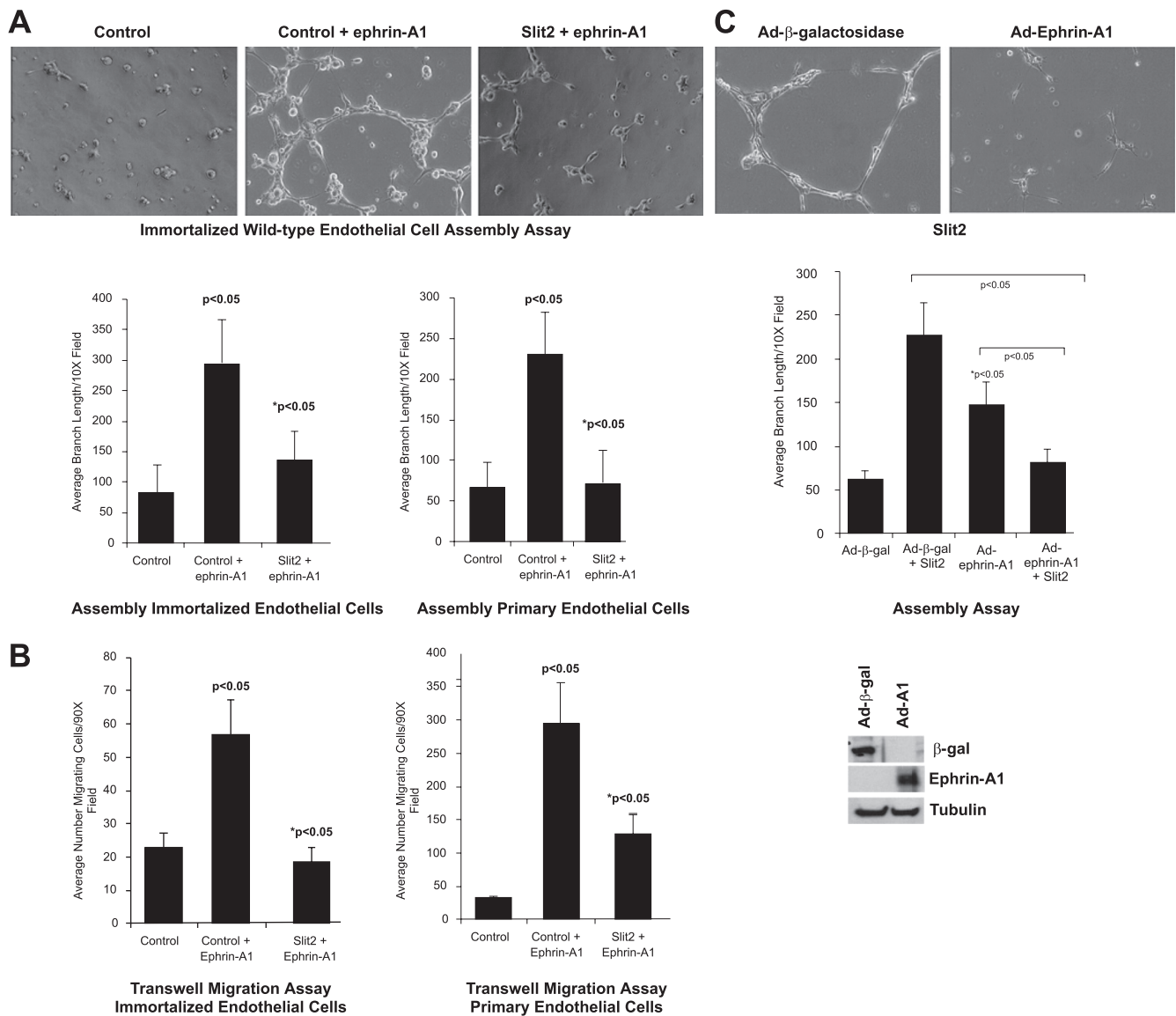


FIG. 3. Ephrin-A1 inhibits Slit2-induced vascular assembly and migration. (A) We plated immortalized or freshly isolated primary MPMEC on a thin layer of growth factor-reduced Matrigel and scored assembly into interconnected vascular networks in response to soluble ephrin-A1-Fc, Slit2, or the combination. While ephrin-A1 potently stimulated assembly in the presence of control medium/IgG, costimulation with medium containing Slit2/recombinant Slit2 significantly inhibited assembly in culture. Results were consistent for immortalized and primary MPMEC. (B) We also assessed MPMEC migration in response to ephrin-A1-Fc, Slit2, and the combination via transwell assay. Consistent with our observations in assembly assays, Slit2 costimulation potently inhibited endothelial cell migration in the presence of ephrin-A1-Fc. (C) To complement these studies, we overexpressed native, membrane-bound ephrin-A1 or control β -galactosidase (β -gal) in wild-type endothelial cells via adenoviral transduction. Ephrin-A1-overexpressing cells did not undergo assembly in response to Slit2 relative to controls, though Ad-ephrin-A1-expressing cells did undergo spontaneous assembly relative to Ad- β -gal controls (*). We confirmed expression of β -galactosidase and ephrin-A1 adenoviral transgenes by immunoblotting. Data are a representation of the averages \pm standard deviations for three to five independent experiments, with replicate samples analyzed in each experiment.

To determine if Slit2 affects ephrin-A1-mediated vascular remodeling from intact vessels *in vivo*, we implanted sponges seeded with control IgG, ephrin-A1-Fc, or ephrin-A1-Fc plus Slit2 subcutaneously into the dorsal flank of recipient mice. Sponges harboring ephrin-A1 stimulated a robust angiogenic response, with a significant increase in TRITC-positive surface blood vessels relative to that for sponges containing IgG control (Fig. 4A and B). We also observed TRITC-positive blood vessels infiltrating sponges containing ephrin-A1, but not con-

trol IgG, in tissue sections. Sponges harboring both ephrin-A1 and Slit2 failed to induce angiogenesis *in vivo* (Fig. 4A and B).

Slit2 promotes angiogenesis through mTORC2-dependent activation of Akt and Rac. In order to determine the molecular mechanism(s) that mediates Slit2-induced angiogenesis and regulates the switch between pro- and antiangiogenic functions of Slit2, we assessed the activation of downstream signaling pathways that are required for vascular remodeling, focusing on Rac-GTPase. Slit2 stimulation enhanced levels of GTP-

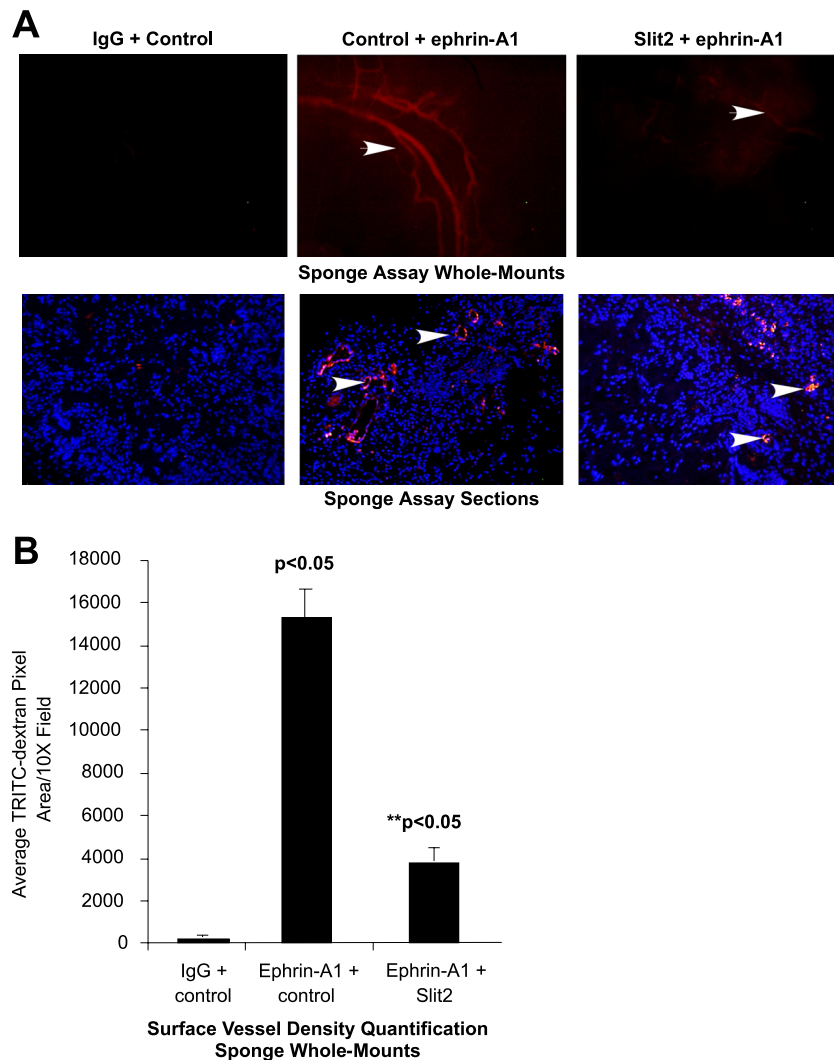
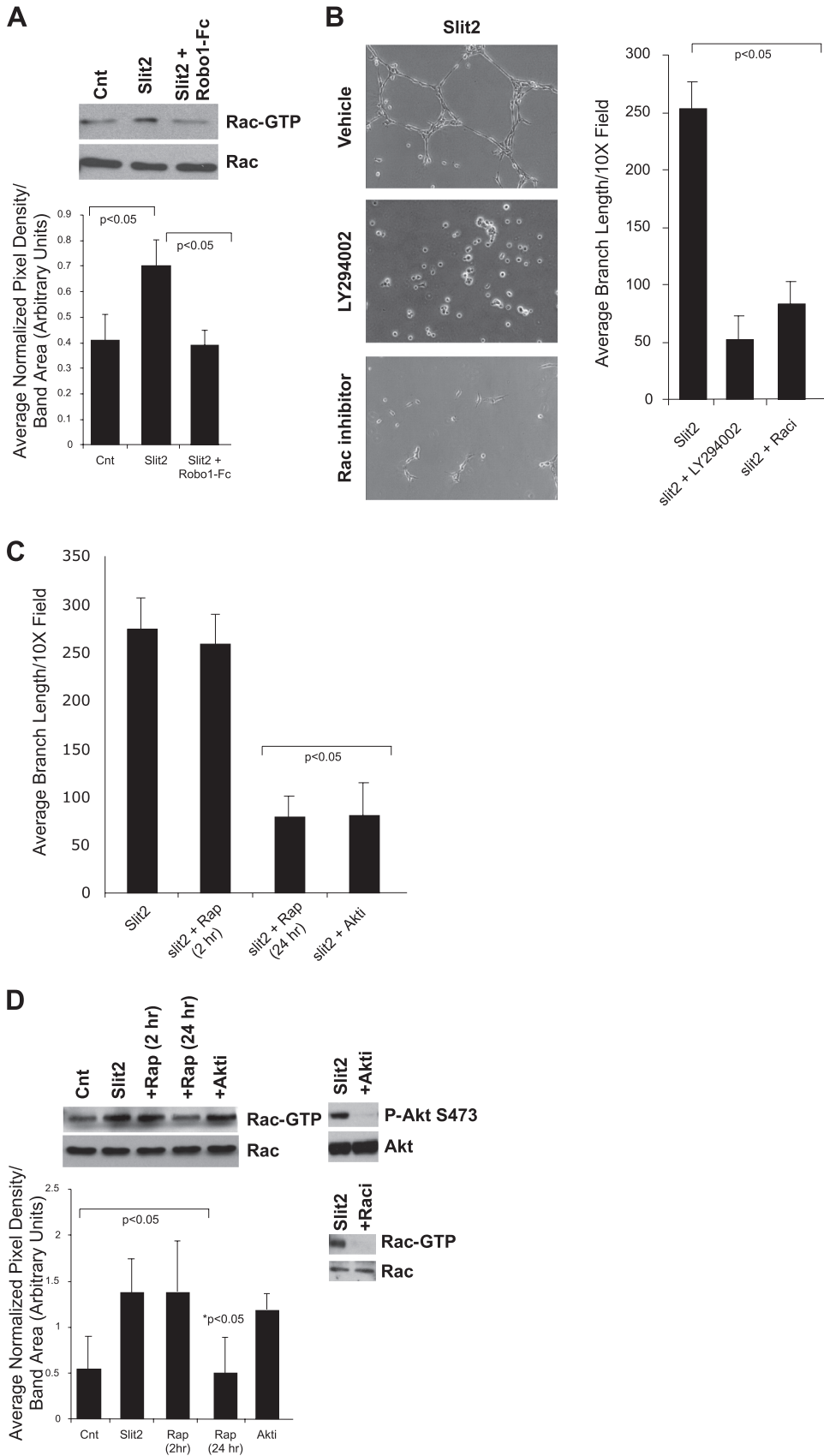


FIG. 4. Ephrin-A1 inhibits Slit2-induced angiogenic remodeling *in vivo*. (A) We loaded Gelfoam sponges with IgG (negative control) versus ephrin-A1-Fc in the presence or absence of control medium/Slit2-containing medium or recombinant Slit2 and subcutaneously implanted the sponges into the dorsal flank of recipient mice. After 7 days, the mice were injected intravenously with TRITC-dextran to label vasculature, and the sponges were excised for analysis. Sponges harboring ephrin-A1-Fc contained significantly more TRITC-positive blood vessels than did control IgG-soaked sponges (arrows, whole mounts; arrowheads, sections). Sponges loaded with both ephrin-A1-Fc and Slit2 displayed a significant reduction in surface vascular density. (B) We quantified surface vessel density in whole mounts based on TRITC-positive pixel area using NIH Image J software analysis. Data are a representation of the averages \pm standard deviations for 10 independent animals total/condition analyzed in 2 independent experiments (5 animals/condition/experiment).

bound, active Rac in wild-type endothelial cells when used as a single agent (Fig. 5A). Moreover, we observed that treatment with a soluble inhibitor of Rac, as well as a pharmacologic inhibitor of the upstream activator PI 3-kinase, impaired vascular assembly induced by single-agent Slit2 (Fig. 5B), providing evidence that this pathway is required for the proangiogenic effects of Slit2.

The serine/threonine kinase mammalian target of rapamycin (mTOR) also activates Rac GTPase, an activity specific to the mTOR complex 2 (mTORC2) (19, 27). In addition, this complex activates Akt (19, 61), which also regulates angiogenesis (50, 64, 65). To determine if mTORC2 and/or Akt are required for Slit2-mediated angiogenesis, we performed vascular assembly assays in cells stimulated with Slit2 in the presence or absence of the mTOR inhibitor rapamycin or an allosteric

inhibitor of Akt (45) (Fig. 5C). Short-term pretreatment (2 h) with rapamycin, a condition under which mTORC1 inhibition is favored, did not affect vascular assembly in response to Slit2 (Fig. 5C). Prolonged pretreatment (24 h) with rapamycin, a condition under which mTORC2 is sensitive to rapamycin at the level of complex assembly (60), as confirmed in endothelium (19), significantly inhibited Slit2-mediated endothelial cell assembly (Fig. 5C). The Akt inhibitor also significantly impaired Slit2-mediated vascular assembly (Fig. 5C). We also observed decreased levels of GTP-bound Rac in Slit2-stimulated cells subjected to prolonged rapamycin, though not with the Akt inhibitor (Fig. 5D) or upon overexpression of a dominant negative Akt construct (see Fig. S2 in the supplemental material). In addition, the soluble Rac inhibitor had no impact on Akt activity (see Fig. S2 in the supplemental material).



These data suggest that Slit2 activates Rac and Akt in parallel, possibly through an mTORC2-mediated mechanism.

As prolonged rapamycin treatment inhibits both mTORC1 and mTORC2, we analyzed primary endothelial cells isolated from mice harboring a floxed (*fl*) *riCTOR* allele (66) and expressing a tamoxifen-inducible estrogen receptor (ER)-*cre* transgene (70). Tamoxifen treatment of *riCTOR^{fl/fl}* endothelial cells in culture significantly reduced expression of *riCTOR*, an mTORC2-specific regulator (reviewed in reference 42), relative to that for vehicle-treated control cells (Fig. 6A). Relative to control cells, *riCTOR*-deficient endothelial cells displayed reduced levels of Akt phosphorylation at serine 473, as well as reduced Rac activation upon stimulation with Slit2 (Fig. 6A). Moreover, *riCTOR* deficiency abrogated endothelial cell assembly in response to Slit2, but not ephrin-A1 (Fig. 6B), consistent with the effect of prolonged rapamycin treatment (Fig. 5C; data not shown for ephrin-A1-Fc). Taken together, these data suggest that Slit2 induces angiogenesis through mTORC2-mediated activation of Akt and Rac.

Ephrin-A1 inhibits Rac and Akt activation by Slit2. Although recent studies correlated impaired vascular remodeling with decreased *src* activation in endothelial cells costimulated with Slit2 and VEGF (34), we did not observe any significant changes in tyrosine-phosphorylated *src* in cells stimulated with ephrin-A1, Slit2, or the combination (Fig. 7A). We did, however, observe that Slit2-induced Rac activation was significantly reduced in the presence of ephrin-A1, to levels below that of untreated cells (Fig. 7B). Consistent with our previous reports, ephrin-A1, like Slit2, stimulates Rac activity as a single agent (6, 23, 31). While single-agent ephrin-A1 stimulation did not increase phospho-Akt S473 levels, costimulation impaired Slit2-induced Akt phosphorylation specifically at serine 473 (Fig. 7C), the target of mTORC2 phosphorylation (25, 32, 61). We did not observe any changes in phosphorylation of the mTORC1 target S6-kinase (Fig. 7C), suggesting that mTOR in complex 2 is specifically required for Slit2-mediated angiogenic remodeling.

Activated Rac and Akt restore angiogenesis in Slit2/Ephrin-A1-costimulated cells. Our analysis of downstream signaling pathways suggests that Slit2-induced Akt and Rac activation may be impaired in the presence of ephrin-A1. To test this hypothesis, we expressed dominant active isoforms of Akt (*myr-Akt* [73]) and Rac (*RacV12* [59]) in primary bovine pulmonary microvascular endothelial cells and scored assembly and migration upon costimulation with Slit2 and ephrin-A1. Relative to results for cells transfected with control green flu-

orescent protein (GFP) plasmid, expression of *myr-Akt* and *RacV12* partially rescued assembly (Fig. 8A) and migration (Fig. 8B) in response to costimulation. Together, these data support a model in which Slit2 stimulates angiogenesis through mTORC2-mediated Akt and Rac activation, which is impaired in the presence of ephrin-A1.

DISCUSSION

Physiologic angiogenesis is a tightly regulated process that involves spatial and temporal coordination between several molecular signaling pathways (e.g., VEGF/VEGF receptor [VEGFR], fibroblast growth factor [FGF]/FGFR, Notch, Ang/Tie, Eph/ephrin, platelet-derived growth factor [PDGF]/PDGFR, transforming growth factor β [TGF- β]/TGF- β receptor). Such molecules cooperate to activate endothelium, promote proliferation, motility, and tubulogenesis, and ultimately recruit mural cells, leading to a stable structure capable of sustaining blood flow. These signaling pathways are exploited by tumors to facilitate neovascularization (reviewed in references 1, 13, and 14). Slit and Robo proteins have recently emerged as key regulators of vascular remodeling (reviewed in reference 43). Here, we report that Slit2 stimulates angiogenesis in cultured endothelial cells and in mice when delivered as a single agent. In the presence of ephrin-A1, however, Slit2 inhibits angiogenesis *in vitro* and *in vivo*. Slit2-induced angiogenesis requires activation of mTORC2/Rac and Akt, which is impaired in the presence of ephrin-A1. Dominant active Akt and Rac mutants partially rescue vascular assembly and migration in costimulated cells. It is possible that constitutively active Akt or Rac enhanced random migration in transwell assays that resulted in some cell movement through the filter. However, since Akt and Rac are activated downstream of Slit2 in parallel rather than by an interdependent mechanism and their activation was inhibited in the presence of ephrin-A1, we hypothesize that restoring activity of at least one of these factors could abrogate the inhibitory effects of ephrin-A1. Indeed, cells expressing *myr-Akt* or *RacV12* displayed a more robust migration in the presence of Slit2/ephrin-A1 than did unstimulated cells. Together, these data suggest that cooperation between the Slit2 and ephrin-A1 pathways regulates a balance between angiogenesis and vascular homeostasis.

While Slit/Robo signaling is known to regulate angiogenesis, the function of these molecules as angiogenic versus angiostatic factors remains controversial. Slit2 interaction with Robo4, the endothelial cell-specific roundabout receptor, was reported

FIG. 5. Slit2-induced angiogenesis requires activation of Rac, mTOR, and Akt. (A) We observed elevated levels of active, GTP-bound Rac GTPase in MPMEC stimulated for 5 min with Slit2 versus control (Cnt), which was abrogated by soluble Robo1-Fc preincubation for 30 min, upon GST-Pak Rac binding domain (RBD) substrate pulldown. Lysates were also probed for Rac expression to validate uniform input. Densitometry analysis for blots is shown in the bar graph. (B) To determine if Slit2-induced angiogenesis requires PI 3-kinase, which functions upstream of Rac, and/or Rac activity, we treated MPMEC with pharmacologic inhibitors and scored vascular assembly in culture. Pretreatment with PI 3-kinase inhibitor LY294002 or soluble Rac inhibitor (Raci) significantly reduced assembly in response to Slit2. (C) Long-term treatment with the mTOR inhibitor rapamycin (Rap) (24 h; inhibits mTORC1 and -2) but not acute treatment (2 h; inhibits mTORC1 only) impaired Slit2-mediated vascular assembly. Pharmacologic inhibition of Akt activity also significantly impaired Slit2-induced vascular assembly. (D) Prolonged treatment with rapamycin also impaired Slit2-induced Rac activation, supporting the hypothesis that Slit2 activates Rac and/or Akt by stimulating mTORC2. We confirmed that the pharmacologic inhibitors of Akt and Rac reduced P-Akt/Rac-GTP levels upon stimulation with Slit2 for 5 (Rac) to 10 (Akt) min. Densitometry analysis for blots is shown in the bar graph. Data are a representation of the averages \pm standard deviations for three to five independent experiments, with replicate samples analyzed in each experiment.

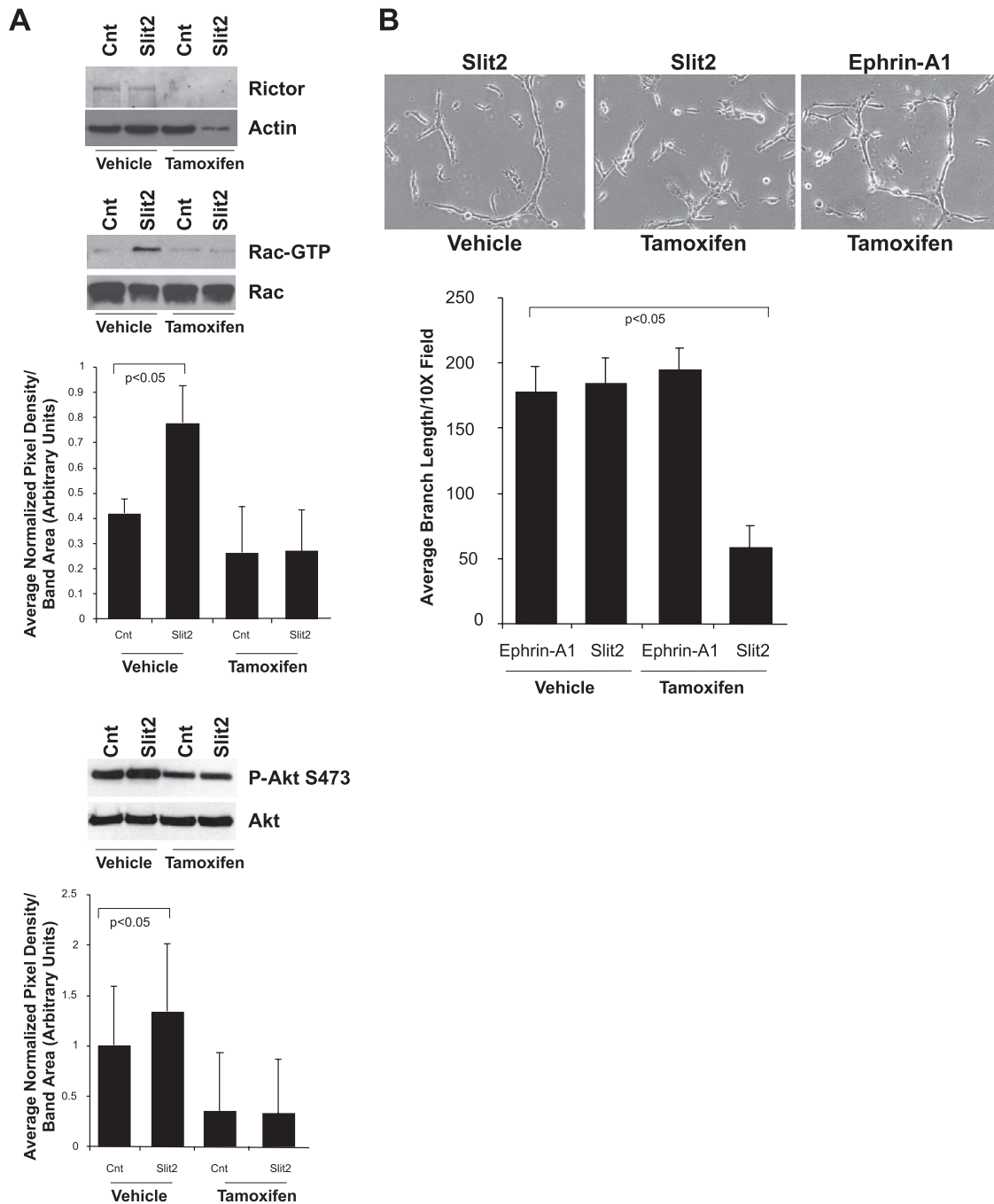


FIG. 6. Loss of rictor expression impairs Slit2-mediated vascular assembly and activation of Akt and Rac. We isolated primary MPMEC from mice harboring a floxed *rictor* allele and expressing a tamoxifen-inducible ER-cre transgene. We treated cells in culture with tamoxifen for 7 days to induce expression of Cre so as to eliminate rictor expression. (A) Tamoxifen treatment of rictor^{fl/fl} endothelial cells in culture resulted in significantly reduced expression of the mTORC2-specific regulator rictor relative to that for vehicle-treated control cells. Relative to control cells, rictor-deficient endothelial cells displayed significantly reduced Rac activation, as well as Akt activation and expression levels, upon stimulation with Slit2 for 5 (Rac) to 10 (Akt) min, as assessed by densitometry analysis shown in the bar graphs below the blots. (B) Rictor deficiency abrogated endothelial cell assembly in response to Slit2, but not ephrin-A1. Data are a representation of the averages \pm standard deviations for four independent rictor^{fl/fl} endothelial cell isolates from two independent experiments.

to inhibit endothelial cell migration (34, 35, 55). Targeted disruption of *robo4* was found to exacerbate pathological angiogenesis (34). This report also provided evidence that Slit2 or Slit3 activation of Robo4 suppresses VEGF-induced endothelial cell migration, assembly, and permeability, likely through a

mechanism involving src family kinases and Rac GTPase (34). Slit2 also impaired vascular smooth muscle cell migration through suppression of Rac (46). These data are consistent with Slit/Robo-mediated axon repulsion (reviewed in reference 20) through repression of cdc42 and/or Rac GTPases by acti-

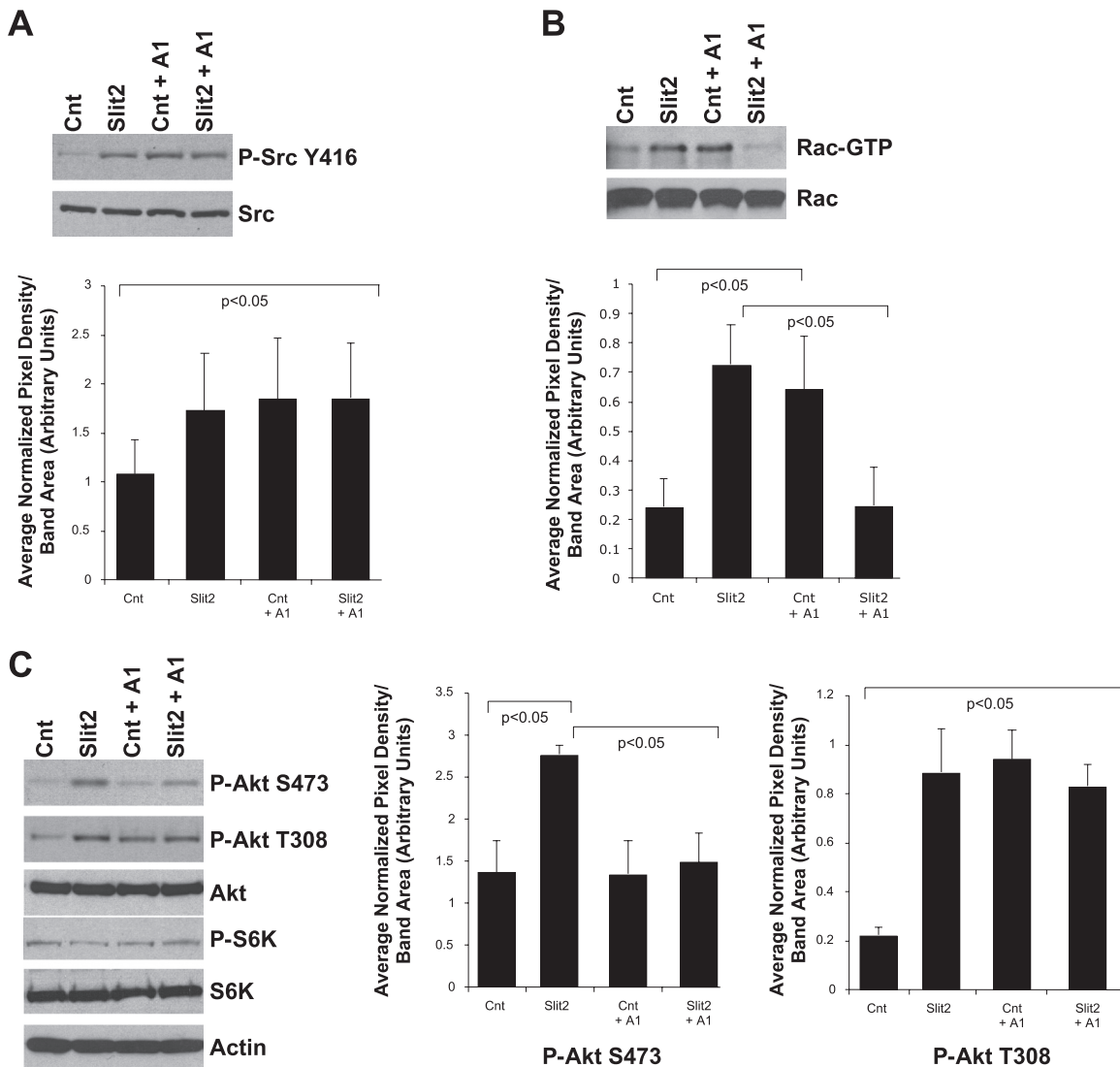


FIG. 7. Ephrin-A1 inhibits Slit2-induced activation of Rac and Akt. (A) Ephrin-A1-Fc costimulation did not affect src activity induced by Slit2. (B) While both Slit2 and ephrin-A1-Fc stimulate Rac activity in MPMEC, costimulation completely abolishes Rac activation, reducing levels of GTP-bound, active Rac below those observed in unstimulated cells. (C) Levels of Akt phosphorylation at serine 473, a substrate for mTORC2, but not threonine 308 (target of PDK1 upon PI 3-kinase activation) were reduced in ephrin-A1-Fc/Slit2-costimulated MPMEC relative to those for cells stimulated with Slit2 alone. Densitometry analyses are shown in bar graphs below (A and B) or adjacent to (C) blots. Cells were stimulated for 5 (Rac) to 10 (all others) min with the indicated factors. Data are a representation of the averages \pm standard deviations for at least three independent experiments/condition.

vation of GTPase activating proteins (GAPs), such as Vilsse/Cross GAP and Slit-Robo GAPs (srGAPs) (22, 30, 48).

Ample evidence also supports a proangiogenic function for Slit2 (reviewed in reference 43), which may be regulated by differential signaling between Robo1 and Robo4 (38, 63). Evidence supporting the proangiogenic function of Slit2 includes the observation that histone deacetylase 5 (HDAC5) inhibits angiogenesis through transcriptional repression of Slit2 and that exogenous Slit2 antagonizes the antiangiogenic effects of HDAC small interfering RNA (siRNA) knockdown (69). In addition, Robo4 activation of cdc42 and Rac in zebrafish was linked to angiogenic remodeling (37), consistent with our observation that Slit2 stimulates Rac activity in endothelium. Our data indicate that Slit2 stimulates activity of mTORC2 and Akt

in endothelium to stimulate angiogenesis. These data are consistent with reports showing that Akt1-deficient endothelial cells display defective sprouting and migration (16), as well as defective vascular remodeling upon inducible endothelial cell-specific expression of dominant active myr-Akt (57, 68). Interestingly, treatment with rapamycin almost completely blocked the formation of pathological blood vessels in myr-Akt1 transgenic mice (57), highlighting the importance of mTOR and Akt in angiogenic remodeling *in vivo*. Indeed, rictor knockdown in endothelium impaired migration, as well as activity, of both Rac and Akt (19). Thus, Slit2 activation of mTORC2/Akt could stimulate Rac-mediated angiogenesis, which could then be inhibited by ephrin-A1 costimulation.

Several studies have demonstrated that ephrin-A1 is re-

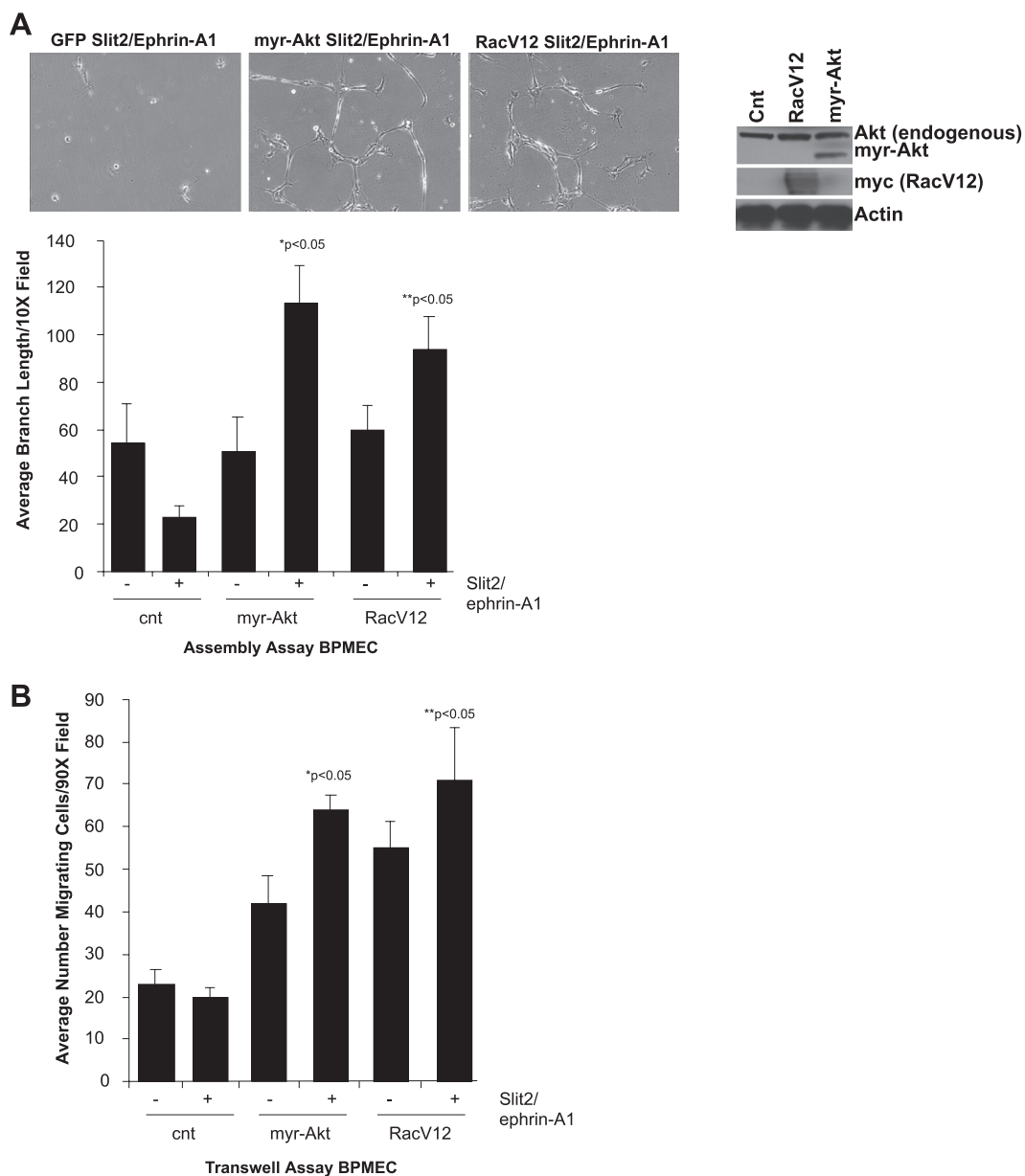


FIG. 8. Dominant active Akt and Rac variants rescue assembly and migration in ephrin-A1/Slit2-costimulated cells, supporting a model in which ephrin-A1 inhibits Slit2-mediated angiogenesis at the level of Akt and Rac. (A) Bovine pulmonary microvascular endothelial cells (BPMEC) were transfected with control GFP plasmid (cnt) or plasmids harboring a dominant active mutant of Akt (myr-Akt) or Rac (RacV12). Relative to results for control cells, myr-Akt or RacV12 expression enabled endothelial cells to assemble in response to ephrin-A1/Slit2. We confirmed expression of myr-Akt and myc-tagged RacV12 by immunoblotting. (B) We observed similar results for migration in response to ephrin-A1/Slit2 in transwell assays. Data are a representation of the averages \pm standards deviations for at least three independent experiments/condition. The *P* values shown compare control samples versus myr-Akt- or RacV12-expressing samples in the presence of Slit2/ephrin-A1, as well as myr-Akt- and RacV12-expressing samples that were unstimulated versus samples that were stimulated with Slit2/ephrin-A1.

quired for angiogenesis and tumor neovascularization, particularly upon stimulation of EphA2 (4, 6, 8–10, 15, 18, 23, 51, 53, 54). How, then, does ephrin-A1 inhibit angiogenesis in the context of Slit2? A recent study demonstrated that EphA2 serine 897 is phosphorylated (P-EphA2 S897) by Akt in response to multiple growth factors/serum, which links ligand-independent EphA2 signaling to increased cell motility (49). Ephrin-A1 blocks growth factor/serum-induced P-Akt S473 and P-EphA2 S897 concomitant with decreased cell migration.

We did not detect EphA2 serine phosphorylation, using a phospho-Akt substrate detection reagent (49), in Slit2-stimulated, ephrin-A1-stimulated, or costimulated endothelial cells under serum-free or normal endothelial cell growth conditions that we tested (data not shown), though ephrin-A1 activation of EphA2 could inhibit Slit2-induced Akt activity through a similar mechanism in endothelium. It is also possible that ephrin-A1 could inhibit mTOR activity in endothelium, as was recently observed in retinal axons in the context of mTORC1

(52), which could in turn impair downstream activation of Akt and/or Rac. Alternatively, ephrin-A1 could activate a serine/threonine phosphatase, which could inhibit Akt and/or mTOR function, as recently described by Yang et al. (74). Further investigation is required to identify the precise target of inhibition.

In summary, our data suggest that Slit2 alone stimulates angiogenesis through activation of mTORC2/Rac and Akt in endothelial cells. In the presence of ephrin-A1, however, Slit2 inhibits angiogenesis. Our data provide a plausible mechanism for the switch between pro- and antiangiogenic functions of Slit2, suggesting that the complex roles of Slit-Robo signaling in angiogenesis involve context-dependent mechanisms.

ACKNOWLEDGMENTS

We thank Carlos Arteaga and Mark Magnuson at Vanderbilt University for sharing the myr-Akt construct and floxed rictor mice, respectively. We thank Keunwook Lee at Vanderbilt University for assistance with experiments involving floxed rictor mice. We also thank Amanda Beauchamp and Waldemar Debinski at Wake Forest University School of Medicine for advice and protocols on analysis of soluble ephrin-A1.

This work was supported by a VA Merit Award through the Department of Veterans Affairs and NIH grants CA95004 and CA114301 to J. Chen and by NIH grant CA1179151 to D. M. Brantley-Sieders. Rebecca Cook is supported by NIH grant CA143126. Jane Y. Wu is supported by NIH grants CA114197 and CA107193 and by the James S. McDonnell Foundation. Mark Boothby is supported by NIH grant AI068149.

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