Original Article

Sphingosine 1-phosphate receptor 1 governs endothelial barrier function and angiogenesis by upregulating endoglin signaling

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Background: The sphingosine 1-phosphate (S1P)/S1P receptor (S1pr) 1 signaling plays an essential role in regulating vascular integrity and angiogenesis. We have previously shown that cell-surface expression of endoglin (Eng) is sustained by S1P/S1pr1 signaling in endothelial cells (ECs). However, whether S1pr1 mediates Eng signaling, or vice versa, remains unknown.

Methods: S1pr1 inhibitors were used to study whether pharmacological inhibition induces basal vascular leakage *in vivo*. An acute respiratory distress syndrome (ARDS) model was used to study whether S1pr1 inhibition evoked greater inflammation in lungs. A S1pr1 inhibitor, a bone morphogenetic protein 9 (BMP9) blocking antibody, or lentivirus-mediated expression of soluble extracellular domain of Eng (sEng) were used to test whether blocking both S1P/S1pr1 and BMP9/Eng signaling axes would impose any interaction in retinal angiogenesis. To clarify whether S1P and BMP9 function in a linear pathway, a study of trans-endothelial electrical resistance (TEER) measurement was carried out using a mouse islet EC line MS1; time course studies were executed to exam downstream effectors of S1P and BMP9 signaling pathways in ECs; two stable MS1 cell lines were generated, one with overexpression of human S1PR1 and the other with knockdown of Eng, to validate S1pr1 and Eng were the key players for the crosstalk. Inhibitor of extracellular regulated protein kinases (ERK) was used to check whether this signaling was involved in S1P-induced cell-surface localization of Eng.

Results: The present study elucidated that S1pr1 and Eng are both pivotal for angiogenesis in the postnatal mouse retina, and that the activation of S1pr1 or Eng increases vascular barrier function. Activation of S1pr1 enhanced the phosphorylation of Smad family members 1, 5, and 8 (pSmad1/5/8), while the inhibition of S1pr1 reduced the levels of pSma1/5/8 induced by BMP9 treatment. Activation or loss of Eng did not affect S1pr1 signaling. Moreover, activation of ERK was involved in promoting EC-surface expression of Eng by S1pr1.

Conclusions: Our data demonstrates for the first time that there exists a linear pathway of S1pr1-Eng

signaling axis in ECs, which governs vascular homeostasis. Functional BMP9/Eng signaling requires S1P/S1pr1 activation, and S1pr1 signaling acts as a vascular protection mechanism upstream of Eng.

Keywords: S1pr1; endoglin (Eng); endothelial cells (ECs); angiogenesis; vascular integrity

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Introduction

Endothelial cells (ECs) form the inner layer of vessel walls and the lumens of capillary beds throughout the body. The EC monolayer imposes a natural 'barrier' to enclose the blood or lymph stream, and the regulation of EC barrier function is pivotal in multiple physiological or pathological events (1), including gas exchange, tissue regeneration, angiogenesis, and inflammation, etc. Although vascular barrier function is one of the most studied topics in EC biology, our understanding of the molecular mechanisms involved in regulating EC integrity is incomplete.

Sphingosine 1-phosphate (S1P) is a simple, blood-borne, lysophospholipid molecule that is essential for vascular homeostasis (2). S1P exerts signaling power by binding to its cognate G protein-coupled receptors, namely S1P receptors (S1prs) 1 to 5 (3). S1pr1 is highly expressed in ECs (4). S1pr1 prevents vascular endothelial (VE)-cadherin internalization , thus counteracts vascular endothelial growth factor (VEGF) function and stabilizes EC adherens junctions (AJs) (5). Knockout (KO) of this gene with a constitutively active EC-specific Cre recombinase is embryonic lethal due to defective development of the vascular system (6). Bred with a more sophisticated Cre mouse strain (*Cdh5*-Cre-ER^{T2}), EC-specific gene deletion can be achieved at postnatal and adult stages (7). EC-specific KO of S1pr1 in the developing mouse retina induces vascular leakage and a hyper-sprouting phenotype (5,8). Deletion of endothelial S1pr1 during adulthood severely damages pulmonary vascular integrity and causes edema, but results in very subtle damage to the function of the blood-brain barrier (9,10). These studies indicate that endothelial S1pr1 is indispensable for vascular integrity and angiogenesis. However, signaling or effector proteins downstream of endothelial S1pr1 have not yet been fully elucidated.

Angiogenesis refers to the growth of new blood vessels from pre-existing vessels and may be viewed as two separate but balanced phases (11). The first is an activation phase characterized by increased vascular permeability, basement membrane degradation, as well as EC proliferation and migration. The second is a resolution phase, with characteristic events like inhibition of endothelial proliferation or migration, reconstitution of basement membrane, and stabilization of the newly formed vessels by recruitment of pericytes and vascular smooth muscle cells (VSMCs) to the nascent endothelial tubes. Embryos of *S1pr1*-KO genotype die at around E13.5 due to massive hemorrhage and defective coverage of VSMCs over ECs (12), whereas EC-specific KO in the postnatal retina or brain results in defective angiogenesis but normal coverage of pericytes and VSMCs (5,8), suggesting endothelial S1pr1 plays a primary role in both phases of angiogenesis.

The transforming growth factor (TGF)-β signaling pathway is another well-studied biological phenomenon required for angiogenesis (13). Regulation of angiogenesis by the TGF-β pathway is complex and has demonstrated a signaling switch from being mostly anti- to pro-angiogenic depending on which of the TGF-β receptors are activated (13,14). Activation of the pro-angiogenic pathway involves the predominantly endothelial TGF-β type I receptor activin receptor-like kinase (ALK) 1 and co-receptor endoglin (Eng), which was previously known as CD105 (cluster of differentiation number 105) (15).

Binding with a putative blood-borne ligand, bone morphogenetic protein (BMP) 9, leads to the activation of ALK1 and subsequent phosphorylation of downstream transcription factors Smad family members 1, 5, and 8 (Smad1/5/8), resulting in the promotion of angiogenesis (16). Eng favors and amplifies the activation of Smad1/5/8 by ALK1 (17,18). Similar to S1pr1, Eng also regulates vascular maturation and angiogenesis. Eng mutations cause hereditary hemorrhagic telangiectasia (HHT) type I, an autosomal dominant vascular dysplasia characterized by recurrent bleeding and arterio-venous malformation (AVM) (19). Global KO of Eng results in embryonic lethality at approximately E10.5 due to defective

angiogenesis, but not vasculogenesis (20,21). EC-specific KO of Eng in postnatal pups results in defective maturation of vessels, AVM, and hyper-sprouting in various tissues (22,23). These results indicate that endothelial Eng is essential for angiogenesis and vascular patterning.

To certain extent, EC-specific KO of S1pr1 and Eng share some angiogenic phenotypes, such as defects in vascular remodeling and hyper-sprouting resulting from imbalanced vascular endothelial growth factor receptor (VEGFR) 2 signaling (5,8,23), suggesting that S1pr1 and Eng might govern angiogenesis via a linear pathway. In other words, these two receptors may regulate each other's signal transduction in ECs. Indeed, we have identified some specific membrane proteins regulated by S1P/S1pr1 signaling pathway. Eng is one of the newly identified membrane proteins regulated by this signaling (10). Genetic KO or pharmacological inhibition of endothelial S1pr1 reduces EC-surface expression of Eng, while activation of S1pr1 increases plasma membrane localization of Eng. Therefore, we hypothesize that S1pr1 regulates vascular function through modulation of the Eng signaling pathway. In the present report, we investigate the relationship between S1pr1 and Eng signaling *in vivo* and *in vitro*. The present study is a continuation of the previous publication. Our data demonstrated that both receptors might mediate vascular integrity and angiogenesis through a linear pathway in which S1pr1 enhances Eng downstream signaling via the activation of extracellular regulated protein kinases (ERK). Our results revealed a crosstalk between S1pr1 and the Eng signaling pathway in ECs. Activation of S1pr1 signaling may provide a novel therapeutic approach to treat HHT patients.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6679/rc).

Methods

Reagents and chemicals

The reagents and chemicals were mostly purchased from Thermo Fisher Scientific (Shanghai, China) unless otherwise indicated. S1P, SEW2871, NIBR0213, FTY720, and W146 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Recombinant human BMP9 was purchased from R&D systems (Minneapolis, MN, USA). The ERK inhibitor SCH772984 and the AKT (protein kinase-B, PKB, also called AKT) inhibitor MK-2206 were purchased from Selleck (Shanghai, China).

Animals

Wild type C57BL/6 male mice were purchased from Shilake (Shanghai, China). The mice were kept in a specific pathogen free (SPF) grade animal facility at the Wenzhou Medical University, with food and water *ad libitum*. All animal protocols applied in this study were approved by the institutional care and use committee (IACUC) of Wenzhou Medical University (No. WYDW2017-0111) and complied with NIH (National Institute of Health) Guidelines for the care and use of animals (24). A protocol was prepared before the study without registration.

Cell culture

MS1, a mouse islet EC line, was purchased from the Cell Bank of the Chinese Academy of Science in Shanghai. HPMVEC (human pulmonary microvascular endothelial cell) was purchased from Lonza (Portsmouth, USA). MS1 cells were cultured with DMEM (Dulbecco's Modified Eagle Medium) supplemented with 5% Gibco^{TM} fetal bovine serum (FBS; Thermo Fisher Scientific, Shanghai, China), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 ℃ in a 5% CO_2 (Carbon Dioxide), 95% air environment in humidified incubators. HPMVEC was cultured in EGM (Endothelial Cell Growth Medium)-2^{TM} (Lonza, Portsmouth, USA) according to the manufacturer's instruction.

Trans-endothelial electrical resistance (TEER)

About 5×10^4 MS1 cells were platted in a Transwell (pore size 0.4 μm) of 12-well plates and allowed to grow to confluence. The cells were starved in serum-free media for 2 h and then two-step treatment experiment was carried out as follows: (I) the Transwells were divided into two groups, with one pretreated with BSA (Bovine Serum Albumin) and the other with S1P for 30 min; (II) within each group, half of the wells were treated BSA and the other half were treated 10 ng/mL BMP9 (Bone Morphogenetic Protein 9) for 30 min or 1 hour. The TEER of the cultured MS1 monolayer was measured using a Millicell-ERS resistance meter (Millipore, USA) according to the manufacturer's instructions. A blank Transwell containing culture medium (without any cells) was used as blank control.

Lentivirus production

PCR (Polymerase Chain Reaction) cloning was used to establish the shuttle plasmids of human S1PR1 [an EGFP (Enhanced Green Fluorescent Protein) tag at C-terminus] and soluble Eng (sEng, whose amino acid sequence corresponds to the extracellular fragment of human endoglin) in the expression vector pCDH-CMV-MCS-EF1a-Puro or pCDH-CMV-MCS-EF1a-EGFP (System Biosciences, Palo Alto, CA, USA). The inserts were located between the NheI and NotI sites of these two vectors. The hairpin shRNA (short hairpin Ribonucleic Acid) vector for mouse Eng (pLKO.1-shEng) was purchased from Sigma (Alexandria, USA). The lentivirus packaging was performed using the third generation packaging system (System Biosciences, Palo Alto, CA, USA) according to manufacturer's instructions. Recombinant lentiviruses were purified by low temperature ultra-high speed centrifugation (4 ℃, 50,000 g, 2 h), and the final lentiviral titer was approximately 10^8 p.f.u. (plaque forming unit) per mL.

Lentiviral infection to generate stable MS1 cell-lines

The MS1 cells were seeded in a six-well plate (5×10^5/well) the day before infection. After culturing overnight, the lentivirus expressing S1PR1 or shEng was added to the six-well plate at a multiplicity of infection (MOI) equal to 0.1. After 48 h, the culture medium was replaced with complete medium containing 0.3 μg/mL puromycin to select the puro-resistant clones.

Inoculation of neonatal pups with lentivirus

The mouse model of lentiviral infection was obtained by intraperitoneal (i.p.) injection of purified lentiviral (10 μL each time in 20 second, using insulin syringe, once a day) from the first day to the third day after birth. The mice were then given a rest for 4 days. At postnatal day 7 (P7), the retinas and livers were surgically isolated for further analysis.

Postnatal retinal angiogenesis

Postnatal day 4 (P4) pups were i.p. injected with 15–20 μL of phosphate buffered saline (PBS), anti-BMP9 (BMP9 blocking antibody, 200 mg/mL, sc-27821, Santa Cruz Biotechnologies, Shanghai, China), and NIBR0213 (30 mg/kg), respectively. Then eyeballs from P7 pups were isolated and fixed with 4% paraformaldehyde (PFA) 4 h at 4 ℃ and surgical dissection of retina was performed under stereoscopic microscope. The isolated retinas were stained with Alexa Fluor® 488 isolectin GS-IB_4 (Thermo Fisher Scientific, Shanghai, China) mounted on a microscope slide in the presence of an anti-fluorescence quenching agent. The retinal blood vessels were observed and photographed using the EVOS FL Auto imaging system (Thermo Fisher Scientific, Shanghai, China). Angiotool 0.5a software (https://ccrod.cancer.gov/confluence/display/ROB2/Home) was employed to analyze angiogenesis-related parameters, include vessels percentage area (the percentage of area occupied by vessels inside the explant area), junctions density (the number of vessel junctions normalized per unit area), and average lacunarity (mean lacunarity overall size boxes) (25).

Assay of vascular leakage

To observe the effect of S1pr1-specific inhibition on pulmonary and tracheal vascular permeability, wild type (WT) adult mice received via oral gavage with either the S1prs functional antagonist [FTY720 (5 mg/kg)] or the S1pr1 specific inhibitor [NIBR0213 (60 mg/kg)] once. 24 h later, a vascular permeability assay was performed as previously described and lung tissues were collected for further analysis.

Acute respiratory distress syndrome (ARDS) mouse model

To investigate the effects of the S1pr1 specific inhibitor, NIBR0213, on the ARDS mouse model, NIBR0213 (30 mg/kg) was gavaged 6 h before Lipopolysaccharides (LPS)airway aspiration. Briefly, 6–8 weeks old male mice were anesthetized with an intraperitoneal injection of 5% chloral hydrate at a dose of 10 μL/g. The tracheas were then surgically exposed and tracheal cartilages were identified. LPS (10 mg/kg) was injected into the trachea between the third and fourth cartilage. After 24 h, the mice were sacrificed and the trachea was intubated. Repeated rinse with 1 mL cold PBS was performed three times to collect the bronchoalveolar lavage fluid (BAF). Cells in the BAF were removed by centrifugation (8,000 rpm, 4 ℃, 10 min). The concentrations of interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α, in the BAF were determined by enzyme-linked immunosorbent assay (ELISA).

Immunofluorescence

HPMVECs grown on cover slides (diameter: 14 mm) were fixed with 4% paraformaldehyde (PFA) at room temperature

(RT) for 15 min and then washed three times with PBS. After blocked with 2% goat serum/PBS at RT for 30 min, these cells were incubated overnight with mouse antibody against Endoglin (Clone 266, BD Biosciences, Shanghai, China). The following day, a secondary antibody Alexa Fluor® 488 goat anti-mouse immunoglobulin G (IgG) (1:500 in PBS) was used to detect the primary mouse antibody. Counterstaining was performed using 4, 6'-diamidino-2-phenylindole (DAPI), and the cells were examined using the EVOS FL Auto imaging system.

Mouse livers were fixed and washed similarly except that they were permeabilized with 0.1% Triton X-100/PBS before the blocking step. A rabbit antibody against GFP (green fluorescent protein) (Genescript, Nanjing, China) and a secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG) were used to detect the lentivirus-mediated expression of GFP in the mouse livers.

Western blot

Proteins were extracted from cultured cells using RIPA (Radio Immunoprecipitation Assay) lysis buffer containing protease inhibitor cocktail (Sigma, Alexandria, USA) and phosphatase inhibitors (Roche, New Jersey, USA), and quantitated with BCA (bicinchoninic acid) protein assay (Beyotime, Shanghai, China). Protein samples (50 μg) were separated with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto PVDF (Polyvinylidene Fluoride) membranes. The membranes were blocked in 5% BSA/PBS and incubated with primary antibodies purchased from Cell Signaling Technology (Boston, MA, USA, including antibodies against ERK42/44, phospho-ERK42/44, Smad1, and phospho-Smad1/5/8), Santa Cruz Biotchnoloogies [Shanghai, China, including antibody against S1pr1 and Vascular endothelium (VE)-cadherin] and Sigma (St. Louis, MO, USA, including antibody against vinculin and actin) diluted in 5% BSA/PBS at 4 ℃ overnight. After extensive washing to remove unbound primary antibodies, the membranes were incubated with HRP (Horseradish Peroxidase)-labelled secondary antibodies against rabbit or mice at RT for 2 h. The resulting membranes were developed with Immobilon Western Chemiluminescent HRP Substrate Kit (Millipore) and imaged with the KwikQuant Imager System (Kindle Biosciences LLC, Greenwich, CT, USA). ImageJ software (National Institutes of Health, USA) was used to obtain densitometric values of protein bands for further analysis.

Statistical analysis

All cell culture experiments were performed at least three times in triplicate. There were at least three mice in each group for the animal studies. Data were expressed as mean ± SEM (Standard Error of Mean) and plotted with bar chart. The Student's *t*-test, one-way ANOVA (Analysis of Variance), and two-way ANOVA were employed to analyze data with Prism 7 software (Graphpad, San Diego, CA, USA). $P<0.05$ was considered statistically significant.

Results

Pharmacological inhibition of S1pr1 disrupts vascular integrity and enhances the inflammatory response

Genetic studies have shown that loss of S1P or S1pr1 deteriorates vascular integrity and barrier function (9,10,26,27). To study whether pharmacological inhibition of S1pr1 has a similar effect, we used two commercially available inhibitors, FTY720 (5 mg/kg) and NIBR0213 (60 mg/kg), to treat WT mice by oral gavage. FTY720 is phosphorylated *in vivo* to generate an active drug FTY720-phosphate, a S1P analog that degrades all S1P receptors except S1pr2 (28). NIBR0213 is a potent S1pr1-selective antagonist, which directly binds to S1pr1 and inhibits downstream signaling (29). Evans blue dye was administrated 24 h after treatment via tail vein injection, and dye extravasation assay was performed to examine the barrier function of thoracic capillary beds. We observed profound interstitial accumulation of the blue dye in the mouse lungs and tracheas after treatment with either FTY720 or NIBR0213 (*Figure 1A*). Treatment with FTY720 induced S1pr1 degradation by ~60% whereas NIBR0213 treatment did not affect S1pr1 expression (*Figure 1B*, S1pr1). VE-cadherin, an endothelial marker for adherens junction (AJ), was also not significantly down-regulated by both treatments (*Figure 1B*, VE-cadherin). Consistent with previous reports (9,30), these data indicate that both inhibitors effectively induce basal vascular leakage *in vivo* without drastically affecting paracellular junctions of ECs.

Next, we assessed the inflammatory responses, which are an indirect manifestation of vascular integrity, after S1pr1 inhibition in a classic mouse model of ARDS. The mouse model was established with intratracheal administration of LPS after NIBR0213 treatment. Histological analysis of lung sections indicated increased leukocyte infiltration and edema in LPS-treated lungs, and pretreatment with

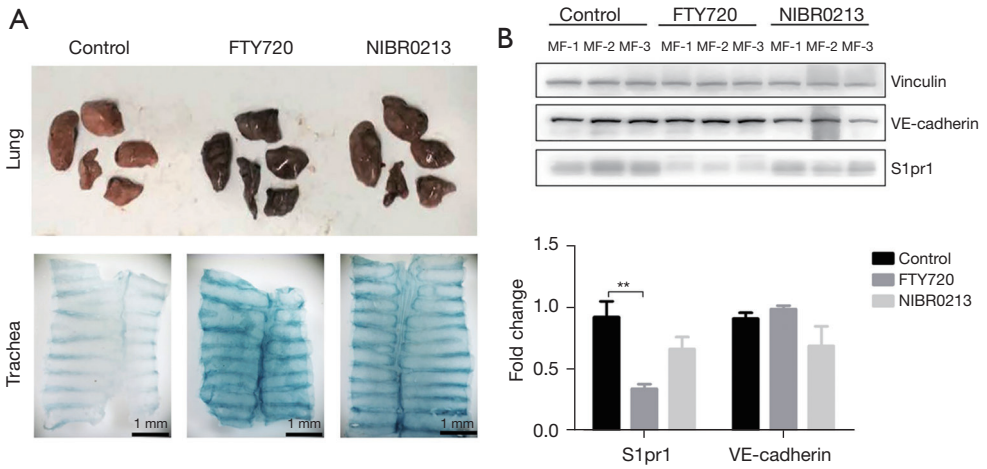

Figure 1 S1pr1 inhibition increased pulmonary and tracheal vascular permeability in adult mice. (A) Adult male mice were randomly divided into three groups according to various treatments, FTY720, NIBR0213, and PBS (as control), with three mice in each group. 24 h after treatment, the mice were subject to Evans blue dye extravasation assay to examine the pulmonary and tracheal vascular integrity. Representative images of mouse lungs and tracheas as indicated were taken after the dye was flushed out of circulation by perfusion. Compared with the control group, treatment with either FTY720 or NIBR0213 increased extravasation of the dye into the interstitial space in these two tissues. (B) The mouse lungs (n=3) were harvested and membrane fractions (MF) were isolated for Western blot to determine the expression of VE-cadherin and S1pr1 after FTY720 and NIBR0213 treatment as indicated. Expression of the housing keeping gene, vinculin, was used as a loading control. The low panel is a histogram of the quantitative results of Western blotting. Control *vs.* FTY720, **, P<0.01. S1pr1, sphingosine 1-phosphate receptor 1; FTY720, phosphorylated *in vivo* to generate an active drug FTY720-phosphate, a S1P analog that degrades all S1P receptors except S1pr2; NIBR0213, a potent S1pr1-selective antagonist; PBS, phosphate buffered saline; MF, membrane fractions; VE-cadherin, vascular endothelial (VE)-cadherin.

NIBR0213 amplified these phenotypes (*Figure 2A*). To obtain an objective view of the phenotype, we measured three pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β in BAF with ELISA. As expected, the levels of all three cytokines were the highest in the NIBR0213-treated ARDS mouse model (*Figure 2B*), indicating that S1pr1 inhibition further damaged the vascular barrier function under inflammatory conditions. Taken together, these data suggest that S1pr1 signaling is essential to maintain vascular integrity under basal and inflammatory conditions.

Inhibition of the S1P/S1pr1 and BMP9/Eng axes induces the hyper-sprouting phenotype in a mouse model of retinal angiogenesis

We have previously reported that plasma membrane localization of Eng is regulated by S1pr1 in microvascular ECs (10), suggesting S1pr1 might regulate BMP9/Eng signaling. Animal studies have also shown that genetic ablation of endothelial S1pr1 or Eng exhibits a similar postnatal hyper-sprouting phenotype of the developing retinal vascular network (5,8,22,23). These two facts propelled us to ask whether blocking both S1P/S1pr1 and BMP9/Eng signaling axes would impose any interaction. Consistently, treatment with either a BMP9 blocking antibody (Anti-BMP9) or NIBR0213 caused a comparable hyper-sprouting phenotype of the retinal neovasculature, while treatment with both drugs significantly increased vessel density and tip cells of leading edges of vessels, and shortened the radius of the vessel area (*Figure 3A*). Further analysis of vessel images with the Angiotool (National Institute of Health National Cancer Institute, Gaithersburg, Maryland, USA) software (25) showed more severe vascular defects with higher levels of vessel percentage area and junction density in the combined Anti-BMP9 and NIBR0213 treatment group (*Figure 3B*), suggesting the combined treatment is more effective than monotherapy. Previous studies have indicated that BMP9 and BMP10 are the physiological ligands of Eng, so we employed a lentivirus-mediated expression of sEng (Figure S1), which preserves the ligand-binding domians, to deplete the two ligands in the circulation, thereby blocking the downstream

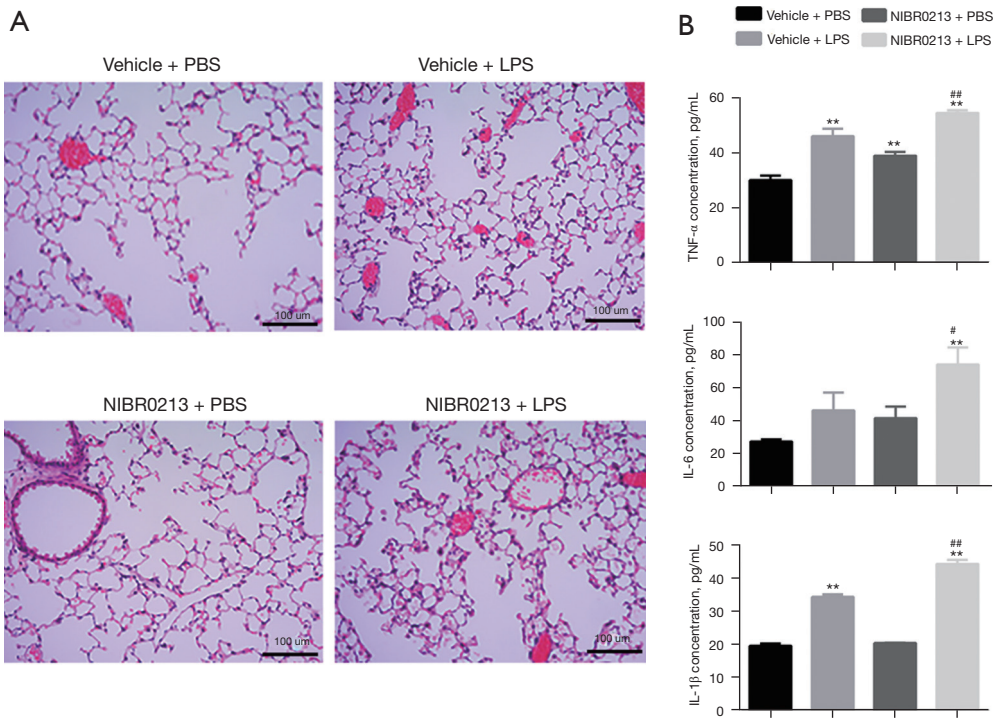

Figure 2 S1pr1 inhibition led to increased inflammation in LPS-induced ARDS mouse model. (A) Representative H&E (Hematoxylin-Eosin) stain images of mouse lung sections treated with NIBR0213 or vehicle in LPS-induced ARDS model. Magnification of images, 100×. (B) Approximately 1ml of BAF was collected from the mice lungs (n=3) as indicated, and the concentrations of IL-1β, IL-6, and TNF-a in BAF were determined using ELISA. The other three groups were compared to the vehicle + PBS group, **, P<0.01; NIBR0213 + LPS *vs.* vehicle + LPS; #, P<0.05; ##, P<0.01. S1pr1, sphingosine 1-phosphate receptor 1; LPS, Lipopolysaccharides; ARDS, acute respiratory distress syndrome; H&E, Hematoxylin-Eosin; NIBR0213, a potent S1pr1-selective antagonist; BAF, bronchoalveolar lavage fluid; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline.

signaling of endothelial Eng (31,32). Indeed, overexpression of sEng induced hyper-sprouting of P7 retinal vessels (*Figure 3C,3D*). In summary, inhibition of the S1P/S1pr1 or BMP9/Eng signaling axis led to a similar angiogenic phenotype, and inhibition of both axes did not have an obvious additive hyper-sprouting phenotype. These data might suggest these two signal axes function in a linear pathway.

Treatment with S1P and BMP9 enhances the TEER of the EC monolayer

To clarify whether S1P and BMP9 may function in a linear pathway, TEER measurement was carried out using a monolayer of mouse EC line MS1 following a combination of S1P and BMP9 treatments. The TEER of MS1 cells treated with S1P or BMP9 was gradually enhanced and eventually reached a plateau over a 1 h period; the combined treatment with S1P and BMP9 did not obviously have an additative effect (*Figure 4*). Again, these data suggest that S1P and BMP9 may transduce signals in a linear pathway.

Activation of S1pr1 potentiates BMP9 downstream signaling in ECs

To evaluate the interactions between S1P/S1pr1 and BMP9/Eng axes, we aimed to identify unique signaling molecules downstream of both axes. Time course studies showed that phosphorylation of Smad1/5/8 (p-Smad1/5/8) was induced only after BMP9 stimulation in human pulmonary microvascular ECs (HPMVEC; *Figure 5A*, p-Smad1/5/8). On the other hand, phosphorylation of p42/44 ERK (p-ERK42/44) was observed in HPMVECs treated with S1P (*Figure 5A*, p-ERK42/44). Similar results were obtained

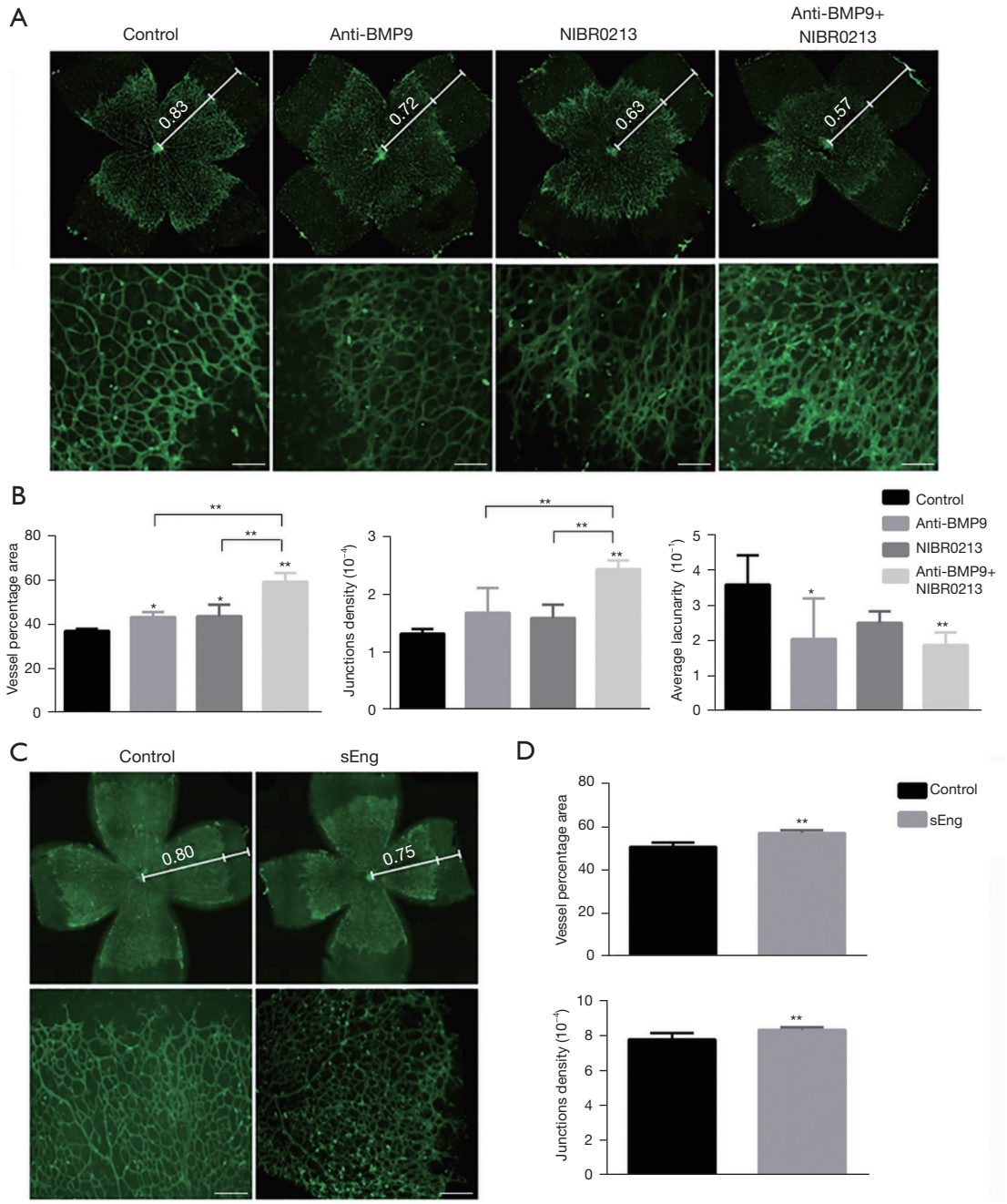

Figure 3 Inhibiting S1pr1, BMP9, and Endoglin signaling disrupted the steady growth of mouse retinal neovasculature. (A) Retinal vessels of the P7 newborn pups (N=6) with various treatments as indicated, were whole mount stained with Alexa Fluor® 488-conjugated IB4 and imaged under fluorescent microscopy. Two radii from the macula are present as the white line, to the edges of the retina and vasculature, respectively. The number defines the ratio between the short radius over the long one, representing the vessel percentage area. Scale bars, 200 μm. (B) Histographic representation of indexes of angiogenesis of panel A, including vessel percentage area, junctions density and average lacunarity. Magnification, 100×; One-way ANOVA, *, P<0.05, **, P<0.01. (C) Representative images of whole mount stain of the retinal vessels from P7 pups (n=6) infected with the control or sEng lentivirus. Scale bars, 200 μm. (D) Histography of the vessel percentage area and junctions density of retinal vasculature of panel C. Magnification, 100×; Student *t*-test, **, P<0.01. S1pr1, sphingosine 1-phosphate receptor 1; BMP9, bone morphogenetic protein 9; P7, postnatal day 7; sEng, soluble extracellular domain of endoglin.

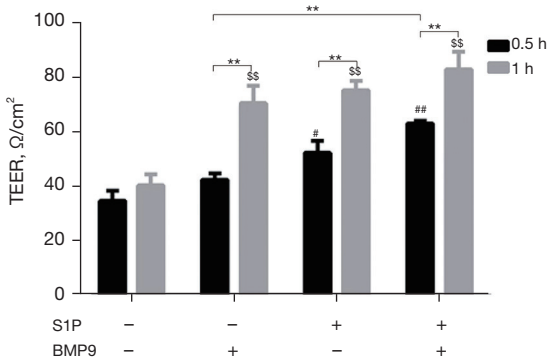

Figure 4 Treatment with S1P and BMP9 increased endothelial integrity measured by TEER assay. MS1 cells plated on a Transwell were allowed to grow to confluence and then treated with various combinations of S1P and BMP9 as indicated. Two-way ANOVA, **, P<0.01; *vs.* 30 min control group, #, P<0.05, ##, P<0.01; *vs.* 1 h control group, $$, P<0.01. –, in the absence of S1P or BMP9; +, in the presence of S1P or BMP9. S1P, sphingosine 1-phosphate; BMP9, bone morphogenetic protein 9; TEER, trans-endothelial electrical resistance; MS1, a mouse islet EC line.

from the MS1 cells, although the basal level of p-ERK42/44 was high in these cells (data not shown). Next, a two-step treatment method was used to study the interaction between S1P/S1pr1 and BMP9/Eng signaling. Again, time course studies were used to show that pretreatment with S1P increased p-Smad1/5/8 levels activated by BMP9 in HPMVECs (*Figure 5B*, 1 h) as well as in MS1 cells (*Figure 5C*, 0.5, 1, 2 h). However, pretreatment with BMP9 did not affect the activation of p-ERK42/44 by S1P (data not shown). These data indicate that S1P acts upstream of BMP9 signaling in ECs and p-Smad1/5/8 is a valid readout for the assessment of the interaction between the two axes.

To validate S1pr1 is the S1P receptor that enhances BMP9 signaling, we generated a stable MS1 cell line overexpressing a C-terminal GFP-tagged human S1PR1 (Figure S2) for further validation. Overexpression of S1PR1 greatly increased p-Smad1/5/8 in the two-step treatment time course study (*Figure 6A*, 0.25, 0.5, 1 h). Moreover, a S1pr1-selective agonist SEW2871 (33) had a similar enhancement on p-Smad1/5/8 in MS1 cells stimulated by BMP9 (*Figure 6B*, 0.25, 0.5, 1 h), whereas treatment with a S1pr1-specific antagonist W146 (34) prior to BMP9 incubation almost abolished the phosphorylation of Smad1/5/8 (*Figure 6C*, 0.25, 0.5, 1 h). These data demonstrate that S1pr1 is the S1P receptor potentiating BMP9 signaling.

Eng is required for S1pr1-potentiated BMP9 signaling

To verify the vital role of Eng involved in BMP9 signaling potentiated by S1pr1 activation, we generated another stable MS1 cell line, in which Eng was knocked down (KD) by lentivirus-mediated expression of shRNA (shEng MS1) (*Figure 7A*). BMP9 stimulation enhanced the levels of p-Smad1/5/8 in both the control and shEng MS1 cells, while S1pr1-potentiated phosphorylation of Smad1/5/8 activated by BMP9 was diminished in the shEng MS1 cells (*Figure 7B*,*7C*). These data clearly indicate that S1pr1 activation enhances endothelial BMP9 signaling via BMP9 and Eng engagement.

Activation of mitogen activated protein (MAP) kinase is essential for S1P-induced EC-surface localization of Eng

We have previously shown that the endothelial S1P/S1pr1 axis enhances cell-surface of Eng (10). In order to interrogate mechanisms dictating S1P-induced cell-surface localization of Eng, we treated HPMVECs with ERK or AKT inhibitors before S1P stimulation. As expected, Eng was highly enriched at the cell-surface and cell-cell junctions after S1P treatment (*Figure 8A*). However, pretreatment with an ERK inhibitor diminished the membrane localization of Eng induced by S1P stimulation (*Figure 8B*). Pretreatment with an AKT inhibitor generated similar results (data not shown). In summary, the MAP kinase pathway is involved in cell-surface localization of Eng induced by S1P stimulation in ECs.

Discussion

Previous studies have demonstrated that the S1P/S1pr1 signaling is critical for preventing vascular permeability in various mouse models (9,10,26,27,30,35,36). Brain endothelial S1pr1 maintains the blood–brain barrier (BBB) by regulating the proper localization of tight junction proteins. Endothelial-specific S1pr1 knockout mice showed BBB breach, allowing small molecules (<10 kD) leaking into brain parenchyma (9). In this study, we also found that pharmacological inhibition of S1pr1 increased basal and pathological vascular leakage in the continuous capillary beds (*Figure 1A*). The basal vascular defect occurred with relatively normal expression of VE-cadherin, which is the dominant endothelial AJ protein (37). Given that S1P/S1pr1 signaling promotes AJ assembly and facilitates tight junction formation, this result is rather intriguing. A

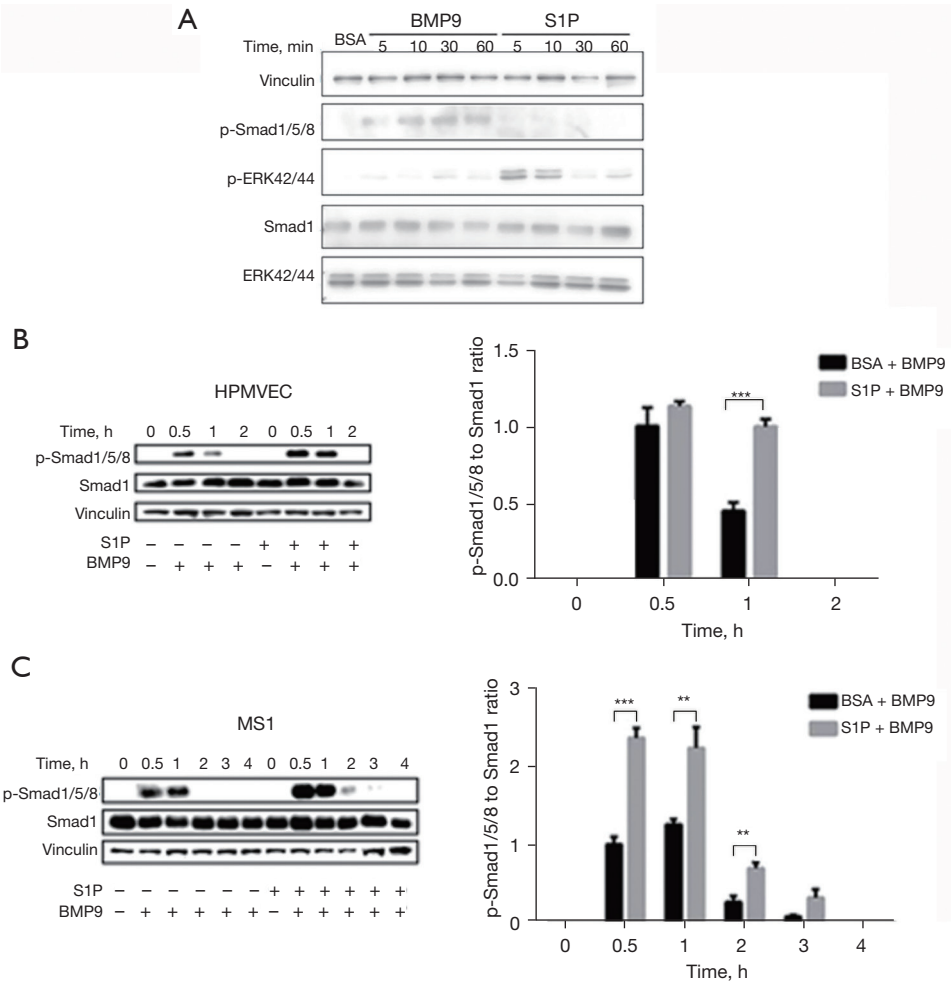

Figure 5 Activation and interaction of S1P and BMP9 downstream signaling in endothelial cells. (A) Confluent HPMVEC cells were treated with S1P (200 nM) or BMP9 (10 ng/mL) as indicated. Expressions of p-ERK42/44, total ERK42/44, p-Smad1/5/8, Smad1, and vinculin were analyzed using Western blot. Treatment with S1P induced phosphorylation of ERK proteins, whereas BMP9 only activated the phosphorylation of Smad1/5/8. (B,C) Expressions of Smad1 and p-Smad1/5/8 were analyzed using Western blot in HPMVEC cells (B) and MS1 cells (C) pretreated with BSA or S1P (200 nM) for 30 min followed with BMP9 (10 ng/mL) as indicated. Vinculin was used as a loading control. The right panels illustrate the ratio of p-Smad1/5/8 over Smad1 with histograms at each time point as indicated. Student *t*-test, **, P<0.01; ***, P<0.001. –, in the absence of S1P or BMP9; +, in the presence of S1P or BMP9. S1P, sphingosine 1-phosphate; BMP9, bone morphogenetic protein 9; HPMVEC, human pulmonary microvascular endothelial cell; ERK, extracellular regulated protein kinases; MS1, a mouse islet EC line; BSA, bovine serum albumin.

recent study indicated that EC-selective adhesion molecule (ESAM) is essential for vascular barrier function besides VE-cadherin (38). Since we have shown previously that S1pr1 regulates the pulmonary expression of ESAM rather than that of VE-cadherin (10), it may be postulated that inhibition of S1pr1 signaling causes AJ disassembly and diminished expression of ESAM, leading to a breakdown of vascular barrier function in the mouse lungs. However, the mechanism through which these two membrane proteins mediate vascular barrier downstream of S1pr1 signaling requires further investigation.

Mouse genetic studies have revealed phenotypic overlaps and differences between postnatal EC-specific KO of S1pr1 and Eng mice. Both mouse models share phenotypes such as hyper-sprouting and delayed vascular maturation (5,8,22,23). EC-specific KO of Eng mice has some unique

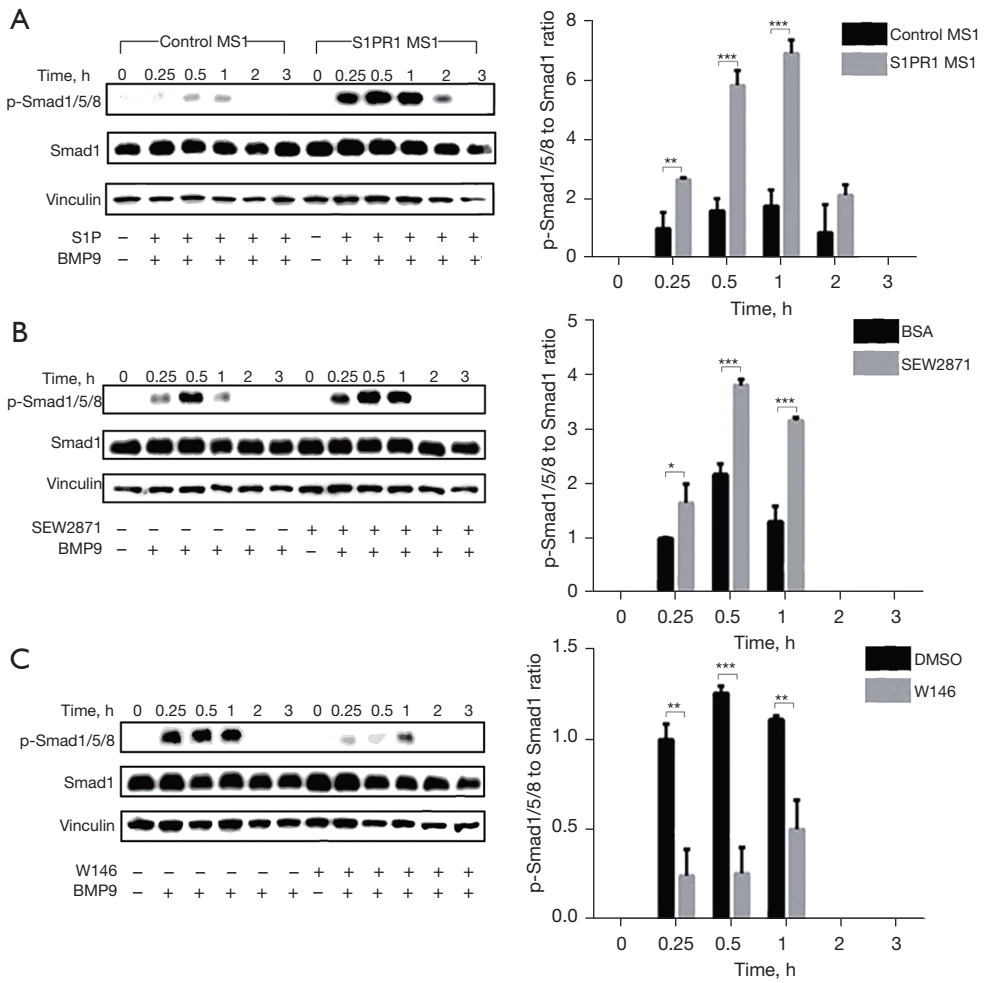

Figure 6 Regulation of BMP9-stimulated phosphorylation of Smad1/5/8 by S1pr1 signaling in endothelial cells. MS1 stably expressing GFP-tagged human S1PR1 (hS1P1) and control cells were starved and pretreated with S1P (200 nM) for 30 min. Next, BMP9 (10 ng/mL) treatment was carried out in a time course study as indicated (A). Parental MS1 cells were also starved and pretreated with agonist SEW2871 (B) or antagonist W146 (C) for 30 min. Subsequently, the cells were treated with BMP9 (10 ng/mL) for various time periods as indicated. Western blotting was used to detect p-Smad1/5/8, Smad1, and vinculin (representative images are shown in the left panels). The right panels illustrate histograms of quantification of p-Smad1/5/8 levels, while Smad1 served as an internal control. Student *t*-test, *, P<0.05; **, P<0.01; ***, P<0.001. –, in the absence; +, in the presence. BMP9, bone morphogenetic protein 9; S1pr1, sphingosine 1-phosphate receptor 1; MS1, a mouse islet EC line; GFP, green fluorescent protein; S1P, sphingosine 1-phosphate; SEW2871, a S1pr1-selective agonist; W146, a S1pr1-specific antagonist.

manifestations; for instance, diluted vessels and AVMs, but no basal vascular leakage of pre-existing capillary beds or post-capillary venules have been reported (22,23). In mouse models of carcinogenic xenografts, endothelial deletion of S1pr1 or Eng undermines the tumor vascular barrier and promotes metastasis (36,39). Moreover, germline KO of Bmp9 increases tumor growth and metastasis owing to increased tumor angiogenesis (40), while EC-specific KO of Alk1 deteriorates vascular barrier function in a hyperglycemic mouse model (41). These findings suggest that the BMP9/Eng/ALK1 pathway, like the S1P/S1pr1 pathway, is indispensible for vascular integrity under pathological conditions. We tested whether these two pathways may mediate vascular integrity in a coordinated fashion *in vivo* (*Figure 3*) and *in vitro* (*Figure 4*). Inhibition of S1pr1, BMP9, or Eng induces hyper-sprouting in postnatal

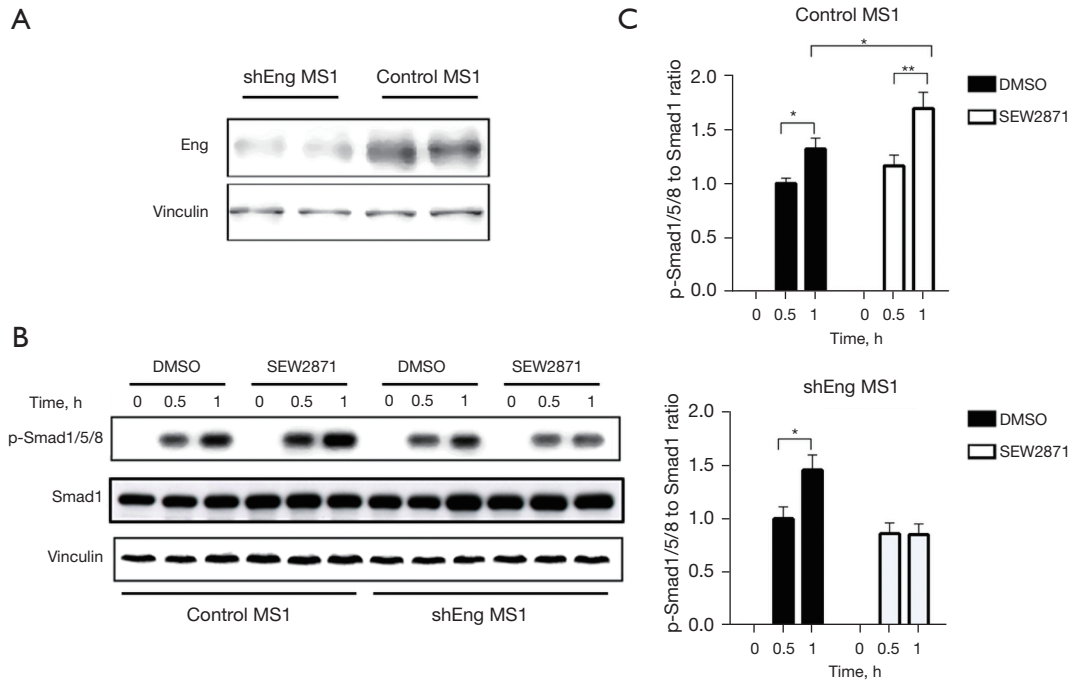

Figure 7 Knockdown of Endoglin abolished S1pr1-enhanced BMP9 signaling in endothelial cells. (A) A representative Western blot image showing diminished expression of Endoglin in Eng knockdown (lentivirus-mediated expression of shRNA against Eng, shEng) and control MS1 cells. Vinculin was used as a loading control. (B) Time course experiment of BMP9 signaling after S1pr1 activation in the shEng and control MS1 cells. After incubation with SEW2871 (100 nM) for 30 min, these cells were treated with BMP9 (10 ng/mL) for various periods of time as indicated. Expressions of p-Smad1/5/8, Smad1, and vinculin were determined using Western blotting. (C) Histographic presentation of p-Smad1/5/8 expression in panel B. Smad1 was used as a loading control. Two-way ANOVA, *, P<0.05; **, P<0.01. S1pr1, sphingosine 1-phosphate receptor 1; BMP9, bone morphogenetic protein 9; Eng, Endoglin; shRNA, short hairpin ribonucleic acid; shEng, lentivirus-mediated expression of shRNA against Eng; MS1, a mouse islet EC line; SEW2871, a S1pr1-selective agonist; DMSO, dimethyl sulfoxide.

mouse retina. Treatment with either S1P or BMP9 enhances TEER of the monolayer of MS1 cells. However, no compelling additive effects were observed when both pathways were inhibited and activated, indicating that S1pr1 and Eng may orchestrate vascular homeostasis via a linear pathway.

Previously we identified Eng as a transmembrane target regulated by the S1P/S1pr1 signaling (10). In this study, we found two unique downstream effectors of S1P and BMP9 in the microvascular ECs, p-ERK42/44 and p-Smad1/5/8, respectively (*Figure 5*). Our data indicates that activation of S1pr1 increases p-Smad1/5/8 stimulated by BMP9, whereas S1P-induced p-ERK42/44 expression was not affected by BMP9 pre-treatment (*Figure 6*). Moreover, Eng is essential for S1pr1-potentiated increase of p-Smad1/5/8, and inhibition of ERK abolished the enhanced expression of Eng on the EC surface by S1pr1 (*Figures 7,8*). It has been suggested that both S1pr1 and Eng regulate angiogenesis and vascular maturation by counteracting VEGFA (vascular endothelial growth factor A)/VEGFR2 (Vascular Endothelial Growth Factor Receptor 2) signaling (5,23), and Eng has been shown to physically associate with VEGFR2 in the endosome and promote its degradation (23). Together, these results suggest that S1pr1 restricts VEGFR2 signaling through Eng. Combined activation of S1pr1 and Eng signaling transduction could therefore be conceptually beneficial to treat peripheral vascular disorders, particularly HHT.

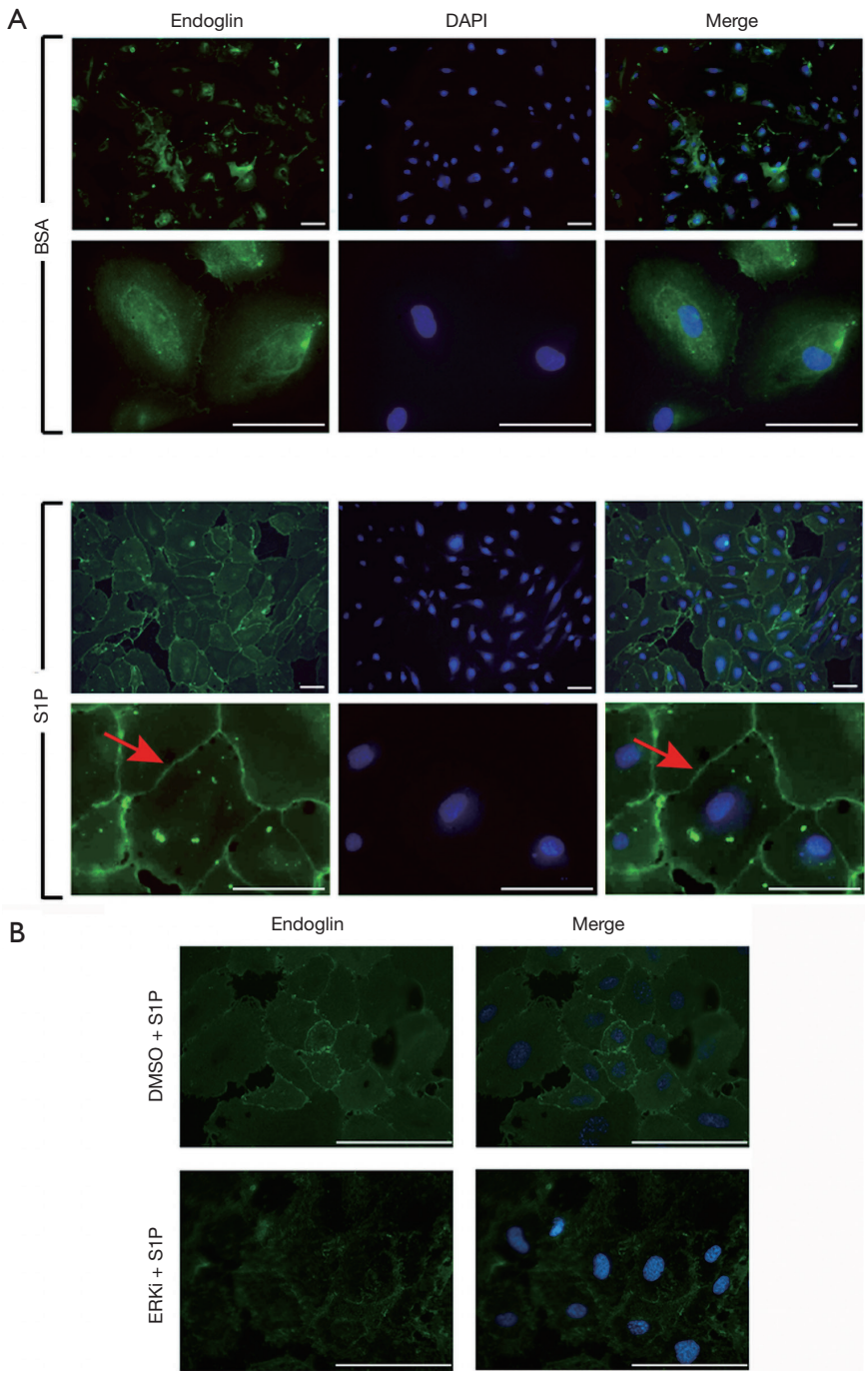

Figure 8 S1P-induced cytoplasmic membrane localization of Endoglin dependent on ERK activation in endothelial cells. (A) HPMVECs were treated with BSA or 200 nM S1P for 30 min and then were fixed prior to immunofluorescent staining of Endoglin. Nuclei were stained with DAPI. Red arrows indicate the enrichment of Endoglin on the plasma membrane. Magnification of BSA or S1P group, top panel 100×, lower panel 400×; Scale bars, 200 μm. (B) HPMVECs incubated with DMSO or 1 μM SCH772984 for 30 min were treated with S1P after words. The cells were processed as described in panel A and analyzed with fluorescent microscopy. Magnification, 400×; Scale bars, 200 μm. S1P, sphingosine 1-phosphate; ERK, extracellular regulated protein kinases; HPMVEC, human pulmonary microvascular endothelial cell; BSA, bovine serum albumin; DAPI, 4, 6'-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; SCH772984, a specific ERK inhibitor.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-21-6679/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-21-6679/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6679/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal protocols applied in this study were approved by the institutional care and use committee (IACUC) of Wenzhou Medical University (No. WYDW2017-0111) and complied with NIH (National Institute of Health) Guidelines for the care and use of animals.

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