Gα_s Relays Sphingosine-1-Phosphate Receptor 1 Signaling to Stabilize Vascular Endothelial-Cadherin at Endothelial Junctions to Control Mouse Embryonic Vascular Integrity

Ximing Shao ^{a,b,1}, Ke Liu ^{a,b,1}, Yi Fan ^a, Zhihao Ding ^b, Min Chen ^c, Minyan Zhu ^c, Lee S Weinstein ^e, Hongchang Li ^{b,*}, Huashun Li ^{a,c,d,*}

^a West China Developmental and Stem Cell Institute, West China Second Hospital, and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China

^b Shenzhen Key Laboratory for Molecular Biology of Neural Development, Laboratory of Developmental and Regenerative biology, Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, Guangdong 518055, China

^c SARITEX Center for Stem Cell Engineering Translational Medicine, Shanghai East Hospital, Tongji University School of Medicine, Shanghai 200123, China

^d Nerdbio Inc., SIP Biobay, Jiangsu 215213, China

^e National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

¹ These authors contributed equally to this work.

* Corresponding authors. Tel: +8618207554971, fax: +86075586585222 (Hongchang Li); Tel: +8618981921691 (Huashun Li).

E-mail addresses: hc.li@siat.ac.cn (Hongchang Li); huashunli@tongji.edu.cn (Huashun Li).

Abbreviation footnote: AJ, adherens junction; CTx, cholera toxin; EC, endothelial cell; FSK, Forskolin; GPCR, G protein-coupled receptor; HUVEC, human umbilical vein endothelial cell; KD, knockdown; KO, knockout; S1PR1, sphingosine-1-phosphate receptor 1; VE, vascular endothelial.

ABSTRACT

Sphingosine-1-phosphate receptor 1 (S1PR1), a G protein-coupled receptor (GPCR), controls vascular stability by stabilizing vascular endothelial (VE)-cadherin junctional localization and inhibiting vascular endothelial growth factor receptor 2 (VEGFR2) signaling. However, the molecular mechanisms that link S1PR1 signaling to intracellular effectors remain unknown. In this study, we demonstrate that the heterotrimeric G protein subfamily member $G\alpha_s$, encoded by *GNAS*, acts as a relay mediator of S1PR1 signaling to control vascular integrity by stabilizing VE-cadherin at endothelial junctions. The endothelial cell-specific deletion of $G\alpha_s$ in mice causes early embryonic lethality with massive hemorrhage and a disorganized vasculature. The immunostaining results revealed that $G\alpha_s$ deletion remarkably reduces the junctional localization of VE-cadherin, whereas the mural cell coverage of the vessels is not impaired. In addition, we found that $G\alpha_s$ depletion blocked the S1PR1 activation induced VE-cadherin stabilization at junctions, supporting that $G\alpha_s$ acts downstream of S1PR1 signaling. Thus, our results demonstrate that $G\alpha_s$ is an essential mediator to relay S1PR1 signaling and maintain vascular integrity.

KEYWORDS

$G\alpha_s$; VE-cadherin; S1PR1 signaling; adherens junctions; vascular integrity

INTRODUCTION

Endothelial adherens junction (AJ), which connects adjacent endothelial cells, has an important role in the control of vascular permeability and integrity (Giannotta et al., 2013). Many adhesion molecules have been shown to participate in the formation of endothelial AJs. One of the key molecules is vascular endothelial (VE)-cadherin, which is specifically expressed in endothelial cells and is crucial for endothelial AJ assembly and dynamics (Lampugnani et al., 1992; Dejana, 1996; Giannotta et al., 2013). Multiple genetic studies show that the dynamic control of VE-cadherin in endothelial cells is crucial for maintaining proper vascular stability and permeability (Carmeliet et al., 1999; Corada et al., 1999; Gory-Faure et al., 1999; Montero-Balaguer et al., 2009; Harris and Nelson, 2010). Knocking out VE-cadherin in mice causes lethality near embryonic days 9.5-10.5 (E9.5-E10.5) due to severe vascular remodeling defects (Gory-Faure et al., 1999). Furthermore, the association of VE-cadherin with β -catenin has been shown to be essential for the cellular control of endothelial permeability (Carmeliet et al., 1999). Moreover, blocking VE-cadherin function in adult mice with antibodies dramatically increases the permeability and induces severe hemorrhages (Corada et al., 1999). In addition to these findings in mice, studies in zebrafish also reveal that the role of VE-cadherin in the control of vascular integrity is conserved. Knocking down zebrafish VE-cadherin expression changes the vascular integrity in a concentration-dependent manner (Montero-Balaguer et al., 2009).

Previous findings demonstrate that the sphingosine-1-phosphate receptor-1 (S1PR1), a G-protein-coupled receptor (GPCR) for the bioactive lipid S1P, is an important regulator of vascular stabilization and permeability (Liu et al., 2000; Allende et al., 2003; Rosen et al., 2009; Gaengel et al., 2012). Both global and endothelial cell (EC)-specific knockout of S1PR1 result in deficient mural cell coverage of the dorsal aorta and other vessels, leading to severe hemorrhage and embryonic lethality at E12.5–E14.5 of gestation (Liu et al., 2000; Allende et al., 2003). However, the ablation of S1PR1 signaling during the early embryonic stage around E11.5 only mildly impacts the mural cell coverage of the aorta but leads to significant aortic hyperbranching, suggesting that the mural cell abnormalities of dorsal aorta in the S1PR1 knockouts were likely due to

endothelial hypersprouting. Moreover, endothelial knockout of S1PR1 also damages the AJs between endothelial cells by destabilizing VE-cadherin at these junctions (Gaengel et al., 2012). This finding suggests that S1PR1 directly maintains vascular stability in endothelial cells (Gaengel et al., 2012). These results are consistent with previously characterized S1PR1 functions in the Rho- and Rac-dependent assembly of VE-cadherin in cultured endothelial cells (Lee et al., 1999). Thus, VE-cadherin is likely an effector of S1PR1 signaling that controls vascular stability and permeability. Nevertheless, which G protein links the S1PR1 signaling to VE-cadherin is unclear. *In vitro* studies revealed that G_i , a subtype of $G\alpha$ proteins, is associated with S1PR1 (Windh et al., 1999). However, whether G_i coupled S1PR1 signaling is involved in AJ assembly remains elusive (Lee et al., 1999). Here, we demonstrate that $G\alpha_s$ acts downstream of S1PR1 to control vascular integrity by stabilizing the junctional localization of VE-cadherin. The EC-specific deletion of $G\alpha_s$ in mice driven by Tie2-Cre causes severe hemorrhage and embryonic lethality around E11.5, which partly phenocopies the S1PR1 knockout mice. The EC-specific deletion of $G\alpha_s$ does not impair the mural cell coverage but remarkably reduces the junctional localization of VE-cadherin in embryos at E10.0-E11.0, which is consistent with a previously identified phenotype caused by S1PR1 knockout in embryonic vascular development. In addition, treatment with a S1PR1 agonist (SEW2871) does not reverse the junctional reduction of VE-cadherin, but $G\alpha_s$ downstream agonist (Forskolin) can notably rescue the junctional deficiency of VE-cadherin in $G\alpha_s$ -deficient endothelial cells. Thus, our findings demonstrate that $G\alpha_s$ is a relay mediator that links the membrane bound GPCR-S1PR1 to its intracellular effector VE-cadherin to control vascular integrity.

99 RESULTS

100 EC-specific Deletion of $G\alpha_s$ Leads to Embryonic Lethality

101 To study the role of $G\alpha_s$ in angiogenesis during embryonic development, we first
102 examined the expression of $G\alpha_s$ in vascular endothelial cells. By immunofluorescence
103 staining, we detected that $G\alpha_s$ expresses in PECAM1-positive endothelial cells in the
104 dorsal aorta as early as E10.0 (Figs. 1A and S1), indicating that $G\alpha_s$ may play important
105 roles in embryonic vascular development.

106 We next generated EC-specific $G\alpha_s$ knockout (KO) mice ($G\alpha_s^{flox/flox}; Tie2-Cre$) by
107 crossbreeding $Tie2-Cre$ mice with homozygous floxed $G\alpha_s$ mice ($G\alpha_s^{flox/flox}$). No viable
108 pups with $G\alpha_s^{flox/flox}; Tie2-Cre$ (EC- $G\alpha_s$ KO) were born, indicating that the disruption of
109 $G\alpha_s$ causes embryonic lethality. To determine the timing of lethality, we analyzed
110 embryos at different embryonic stages during gestation. All EC- $G\alpha_s$ KO embryos can live
111 up to E10.0. However, embryonic lethality gradually increased, and only one out of 6
112 EC- $G\alpha_s$ KO embryos survived at E11.5 (Fig. 1D and Table 1). The $G\alpha_s$ knockout
113 specificity and efficiency were further examined by genomic PCR and
114 immunofluorescence staining of dissociated embryonic cells. As shown, only embryos
115 carrying $Tie2-Cre$ and floxed alleles demonstrated recombination (Fig. S2) and the $Tie2$ -
116 Cre mediated knockout of $G\alpha_s$ dramatically reduced the $G\alpha_s$ expression in PECAM1-
117 positive endothelial cells as early as E9.5 (Fig. 1B and C). All of these results suggest
118 that $G\alpha_s$ expression is required in endothelial cells for embryonic development.

119 EC-specific Deletion of $G\alpha_s$ Causes Severe Embryonic Vascular Defect

120 To determine the cause of $G\alpha_s$ knockout-induced embryonic lethality, we examined the
121 EC- $G\alpha_s$ KO embryos at E10.0, E11.0, and E11.5. Prior to E10.0, the control and mutant
122 embryos were morphologically similar. However, by E11.0 and E11.5, the size of EC-
123 $G\alpha_s$ KO embryos largely reduced, most likely due to retarded embryonic development.
124 Strikingly, EC- $G\alpha_s$ KO embryos displayed dramatic vascular developmental defects.
125 Starting at E11.0, we found massive hemorrhage in multiple regions in EC- $G\alpha_s$ KO
126 embryos, including the forebrain, cervical, and thoracic region (Fig. 1E). Hemorrhage in
127 EC- $G\alpha_s$ KO embryos mostly occurred between E11.0 and E11.5 (Fig. 1F). Due to the

pooling of blood, the embryos and yolk sacs of the mutant were pale in comparison to littermate controls (Fig. 1E). We also found that the EC- $G\alpha_s$ KO dorsal aorta was ruptured in some regions of the embryos, which might be a major cause of bleeding (Fig. S3). Consistently, isolectin B4 immunostaining shows that EC- $G\alpha_s$ KO embryos at E11.0 failed to develop a well-organized intersomitic vasculature and instead formed truncated networks (Fig. 1G). Thus, these results suggest that $G\alpha_s$ is required for embryonic vascular development.

EC-specific Deletion of $G\alpha_s$ Does Not Affect Embryonic Vascular Mural Cell Coverage

The proper recruitment of mural cells (pericytes and vascular smooth muscle cells) to nascent blood vessels is crucial for vessel maturation (Jain, 2003; Gaengel et al., 2009). We then sought to determine whether $G\alpha_s$ signaling affects mural cell coverage on newly formed vessels. To this end, we performed immunostaining for α -smooth muscle actin (α -SMA), a marker of vascular smooth muscle cells (vSMCs), in both control and mutant embryos at E11.0. We found that α -SMA-positive vSMCs properly enveloped PECAM1-positive endothelial cells in both the control and EC- $G\alpha_s$ KO dorsal aorta (Fig. 2A), indicating that $G\alpha_s$ deletion does not affect vSMCs recruitment. Next, we examined the recruitment of pericytes to the dorsal aorta and the neural tube capillaries in EC- $G\alpha_s$ KO embryos by immunostaining for NG2, which has been extensively used as a marker for pericytes. In both the dorsal aorta and the neural tube capillaries, NG2 positive cells were observed to closely attach to endothelial cells, and $G\alpha_s$ deletion did not cause a detectable defect in the pericyte coverage (Fig. 2B-D). These results suggest that the EC-specific deletion of $G\alpha_s$ does not impair embryonic vascular mural cell coverage.

Endothelial Loss of $G\alpha_s$ Reduces VE-cadherin Localization at AJs

The formation of proper endothelial AJs is crucial for vascular integrity (Giannotta et al., 2013). The discontinuous dorsal aorta and the truncated intersomitic vascular network in EC- $G\alpha_s$ KO embryos suggest that strong adhesive defects exist in $G\alpha_s$ mutant endothelial AJs. N-cadherin and VE-cadherin have been shown to be major components of endothelial AJs, and both are important for maintaining vascular integrity (Giannotta et

al., 2013). Thus, we examined these two molecules using immunofluorescence staining. Our data show that N-cadherin was equally enriched around PECAM1-positive endothelial cells in both the control and EC- $G\alpha_s$ KO dorsal aorta at E11.0 (Fig. 3A), suggesting that an endothelial loss of $G\alpha_s$ does not affect N-cadherin localization. In contrast, $G\alpha_s$ deletion remarkably reduced the VE-cadherin localization in the dorsal aorta, especially at E11.0 (Fig. 3B and C). Furthermore, the VE-cadherin in the EC- $G\alpha_s$ KO dorsal aorta diffused in endothelial cells, while the VE-cadherin in the control dorsal aorta mostly accumulated at the endothelial junctions (Fig. 3D). Next, we examined the impact of $G\alpha_s$ deletion on the VE-cadherin subcellular distribution in capillaries. Our data show that control endothelial junctions were fully covered with VE-cadherin. In contrast, VE-cadherin was discontinuously attached at cell contact locations in the EC- $G\alpha_s$ KO capillaries (Fig. 3E). Similar defects caused by $G\alpha_s$ deletion were also observed in yolk sacs. $G\alpha_s$ knockout causes a dramatic loss of VE-cadherin at the endothelial junctions of yolk sacs (Fig. 3F). These results strongly suggest that $G\alpha_s$ controls vascular integrity by recruiting or stabilizing VE-cadherin at endothelial AJs.

Effect of $G\alpha_s$ Signaling on Endothelial Cell Tube Formation *in vitro*

To further characterize the role of $G\alpha_s$ in the formation of the vascular network, we established an *in vitro* tube formation assay (Ponce, 2009). Two $G\alpha_s$ knockdown (KD) human umbilical vein endothelial cell (HUVEC) lines were generated using RNA interference technology (Moore et al., 2010). Western blot results showed that the expression of $G\alpha_s$ was dramatically reduced in these cells (Fig. 4A). Next, both control and $G\alpha_s$ KD cells were plated on Matrigel, and tube-like structure formation was carefully examined. Compared to control cells, the capillary-like tube formation was significantly reduced in $G\alpha_s$ KD cell lines (Fig. 4B). Further quantification analysis shows that $G\alpha_s$ knockdown reduced both the tube number and total tube length up to nearly 50% (Fig. 4C). Then we wonder whether activation of $G\alpha_s$ has an opposite effect on tube formation. We used cholera toxin (CTx) to directly activate $G\alpha_s$ (O'Neal et al., 2005; Chiou and Zennadi, 2015). Surprisingly, treatment of HUVECs with CTx inhibited the tube formation in a concentration-dependent manner (Fig. S4). Together, as inhibition

and hyper-activation of $G\alpha_s$ both inhibit the *in vitro* tube formation, we propose that fine-tuning of $G\alpha_s$ signaling is critical for vessel formation.

Depletion of $G\alpha_s$ in HUVECs Leads to Decreased Junctional VE-cadherin

To determine the effect of $G\alpha_s$ depletion on VE-cadherin *in vitro*, we first analyzed VE-cadherin expression levels in established HUVEC lines by Western blot. As indicated, the protein levels of VE-cadherin were comparable between control and $G\alpha_s$ KD cells (Fig. 4D). We then used immunofluorescent staining to observe the localization of VE-cadherin upon $G\alpha_s$ knockdown. In control cells, VE-cadherin linearly accumulated at cell-cell contacts. In contrast, little VE-cadherin staining signal was detected at the contacts of $G\alpha_s$ KD cells (GFP positive cells), supporting that $G\alpha_s$ is required for the junctional localization of VE-cadherin *in vitro* (Fig. 4E and F).

$G\alpha_s$ Acts Downstream of S1PR1 Signaling to Stabilize VE-cadherin at the Endothelial Cell-cell Junctions

The activation of S1PR1 signaling in endothelial cells has been shown to stabilize the junction localization of VE-cadherin (Lee et al., 1999). The inhibition or inactivation of S1PR1 signaling reduces the junctional localization of VE-cadherin (Gaengel et al., 2012), which is similar to the phenotype caused by $G\alpha_s$ deletion in endothelial cells. Thus, we hypothesize that $G\alpha_s$ is likely a downstream mediator of S1PR1 signaling. To test this hypothesis, control and $G\alpha_s$ KD HUVECs were first cultured in medium without serum, followed by treatment with a S1PR1-specific agonist SEW2871. Our data show that the activation of S1PR1 signaling by SEW2871 treatment strongly promoted the junctional localization of VE-cadherin in control cells but not in $G\alpha_s$ KD cells (Fig. 5A and B). These results suggest that $G\alpha_s$ is required for S1PR1 signaling transduction and that $G\alpha_s$ acts downstream of S1PR1 to control the junctional localization of VE-cadherin. $G\alpha_s$ signaling transduction has been extensively demonstrated to largely occur *via* the activation of adenylyl cyclase to generate cAMP (Spiegel and Weinstein, 2004; Li et al., 2013). We sought to determine whether a lack of a sufficient cAMP signal was responsible for the $G\alpha_s$ knockdown-induced junctional reduction of VE-cadherin. To test this hypothesis, we first measured the basal level of cAMP in control and $G\alpha_s$ KD cells

upon stimulation with SEW2871. Strikingly, we found that SEW2871 significantly elevated the cAMP above the basal level in HUVECs and this effect can be completely blocked by addition of S1PR1 antagonist (W146), suggesting that S1PR1 activation can increase the basal cAMP level (Fig. S5). In $G\alpha_s$ KD cells, however, the cAMP level cannot be elevated by SEW2871 (Fig. 5C), suggesting that $G\alpha_s$ acts as a downstream effector for S1PR1 signaling transduction. Then, we applied adenylyl cyclase activator Forskolin (FSK) to $G\alpha_s$ KD cells and control cells. Our results show that FSK treatment strongly promotes VE-cadherin junctional enrichment in $G\alpha_s$ KD cells as in control cells either with or without SEW2871 treatment (Fig. 5A and B). Together, these results suggest that $G\alpha_s$ controls the junctional localization of VE-cadherin *via* cAMP signaling.

S1PR1-activation induced endothelial AJ assembly was previously shown to be associated with the cortical reorganization of cytoskeletal actin (Lee et al., 1999). Therefore, we examined the effect of $G\alpha_s$ depletion on this dynamic process. As shown, $G\alpha_s$ depletion significantly attenuated the SEW2871-induced cortical actin formation, but enhancing cellular cAMP with FSK treatment completely restored this defect in $G\alpha_s$ KD cells (Fig. 5D and E). These results suggest that $G\alpha_s$ is a downstream effector of S1PR1 signaling and activates adenylyl cyclase to generate cAMP to control cortical actin organization as well as the junctional localization of VE-cadherin.

DISCUSSION

Heterotrimeric G proteins, which are composed of α , β , and γ subunits, mostly remain trimers and segregate into $G\alpha$ and $G\beta\gamma$ upon the activation of GPCR in response to an external ligand. Heterotrimeric G proteins are the largest family of signaling transducers and involved in almost every developmental and physiological process from single cell oocyte maturation to multi-cell organogenesis (Hampoelez and Knoblich, 2004; Malbon, 2005; Baumbach et al., 2014). Recent studies demonstrate that G proteins have critical but distinct roles in the maintenance of endothelial permeability and integrity by controlling AJ dynamics. $G\alpha_{13}$ facilitates the disassembly of AJs and thereby increases endothelial permeability by activating Src phosphorylation-dependent VE-cadherin internalization (Gong et al., 2014). $G\beta\gamma$ contributes to the re-annealing of AJs and thereby reduces endothelial permeability (Knezevic et al., 2009). Here, we demonstrate that $G\alpha_s$ is crucial for the maintenance of vascular integrity. The EC-specific deletion of $G\alpha_s$ caused severe vascular leakage and reduced the junctional localization of VE-cadherin without impairing mural cell coverage, which resembles the phenotype caused by the deletion of S1PR1 (Gaengel et al., 2012).

S1PR1 is a GPCR and required to maintain the vascular integrity in response to the blood-borne bioactive lipid S1P (Rosen et al., 2009). The embryos of S1P and S1PR1 deletion both display severe hemorrhage and die between E11.5 and E13.5 (Liu et al., 2000; Mizugishi et al., 2005), suggesting that both S1P and S1PR1 are required for maintaining vascular integrity. A more detailed study shows that the ablation of S1P/S1PR1 signaling during the early embryonic stage around E11.5 does not impair the mural cell coverage of hindbrain vasculature but disorganizes junctional VE-cadherin (Gaengel et al., 2012). Nevertheless, the link between S1PR1 and its effector VE-cadherin is unknown. S1PR1 has been shown to signal exclusively through G_i , a subtype of $G\alpha$ proteins (Windh et al., 1999), and also has been shown to inhibit isoproterenol-stimulated cAMP accumulation through a pertussis toxin-sensitive G_i -mediated pathway (Means et al., 2008). However, pertussis toxin treatment does not inhibit S1P-stimulated translocation of VE-cadherin to cell-cell junctions, suggesting that a non- G_i pathway is involved (Lee et al., 1999). In the present study, we provide evidence that $G\alpha_s$ acts

downstream of S1PR1 signaling to control vascular integrity. As mentioned above, the mutant embryos harboring an EC-specific deletion of $G\alpha_s$ show severe hemorrhage and die around E11.5. A loss of $G\alpha_s$ in endothelial cells reduces the junctional localization of VE-cadherin and does not impair mural cell coverage. Thus, EC- $G\alpha_s$ KO embryos display vascular developmental and endothelial junctional defects similar to those in S1PR1 knockout mice, suggesting a close relationship between these two signaling pathways. Moreover, the inactivation of $G\alpha_s$ blocks the adherens junctional stabilization of VE-cadherin and cortical actin formation induced by the treatment with the S1PR1-specific agonist SEW2871. This finding indicates that $G\alpha_s$ acts downstream of S1PR1 signaling to control vascular integrity. Furthermore, we show that the activation of S1PR1 by SEW2871 elevates the basal cAMP level through $G\alpha_s$ -mediated pathway, supporting that $G\alpha_s$ is required for S1PR1 signaling transduction. Based on these observations, we expect an association between $G\alpha_s$ and S1PR1. By a co-IP assay, however, we failed to identify the interaction between these two proteins. Thereby, how $G\alpha_s$ relays S1PR1 signaling is still unclear. The most possible explanation is that $G\alpha_s$ binds to S1PR1 in a very dynamic manner which cannot be detected by co-IP assays. Further technologies need to be introduced to explore this possibility.

In summary, we demonstrate that $G\alpha_s$ regulates endothelial junctional VE-cadherin dynamics at downstream of S1PR1 signaling to control vascular integrity. This finding has advanced our understanding of the role of S1P/S1PR1 signaling in maintaining vascular integrity during embryonic vascular development. S1P/S1PR1 signaling is involved in multiple diseases, such as cancer and cardiovascular diseases (Deng et al., 2012; Gaengel et al., 2012; Cannavo et al., 2013; Sarkisyan et al., 2014; Zhou et al., 2014). The identification of $G\alpha_s$ as a mediator of this pathway will provide a novel option for drug discovery and new applications of established drugs in cancer therapy (Lappano and Maggiolini, 2011; Liu et al., 2012).

MATERIALS AND METHODS

Mouse strains

Mice with floxed $G\alpha_s$ allele (Chen et al., 2005a; Chen et al., 2005b) and *Tie2-Cre* transgenic mice (Kisanuki et al., 2001) were reported previously. For deletion of the $G\alpha_s$ in embryonic endothelial cells, the female $G\alpha_s^{flox/flox}$ mice were crossed with male *Tie2-Cre* mice. The resulting progeny ($G\alpha_s^{flox/+}$; *Tie2-Cre* mice) were further mated with female $G\alpha_s^{flox/flox}$ mice to generate EC- $G\alpha_s$ knockout embryos. Genotyping was performed as described (Kisanuki et al., 2001; Chen et al., 2005b; Sakamoto et al., 2005). All animal procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University and Shenzhen Institutes of Advanced Technologies, Chinese Academy of Sciences.

Embryo dissection, embryonic cells dissociation and immunostaining

Noon of the day when vaginal plug was observed was defined as E0.5. Timed pregnant mice were harvested and followed by dissection of extraembryonic and embryonic tissues. The yolk sac was opened and the embryo was examined for gross phenotypes. For immunostaining in embryonic cells, embryos at E9.5 were isolated into ice-cold PBS and were treated with 0.05% Trypsin-EDTA (HyClone, USA) for 10 min at 37°C with occasional swirling and trituration. The trypsin was inactivated by adding FBS to 10% and the dissociated cells were immediately spun onto coverslip at 2000 rpm for 5 min. Then the cells were fixed and stained with anti- $G\alpha_s$, anti-PECAM1 and DAPI. Immunostaining in whole mount was performed as previously described (Franco et al., 2008). In brief, embryos were fixed in 4% paraformaldehyde-PBS overnight at 4°C, dehydrated and rehydrated in a series of methanol (25%/PBS, 50%/PBS, 75%/PBS, 100%), blocked in blocking buffer (1% BSA, 0.1% triton X-100, PBS) for 2 h at room temperature, and incubated in blocking buffer containing biotinylated-isolectin B4 for 48 h at 4°C, followed by incubation with second antibodies in blocking buffer overnight at 4°C. For immunostaining in frozen tissue sections, embryos and yolk sacs fixed in 4% paraformaldehyde-PBS were transferred to PBS supplemented with 30% of sucrose and incubated overnight. The tissues were then embedded in OCT compound (Leica,

Germany) at -80°C and cryo-sectioned at 25 µm. Staining of frozen sections was performed as previously described (Gaengel et al., 2012). The reagents and antibodies used are as following: biotinylated-isolectin B4 (1:50, Vector Labs, Canada), anti-PECAM1 antibodies (1:100, BD Bioscience, USA), anti- α -smooth muscle actin antibodies conjugated to Cy3 (1:100, Sigma, USA), anti-VE-cadherin antibodies (1:100, BD Bioscience), anti- $G\alpha_s$ antibodies (1:100, BD Bioscience), anti-NG2 antibodies (1:200, Millipore, Germany), anti-N-cadherin antibodies (1:100, BD Bioscience), DAPI (Invitrogen, USA). Images were acquired using a LSM510 (Carl Zeiss, Germany) confocal microscope or a Leica SD AF Confocal System. Evaluation of mural cell coverage was performed as previously described (Gaengel et al., 2012).

Cell culture and immunocytochemistry

HUVECs (passage 2-8) were purchased from PromoCell (Germany) and cultured in collagen I-coated tissue culture dishes in Medium 200 supplemented with LSGS (Invitrogen). For immunofluorescence, monolayer-cultured HUVECs were washed in ice-cold PBS, fixed in 4% PFA at 4°C for 20 min, then blocked in blocking buffer (PBS, 3% BSA, 2% goat serum and 0.2% Triton X-100) and stained with VE-cadherin (1:100, Cell Signaling Technology, USA) antibodies or Alexa555-phalloidin (1:50, Invitrogen). Images were acquired by using a LSM510 (Carl Zeiss) confocal microscope or a Leica SD AF confocal System. For drug treatment, HUVECs were incubated in starvation medium for 4 h and subsequently treated with 2 µmol/L SEW2871 (Millipore), 10 µmol/L FSK (Sigma) or a combination of SEW2871 and FSK for 30 min. Quantification of the levels of VE-cadherin or F-actin at cell-cell contacts was performed using Leica MM AF software (Powered by MetaMorph) as previously described (Noda et al., 2010).

Lentivirus-mediated shRNA knockdown of $G\alpha_s$

pGIPZ-lentiviral shRNA mir vectors targeting human $G\alpha_s$ gene (RHS4430-98852132; RHS4430-101029777) and non-silencing pGIPZ control vector (RHS4346) were purchased from Open Biosystems (USA).

For production of infectious lentiviral particles, a vector mix (pGIPZ vector, psPAX2, pMD2.G) was transfected into HEK293T cells at ~80% confluency using MegaTran 1.0

(OriGene Technologies, USA). The viral supernatant was collected 48 hours post transfection, filtered to remove particulate, and concentrated by ultracentrifugation. To generate $G\alpha_s$ knockdown stable cell lines, HUVECs grown at about 30% confluence were infected with lentivirus. After 48 h of infection, transfection positive cells were selected by 1 μ g/mL puromycin for additional 4 days and subsequently used for further analysis.

Morphogenesis assay on matrigel

Morphogenesis on matrigel was performed as described (Ponce, 2009). Briefly, 1.0×10^5 cells/well of HUVECs were plated in 24-well plates precoated with 250 μ L Matrigel Matrix (BD Bioscience). At 12 h after seeding, representative images were acquired. For CTx (Sigma) treatment, HUVECs were serum starved, and then seeded with CTx on Matrigel Matrix (growth factor-reduced, BD Bioscience). Tube number and total tubular length were measured by using Image-Pro Plus 6.0 software.

Western blot analysis

Western blot was performed as described previously (Shi et al., 2013). Briefly, HUVECs grown at ~95% confluence in 10 cm dishes were harvested and lysed with RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L PMSF) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Scientific, USA). Anti- $G\alpha_s$ (BD Bioscience), Anti-VE-cadherin (Cell Signaling Technology) and anti- β -actin (Santa Cruz Biotechnology, USA) antibodies were used to detected endogenous proteins of VE-cadherin, $G\alpha_s$ and β -actin.

Enzyme-linked immunoassay for cAMP

HUVECs grown at ~95% confluence in 6-well tissue culture plates were serum starved for 4 h, then incubated with or without 10 μ mol/L W146 (Sigma) for 1 h and stimulated with 2 μ mol/L SEW2871, 10 μ mol/L FSK or a combination of SEW2871 and FSK for 30 min. The cAMP level in the HUVECs was assayed following the manufacturer's protocol (Sigma, CA200).

Statistical analysis

376 Statistical analysis was performed using Student's t-test. Results shown are the mean $\pm$
377 SD from at least three independent experiments. $P < 0.05$ was considered statistically
378 significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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512 **Table 1** Genotypes of progeny from crosses between male $G\alpha_s^{flox/+}$; *Tie2-Cre* and female
513 $G\alpha_s^{flox/flox}$ mice

Age	Litter (pup)	Number of embryos				
		$G\alpha_s^{flox/flox}$; <i>Tie2-Cre</i>	$G\alpha_s^{flox/+}$; <i>Tie2-Cre</i>	$G\alpha_s^{flox/flox}$	$G\alpha_s^{flox/+}$	%Exp ^a
E9.5	5 (52)	13 (25%)	14	12	13	25
E10.0	5 (56)	15 (27%)	13	13	15	25
E11.0	11 (107)	19 (18%)	30	31	27	25
E11.5	6 (52)	6 (12%)	15	14	17	25

514 ^aThe percent of embryos expected (%Exp) is indicated if Mendelian ratios were obtained.

515

Figure Legends

Fig. 1. Phenotypes of $G\alpha_s$ endothelial-specific knockout embryos.

A: Double immunofluorescence staining of sections of E10.0 embryos using antibodies against $G\alpha_s$ and PECAM1 (an endothelial cell marker). The nuclei were stained with DAPI. The maximum projection of the z-stacks is shown at the top, and boxed region within the upper panel is magnified and shown using single confocal plane at the bottom. $G\alpha_s$ was visualized in the endothelial cells of the dorsal aorta (white arrows). da, dorsal aorta. **B:** Trypsin-dissociated E9.5 embryonic cells of control and mutant (EC- $G\alpha_s$ KO) were spun onto coverslip and stained with anti- $G\alpha_s$, anti-PECAM1 and DAPI. The dashed line encircles PECAM1 positive cells. Tie2-Cre mediated knockout of $G\alpha_s$ results in loss of $G\alpha_s$ expression in the endothelial cells. **C:** Quantification of $G\alpha_s$ intensity of PECAM1 positive cells in **B**. The intensity of $G\alpha_s$ staining was normalized to the average intensity of controls. Values are presented as means $\pm$ SD of > 40 cells from 3 embryos. *** $P < 0.001$. **D:** Histograms show percentages of EC- $G\alpha_s$ KO embryos alive with respect to total number of EC- $G\alpha_s$ KO embryos. Alive embryos were defined as those having a detectable heartbeat. **E:** Pictures of freshly isolated control and mutant embryos at E11.0 and E11.5. A higher magnification of boxed regions within the upper panels is shown at the bottom. The hemorrhages in the dorsal aorta and forebrain are indicated by the yellow arrows. **F:** Histograms show the percentages of EC- $G\alpha_s$ KO embryos having hemorrhages with respect to the total number of EC- $G\alpha_s$ KO embryos. **G:** Whole-mount isolectin B4 immunostaining of control and EC- $G\alpha_s$ KO embryos at E11.0. The truncated intersomitic vessels were detected in the mutant embryos (white arrows).

Fig. 2. EC-specific deletion of $G\alpha_s$ has no effect on mural cell coverage of vessels.

A: Co-immunostaining of sections of control and EC- $G\alpha_s$ KO embryos at E11.0 using antibodies against PECAM1 and α -SMA (a marker for vSMCs). da, dorsal aorta. **B and C:** Co-immunostaining of sections of control and mutant embryos at E11.0 using antibodies against PECAM1 and NG2 (a marker for pericytes). Higher magnification of boxed regions in **C** is shown on the right, respectively. The mural cell coverage of either dorsal aortae (**B**) or neural tube capillaries (**C**) is identical in control and EC- $G\alpha_s$ KO embryos.

The nuclei were stained with DAPI. Arrows point at pericytes. **D:** Quantification of relative mural cell densities in the dorsal aortae (**B**) and neural tube capillaries (**C**) of control and EC- $G\alpha_s$ KO embryos ($n = 3$). Values are expressed as a ratio of the fluorescent signal for the mural cell marker NG2 and the vessel marker PECAM1 and are presented as means $\pm$ SD. n.s. indicates no significance between two groups.

Fig. 3. Endothelial loss of $G\alpha_s$ reduces VE-cadherin localization at AJs.

A: PECAM1 and N-cadherin co-staining of the dorsal aorta in control and EC- $G\alpha_s$ KO embryos at E11.0. da, dorsal aorta. **B:** VE-cadherin immunostaining of control and mutant dorsal aorta from embryos at E10.0 and E11.0, respectively. **C:** Quantification of VE-cadherin immunofluorescence intensity in **B** ($n = 3$). The intensity of VE-cadherin staining was normalized to the average intensity of controls at E11.0. Values are presented as means $\pm$ SD. * $P < 0.05$, *** $P < 0.001$. **D:** VE-cadherin and Isolectin B4 (a vessel marker) co-staining of the dorsal aorta in control and EC- $G\alpha_s$ KO embryos at E11.0. The images within the white squares are expanded on the right. A marked decrease in the level of VE-cadherin at cell-cell contacts was detected in EC- $G\alpha_s$ KO mutant (white arrows). **E:** Immunofluorescence staining of VE-cadherin in the neural tube capillaries of control and EC- $G\alpha_s$ KO embryos at E11.0. The images within the white squares are expanded on the right. In contrast to the abundant accumulation of VE-cadherin at cell-cell contacts in control embryos, reduced, discontinuous, and dot-like VE-cadherin was detected in mutant cell-cell contacts (white arrows). **F:** Immunofluorescence staining of VE-cadherin and α -SMA on cross-sections of control and EC- $G\alpha_s$ KO yolk sacs at E10.5. In yolk sacs, junctional VE-cadherin signal was significantly decreased in mutant (white arrows). The nuclei were stained with DAPI.

Fig. 4. Depletion of $G\alpha_s$ impairs endothelial cell tube formation and leads to decreased junctional VE-cadherin *in vitro*.

A: Western blot analysis of $G\alpha_s$ expression in control and $G\alpha_s$ knockdown HUVECs, indicating a ~90% decrease of $G\alpha_s$ expression in $G\alpha_s$ KD cells. The two bands represent long and short $G\alpha_s$ isoforms, respectively. shRNA-scramble, non-silencing lentiviral vector as a negative control. **B:** Tube formation of control and $G\alpha_s$ knockdown HUVECs

on Matrigel. $G\alpha_s$ -depleted HUVECs exhibited dramatically reduced tube formation ability. **C:** Quantification of tube number and total tube length of capillary-like structures shown in **B**. Values are presented as means $\pm$ SD of three independent experiments. ** $P < 0.01$. **D:** Western blot analysis of VE-cadherin expression in control and $G\alpha_s$ knockdown HUVECs. No significant differences were found between control and $G\alpha_s$ KD cells. **E:** VE-cadherin immunostaining in control and $G\alpha_s$ knockdown HUVECs. GFP-positive cells indicate the cells transfected with scramble shRNA or $G\alpha_s$ -targeted shRNA. White arrowheads show the linear cell-cell contacts of transfection-positive cells. **F:** Quantification of VE-cadherin levels at cell-cell contacts indicated in **E**. Values are expressed as a percentage relative to that in control cells and are shown as means $\pm$ SD of > 40 contacts. *** $P < 0.001$.

Fig. 5. $G\alpha_s$ acts downstream of S1PR1 to stabilize VE-cadherin at cell-cell junctions.

A and D: Effect of SEW2871, FSK or both compounds treatment on the VE-cadherin junctional localization (**A**) and cortical actin formation (**D**) in control and $G\alpha_s$ knockdown HUVECs. Control and $G\alpha_s$ knockdown HUVECs were starved and subsequently treated with or without indicated drugs for 30 min, followed by immunostaining of VE-cadherin (**A**) or phalloidin (**D**). The nuclei were stained with DAPI. Note that knockdown of $G\alpha_s$ diminished the SEW2871-induced junctional VE-cadherin staining at cell-cell contacts (white arrows) and cortical actin formation (yellow arrows). **B and E:** Quantification of VE-cadherin levels at cell-cell contacts indicated in **A** and relative amount of cortical actin shown in **D**, respectively. Values are expressed as a percentage relative to that in DMSO-treated control cells and are shown as means $\pm$ SD of > 40 contacts. *** $P < 0.001$. **C:** cAMP levels of control and $G\alpha_s$ knockdown HUVECs starved and treated with indicated drugs for 30 min. Values are presented as means $\pm$ SD of three independent experiments. *** $P < 0.001$. The cells selected by puromycin for 4 days after lentiviral-mediated transduction of shRNA were used for these experiments.

Supplementary Figure Legends

Fig. S1. $G\alpha_s$ is expressed in the dorsal aorta.

A: Co-immunostaining of sections of embryos at E10.5 using antibodies against PECAM1 and $G\alpha_s$. da, dorsal aorta. **B:** No primary antibody against $G\alpha_s$ was added to the tissue as a control.

Fig. S2. Tie2-Cre mediates efficient recombination of the $G\alpha_s$ gene.

PCR analysis was performed using genomic DNA of E9.5 embryos from $G\alpha_s^{flox/+}$; *Tie2-Cre* crossed with $G\alpha_s^{flox/flox}$ mice. The four different genotypes were represented. The DNA fragments corresponding to the floxed (flox), wild-type (wt), or recombined (rec) $G\alpha_s$ gene are indicated.

Fig. S3. EC-specific deletion of $G\alpha_s$ causes the rupture of dorsal aorta.

Co-immunostaining of sections of control and EC- $G\alpha_s$ KO embryos was performed at E11.0 using antibodies against PECAM1 and α -SMA. White arrows show the rupture of the vessel wall. da, dorsal aorta.

Fig. S4. Activation of $G\alpha_s$ by CTx inhibits tube formation *in vitro*.

A: Tube formation of HUVECs treated with CTx at the indicated concentrations. **B:** Quantification of tube number and total tube length of capillary-like structures shown in **A**. Values are presented as means $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Fig. S5. SEW2871 activates S1PR1 signaling to increase the basal level of cAMP.

Effects of S1PR1 agonist (SEW2871) and antagonist (W146) on the basal cAMP levels were investigated in HUVECs. Values are presented as means $\pm$ SD of three independent experiments. *** $P < 0.001$.

A **B** **C** **D** **E** **F**

A **B** **C** **D** **E**

A

PECAM1

$G\alpha_s$

Merge

B

PECAM1

No primary

Merge

PECAM1

 α -SMA

Merge/DAPI

Control

EC-G α_s KO

A **B**