Annexin 2 Regulates Endothelial Morphogenesis by Controlling AKT Activation and Junctional Integrity

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Sprouting angiogenesis is a multistep process that involves endothelial cell activation, basement membrane degradation, proliferation, lumen formation, and stabilization. In this study, we identified annexin 2 as a regulator of endothelial morphogenesis using a three-dimensional in vitro model where sprouting angiogenesis was driven by sphingosine 1-phosphate and angiogenic growth factors. We observed that sphingosine 1-phosphate triggered annexin 2 translocation from the cytosol to the plasma membrane and its association with vascular endothelial (VE)-cadherin. In addition, annexin 2 depletion attenuated Akt activation, which was associated with increased phosphorylation of VE-cadherin and endothelial barrier leakage. Disrupting homotypic VE-cadherin interactions with EGTA, antibodies to the extracellular domain of VE-cadherin, or gene silencing all resulted in decreased Akt (but not Erk1/2) activation. Furthermore, expression of constitutively active Akt restored reduced endothelial sprouting responses observed with annexin 2 and VE-cadherin knockdown. Collectively, we report that annexin 2 regulates endothelial morphogenesis through an adherens junction-mediated pathway upstream of Akt.

Angiogenesis is the formation of new blood vessels from pre-existing structures, where endothelial cells (ECs) undergo morphogenic processes, including cell proliferation, invasion, migration, lumen formation, and sprouting (1–3). Because sprouting angiogenesis is a multistep process, standard two-dimensional cell culture environments cannot completely reflect this dynamic process. One experimental approach to overcome this restriction is to utilize in vitro three-dimensional models of EC invasion, where individual steps of angiogenesis can be reproduced (4–8). In the three-dimensional model we utilized in this study (9, 10), sphingosine 1-phosphate (SIP) together with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) synergize to induce rapid and robust endothelial morphogenesis, specifically EC invasion, which mimics sprout initiation during angiogenesis.

Angiogenic growth factors such as VEGF and bFGF are powerful pro-angiogenic stimuli, and multiple studies have documented the involvement of these growth factors and their receptors in mediating angiogenic events. In addition to polypeptide growth factors, S1P is a biologically active sphingolipid that mediates a variety of cellular responses (11–15) and has emerged as a target of anticancer therapies (16, 17). The downstream signaling activated by S1P has been extensively studied. Cellular responses initiated by S1P are through one or more of its five known G protein-coupled receptors, S1P<sub>1</sub>–S1P<sub>5</sub> (18). In human umbilical vein endothelial cells, which express S1P<sub>1</sub> and S1P<sub>3</sub>, it is known that S1P induced translocation of VE-cadherin, which is the major determinant of adherens junctions, and β-catenin to the endothelial junctions. This phenomenon required the activity of small GTPases Rho and Rac and was mediated by S1P<sub>1</sub> and S1P<sub>3</sub> (19). Cdc42 and Rac1 recently have been reported as key mediators of EC morphogenesis in three-dimensional collagen matrices (20). Moreover, cumulative evidence showed that S1P induced an increase in intracellular calcium concentration (21, 22). This increase in calcium influx occurred due to the release of Ca<sup>2+</sup> through activation of nonselective Ca<sup>2+</sup> channels on plasma membrane and inositol 1,4,5-triphosphate-sensitive channels on endoplasmic reticulum (22–24). In addition to calcium homeostasis, S1P has also been shown to induce membrane ruffles and cell spreading of ECs (25, 26) and to stimulate angiogenesis (18, 27–30).

We report here that annexin 2, a Ca<sup>2+</sup>-regulated membrane-binding protein, was differentially expressed in a proteomic screen designed to dissect downstream targets of S1P that regulate EC invasion. Annexin 2 was found to bind to the cytoskeletal proteins F-actin and nonerythroid spectrin 2 decades ago (31). Until now, it is believed that annexin 2 functions to organize the interface between the cytoplasm and plasma membrane by interacting with membrane phospholipids and actin filaments (32, 33). Recent gene silencing studies indicated a role for annexin 2 in regulating endocytic and secretory events, as well as adherens junction and actin dynamics (34–37). In addition, annexin 2 has also been shown to be associated with and required for the formation of actin-rich tight junctions (38). Here, we show that specific knockdown of annexin 2 in ECs decreased invasion responses and attenuated Akt activation, which is associated with impaired integrity of endothelial adherens junctions. These results indicate a functional requirement for annexin 2 during EC morphogenesis.

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The abbreviations used are: EC, endothelial cell; S1P, sphingosine 1-phosphate; VE, vascular endothelial; bFGF, basic FGF; TEER, transendothelial electrical resistance; B2M, β<sub>2</sub>-microglobulin; GTPγS, guanosine 5′-3′-O-(thio)triphosphate; GF, growth factor.
EXPERIMENTAL PROCEDURES

Endothelial Cell Culture and Invasion—Human umbilical vein endothelial cells (ECs), passage 3–6 (Lonza, Cambrex, MA), were passaged once weekly and cultured on gelatin-coated (1 mg/ml) tissue culture flasks in medium 199 (M199) containing 100 µg/ml heparin (Sigma), 0.4 mg/ml lyophilized bovine hypothalamic extract (Pel-Freeze Biologicals) (39), 15% fetal bovine serum (Lonza), antibiotics, and antimycotics (9). Collagen type I was isolated from tendons of one rat tail by incubation with gentle agitation in 150 ml of 0.1% acetic acid for 1 week. Supernatants were lyophilized, weighed, and resuspended in 0.1% acetic acid at 7.1 mg/ml and stored at 4 °C. In all invasion experiments, collagen matrices were prepared at 25 mg/ml with 1 µM S1P (Avanti Polar Lipids, Alabama) as reported previously (40). Gels (25 µl) were added to half-area (A/2) 96-well plates (Costar) and allowed to equilibrate for 45 min at 37 °C with 5% CO₂. Cells were fed 24 h before the beginning of each experiment. For all three-dimensional invasion experiments, confluent flasks of ECs were washed with 1 × HEPES-buffered saline, trypsinized, and counted. The final cell pellet was resuspended at a density of 30,000 cells per 50 µl in M199 and allowed to attach for 30 min before adding growth media (50 µl/well) containing 100 µg/ml bovine serum albumin (BSA) (Sigma), 5 µg/ml human holo-transferrin (Sigma), 5 µg/ml insulin (Sigma), 4.28 µg/ml sodium oleate (Sigma), and 5 ng/ml sodium selenite (Sigma).

Invasion Quantification—For quantifying the average numbers of invading cells per standardized field, conditioned media were removed, and invasion samples were fixed with 3% glutaraldehyde (Electron Microscopy Sciences) for 10 min, were stained with 0.1% toluidine blue (Sigma) containing 30% paraformaldehyde (Electron Microscopy Sciences), and allowed to dry in the dark prior to quantification. Eyepieces mounted with a vertical reticle displaying a 10 × 10 grid, which covers an area of 6.25, 1, and 0.25 mm² at 4 ×, 10 ×, and 20 ×, respectively, were used for quantifying average numbers of invading cells per standardized field. For each data set, three or four separate fields from each treatment were recorded and averaged.

Immunoblotting and Immunofluorescence—For immunoblotting, total lysates of invading cultures were prepared by removing conditioned media and transferring collagen gels containing invading ECs into boiling 1.5 × Laemmli sample buffer containing 2% β-mercaptoethanol at 95 °C for 10 min. Samples were separated using SDS-polyacrylamide gels and transferred to Immobilon PVDF membranes (Millipore). Antibodies against the following proteins were used for detection: annexin II (AF3928, BD Transduction Laboratories); β₂-microglobulin (M8523, Sigma); actin (CP01, Calbiochem); GAPDH (ab8245, Abcam); pan-cadherin (ab16505, Abcam); connexin43 (C6219, Sigma); calpain S1 (ab28237, Abcam); Akt (9272, Cell Signaling); phosphor-Akt (Ser-473, 4060, Cell Signaling); Erk2 (sc-153, Abcam), phosphor-p44/42 MAPK (p-Erk, 9101, Cell Signaling); Rac1 (ARC03, Cytoskeleton); Cdc42 (610928, BD Transduction Laboratories); VE-cadherin (sc-52751, Santa Cruz Biotechnology); PECAM1 (sc-1505, Santa Cruz Biotechnology); phosphor-VE-cadherin (Tyr-731, 441145G, Invitrogen); β-catenin (sc-7199, Santa Cruz Biotechnology); anti-FLAG M2 (F3165, Sigma); N-cadherin (sc-7939, Santa Cruz Biotechnology); claudin-5 (35-2500, Invitrogen); GFP (41), and HRP-conjugated secondary antibodies (Dako). Densitometric analysis of blots was performed using ImageJ software. For immunofluorescence analyses, ECs cultured on glass coverslips were rinsed with PBS and fixed in 100% cold methanol at −20 °C for 15 min. Nonspecific binding was blocked by incubation with PBS containing 1% BSA, 1% goat serum, and 0.1% Triton X-100 overnight at 4 °C. Following incubation with an anti-VE cadherin monoclonal antibody (Santa Cruz Biotechnology; 1:100 dilution) for 1 h, cells were washed three times. Alexa Fluor 594 goat anti-mouse IgG (Invitrogen; 1:200 dilution) was added for 1 h in PBS containing 1% BSA, 10% goat serum, and 0.1% Triton X-100. Samples were washed three times and mounted on glass slides with an anti-fading, aqueous mounting medium (Biomeda, Foster City, CA).

GTP-Rac1/GTP-Cdc42 Pulldown Assay—Rho GTPase activation assay during EC invasion in three-dimensional collagen matrices was performed as reported previously (42). In brief, EC cultures were extracted using cold detergent lysis buffer of 1% Triton X-100 in Tris-buffered saline, pH 8.0, containing Complete protease inhibitor mixture (Roche Diagnostics), 150 µg/ml high purity collagenase (Sigma), and 100 ng/ml GTPyS (Calbiochem). Lysates were incubated at 4 °C for 60 min to dissolve collagen and clarified by centrifugation at 15,000 × g for 15 min at 4 °C. Supernatants were incubated with GST-PAK-PBD protein-agarose beads (Cytoskeleton) for 45 min at 4 °C. The beads were washed four times. Bound active Rho GTPases were detected using Western blot analyses.

Plasmid Constructs, Gene Expression, and Gene Silencing—Recombinant lentiviral vector encoding an enhanced GFP was previously described (43) and was a kind gift from Dr. George E. Davis (Columbia, MO). For constructing the lentiviral vector that encodes annexin 2 fused to a C-terminal green fluorescent protein (ANXA2-GFP) and a C-terminal FLAG tag (ANXA2-FLAG), annexin 2 was amplified by PCR from EC cDNA and subcloned into pEGFP-N2 (Clontech) and pFLAG-CMV-5a (Sigma), respectively. The constructs were sequenced and tested for expression of the C-terminal GFP-tagged annexin 2 or C-terminal FLAG-tagged annexin 2 in HEK293 cells. ANXA2-GFP and ANXA2-FLAG were amplified by PCR using pEGFP-N2-based ANXA2-GFP vector and pFLAG-CMV-5a clone, respectively, as the templates and subcloned into pENTR4 (Invitrogen). Subsequently, ANXA2-GFP and ANXA2-FLAG were subcloned into pLent6/V5-DEST using the Gateway system (Invitrogen). For constructing the lentiviral vector that encodes myr-Akt, the insert was amplified by PCR using pcDNA3 Myr HA Akt1 (Addgene) as the template and subcloned into pENTR4 (Invitrogen). Subsequently, myr-Akt was subcloned into pLent6/V5-DEST using the Gateway system (Invitrogen) and sequenced. ECs were
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transduced with the indicated constructs using ViraPower lentiviral expression systems (Invitrogen) according to the manufacturer’s instructions. For gene silencing, ECs were infected with pLKO.1-puro lentiviral vectors encoding shRNAs against human annexin A2, β2-microglobulin, GFP, VE-cadherin, and PECAM1 (Sigma, sequences are available in the supplemental material) using ViraPower lentiviral expression system (Invitrogen). For annexin A2 and VE-cadherin knockdown, findings were reproduced by two distinct shRNAs. Invasion assays were conducted 3–7 days following infection. Alternatively, cells infected with lentiviruses were propagated and maintained in 2% of FCS for 7 days or until a monolayer was formed. The amount of FITC-dextran across endothelial monolayers was assessed 1 h later by taking 100-μl aliquots from the outer chambers and then measured using an Infinite M200 microplate reader (Tecan) with excitation/emission at 485/530 nm.

**Transendothelial Electrical Resistance (TEER)**—For measuring the TEER, 50,000 ECs were seeded on top of gelatin-coated Transwell inserts for 24-well plates (0.4-μm pore size; Costar) and transduced with lentiviruses for 8 h. Subsequently, medium containing lentiviruses was replaced with culture medium. TEER was monitored using an Evometer (World Precision Instruments) fitted with a Chopstick electrode every 24 h after lentiviral administration. Results were normalized by the area of the monolayer, and the background TEER of blank inserts was subtracted from the TEER of the EC monolayer.

**RESULTS**

**Identification of Annexin 2 as a Regulator of Endothelial Morphogenesis**—We have undertaken a proteomic screen to identify intracellular targets of S1P that regulate EC invasion and have identified annexin 2 (ANXA2), a Ca2+-regulated membrane-binding protein (supplemental Fig. 1). Because annexin 2 has been implicated in the formation of new blood vessels (44), we next investigated whether annexin 2 is functionally required for EC invasion in collagen matrices. To accomplish this, we used recombinant lentiviruses that expressed short hairpin RNAs (shRNAs) directed to ANXA2, β2-microglobulin (B2M), and GFP (as a scrambled control) to specifically knock down the expression of annexin 2 or β2-microglobulin and subsequently performed invasion assays. The EC invasion assay utilized throughout this study is a defined three-dimensional model of endothelial invasion where robust and rapid responses are triggered by S1P, bFGF, and VEGF (9, 10). Western blot analyses of extracts collected from invading cultures showed selective knockdown of annexin 2 and β2-microglobulin control with respective shRNAs (Fig. 1B). Photographs of invading cultures illustrated that invasion was significantly decreased in ECs expressing ANXA2 shRNA (Fig. 1A). Quantification of the invasion density revealed decreased numbers of invading cells with ANXA2 shRNA treatment (Fig. 1C). Thus, silencing of ANXA2 confirmed a functional involvement of annexin 2 in S1P and growth factor-stimulated EC invasion in three-dimensional collagen matrices.

**Membrane Translocation of Annexin 2 Was Driven by S1P**—Proposed to act as a membrane scaffold protein, most annexin 2 functions are linked to its ability to associate with cellular membranes in a regulated manner (32, 45). We therefore investigated whether the factors that promote invasion in our assay (e.g. S1P or angiogenic growth factors) stimulate annexin 2 localization from cytosol to plasma membrane. Recombinant lentiviruses that express annexin 2 fused to a C-terminal GFP, referred to as ANXA2-GFP, or GFP only were added to each sample, incubated for another 2 h, and washed extensively. Magnetic beads were suspended in 1× Laemmli sample buffer containing 2% β-mercaptoethanol for Western blot analyses.

**FITC-Dextran Permeability Assay**—Endothelial permeability was assessed by quantifying diffusion of FITC-dextran across endothelial monolayers. 50,000 ECs were seeded on top of gelatin-coated Transwell chambers in 24-well plates (0.4-μm pore size; Falcon) and transduced with lentiviruses for 8 h. Subsequently, medium containing lentiviruses was replaced with phenol red-free culture media, and cells were allowed to grow for 72 h, or until a monolayer was formed. Cells were serum-starved for 6 h in phenol red-free M199 (Invitrogen) prior to adding 25 μl of 20 μg/μl FITC-dextran (70 kDa; Sigma) into the upper chamber. The amount of FITC-dextran across endothelial monolayers was assessed 1 h later by taking 100-μl aliquots from the outer chambers and then measured using an Infinite M200 microplate reader (Tecan) with excitation/emission at 485/530 nm.

**Microscopy and Imaging**—For visualization of invasion responses, photographs of invading cells were taken from the top and side views using an Olympus CKX41 microscope equipped with a Q color 3 Olympus camera and 20× objective. For fluorescent imaging, photographs were taken under a Nikon Eclipse TE2000U fluorescence inverted microscope equipped with a CCD camera operated by Metamorph software (Universal Imaging Corp.).

**Subcellular Fractionation**—ECs were serum-starved for 4 h and treated with 1 μM S1P or the combination of 40 ng/ml VEGF and bFGF for 0, 30, and 60 min. EC membranes were prepared by incubating the cells in a lysis buffer (20 mM Hepes, pH 7.4, 20 mM NaCl, 1.5 mM MgCl2, 250 mM sucrose, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, Complete protease inhibitor mixture (Roche Diagnostics) and Halt phosphatase inhibitor mixture (Thermo Scientific)). Lysates were passed 10 times through a 25-gauge needle using a 1-ml syringe and kept on ice for 20 min. After homogenization, lysates were centrifuged at 1000 × g for 5 min at 4 °C to remove unbroken cells. The supernatants were collected and centrifuged at 150,000 × g for 30 min at 4 °C. The resulting supernatants corresponded to cytoplasmic fractions. Pellets, corresponding to the membrane fractions, were resuspended in lysis buffer containing 0.5% Nonidet P-40 and analyzed by Western blotting or utilized for immunoprecipitation.

**Immunoprecipitation**—Protein samples were incubated with 2 μg of the indicated antisera (rabbit anti-VE-cadherin, ab71285, Abcam; rabbit anti-FLAG, F7425, Sigma; and normal rabbit IgG, 2729, Cell Signaling) in 500 μl of lysis buffer containing 0.5% Nonidet P-40 overnight at 4 °C with gentle shaking. 20 μl of protein G Dynabeads (Invitrogen) were added to each sample, incubated for another 2 h, and washed extensively. Magnetic beads were suspended in 1× Laemmli sample buffer containing 2% β-mercaptoethanol for Western blot analyses.
or GFP were treated with S1P or angiogenic growth factors (GF). Fig. 2A shows that ANXA2-GFP was located within the cytoplasm and excluded from the nucleus, whereas GFP was distributed over both the cytoplasm and nucleus. Treatment with S1P resulted in the apparent enrichment of ANXA2-GFP at the plasma membrane (Fig. 2A, arrowheads), whereas GF failed to induce the membrane translocation of ANXA2-GFP and the formation of membrane ruffles. To provide additional biochemical evidence for annexin 2 localization, membranes were prepared from EC monolayers treated with S1P or GF, and then immunoblotted for annexin 2 and various other cytoplasmic and plasma membrane markers. Annexin 2 levels increased in membrane fractions following stimulation with S1P (Fig. 2B), but remained unchanged following stimulation with GF (Fig. 2C). Akt and Erk activation were examined to validate the effects of two stimuli, S1P and GF, respectively. We observed a significant increase in Akt activation with S1P treatment (Fig. 2B) but not with GF treatment (Fig. 2C). In contrast, Erk activation was induced substantially by GF (Fig. 2C) but not S1P (Fig. 2B). Detection of connexin 43 and calpain S1 in membrane and cytoplasmic fractions, respectively, revealed successful partitioning of the indicated subcellular fractions. Thus, S1P induced membrane translocation of annexin 2 and increased Akt activation.

Annexin 2 Depletion Attenuated Akt Activation during EC Invasion—EC morphogenesis is regulated by a broad spectrum of intracellular signaling pathways, many of which are known to be mediated by S1P receptors (47). To more clearly determine the molecular mechanism by which annexin 2 modulates the EC invasion, we explored whether annexin 2 depletion affected any signaling pathways that are involved in angiogenesis and downstream of S1P receptors, including Akt, Erk, and the small GTPases, Cdc42 and Rac1. ECs expressing ANXA2 or B2M shRNA were placed in invasion assays on three-dimensional collagen matrices containing 1 μM S1P and 40 ng/ml GF, and extracts were collected at the indicated times for Western blot analyses. Strikingly, silencing of annexin 2 expression decreased Akt activation (Fig. 3A). However, neither annexin 2 nor β2-microglobulin depletion altered Erk activation during EC invasion (Fig. 3B). To examine Cdc42 and Rac1 activation, cell lysates were incubated with GST-PAK-PBD protein-agarose beads and analyzed for GTP-bound Cdc42 or Rac1. No apparent differences were observed in the activation of the Rac1 or Cdc42 GTPases following annexin 2 or β2-microglobulin knockdown compared with the nontransduced control in extracts collected at 16 h of invasion. Similar results were seen in extracts analyzed at 3 h (data not shown). Altogether, annexin 2 does not appear to modulate Erk, Rac1, or Cdc42 activation. Rather, Akt is coupled with an annexin 2-mediated signaling pathway during EC invasion.

Annexin 2 Modulated Akt Activation by Stabilizing Adherens Junctions—Akt has been demonstrated to serve as a crucial regulator of vascular permeability both in vivo and in vitro (48–50). In addition, annexin 2 recently was reported to contribute to the establishment of mature endothelial adherens

FIGURE 1. Knockdown of ANXA2 interfered with EC invasion. A, photographs illustrating the invasion responses (top view, upper panel; side view, lower panel). ECs were transduced with lentiviruses expressing the indicated shRNAs and selected with puromycin prior to seeding on the surface of collagen matrices containing 1 μM S1P. Cultures were grown in media containing RSII, 50 μg/ml ascorbic acid, and 40 ng/ml VEGF and bFGF as described under “Experimental Procedures.” Cultures were fixed at 24 h and stained with toluidine blue. Black arrowhead indicates monolayer of endothelial cells. Scale bar, 100 μm. B, verification of annexin 2 and B2M protein suppression using extracts collected after 24 h of invasion. Antibodies against annexin 2, B2M, as an experimental control, and actin, as a loading control, were used for Western blot analyses. C, quantification of EC invasion density after 24 h of invasion. Data represent average numbers of invading cells per standardized field (n = 3 fields, Student’s t test; *, p < 0.01). “

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junctions, which are prominent in the maintenance of vascular endothelial integrity (35). Because we observed that membrane translocation of annexin 2 was driven by S1P, which is also known as an endothelial barrier-enhancing agent (51), we determined whether the activation of Akt during EC invasion required the integrity of adherens junctions. Ca$^{2+}$-dependent, homotypic VE-cadherin interactions were disrupted using three different methods as follows: 1) addition of increasing concentrations of EGTA to culture medium to chelate extracellular Ca$^{2+}$; 2) addition of function-blocking antibodies directed to the extracellular domain of VE-cadherin, and 3) gene silencing with VE-cadherin shRNAs. Cells that underwent various treatments were placed in three-dimensional invasion assays in the presence of S1P and GF. All three treatments decreased Akt activation during EC invasion (Fig. 4) and hindered the ability of cells with perturbed adherens junctions to invade (supplemental Fig. 2). Addition of EGTA to culture medium caused attenuated activation of Akt in a dose-dependent manner but did not decrease Erk activation (Fig. 4A). Immunofluorescence analyses revealed that EGTA resulted in a loss of VE-cadherin at intercellular junctions (data not shown). Similar results were obtained by the addi-

FIGURE 2. S1P triggered the membrane translocation of annexin 2. A, fluorescent images of annexin 2 distribution in control, S1P-, or GF-stimulated ECs. ECs were transduced with lentiviruses expressing annexin 2 fused to a C-terminal green fluorescent protein (ANXA2-GFP) or green fluorescent protein only (GFP) and selected with blasticidin. Cells that stably express ANXA2-GFP or GFP were treated without (control) or with 1 μM S1P (S1P) or the combination of 40 ng/ml VEGF and bFGF (GF) for 30 min prior to fixing and imaging with epi-fluorescent microscopy. The right panels are magnified images of the areas boxed in the left panels (Magn.). Scale bar, 10 μm. B and C, biochemical analyses of annexin 2 recruitment to membranes. ECs were stimulated with 1 μM S1P (B) or the combination of 40 ng/ml VEGF and bFGF (GF) (C) for indicated times. Plasma membrane (Membrane) and cytoplasmic (Cytosol) fractions were prepared and immunoblotted with antibodies against the indicated proteins. Blots are representative of four independent experiments. Densitometric analyses were quantified by ImageJ software and expressed as means ± S.E. in arbitrary units (a.u.) (n = 3, Student’s t test; *, p < 0.01, compared with unstimulated controls).
tion of VE-cadherin blocking antibodies (Fig. 4B) or gene silencing of VE-cadherin (Fig. 4C). Incubation of monoclonal VE-cadherin antibodies has been shown to increase paracellular permeability, inhibit VE-cadherin reorganization, and block angiogenesis in vitro (52). VE-cadherin antagonism with CDH5 antibodies resulted in decreased Akt activation during EC invasion. These findings were reproduced using two additional VE-cadherin-specific antisera (BV9, Santa Cruz Biotechnology, and ab71285, Abcam; data not shown). Moreover, expression of VE-cadherin shRNA reduced the level of VE-cadherin protein by ~90% and reduced Akt activation as well (Fig. 4C). Gene silencing of platelet endothelial cell adhesion molecule 1 (PECAM1), another transmembrane homotypic adhesion protein expressed on human umbilical vein endothelial cells, did not alter Akt activation levels. Consistently, Erk activation during EC invasion remained unchanged by either VE-cadherin function-blocking antibodies or shRNA expression. These findings demonstrate that disruption of

FIGURE 3. Loss of annexin 2 leads to attenuation of Akt activation during EC invasion. A and B, Akt and Erk activation in annexin 2- or B2M-depleted cells during EC invasion on three-dimensional collagen matrices. Cultures were allowed to invade for 1 h with the indicated concentrations of EGTA, and extracts were collected for Western blot analyses using antibodies directed to phosphor-Akt (Ser-473) and total Akt (A) or phosphor-p44/42 MAPK (p-Erk) and total Erk (B). Control (ctl) represents cells cultured on collagen matrices in the absence of S1P and growth factors for 0.5 h. C, Rac1 and Cdc42 activation is not altered in annexin 2- or B2M-depleted cells during EC invasion. Puromycin-selected ECs were cultured on the surface of collagen matrices for 16 h. Equal amounts of extracts were prepared and incubated with GST-PAK-PBD protein-agarose beads. Eluates and starting lysates were analyzed by Western blot analyses using antibodies against Rac1, Cdc42, annexin 2, or GAPDH (as a loading control). Blots are representatives of three independent experiments. Densitometric analyses represent means ± S.E. (n = 3).

FIGURE 4. Akt activation during EC invasion requires intact adherens junctions. A, indicated concentrations of EGTA were administered to EC suspensions for 10 min prior to seeding on three-dimensional collagen matrices. Cultures were allowed to invade for 1 h with the indicated concentrations of EGTA, and extracts were collected for Western blot analyses using antibodies directed to phosphor-Akt (Ser-473). Akt, phosphor-p44/42 MAPK (p-Erk), Erk, or GAPDH (as a loading control). B, ECs were incubated with 50 μg/ml VE-cadherin (VE-cad) antibody (sc-52751, Santa Cruz Biotechnology, or 610252, BD Transduction Laboratories), 50 μg/ml isotype control antibody (ab18414, Abcam), or PBS containing 0.1% BSA only (vehicle) for 30 min prior to seeding on collagen matrices. Cells were allowed to invade in the presence of indicated antibodies for 1 h, and extracts were prepared and immunoblotted for phosphor-Akt (Ser-473), Akt, phosphor-p44/42 MAPK (p-Erk), Erk, or actin (as a loading control). C, ECs were transduced with lentiviruses expressing indicated shRNAs for 3 days and subsequently used for invasion assays. Extracts were collected following 1 h of invasion and analyzed by Western blot using the indicated antibodies. Blots are representative of three independent experiments. Densitometric analyses represent means ± S.E. (n = 3, Student’s t test; *, p < 0.05; **, p < 0.01, compared with corresponding controls).
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VE-cadherin interactions caused a decrease in Akt activation during EC sprouting in three-dimensional collagen matrices. This observation is consistent with what we observed following ANXA2 gene knockdown.

Because Akt activation during EC invasion required annexin 2 and intact adherens junctions, we next explored whether annexin 2 regulated endothelial permeability, an endothelium-specific function that is mediated in part by adherens junctions. It has been demonstrated that tyrosine phosphorylation of VE-cadherin is associated with weak junctions and impaired barrier function (53). We observed that phosphorylation of VE-cadherin was higher in annexin 2-depleted cells from invading cultures by Western blot analyses using antibodies directed against phosphotyrosine 731 of VE-cadherin (Fig. 5A). These data suggest that annexin 2 may positively regulate junctional integrity, thereby enhancing barrier function. We next determined whether annexin 2 contributed to regulation of endothelial permeability. To investigate this, the TEER of annexin 2-depleted cells was assessed. Cells were transduced with shB2M, shANXA2, and shVE-cad lentiviruses and seeded on Transwell inserts. The TEER value was monitored 24, 48, and 72 h post-shRNA administration. A decrease in TEER of annexin 2-depleted cells was observed 48 h post-transduction (−18.37%, \( p = 0.038 \), at 48 h, and −36.2%, \( p = 0.007 \), at 72 h, compared with B2M control at the same time point, see Fig. 5B). Specific down-regulation of VE-cadherin also caused a significant decrease in TEER (−48.15%, \( p = 0.006 \), at 48 h, and −53.15%, \( p = 0.002 \), at 72 h, see Fig. 5B) that remained stable between 48 and 72 h. Following quantification, successful knockdown of annexin 2, VE-cadherin and B2M were verified by Western blot analyses using cell extracts collected from cultures (data not shown).

We further analyzed the effect of annexin 2 on the endothelial barrier stabilization in the presence or absence of S1P using FITC-dextran permeability assays. In these experiments, EC monolayers were serum-starved for 6 h and treated with or without 1 μM S1P for 1 h prior to adding FITC-labeled dextran into the upper chambers. Endothelial permeability (fluorescence in the lower chamber) was measured at 1 h after the addition of FITC-dextran. Data presented are average values ± S.E. from representative experiments (\( n = 4 \), Student’s t test; *, \( p < 0.05 \); **, \( p < 0.01 \), compared with B2M controls at the same time point).

FIGURE 5. Annexin 2 contributed to junctional integrity of EC monolayer. A, knockdown of annexin 2 augments tyrosine phosphorylation of VE-cadherin (VE-cad) during EC invasion. ECs treated with (shANXA2) or without (wild type, WT) lentiviruses that express ANXA2 shRNA were used for invasion assays. Extracts were prepared from invading cultures at indicated time points and immunoblotted with phosphor-VE-cadherin (Tyr-731), VE-cadherin, annexin 2, or actin (as a loading control). Control (ctl) represents ECs that were cultured on collagen matrices in the absence of S1P and growth factors for 0.5 h. B, TEER in annexin 2-depleted EC monolayer. ECs were seeded on Transwell inserts and subsequently transduced with lentiviruses expressing indicated shRNAs. The resistance was monitored at the time points indicated after lentiviral transduction. Data were normalized and presented as average TEER values ± S.E. from two experiments (\( n = 3 \), Student’s t test; *, \( p < 0.05 \); **, \( p < 0.01 \), compared with B2M controls at the same time point). C, FITC-dextran flux permeability assay. ECs seeded on Transwell inserts were transduced with lentiviruses expressing shRNAs indicated and grown to confluence. EC monolayers were serum-starved for 6 h and treated with or without 1 μM S1P for 1 h prior to adding FITC-labeled dextran into the upper chambers. Endothelial permeability (fluorescence in the lower chamber) was measured at 1 h after the addition of FITC-dextran. Data presented are average values ± S.E. from representative experiments (\( n = 4 \), Student’s t test; *, \( p < 0.05 \); **, \( p < 0.01 \), compared with B2M controls).

Because Akt activation during EC invasion required annexin 2 and intact adherens junctions, we next explored whether annexin 2 regulated endothelial permeability, an endothelium-specific function that is mediated in part by adherens junctions. It has been demonstrated that tyrosine phosphorylation of VE-cadherin is associated with weak junctions and impaired barrier function (53). We observed that phosphorylation of VE-cadherin was higher in annexin 2-depleted cells from invading cultures by Western blot analyses using antibodies directed against phosphotyrosine 731 of VE-cadherin (Fig. 5A). These data suggest that annexin 2 may positively regulate junctional integrity, thereby enhancing barrier function. We next determined whether annexin 2 contributed to regulation of endothelial permeability. To investigate this, the TEER of annexin 2-depleted cells was assessed. Cells were transduced with shB2M, shANXA2, and shVE-cad lentiviruses and seeded on Transwell inserts. The TEER value was monitored 24, 48, and 72 h post-shRNA administration. A decrease in TEER of annexin 2-depleted cells was observed 48 h post-transduction (−18.37%, \( p = 0.038 \), at 48 h, and −36.2%, \( p = 0.007 \), at 72 h, compared with B2M control at the same time point, see Fig. 5B). Specific down-regulation of VE-cadherin also caused a significant decrease in TEER (−48.15%, \( p = 0.006 \), at 48 h, and −53.15%, \( p = 0.002 \), at 72 h, see Fig. 5B) that remained stable between 48 and 72 h. Following quantification, successful knockdown of annexin 2, VE-cadherin and B2M were verified by Western blot analyses using cell extracts collected from cultures (data not shown).

We further analyzed the effect of annexin 2 on the endothelial barrier stabilization in the presence or absence of S1P using FITC-dextran permeability assays. In these experiments, EC monolayers were serum-starved for 6 h and treated with or without 1 μM S1P for 1 h prior to adding FITC-labeled dextran into the upper chambers. Endothelial permeability (fluorescence in the lower chamber) was measured at 1 h after the addition of FITC-dextran. Data presented are average values ± S.E. from representative experiments (\( n = 4 \), Student’s t test; *, \( p < 0.05 \); **, \( p < 0.01 \), compared with B2M controls).
tion. Similarly, expression of VE-cadherin shRNA augmented the flux across EC junctions compared with B2M controls either with (S1P) or without S1P treatment (32.5%, p = 0.031) as expected. S1P did not significantly strengthen the barrier function of VE-cadherin-depleted cells (16.35%, p = 0.097).

It has been shown that blocking adherens junctions interferes with the correct organization of tight junctions or the expression of tight junction components in various cell systems (54–56). Therefore we further examined whether annexin 2 depletion altered the organization or expression of claudin-5, an EC-specific tight junction protein (57). However, neither expression nor localization of claudin-5 was altered by appreciable knockdown of annexin 2 or VE-cadherin (supplemental Fig. 4). These results indicate that annexin 2 is functionally involved in regulating the integrity of adherens junctions.

**S1P Stimulation Induced the Association of Annexin 2 with VE-cadherin**—The above results show that annexin 2 stabilized adherens junctions, and S1P stimulated membrane recruitment of annexin 2. We therefore reasoned that S1P might promote the association of annexin 2 and VE-cadherin. To test this, we isolated membrane fractions of S1P- or angiogenic GF-stimulated EC monolayers and performed immunoprecipitations using an antibody directed to VE-cadherin. Eluates were probed for annexin 2, VE-cadherin, and proteins known to form a complex at cell-cell contacts (β-catenin and actin) (58). As shown in Fig. 6A, S1P (but not GF) stimulation increased the amount of annexin 2 that immunoprecipitated with VE-cadherin, although equivalent quantities of VE-cadherin were immunoprecipitated in all conditions. Moreover, this increase appeared independent of the association of VE-cadherin with β-catenin and actin. In addition, we performed the reverse immunoprecipitation experiment. Extracts of S1P-treated ECs expressing annexin 2 tagged with a C-terminal FLAG epitope (ANXA2-FLAG) were incubated with an antibody against FLAG to verify the physical interaction of annexin 2 with VE-cadherin, although N-cadherin was not detected in anti-annexin2-FLAG IPs (Fig. 6B).

To demonstrate the co-localization of annexin 2 and VE-cadherin following S1P stimulation, immunofluorescence analyses of VE-cadherin were performed using ECs expressing ANXA2-GFP in the absence or presence of S1P. In the absence of S1P, little annexin 2 was detectable at cell-cell junctions (Fig. 6C). However, ECs stimulated with S1P showed increased co-localization of annexin 2 and VE-cadherin at cell-cell contacts in the presence of S1P. Together, the results indicate that S1P induced annexin 2 translocation to the plasma membrane, where annexin 2 complexed with VE-cadherin.
Akt Activation Restored Annexin 2-mediated Aberration of EC Invasion—We next sought to determine whether annexin 2-mediated attenuation of Akt activation is a crucial event in EC morphogenesis and is responsible for impaired invasion responses. Treatment of ECs with a specific Akt inhibitor, Akt inhibitor X, which selectively inhibits the phosphorylation of Akt and its in vitro kinase activity (59), resulted in a dose-dependent decrease in Akt activation and invasion responses (data not shown). To analyze the potential involvement of Akt in annexin 2-mediated EC morphogenesis, we introduced a constitutively active version of Akt (myr-Akt) into annexin 2-depleted ECs. Recombinant lentiviruses that express myr-Akt or GFP were generated and used to induce Akt activation in ECs stably expressing shRNAs against ANXA2 or B2M. Western blot analyses of extracts collected from invading cultures revealed successful protein expression of myr-Akt and GFP as well as selective knockdown of annexin 2 and B2M control (Fig. 7B). Imaging of invading cultures revealed that the invasion density and length were both restored in annexin 2-depleted ECs that expressed Myr-Akt (Fig. 7C).

Quantification of the invasion density showed that shB2M and GFP co-expressing cells were not hindered in their ability to invade. However, knockdown of annexin 2 (shANXA2) was not rescued by GFP (Fig. 7A). Knockdown of annexin 2 (shANXA2) combined with GFP expression was comparable with ANXA2 silencing alone (Fig. 1C). However, myr-Akt expression partially restored the ability of annexin 2-depleted cells to invade, although it did not enhance invasion in ECs depleted of B2M. Moreover, because specific VE-cadherin reduction diminished Akt activation during EC invasion (Fig. 4C), we also determined whether activated Akt could overcome deficiencies of VE-cadherin expression and rescue invasion responses. Examination of invasion cultures using ECs expressing either VE-cadherin or B2M shRNA revealed that myr-Akt recovered VE-cadherin-mediated impairment of EC invasion as well (supplemental Fig. 5). Besides, myr-Akt also enhanced the endothelial barrier integrity in ECs depleted of B2M, annexin 2, or VE-cadherin in the presence of S1P (Fig. 7D and supplemental Fig. 5C). These results indicate that Akt is activated downstream of an adherens junction-mediated pathway, and Akt activation requires annexin 2 during endothelial morphogenesis.

DISCUSSION

In this study, we demonstrate that annexin 2 regulates endothelial morphogenesis through an adherens junction-mediated pathway upstream of Akt. Annexin 2 is a multifunctional protein and has been reported to be required for cell spreading and adhesion (60, 61). In addition, annexin 2 has also been implicated in developmental mechanisms, such as the establishment of epithelial polarity and the formation of new blood vessels (44, 62). Because annexin 2 is proposed as a membrane scaffold protein, its functions are commonly ascribed to an ability to associate with plasma membrane, which
can be regulated by various factors in different conditions. Intracellular Ca\(^{2+}\) mobilization is the primary signal to induce recruitment of annexin 2 to membranes (33). Here, for the first time, we have shown that membrane translocation of annexin 2 is triggered by S1P, which increases intracellular Ca\(^{2+}\) levels (21, 22). In addition to elevating intracellular Ca\(^{2+}\) concentration, other mechanisms have been reported to target annexin 2 to membranes, including tyrosine phosphorylation and cleavage of annexin 2 (61, 63, 64). Also, cell density may mediate the membrane translocation of annexin 2 in particular circumstances (35). Considerable evidence has revealed that the specificity for membrane targeting of annexin 2 lies in cholesterol-enriched membrane rafts (63, 65). Recently, study of S1P-induced barrier stabilization indicated that annexin 2 was present in membrane rafts as human pulmonary artery endothelial cells were challenged with S1P (66). Our data, together with findings from others, suggest that annexin 2 acts downstream of S1P signaling to regulate various cellular responses, including endothelial morphogenesis. Determining the mechanism by which S1P induces recruitment of annexin 2 to membranes will require further investigation.

ECs are interconnected via three distinct junctional structures, including adherens junctions, tight junctions, and gap junctions. Adherens junctions and tight junctions are mainly responsible for intercellular adhesion via the formation of actin filament-associated protein complexes along transmembrane adhesion sites (67) and both require the presence of annexin 2 (35, 38). Because our previous work showed that a cohort of cell adhesion molecule genes involved in assembly and regulation of adherens junction were up-regulated, whereas a set of genes associated with tight junctions were down-regulated, during EC invasion (10), we speculated that the action of annexin 2 on EC sprouting behaviors was associated with adherens junctions. In this study, we demonstrated a functional role for annexin 2 in adherens junction-related cellular responses, including endothelial permeability on transwell membranes, along with tyrosine phosphorylation of VE-cadherin and Akt activation during EC invasion. Importantly, annexin 2 depletion dampened the endothelial barrier function in the presence and absence of S1P (Fig. 5C), but annexin 2 knockdown did not alter Rac1 activation in response to S1P (supplemental Fig. 3). Akt and Rac1 have been shown to be involved in S1P-mediated enhancement of endothelial barrier integrity (68, 69). Our data, together with findings from others (69), may suggest that Rac1 acts upstream of annexin 2 in S1P-mediated signaling. Alternatively, the role of S1P in Rac1 signaling may be cell type-dependent. In addition, it is intriguing that annexin 2 depletion augmented tyrosine phosphorylation of VE-cadherin during EC invasion, although the mechanism of VE-cadherin phosphorylation has not yet been fully clarified. This finding may be due to either a decrease in Al-associated phosphatases recruited by annexin 2 or enhanced kinase activity in the absence of annexin 2. Moreover, we observed increased association of annexin 2 with VE-cadherin following S1P stimulation, suggesting that annexin 2 may play a role in S1P-enhanced endothelial barrier function. S1P signaling not only regulates the localization of VE-cadherin and other catenins at endothelial junctions but also the expression of these junctional molecules (19, 70, 71). In this study, similar effects of S1P were observed for expression and localization of annexin 2. Hence, with S1P stimulation, the functional connection of annexin 2 with the integrity of endothelial adherens junctions plays a key role in modulating EC invasion.

We have previously shown that inhibition of Akt activation diminished the EC invasion responses induced by the combined application of fluid shear stress and S1P (72). Akt is critical in the regulation of various EC functions and maintenance of vascular integrity. Here, we demonstrate that knockdown of annexin 2 attenuated Akt activation and invasion responses during EC invasion, as did disrupting the homotypic binding of VE-cadherin. Expression of a constitutively active version of Akt partially but significantly restored the impairment of invasion responses as well as endothelial barrier functions caused by depletion of annexin 2 or VE-cadherin. This finding is consistent with the proposed role of Akt in vascular maturation and angiogenesis during wound healing (73), which is mimicked in our three-dimensional in vitro model (10). In addition, Akt was shown to be crucial for the restoration of reactive oxygen species-stressed barrier integrity in human cardiac microvascular ECs (50), which is consistent with our findings that annexin 2 knockdown decreased Akt activation and increased endothelial permeability. However, introducing constitutively active Akt did not fully rescue EC invasion responses blocked by annexin 2 knockdown, whereas constitutively active Akt almost completely rescued EC invasion responses in VE-cadherin-depleted cells. One explanation for this outcome is that annexin 2 is recognized as a multifunctional protein and is implicated in various molecular mechanisms, including (but not limited to) the regulation of actin dynamics as well as endocytic and secretory pathways (34, 36, 37). The expression of activated Akt is insufficient to compensate completely for the depletion of annexin 2 during EC invasion. This suggests other ancillary roles for annexin 2 in endothelial morphogenesis. Furthermore, Akt modulates various signaling pathways, many of which have been shown to be important for angiogenesis in different scenarios (74). The signaling pathways downstream of annexin 2-mediated Akt activation that control EC morphogenesis remain to be elucidated.

In conclusion, we have demonstrated that annexin 2 regulates endothelial morphogenesis via an adherens junction-mediated pathway upstream of Akt, revealing for the first time that annexin 2 regulates Akt activation during sprouting angiogenesis driven by S1P and angiogenic growth factors. Our results provide novel insight into annexin 2 function, which is not only activated downstream of S1P signaling but is also linked to the stabilization of adherens junctions.

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REFERENCES

1. Iruela-Arispe, M. L., and Davis, G. E. (2009) Dev. Cell 16, 222–231
2. Adams, R. H., and Altitude, K. (2007) Nat. Rev. Mol. Cell Biol. 8, 464–478
3. Kalluri, R. (2003) Nat. Rev. Cancer 3, 422–433
4. Davis, G. E., and Camarillo, C. W. (1996) Exp. Cell Res. 224, 39–51
5. Eggington, S., and Gerritsen, M. (2003) Microcirculation 10, 45–61
6. Montesano, R., Pepper, M. S., Vassalli, J. D., and Orci, L. (1992) Exs 61, 129–136
7. Nicosia, R. F., and Villaschi, S. (1999) Int. Rev. Cytol. 185, 1–43
8. Vernon, R. B., and Sage, E. H. (1995) Am. J. Pathol. 147, 873–883
9. Bayless, K. I., Kwak, H. I., and Su, S. C. (2009) Nat. Protoc. 4, 1888–1898
10. Su, S. C., Mendoza, E. A., Kwak, H. I., and Bayless, K. J. (2008) Am. J. Physiol. Cell Physiol 295, C1215–C1229
11. Rivera, J., Proia, R. L., and Olivera, A. (2008) Nat. Rev. Immunol. 8, 753–763
12. Rosen, H., and Goetzl, E. J. (2005)
13. Maciag, T., Cerundolo, J., Ilsley, S., Kelley, P. R., and Forand, R. (1979)
14. Gerke, V., and Moss, S. E. (2004) Nat. Rev. Mol. Cell Biol. 5, 649–461
15. Merrifield, C. J., Rescher, U., Almers, W., Proust, J., Gerke, V., Sechi, A. S., and Moss, S. E. (2001) Curr. Biol. 11, 1136–1141
16. Sanchez, T., and Hla, T. (2004) J. Cell. Biochem. 92, 913–922
17. Visentin, B., Vekich, J. A., Sibbald, B. J., Cavalli, A. L., Moreno, K. M., Kwak, H. I., and Kwon, Y. G. (1999)
18. Lee, O. H., Kim, Y. M., Lee, Y. M., Moon, E. J., Lee, D. J., Kim, J. H., Kim, J. W., and Hla, T. (1999) Cell 99, 301–312
19. Koh, W., Mahan, R. D., and Davis, G. E. (2008) J. Cell Sci. 121, 899–1001
20. Ansell, N., and Hla, T. (1999) J. Biol. Chem. 274, 18997–19002
21. Muraki, K., and Imai, Z. (2001) J. Physiol. 537, 431–441
22. Nilius, B., and Droogmans, G. (2001) Physiol. Rev. 81, 1445–1459
23. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Science 287, 1604–1605
24. Endo, A., Nagashima, K., Kurose, H., Mochnizuki, S., Matsuda, M., and Mochnizuki, N. (2002) J. Biol. Chem. 277, 23747–23754
25. Paik, J. H., Chae, S., Lee, M. J., Thangada, S., and Hla, T. (2001) J. Biol. Chem. 276, 11830–11837
26. English, D., Brindley, D. N., Spiegel, S., and Garcia, J. G. (2002) Biochem. Biophys. Acta 1582, 228–239
27. Hla, T. (2004) Semin. Cell Dev. Biol. 15, 513–520
28. Langois, S., Gingras, D., and Beliveau, R. (2004) Blood 103, 3020–3028
29. Lee, O. H., Kim, Y. M., Moon, E. J., Lee, D. J., Kim, J. H., Kim, K. W., and Kwon, Y. G. (1999) Biochem. Biophys. Res. Commun. 264, 743–750
30. Gerke, V., and Weber, K. (1984) EMBO J. 3, 227–233
31. Rescher, U., and Gerke, V. (2004) J. Cell Sci. 117, 2631–2639
32. Gerke, V., and Moss, S. E. (2002) Physiol. Rev. 82, 331–371
33. Hayes, M. J., Shao, D., Bally, M., and Moss, S. E. (2006) EMBO J. 25, 1816–1826
34. Heyraud, S., Jaquinod, M., Durmott, C., Dambroise, E., Concord, E., Schaal, J. P., Huber, P., and Gulino-Debrac, D. (2008) Mol. Cell. Biol. 28, 1657–1668
35. Harder, T., and Gerke, V. (1993) J. Cell Biol. 123, 1119–1132
36. Knop, M., Aareskjold, E., Bode, G., and Gerke, V. (2004) EMBO J. 23, 2982–2992
37. Yamada, A., Fujita, N., Sato, T., Okamoto, R., Ososho, T., Hirota, T., Morimoto, K., Irie, K., and Takai, Y. (2006) Oncogene 25, 5085–5102
38. Maciag, T., Cerundolo, J., Isley, S., Kelley, P. R., and Forand, R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5674–5678
39. Bayless, K. J., and Davis, G. E. (2003) Biochem. Biophys. Res. Commun. 312, 903–913
40. Bayless, K. J., and Davis, G. E. (2002) J. Cell Sci. 115, 1123–1136
41. Koh, W., Stratman, A. N., Sachardou, A., and Davis, G. E. (2008) Methods Enzymol. 443, 83–101
42. Saunders, W. B., Bohnsack, B. L., Faske, J. B., Anthis, N. J., Bayless, K. J., Hirsch, K. K., and Davis, G. E. (2006) J. Cell Biol. 175, 179–191
43. Ling, Q., Iacovina, A. T., Deora, A., Febrario, M., Simantov, R., Silverstein, R. L., Hempstead, B., Mark, W. H., and Hajjar, K. A. (2004) J. Clin. Invest. 113, 38–48
44. Gerke, V., Creutz, C. E., and Moss, S. E. (2005) Nat. Rev. Mol. Cell Biol. 6, 449–461
45. Merrifield, C. J., Rescher, U., Almers, W., Proust, J., Gerke, V., Sechi, A. S., and Moss, S. E. (2001) Curr. Biol. 11, 1136–1141
46. Sanchez, T., and Hla, T. (2004) J. Cell. Biochem. 92, 913–922
47. Li, C., and Hajjar, K. A. (2005) Circ. Res. 97, 1552–1555
48. Chen, S., Somanath, P. R., Razorenova, O., Chen, W. H., Hay, N., Bornstein, P., and Byzova, T. V. (2005) Nat. Med. 11, 1188–1196
49. Somanath, P. R., Razorenova, O. V., Chen, J., and Byzova, T. V. (2006) Angiogenesis 11, 277–288
50. Somanath, P. R., Razorenova, O. V., Chen, J., and Byzova, T. V. (2006) Cell Cycle 5, 512–518