Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Sphingosine-1-Phosphate Receptor-1 Controls Venous Endothelial Barrier Integrity in Zebrafish

Chiara Tobia, Paola Chiodelli, Stefania Nicoli, Patrizia Dell'Era, Simone Buraschi, Stefania Mitola, Efrem Foglia, Pieter B. van Loenen, Astrid E. Alewijnse and Marco Presta

Arterioscler Thromb Vasc Biol. 2012;32:e104-e116; originally published online July 26, 2012;
doi: 10.1161/ATVBAHA.112.250035
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

Sphingosine-1-Phosphate Receptor-1 Controls Venous Endothelial Barrier Integrity in Zebrafish

Chiara Tobia, Paola Chiodelli, Stefania Nicoli, Patrizia Dell'Era, Simone Buraschi, Stefania Mitola, Efrem Foglia, Pieter B. van Loenen, Astrid E. Alewijnse, Marco Presta

Objective—Endothelial sphingosine-1-phosphate (S1P) receptor-1 ($S1P_1$) affects different vascular functions, including blood vessel maturation and permeability. Here, we characterized the role of the *zS1P$_1$* ortholog in vascular development in zebrafish.

Methods and Results—*zS1P$_1$* is expressed in dorsal aorta and posterior cardinal vein of zebrafish embryos at 24 to 30 hours postfertilization. *zS1P$_1$* downregulation by antisense morpholino oligonucleotide injection causes early pericardial edema, lack of blood circulation, alterations of posterior cardinal vein structure, and late generalized edema. Also, *zS1P$_1$* morphants are characterized by downregulation of vascular endothelial cadherin (*VE-cadherin*) and Eph receptor *EphB4a* expression and by disorganization of zonula occludens 1 junctions in posterior cardinal vein endothelium, with no alterations of dorsal aorta endothelium. *VE-cadherin* knockdown results in similar vascular alterations, whereas *VE-cadherin* overexpression is sufficient to rescue venous vascular integrity defects and *EphB4a* downregulation in *zS1P$_1$* morphants. Finally, *S1P$_1$* small interfering RNA transfection and the $S1P_1$ antagonist (R)-3-amino-(3-hexylphenylamino)-4-oxobutylphosphonic acid (W146) cause EPHB4 receptor down-modulation in human umbilical vein endothelial cells and the assembly of zonula occludens 1 intercellular contacts is prevented by the EPHB4 antagonist TNYL-RAW peptide in these cells.

Conclusion—The data demonstrate a nonredundant role of *zS1P$_1$* in the regulation of venous endothelial barrier in zebrafish and identify a *S1P$_1$/VE-cadherin/EphB4a* genetic pathway that controls venous vascular integrity. **(*Arterioscler Thromb Vasc Biol*. 2012;32:e104-e116.)**

Key Words: sphingosine-1-phosphate ■ endothelial barrier ■ zebrafish ■ VE-cadherin ■ Eph receptor

The lysophospholipid sphingosine-1-phosphate (S1P) is a signaling molecule that acts through the binding to a family of 7-transmembrane G-protein–coupled receptors that include 5 high-affinity members ($S1P_{1–5}$).[1] S1P receptors modulate a variety of biological processes, including cell adhesion, migration, and endocytosis with different and sometimes opposing effects on the regulation of cellular functions.[2] In particular, endothelial $S1P_1$ is involved in cytoskeletal rearrangement and formation of intercellular adherens junctions, its exposure to blood-borne S1P being required for the maintenance of blood–barrier integrity in defined vascular beds.[3,4] Also, genetic deletion demonstrates the nonredundant role of $S1P_1$ in vascular maturation during embryogenesis, leading to embryonic lethality in knockout mice.[5] Thus, chemical modulators of $S1P_1$ activity have been developed as putative therapeutic molecules acting to restore endothelial barrier integrity in various pathological conditions characterized by the disruption of endothelial cell–cell interaction in different organs, including lung, heart, kidney, and brain.[4]

Endothelial barrier integrity is dependent upon a complex network of molecular interactions involving adherens and tight junctions.[6] S1P may affect endothelial cell–cell junctions by regulating assembly and expression of the major adherens junction component, vascular endothelial adhesion molecule VE-cadherin, and formation of zonula occludens 1 $(ZO1)^+$ tight junctions.[7–10] However, the molecular and functional bases of the impact of the $S1P/S1P_1$ receptor system on endothelial barrier integrity remain to be fully elucidated.

The teleost zebrafish (*Danio rerio*) represents a promising experimental model for in vivo analysis of the molecular and cellular mechanisms underlying blood vessel development and vascular dysfunctions.[6] When compared with other vertebrate model systems, zebrafish offers many advantages, including ease of experimentation, drug administration, amenability to in vivo manipulation, and feasibility of reverse and forward genetic approaches.[11] Also, zebrafish possesses a complex circulatory system similar to that of mammals and the optical transparency and ability to survive for several days without a

Received on: October 24, 2011; final version accepted on: July 13, 2012.

From the Department of Biomedical Sciences and Biotechnology, Unit of General Pathology and Immunology, School of Medicine, University of Brescia, Italy (C.T., P.C., P.D., S.N., S.B., S.M., M.P.); Department of Biology, University of Milan, Italy (E.F.); Department of Pharmacology and Pharmacotherapy, Academic Medical Center, Amsterdam, The Netherlands (P.B.v.L., A.E.A.); Department of Internal Medicine, Yale University School of Medicine, New Haven, CT (S.N.); and Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, PA (S.B.).

Correspondence to Marco Presta, General Pathology, Department of Biomedical Sciences and Biotechnology, Viale Europa 11, 25123 Brescia, Italy. E-mail presta@med.unibs.it

Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.112.250035**

functioning circulation make the zebrafish embryo amenable for vascular biology studies.[12]

Expression of the zebrafish ortholog of the *S1P$_1$* receptor (*zS1P$_1$*) has been observed in the zebrafish embryonic brain,[13] whereas zebrafish mutants of the *S1P$_2$* receptor ortholog *miles apart* (*mil*) show organogenesis defects of the heart,[14] demonstrating that bioactive lipid receptors play different roles in zebrafish development. In the present work, we performed the characterization and functional analysis of *zS1P$_1$* receptor during vascular development in zebrafish. *zS1P$_1$* is transiently expressed in the axial vasculature of the trunk of zebrafish embryos. *zS1P$_1$* knockdown caused defects of venous vascular integrity, including *VE-cadherin* downregulation and disorganization of endothelial junctions, lack of blood circulation, and late generalized edema. In association with these defects, *zS1P$_1$* morphants showed the downregulation of the Eph receptor *EphB4a* in venous axial endothelium. Similar defects were observed in *VE-cadherin* morphants and genetic evidence indicate that *VE-cadherin* is a *zS1P$_1$*-regulated element that controls venous endothelial barrier integrity by acting upstream of *EphB4a*. Finally, *S1P$_1$* small interfering RNA (siRNA) transfection and the specific S1P$_1$ antagonist W146[15] cause EPHB4 receptor downmodulation in human umbilical vein endothelial cells (HUVECs). Furthermore, a selective EPHB4 antagonist disrupts ZO1$^+$ intercellular contacts in these cells. Together, the results provide evidence for a nonredundant role for *S1P$_1$* in the regulation of blood–barrier integrity via a novel *S1P$_1$/VE-cadherin/EphB4a* cascade that controls venous endothelial cell–cell junction assembly.

Materials and Methods

Reagents

S1P, dihydro-S1P (dhS1P), and W146 were from Avanti Polar Lipids Inc (Alabaster, AL). 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole (SEW2871) was from Calbiochem (Nottingham, UK). Racemic 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol mono (dihydrogen phosphate) ester (FTY720-P) and [2-(4-(5-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-yl amino) ethanol (CYM-5442) were synthesized as described.[16,17] TNYL-RAW peptide (YNYLFSPNGPIARAW) and scrambled peptide SCR-WTL (WTLAIFARNYNGPSP)[18,19] were kindly provided by Dr O. Salvucci (Bethesda, MD).

Zebrafish Stocks

Wild-type AB and transgenic tg(fli1:enhanced green fluorescent protein [EGFP])^{y120} zebrafish lines were maintained under standard conditions,[21] and embryos were staged by hours postfertilization (hpf), as described.[22] When specified, fertilized eggs were maintained in fish water containing 250 mmol/L D-mannitol (Sigma, St. Louis, MO) or treated with 0.66 mg/mL tricaine at different developmental stages and allowed to develop at 28.5°C.

Whole-Mount In Situ Hybridization

Digoxigenin-labeled RNA probes were transcribed from linear cDNA constructs (Roche Applied Science, Indianapolis, IN). Whole-mount in situ hybridization (ISH) was performed as described.[23] For sectioning, fixed embryos were dehydrated in ethanol series, cleared in xilol, and paraffin embedded overnight.

Morpholino-Mediated Knockdown of *zS1P$_1$* and *VE-cadherin*

Antisense morpholino oligonucleotides (MO) (Gene Tools, Corvallis, OR) were directed against the 5′ untranslated region spanning the *zS1P$_1$* ATG start codon (*zS1P$_1$*-MO: 5′-AGTGTCTGGCGATTAGGT CATCCAT-3′) or were designed to complement exon–intron boundaries of the zebrafish *VE-cadherin* gene (*VE-cadherin*-MO: 5′-TTT ACAAGACCGTCTACCTTTCCAA-3′). Standard MO (Std-MO: 5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used as control. Routinely, MOs were microinjected in 4.0 nL volume into 1- to 4-cell stage embryos at the concentration of 0.4 pmoles/embryo for *zS1P$_1$*-MO or 1.0 pmoles/embryo for *VE-cadherin*-MO. A subset of embryos was coinjected with 0.25 to 0.4 pmoles/embryo of *zS1P$_1$*-MO and human *S1P$_1$* mRNA (70–100 pg/embryo), murine *VE-cadherin* mRNA (40–60 pg/embryo), or human *VEGF-A* mRNA (100 pg/embryo). *zS1P$_1$*-MO shows no significant homology with human *S1P$_1$* mRNA and murine *VE-cadherin* mRNA sequences.

Microangiography

Tetramethylrhodamine isothiocyanate–dextran (molecular weight 2.0×10^6; Invitrogen, Carlsbad, CA) was dissolved in double distilled water at 20 mg/mL and microinjected into the sinus venosus of zebrafish embryos at 50 hpf as described.[24]

Microscopy

Embryos were mounted in agarose-coated dishes and photographed under an epifluorescence Leica MZ16 F stereomicroscope (1× Plan Apo objective; NA, 0.141) equipped with a DFC480 digital camera and ICM50 software version 2.8.1 (all from Leica, Wetzlar, Germany). Confocal images were acquired with an LSM 50 META confocal laser microscope (Carl Zeiss, Germany). When specified, dechorionated 28 hpf zebrafish embryos were fixed overnight at 4°C with 1.5% glutaraldehyde plus 4% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3. Then, embryos were postfixed for 1 hour in sodium-cacodylate–buffered 1% osmium tetroxide, dehydrated in a graded ethanol series, transitioned to propylene oxide, and embedded in Epon 812-Araldite. Semithin sections (0.8 μm) were obtained using a Reichert UltracutE ultramicrotome, stained with gentian violet and basic fuchsin, observed under a Leica DM 6000B microscope and photographed with a digital camera.

ZO1 Immunofluorescence Analysis of Zebrafish Embryos

Control and MO-treated tg(fli1:EGFP)^{y1} embryos were fixed at 30 hpf in 4% paraformaldehyde for 2 hours at room temperature. Whole-mount immunofluorescence analysis was performed as described[25] using a mouse anti-ZO1 antibody (Invitrogen) followed by Alexa Fluor 594 anti-mouse IgG (Invitrogen) and embryos were analysed by confocal laser microscopy.

zS1P$_1$ Transfection in Mammalian Cells and cAMP Assay

The polymerase chain reaction (PCR) fragment with the full-length coding sequence of *zS1P$_1$* was cloned from the total RNA isolated from zebrafish embryos at 24 hpf (primer set: forward, 5′-TCCATGATGCAGTTTTTGGA-3′; reverse, 5′-AGTTCCATCCCTCCAGTTT-3′) into the pCRII-TOPO vector (Invitrogen). Next, the *zS1P$_1$* coding region was subcloned from pCRII-TOPO into the pcDNA5/FRT/TO vector (Invitrogen) using BamHI and XbaI (Fermentas Life Sciences, St. Leon-Rot, Germany). Chinese hamster ovary–FlpIn cells (Invitrogen) were maintained in Ham-F12 with L-glutamine, supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% charcoal-stripped fetal calf serum (Invitrogen). Ten μg of plasmid DNA were used for transfection into Chinese hamster ovary–FlpIn cells using Lipofectamine 2000 (Invitrogen). After 24 hours, transfected cells were washed with serum-free medium and serum starved for

16 hours. Next, transiently transfected cells were detached from the surface using cell dissociation buffer (Invitrogen), washed once with Hanks balanced salt solution, and resuspended in 5.0 mmol/L Hepes buffer, containing Hanks balanced salt solution with 0.05% fatty acid–free BSA (Sigma). Stimulation mixtures consisted of stimulation buffer with 3 μmol/L forskolin, 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX) (both from Sigma) and the concentration response curve of the indicated compounds. Cells (2500 per well) were added to the stimulation mixtures in a 1:1 ratio in a 384-well Optiplate (Perkin-Elmer, Zaventem, Belgium) and stimulated for 30 minutes. The LANCE cAMP 384 kit (Perkin-Elmer) was used to determine the concentration of cAMP accumulated during stimulation according to the manufacturer's protocol in a total volume of 20 μL. Measurements were performed 3 hours after adding detection buffer and antibody mixture using a Wallac 1420 Victor^2 (PerkinElmer). Data are expressed as the potency of the indicated compound to inhibit forskolin–induced cAMP accumulation (calculated as pEC_{50} value, here defined as the negative logarithm of the EC_{50} value).

Quantitative RT-PCR Analysis

Total RNA was isolated from zebrafish embryos using Trizol reagent (Invitrogen). Approximately 2 μg of total RNA were retrotranscribed using M-MLV Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed with a Biorad iCycler iQ Real-Time PCR Detection System using iQ SYBR Green Supermix (Biorad, Hercules, CA) according to manufacturer's instructions. Primers sequence were as follows: *EphB4* (forward: 5′-GGGGCCTGCAGGAGAGACCA-3′, reverse: 5′-GATCCGCGCTCCTTCTCGCA-3′); *VE-cadherin* (forward: 5′-TGACGAGGAGGGCGGAGGAG-3′, reverse: 5′-AGGTCCCACA CTGGGCCGAA-3′); *β-actin* (forward: 5′-CGAGCAGGAGATG GGAACC-3′, reverse: 5′-CAACGGAAACGCTCATTGC-3′).

Samples were analyzed in triplicate, normalized against the threshold cycle (C_t) of *β-actin*, and expressed as changes with respect to the levels of the gene under investigation in control samples.

HUVEC Cultures

HUVECs were grown in M199 medium (Invitrogen) supplemented with 20% fetal calf serum (Invitrogen), endothelial cell growth factor (100 μg/mL, Sigma), and porcine heparin (100 μg/mL, Sigma). HUVECs were used at early passages (I–V) and grown on plastic surface coated with porcine gelatin (Sigma). When indicated, HUVECs were transfected with control-scrambled siRNA or with specific *S1P$_1$* siRNAs (TAGCATTGTCAAGCTCCTAAA, *S1P$_1$* siRNAa; and CTCGGTCTCTGACTACGTCAA, *S1P$_1$* siRNAb; both from Qiagen, Chatsworth, CA) using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Transfection efficiency was equal to 70% to 80% as shown by transfection with a fluorescein-conjugated control siRNA.

Western Blot Analysis of HUVECs

HUVECs were incubated with 30-μmol/L W146 for 48 hours, and cells lysates were probed with anti-EPHB4 antibody in a Western blot (Santa Cruz Biotechnology). Uniform loading of the gels was assessed using anti-focal adhesion kinase antibodies. In the second set of experiments, *S1P$_1$* siRNA-transfected HUVECs were analyzed 96 hours after transfection by Western blotting with anti-EPHB4 and anti-$S1P_1$ antibodies (Santa Cruz Biotechnology). The corresponding immunoreactive bands were quantified by computerized image analysis and data were normalized for the intensity of the control tubulin band in the same sample.

Immunofluorescence Analysis of HUVECs

Cells were seeded on gelatine-coated coverslips in complete cell culture medium and allowed to reach confluence. Then, confluent cells were incubated for 24 hours in the absence or in the presence of TNYL-RAW or SCR-WTL (both at 100 μmol/L), washed, fixed in 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton-X100, and blocked with 10% BSA/0.1% Triton-X100 in PBS. Then, cells were incubated with the mouse anti-ZO1 antibody followed by incubation with Alexa Fluor 488 anti-mouse IgG (Invitrogen) and nuclear counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Then, cells were photographed using a LSM510 Meta confocal microscope equipped with a Plan-Apochromat ×63/1.4 NA oil objective.

Results

Vascular Expression of *zS1P$_1$*

The complete coding sequence of *zS1P$_1$* (GenBank accession no. NM_131691) was cloned from the total RNA isolated from zebrafish embryos at 24 hpf. In keeping with previous observations,[13] *zS1P$_1$* encodes for a putative 362 amino acid protein highly homologous to human and murine $S1P_1$ receptors (70% and 71% similarity, respectively). Accordingly, both the selective $S1P_1$ receptor agonists SEW2871[26] and CYM5442[17] and the nonselective S1P receptor agonists S1P, dhS1P, and synthetic FTY720-P[27] potently inhibit forskolin-induced cAMP accumulation in Chinese hamster ovary–FlpIn cells transiently transfected with the *zS1P$_1$* cDNA with the following ranking order: FTY720-P>dhS1P≈S1P>CYM5442≈SEW2871 (Table). Similar results were obtained for Chinese hamster ovary–FlpIn cells transiently transfected with the human *S1P$_1$* cDNA (Table), thus, confirming that *zS1P$_1$* encodes for the bona fide zebrafish ortholog of the human *S1P$_1$* gene.

On this basis, we analyzed the expression pattern of the *zS1P$_1$* gene in zebrafish embryos by whole-mount ISH. In agreement with previous observations,[13] *zS1P$_1$* expression is restricted to the diencephalon at early somitogenesis (data not shown) and is widespread in the brain and neural tube at subsequent stages of development (Figure 1). Interestingly, whole-mount ISH showed also a transient but significant expression of *zS1P$_1$* transcript in the axial vasculature of the trunk of zebrafish embryos. This vascular pattern expression occurs at 24 hpf (Figure 1A and 1B), is maintained at 26 hpf (Figure 1D and 1E), and decreases at 30 hpf (Figure 1G and 1H), being lost at 48 hpf (data not shown). Analysis of the cross sections of the trunk of 24, 26, and 30 hpf embryos confirmed that, in addition to the neural tube, *zS1P$_1$* marks the

Table. Pharmacological Characterization of zS1P$_1$ Receptor

Receptor Agonist*	*zS1P$_1$*(pEC_{50})†	*hS1P$_1$*(pEC_{50})†
S1P	9.0±0.3	9.1±0.2
dhS1P	9.2±0.1	9.7±0.1
FTY720-P	10.5±0.1	10.8±0.4
SEW2871	7.5±0.2	7.9±0.1
CYM-5442	7.7±0.1	8.3±0.1

zS1P$_1$ indicates zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1; dhS1P, dihydro-S1P.

*Chinese hamster ovary–FlpIn cells were transiently transfected with zebrafish *zS1P$_1$* or human *hS1P$_1$* receptor cDNAs and treated with the indicated receptor agonists.

†Data are expressed as the potency of the indicated compound to inhibit forskolin-induced cAMP accumulation (calculated as pEC_{50} value, here defined as the negative logarithm of the EC_{50} value) and represent the mean±SEM of 2 to 3 independent experiments. No effect was observed in mock-transfected cells (data not shown).

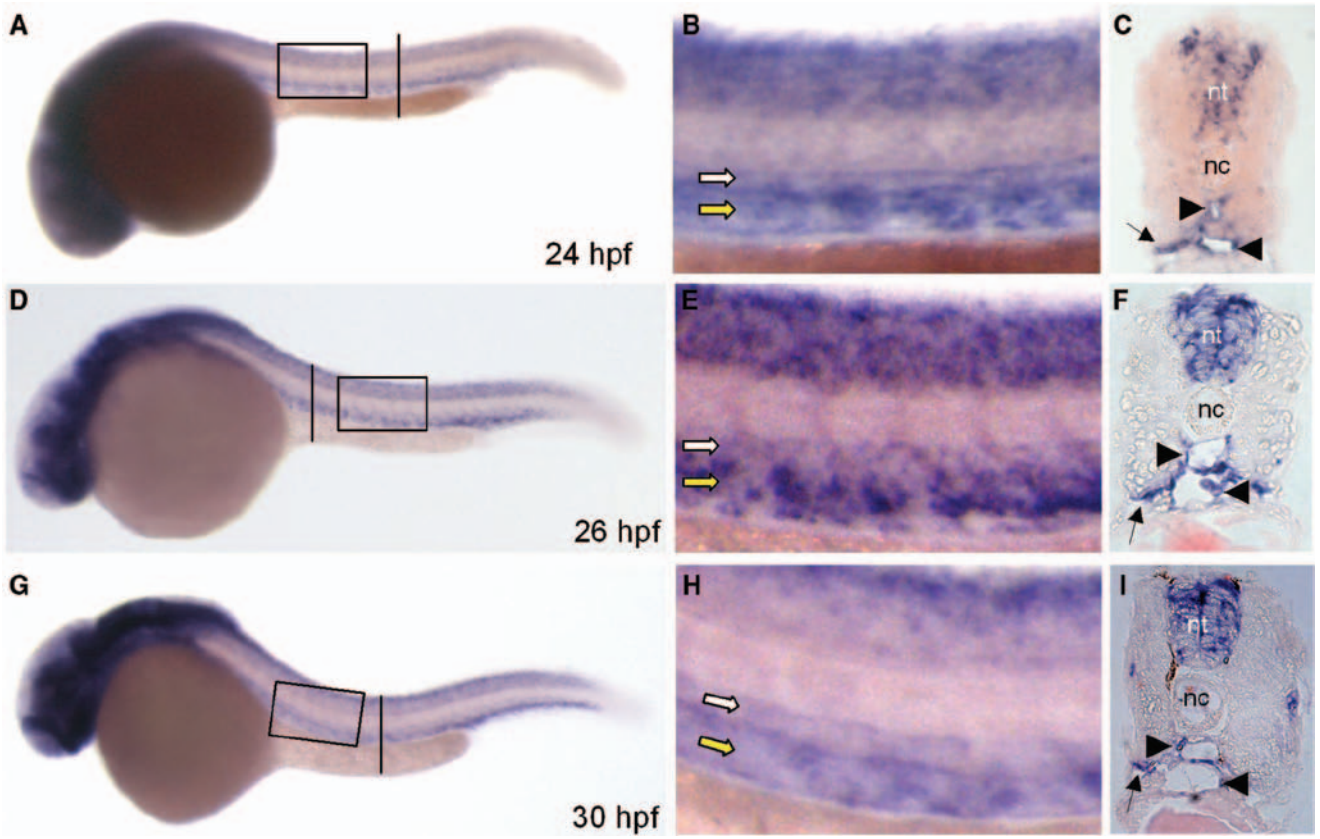

Figure 1. Vascular expression of the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P$_1$*). **A**, **D**, and **G**, *zS1P$_1$* expression was analyzed in zebrafish embryos by in situ hybridization at the indicated developmental stages. Embryos are anterior to the left and lateral to the top. **B**, **E**, and **H**, High magnification of the area in black boxes in **A**, **D** and **G**. **C**, **F**, and **I**, Transverse sections through the trunk highlighted as vertical black bars in **A**, **D**, and **G**. *zS1P$_1$* is expressed by the axial vasculature of the trunk (**white arrow** indicates dorsal aorta [DA]; **yellow arrow**, posterior cardinal vein [PCV]). Transverse sections confirm *zS1P$_1$* expression in DA and PCV (**black arrowheads**), in the basal aspect of the somites (**black arrows**), and in neural tube (nt). nc indicates notocord; hpf, hours postfertilization.

endothelium of the main medial dorsal aorta (DA) and posterior cardinal vein (PCV), as well as the basal aspect of the somites (Figure 1C, 1F, and 1I).

Endothelial differentiation in zebrafish is under the control of the *sonic hedgehog/vascular endothelial growth factor* (*shh*/*vegf*) genetic pathway.[28] Endothelial expression of *zS1P$_1$* in the axial vasculature of the trunk of 26 hpf embryos was abrogated by treatment with cyclopamine, a potent inhibitor of *shh* signaling[29] (data not shown) or after downregulation of *vegf* expression by antisense MO injection (Figure 2). Specificity of the effect was demonstrated by the lack of effect of both treatments on somitic and central nervous system expression of *zS1P$_1$*, thus, indicating that endothelial *zS1P$_1$* expression is under the control of the *shh*/*vegf* cascade.[30]

MO Knockdown of *zS1P$_1$* Function Results in Venous Vascular Barrier Defects

To assess the functional role of *zS1P$_1$* in zebrafish vascular development, we used an antisense MO knockdown approach.[31] To this purpose, a MO was designed directed against the 5′ untranslated region spanning the *zS1P$_1$* ATG start codon to inhibit protein translation (*zS1P$_1$*-MO). Next, tg(*fli1*:EGFP)^{y1} transgenic zebrafish embryos in which EGFP expression is driven by the promoter of the pan-endothelial marker *fli-1* were injected at the 1- to 4-cell stage with different doses of *zS1P$_1$*-MO (ranging from 0.1–0.4 pmoles/embryo) or of control std-MO and the development of EGFP–labeled blood vessels was directly observed in live embryos.

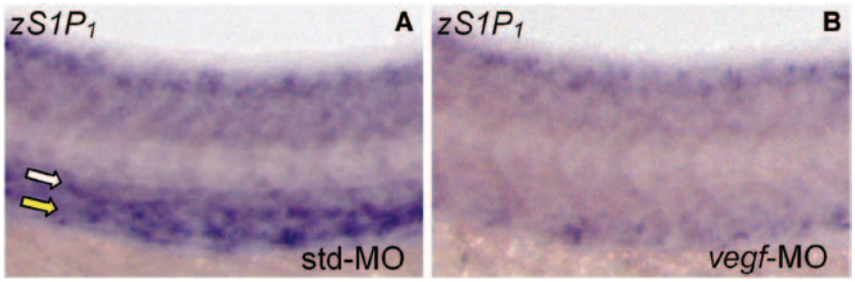

Figure 2. Vascular zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P$_1$*) expression is mediated by *vascular endothelial growth factor* (*vegf*). *zS1P$_1$* expression was assessed by in situ hybridization in standard-MO (std-MO) (**A**) and *vegf*-MO–injected embryos (**B**) at 28 hpf. Note the absence of *zS1P$_1$* transcript in dorsal aorta (DA) and posterior cardinal vein (PCV) of *vegf* morphants. **White arrow** indicates dorsal aorta; **yellow arrow**, PCV.

Whole–mount microscopic analysis of 24-hpf embryos injected with 0.4 pmoles/embryo of *zS1P*$_1$-MO revealed the presence of pericardial edema and enlargement of the PCV (Figure 3C and 3D) whose altered structure was confirmed by the analysis of histological semithin cross sections of the trunk of *zS1P*$_1$ morphants (Figure 4). At 2.5 days postfertilization (dpf), intersomitic vessels of all *zS1P*$_1$-MO–injected embryos (n=181) appear slightly thinner than in control Std-MO–injected embryos (Figure 3J). Also, *zS1P*$_1$ downregulation was associated with minor defects in the development of the duct of Cuvier and of the subintestinal vein basket in all mophants (Figure 3K and 3L). In keeping with the modest impact of *zS1P*$_1$ downregulation on blood vessel development, no differences in the expression of *shh*, *vegf*, *kdr,* and *fli-1* genes were observed in *zS1P*$_1$-MO–injected versus Std-MO–injected embryos (data not shown). These data support previous observations in *S1P*$_1$-null mice indicating that this S1P receptor is not essential for vasculogenic and angiogenic events

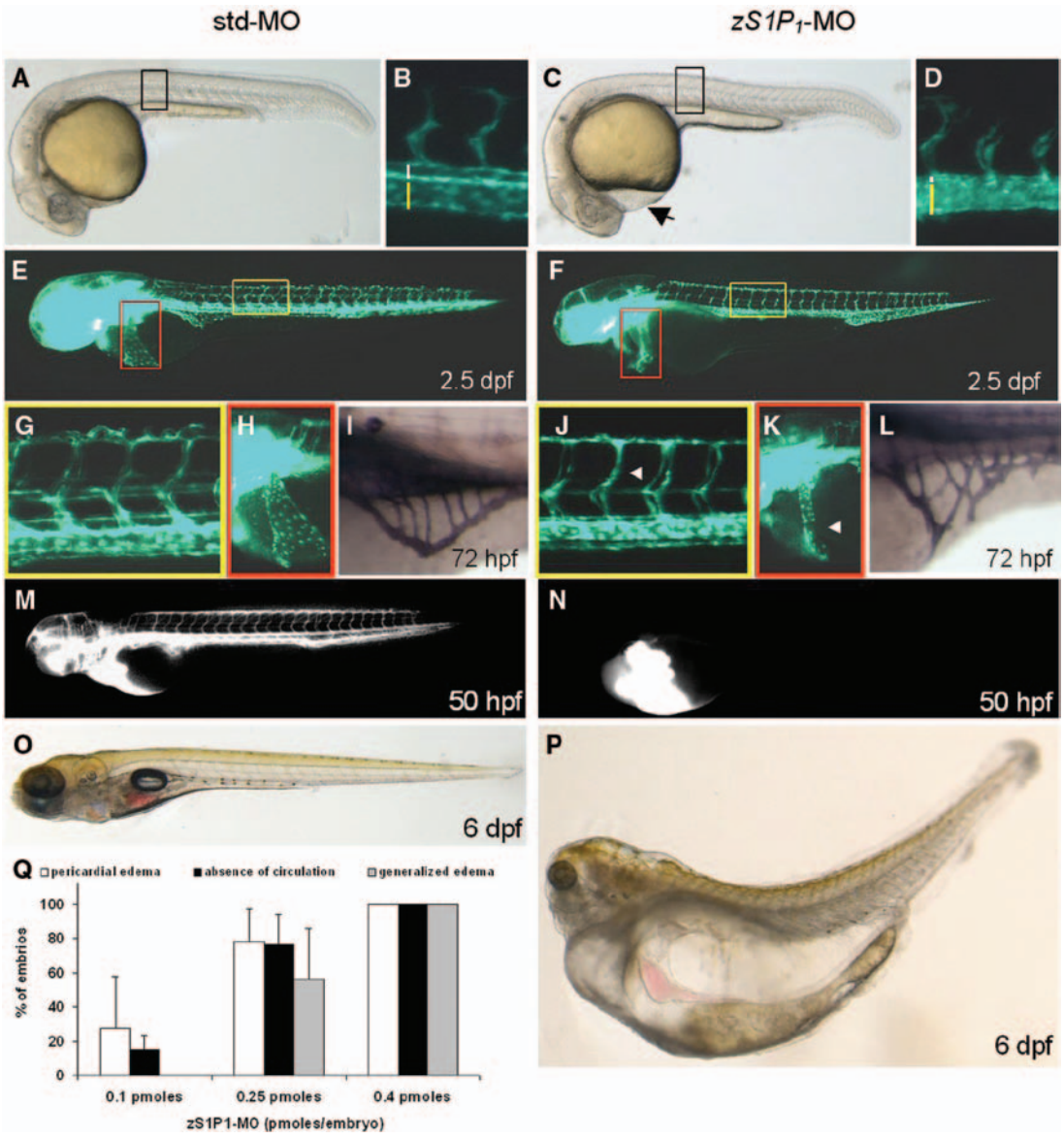

Figure 3. Vascular defects in the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P*$_1$) morphants. Vascular morphology in tg(*fli1*:EGFP)^{y1} embryos injected with standard-MO (std-MO) or *zS1P*$_1$-MO. Morphological appearance of control (**A**) and *zS1P*$_1$ morphant (**C**) embryos at 24 hours postfertilization (hpf). The pericardial edema in *zS1P*$_1$ morphants is indicated by a **black arrow** in **C**. Trunk regions highlighted by black boxes are shown at higher magnification in **B** and **D** where dorsal aorta (DA) and posterior cardinal vein (PCV) are indicated by white and yellow bars, respectively; note the abnormal size of PCV in *zS1P*$_1$ morphants (**D**). **E** and **F**, Epifluorescence microscopy of std-MO– and *zS1P*$_1$-MO–injected tg(*fli1*:EGFP)^{y1} embryos at 2.5 days postfertilization (dpf). Trunk and Duct of Cuvier regions highlighted by yellow and red boxes, respectively, are shown at higher magnification in (**G**), (**H**), (**J**), and (**K**); **white arrowheads** point to thinner intersomitic vessels (**J**) and to poorly developed Duct of Cuvier (**K**) in *zS1P*$_1$ morphants. **I** and **L**, Alkaline phosphatase staining of 3-dpf embryos revealed alterations of subintestinal vessel organization in *zS1P*$_1$ morphants (**L**). **M** and **N**, *zS1P*$_1$ knockdown causes absence of blood circulation as shown by microangiography performed at 50 hpf. **O** and **P**, Morphological appearance of control and *zS1P*$_1$ morphant larvae at 6 dpf; note the generalized edema in *zS1P*$_1$ morphants (**P**). **Q**, Dose-dependent effects of *zS1P*$_1$ knockdown: white bars, pericardial edema; black bars, absence of blood circulation; gray bars, late generalized edema (each point is the mean±SD of 97–181 embryos per group).

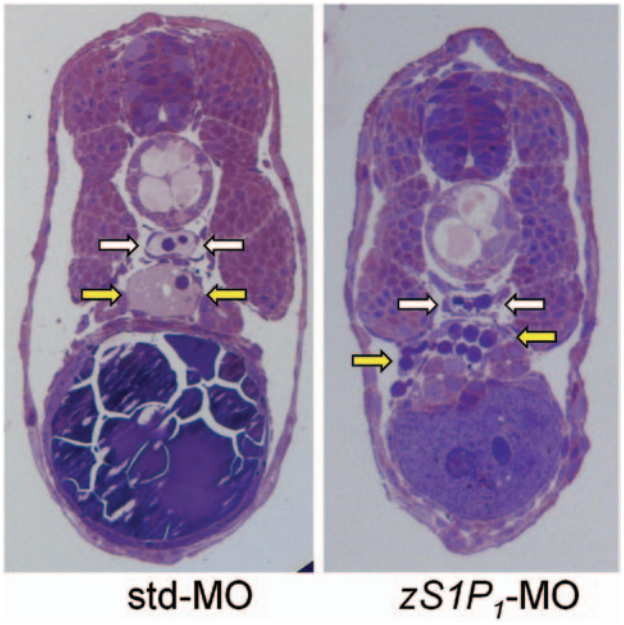

Figure 4. Alterations of posterior cardinal vein (PCV) morphology in the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P$_1$*) morphants. Histological semithin cross sections of the trunk of 28 hours postfertilization embryos demonstrate the altered structure of the PCV in *S1P$_1$* morphants when compared with standard-MO (std-MO)–injected embryos. **White arrows** indicate dorsal aorta; **yellow arrows**, PCV.

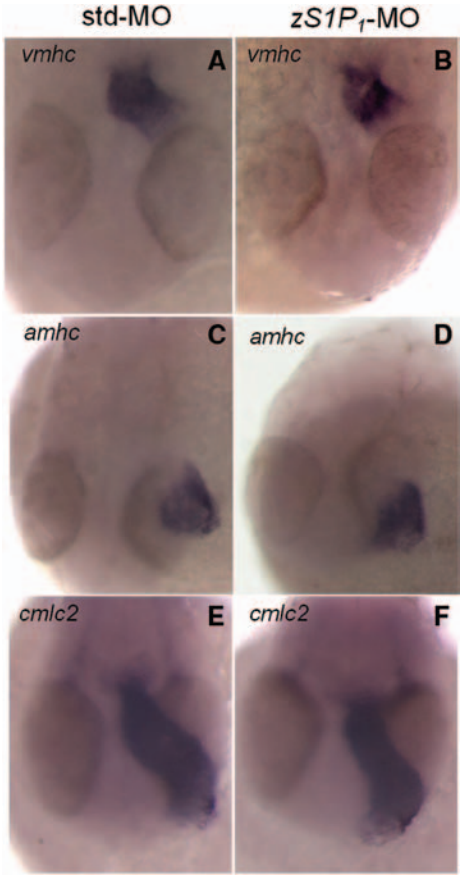

Figure 5. Effect of the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P$_1$*) knockdown on cardiac myogenesis gene expression. Standard-MO (std-MO)– and *zS1P$_1$*-MO–injected embryos (n=14) were grown in fish water containing 250 mmol/L D-mannitol and analyzed at 28 hours post fertilization (hpf) for the expression of the cardiac myogenesis gene markers *ventricular myosin heavy chain* (*vmhc*; **A** and **B**), *atrial myosin heavy chain* (*amhc*; **C** and **D**), and *cardiac myosin light chain 2* (*cmlc2*; **E** and **F**). No alterations were observed in z*S1P$_1$* morphants when compared with controls. At later stages of development (48 hpf), minor defects in *cmlc2* expression were observed only in a limited number of z*S1P$_1$* morphants (11/33) (data not shown).

during embryogenesis.[5] However, despite minor alterations in vascular development, the presence of a beating heart with no major developmental defects (Figure 5) and of a patent lumen in both DA and PCV cross sections (Figure 4), *zS1P$_1$*-MO–injected embryos showed no apparent blood circulation, as confirmed by the absence of tetramethylrhodamine isothiocyanate–dextran perfusible vessels at 50 hpf (Figure 3N). Of note, 6 dpf *zS1P$_1$*-MO–injected embryos showed a remarkable generalized edema never observed in control embryos (Figure 3P). The incidence of phenotypic alterations (early pericardial edema, lack of blood circulation, and late generalized edema) was directly related to the dose of injected *zS1P$_1$*-MO, 100% of the embryos showing all these defects when injected with 0.4 pmoles/embryo (Figure 3Q). The appearance of the generalized edema, already evident at 4 dpf, was fully prevented when *zS1P$_1$* morphants were grown in fish water containing 250 mmol/L D-mannitol. Nevertheless, blood circulation was not restored (data not shown).

To confirm the specificity of the vascular and circulatory defects in *zS1P$_1$* morphants, *zS1P$_1$*-MO was coinjected with a MO-resistant form of the human *S1P$_1$* mRNA. In 2 independent experiments, analysis of the embryos at 30 hpf showed the ability of the human transcript to rescue the *zS1P$_1$*-knockdown phenotype, as assessed by the presence of normal blood flow in 76% of rescued embryos when compared with 36% of *zS1P$_1$* morphants ($P<0.001$; Fisher exact test; Figure 6A). Also, analysis of the embryos at 6 dpf showed the ability of the human transcript to rescue the generalized edema observed in *zS1P$_1$* morphants. In fact, only 34% of *zS1P$_1$* morphants coinjected with the human *S1P$_1$* mRNA developed generalized edema when compared with 57% of *zS1P$_1$* morphants ($P<0.001$; Fisher exact test; Figure 6B).

S1P$_1$ receptor and its ligand S1P play an important role in the control of vascular barrier by affecting endothelial cell–cell junctions,[15] including the expression of the adherens junction component *VE-cadherin*.[10] The phenotype observed in *zS1P$_1$* morphants prompted us to assess the possibility that *zS1P$_1$* may control endothelial barrier integrity in zebrafish. On this basis, the effect of *zS1P$_1$* downregulation on the organization of endothelial cell–cell junctions was investigated by whole-mount ISH of *VE-cadherin*[32] expression in the axial vasculature of the trunk and by immunofluorescence analysis of ZO1, a tight junction–associated protein functionally critical in regulating S1P-mediated endothelial barrier integrity.[33] As shown in Figure 7B, *zS1P$_1$* knockdown caused the selective downregulation of *VE-cadherin* in the PCV but not in the DA of *zS1P$_1$* morphants. In keeping with the ISH data, the RT-PCR analysis showed a decrease of steady state *VE-cadherin* mRNA levels in *zS1P$_1$* morphants when compared with control embryos (31%±12%, average of the 2 independent experiments).

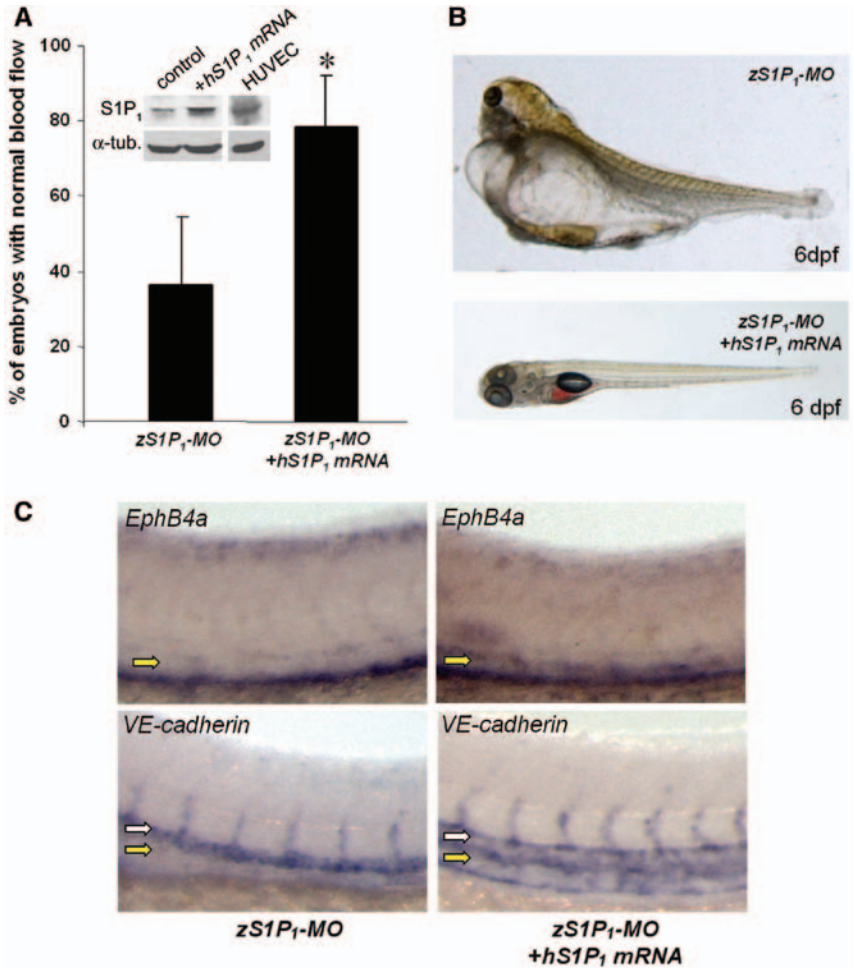

Figure 6. Human *hS1P*$_1$ overexpression rescues vascular defects in the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P*$_1$) morphants. *zS1P*$_1$ morphants were injected with *hS1P*$_1$ mRNA and lack of blood flow (**A**) and generalized edema (**B**) were analyzed at 30 hours postfertilization and 6 days postfertilization (dpf), respectively. *hS1P*$_1$ overexpression caused a significant increase in the percentage of embryos with normal blood flow (122/161 [76%] vs 42/117 [36%] *zS1P*$_1$ morphants injected or not with *hS1P*$_1$ mRNA, respectively; $P<0.001$, Fisher exact test) and in the percentage of embryos devoid of a significant generalized edema (100/152 [66%] vs 48/112 [43%] *zS1P*$_1$ morphants injected or not with *hS1P*$_1$ mRNA, respectively; $P < 0.001$, Fisher exact test). Western blot analysis confirmed the increased expression of S1P$_1$ protein in 24 hpf zebrafish embryos injected with *hS1P*$_1$ mRNA when compared with noninjected animals (inset in **A**; human umbilical vascular endothelial cells [HUVEC] extract was used as a control and uniform loading of the gel was assessed with anti–α-tubulin [α-tub] antibodies). **C**, The loss of *EphB4a* and vascular endothelial cadherin (*VE-cadherin*) expression in posterior cardinal vein (PCV) of *zS1P*$_1$ morphants (43/60 [72%] and 33/44 [75%] embryos in two different experiments) was reduced to 4/17 (24%) and 9/36 (25%) embryos coinjected with the human *S1P*$_1$ mRNA ($P<0.001$, Fisher exact test). **White arrow** indicates DA; **yellow arrow**, PCV.

Also, confocal microscopy analysis of the vasculature of the trunk of control and *zS1P*$_1$ morphants at 30 hpf revealed a complete disorganization of the ZO1$^+$ endothelial junctions in the PCV of all the *zS1P*$_1$ morphants examined (n=5) (Figure 7D). Instead, no effect on ZO1 immunolocalization was observed in the DA of *zS1P*$_1$ morphants (Figure 7D) and in both axial vessels of std-MO–injected embryos (Figure 7C). Together, the data indicate a nonredundant

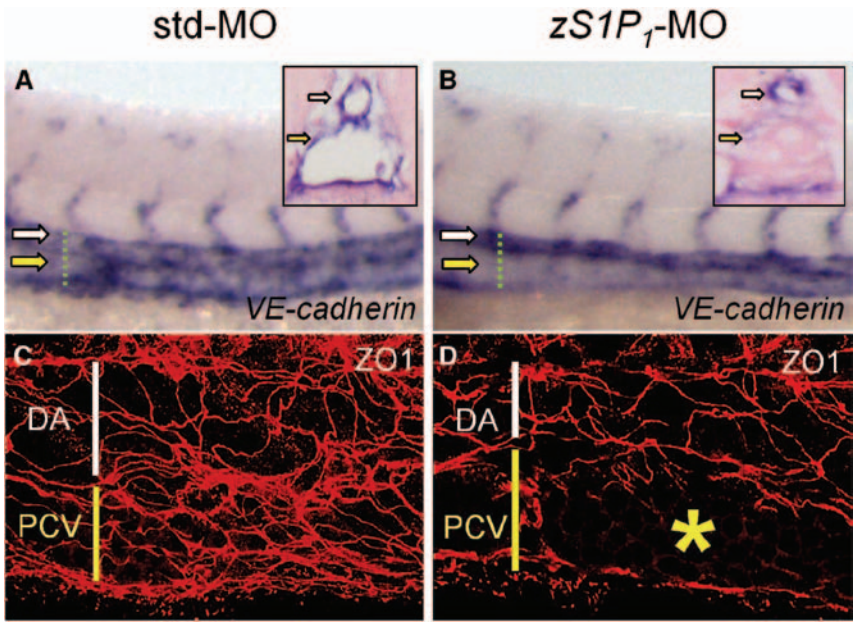

Figure 7. The zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P*$_1$) knockdown affects Vascular endothelial cadherin (*VE-cadherin*) expression and zonula occludens 1 (ZO1) immunolocalization in posterior cardinal vein (PCV). Standard-MO (std-MO)– and *zS1P*$_1$-MO–injected embryos were analyzed at 28 hpf for the expression of *VE-cadherin* by in situ hybridization (**A** and **B**) and immunostained for ZO1 at 30 hours postfertilization (**C** and **D**). Dorsal aorta (DA) and PCV are indicated by white and yellow symbols, respectively. zS1P$_1$ morphants were characterized by the downregulation of *VE-cadherin* expression (33/44 embryos examined) and altered ZO1 organization in PCV (asterisk in **D**) but not in DA (**D**) (5/5 embryos examined). In contrast, normally organized ZO1$^+$ junctions were observed in both axial vessels of control embryos (**C**). Transverse sections highlighted as vertical green dotted bars confirm *VE-cadherin* downregulation in PCV of *zS1P*$_1$ morphants when compared with controls (insets in **A** and **B**).

vascular function for *zS1P$_1$* that affects venous endothelial cell–cell barrier in zebrafish.

Downregulation of Venous Endothelial *EphB4a* Expression in *zS1P$_1$* Morphants

The selective effect of *zS1P$_1$* knockdown on PCV structure, *VE-cadherin* expression, and ZO1 organization prompted us to investigate the expression of arterial and venous markers in the axial vessels of the trunk in *zS1P$_1$*-MO–injected embryos. As shown in Figure 8, *zS1P$_1$* downregulation does not affect the expression of the specific arterial markers *ephrinB2*[30] and *crlr*[34] in the DA of *zS1P$_1$* morphants. Also, *zS1P$_1$*-MO injection does not alter the expression of the venous markers *flt4*[35] and *dab2*[36] in the PCV. Thus, in keeping with previous observations in *S1P$_1$* null mice,[5] the data indicate that *zS1P$_1$* knockdown does not affect arterial/venous differentiation of endothelial cells in zebrafish. However, *zS1P$_1$* downregulation caused the loss of the expression of the Eph receptor *EphB4a* in the PCV of *zS1P$_1$*-MO–injected embryos (Figure 8J), as confirmed by the decrease of steady state *EphB4a* mRNA levels in *zS1P$_1$*-null embryos when compared with controls (38%±9%, average of the 2 independent quantitative RT-PCR experiments). Of note, *zS1P$_1$* downregulation did not affect the expression of *EphB4a* in the gut of zebrafish embryos, thus demonstrating the specificity of the effect of *zS1P$_1$*-MO on *EphB4a* expression in PCV (asterisk in Figure 8I and 8J).

The loss of *EphB4a* and *VE-cadherin* expression in venous endothelium of *zS1P$_1$* morphants (72% and 75% of *zS1P$_1$* morphants, respectively) was significantly reduced to 24% and 25% for the 2 genes in embryos coinjected with the human *S1P$_1$* mRNA (Figure 6C), thus confirming the specificity of the effect ($P<0.001$, Fisher exact test). Also, heart beating arrest after treatment with a high dose of ethyl-m-aminobenzoate (tricaine) did not affect *EphB4a* and *VE-cadherin* expression in zebrafish embryos (n=22), thus, indicating that the observed downregulation of these genes in *zS1P$_1$* morphants is not the direct consequence of the lack of blood flow (Figure 9). Finally, coinjection of the human *S1P$_1$* mRNA fully rescued ZO1 organization in the PCV of *zS1P$_1$* morphants (data not shown). These observations point to a role for S1P$_1$ receptor in the control of venous endothelial cell functions and gene expression in zebrafish.

VE-cadherin Acts Upstream of *EphB4a* in Modulating Venous Vascular Barrier Organization

Previous observations had demonstrated a tight relationship between the expression of the members of the cadherin family of adhesion molecules and Eph receptors in epithelial cells.[37] On this basis, we addressed the possibility that the venous endothelial barrier defects in zebrafish embryos lacking *zS1P$_1$* activity might be the consequence of defects in a putative *zS1P$_1$*/*VE-cadherin*/*EphB4a* pathway.

To assess this hypothesis, we investigated the effect of *VE-cadherin* downregulation on *EphB4a* expression and vascular integrity in zebrafish morphants.[32] As shown in Figure 10, embryos injected with *VE-cadherin*–MO (1.0 pmoles/embryo) showed downregulation of *EphB4a* expression (24/24 embryos), pericardial edema and lack of blood circulation (28/30 embryos, data not shown), and

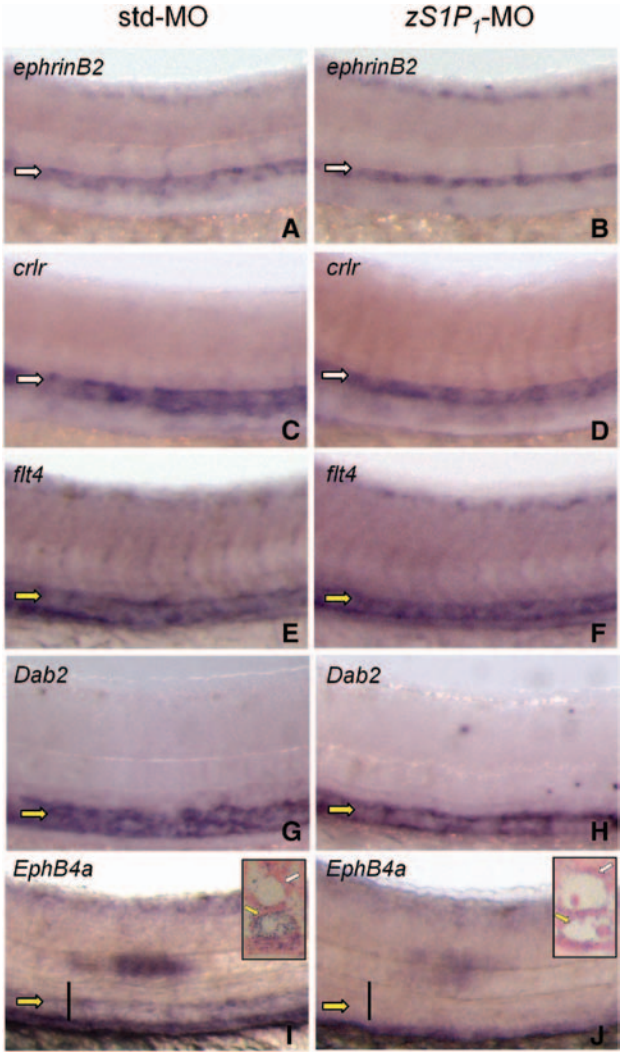

Figure 8. The zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P$_1$*) knockdown causes *EphB4a* downregulation in posterior cardinal vein (PCV). Standard-MO (std-MO)– and *zS1P$_1$*-MO–injected embryos were analyzed at 28 hours postfertilization for the expression of the indicated markers by in situ hybridization (lateral view of the trunk region; dorsal aorta (DA), **white arrow**; PCV, **yellow arrow**). *zS1P$_1$* morphants were characterized by the lack of expression of *EphB4a* in the PCV (**J**; 43/60 embryos examined). In contrast, arterial markers *ephrinB2* (**B**) and *crlr* (**D**) and venous markers *flt4* (**F**) and *dab2* (**H**) were expressed in 9/10, 9/12, 11/12, and 14/18 *zS1P$_1$* morphants, respectively. All genes were instead normally expressed in all control embryos examined (**A**, **C**, **E, G**, and **I**; n=15–30). Note that *EphB4a* expression, downregulated in the PCV, is maintained in the gut of all the *zS1P$_1$* morphants (asterisk in **I** and **J**). Transverse sections highlighted as vertical black bars confirm *EphB4a* downregulation in the PCV of *zS1P$_1$* morphants when compared with controls (insets in **I** and **J**).

late generalized edema (19/22 embryos) with no changes in vascular *zS1P$_1$* expression (13/13 embryos). Also, *VE-cadherin* morphants revealed a complete disorganization of the ZO1$^+$ endothelial junctions in PCV and DA of all the embryos examined (Figure 10H). These data extend previous observations about the role of *VE-cadherin* on vascular stability in zebrafish.[32,38] On this basis, to assess whether exogenous *VE-cadherin* is sufficient to rescue *EphB4a* expression and vascular integrity in the absence of *zS1P$_1$* activity, *zS1P$_1$*

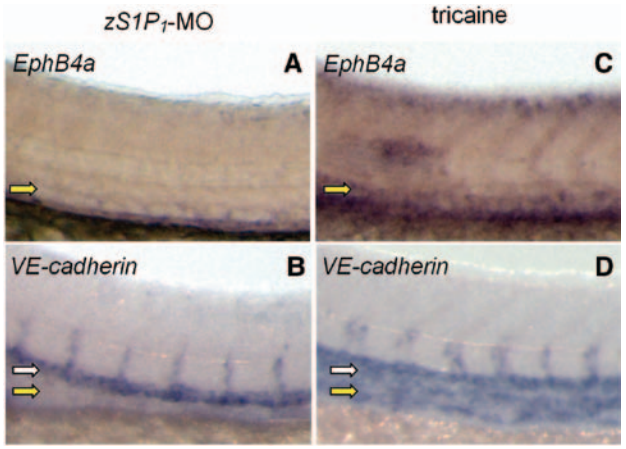

Figure 9. *EphB4a* and vascular endothelial cadherin (*VE-cadherin*) downregulation in $zS1P_1$ morphants is not a result of the absence of blood circulation. In situ hybridization at 28 hour postfertilization revealing *EphB4a* and *VE-cadherin* expression in the posterior cardinal vein (PCV) of tricaine-treated embryos (**C** and **D**) and absent in the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 ($zS1P_1$)-MO–injected embryos (**A** and **B**). **White arrow** indicates DA; **yellow arrow**, PCV.

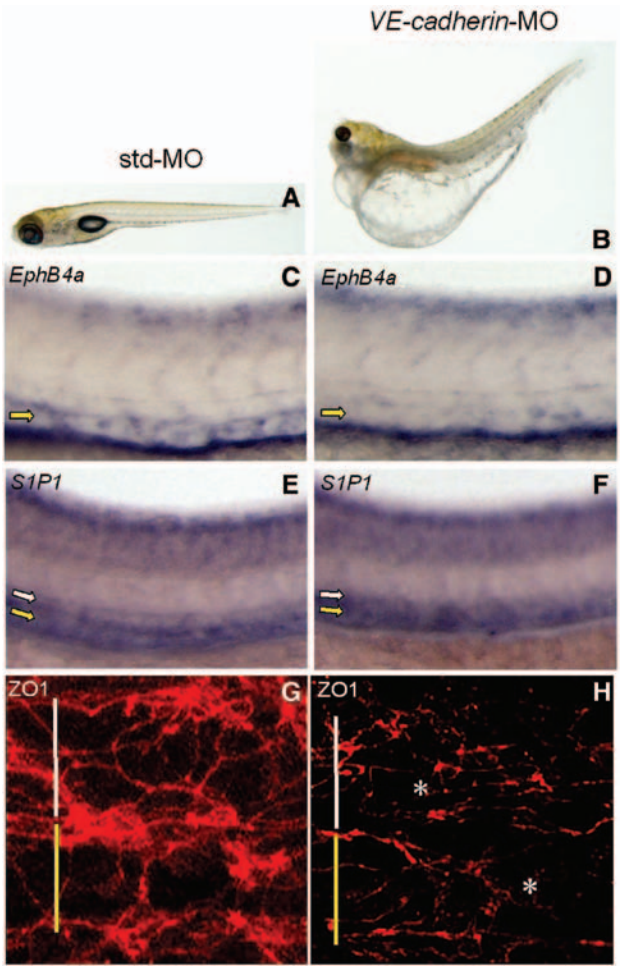

Figure 10. Vascular endothelial cadherin (*VE-cadherin*)–MO injection phenocopies the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 ($zS1P_1$) knockdown. Standard-MO (std-MO)– and *VE-cadherin*-MO–injected embryos were analyzed for the appearance of generalized edema at 6 dpf (**A** and **B**), *EphB4a* and $zS1P_1$ expression by in situ hybridization at 28 hours postfertilization (hpf) (**C**–**F**) and immunostained with anti-ZO1 antibody at 30 hpf (**G** and **H**). Dorsal aorta (DA) and posterior cardial vein (PCV) are indicated by white and yellow symbols, respectively. *VE-cadherin* morphants were characterized by presence of generalized edema (**B**, 19/22 embryos), downregulation of *EphB4a* (**D**, 24/24 embryos), and altered zonula occludens 1 (ZO1) organization in PCV and DA (asterisks in **H**). In contrast, normally organized ZO1$^+$ junctions were observed in both axial vessels of control embryos (**G**). *VE-cadherin* downregulation does not affect $zS1P_1$ expression in DA and PCV (**E** and **F**).

morphants were injected with the mRNA encoding for murine *VE-cadherin*.[32] As shown in Figure 11B and 11C, the loss of *EphB4a* expression in venous endothelium of $zS1P_1$ morphants was significantly reduced in embryos coinjected with the murine *VE-cadherin* mRNA (78% versus 41% of morphants, respectively; $P<0.001$, Fisher exact test). Also, injection of *VE-cadherin* mRNA caused a significant decrease in the number of $zS1P_1$ morphants showing early pericardial edema, lack of blood circulation, and late generalized edema (Figure 11A). Accordingly, *VE-cadherin* mRNA overexpression rescued the organization of ZO1$^+$ endothelial junctions in the PCV of $zS1P_1$ morphants (Figure 11E). At variance, no rescue was observed after injection of $zS1P_1$ morphants with *VEGF-A* mRNA, thus confirming the specificity of the effect (data not shown). These results suggest that the loss of *EphB4a* expression in $zS1P_1$ morphants is a result of the *VE-cadherin* downregulation in PCV endothelium downstream of $zS1P_1$, pointing to the existence of a $zS1P_1$/*VE-cadherin*/*EphB4a* pathway modulating venous vascular barrier organization in zebrafish embryo.

EPHB4 Controls Barrier Integrity in HUVECs

The segregation of distinct arterial and venous vessels is disrupted by *EphB4a* knockdown in zebrafish,[39] hampering the possibility to use *EphB4a* morphants to directly assess the role of this receptor in PCV integrity. Also, *EphB4a* overexpression severely affects the general development of $S1P_1$ morphants (data not shown), forbidding phenotypic rescue experiments in these embryos.

To overcome these limitations and to confirm that a S1P/Eph receptor cross talk may modulate intercellular contacts also in mammalian venous endothelium, we took advantage of HUVECs that express both $S1P_1$[40] and the Eph receptor EPHB4.[19] As anticipated, a 1-hour pretreatment of confluent HUVECs with 30 μmol/L of the selective $S1P_1$ antagonist W146[15] prevents ZO1$^+$ intercellular junction assembly triggered by a 2-hour treatment with 62.5 nmol/L S1P (data not shown) and causes a potent inhibition of *EPHB4* expression in both control and S1P-treated HUVECs as assessed by quantitative RT-PCR (87%±2% and 95%±1% inhibition, respectively; n=2). Accordingly, W146 treatment induces a significant inhibition of the levels of EPHB4 receptor protein in these cells (Figure 12A).

Only a limited inhibition of EPHB4 protein levels was observed after transfection with either 1 of the 2 different $S1P_1$ siRNAs, each causing a partial downregulation of $S1P_1$ expression (Figure 12B). However, the simultaneous

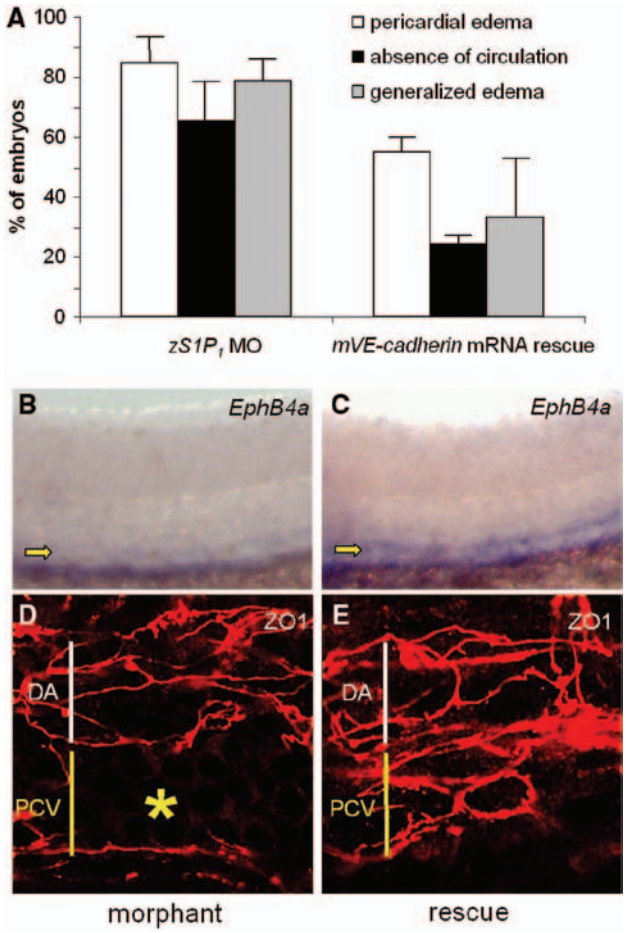

Figure 11. Murine vascular endothelial cadherin (*VE-cadherin*) overexpression rescues vascular defects in the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (*zS1P$_1$*) morphants. **A**, z*S1P$_1$* morphants were left untreated (n=72) or were injected with murine *VE-cadherin* mRNA (n=123), and their phenotype was analyzed for the presence of pericardial edema and lack of blood flow at 48 hours post fertilization (hpf) and late generalized edema at 5 dpf. *mVE-cadherin* overexpression rescued all these vascular alterations in *zS1P$_1$* morphants. **B–E**, Control and *mVE-cadherin* mRNA-–injected z*S1P$_1$* morphants were analyzed at 28 hpf for the expression of *EphB4a* (**B** and **C**) and immunostained for zonula occludens 1 (ZO1) at 30 hpf (**D** and **E**). Dorsal aorta (DA) and posterior cardinal vein (PCV) are indicated by white and yellow symbols, respectively. The loss of *EphB4a* in PCV of *zS1P$_1$* morphants (60/77 [78%] embryos in 3 different experiments) was reduced to 31/76 (41%) embryos coinjected with *mVE-cadherin* mRNA (**C**; $P<0.001$, Fisher exact test). Also, normal ZO1 immunolocalization was observed in the PCV of all the observed *mVE-cadherin* mRNA–injected z*S1P$_1$* morphants (**E**).

transfection with both *S1P$_1$* siRNAs allowed a more significant downregulation of S1P$_1$ receptor and resulted in a remarkable decrease of EPHB4 protein levels (Figure 12B), with ≈25% to 30% of the receptor levels detected in cells transfected with the scrambled siRNA as assessed by computerized image analysis of the immunoreactive bands (Figure 12C). Thus, in agreement with the data obtained with *zS1P$_1$* morphants, these results indicate that the suppression of S1P$_1$ receptor expression (by siRNA transfection) or activity (by W146 treatment) induces a significant downregulation of EPHB4 in HUVEC.

On this basis, we investigated the effect of the selective EPHB4 receptor antagonist TNYL-RAW peptide and of the control-scrambled peptide SCR-WTL[18,19] on ZO1$^+$ intercellular contact organization in HUVECs. Similar to W146 (data not shown), TNYL-RAW inhibited ZO1 organization in cell–cell junctions of confluent HUVEC monolayers maintained in serum-containing cell culture medium, whereas no effect was exerted by the control-scrambled peptide SCR-WTL (Figure 12D). In keeping with our observations in zebrafish embryos, these data support a direct functional role for the Eph receptor EPHB4 in mediating S1P$_1$-dependent organization of ZO1$^+$ intercellular junctions in venous endothelium.

Discussion

In this study, we report the characterization and functional analysis of *S1P$_1$*, a major endothelial S1P receptor, in the development of vascular system in zebrafish embryo. Here we show that *S1P$_1$* plays a nonredundant role in the regulation of endothelial barrier in zebrafish via a novel *S1P$_1$/VE-cadherin/EphB4a* genetic pathway that controls venous vascular integrity.

In agreement with previous observations,[13] pharmacological characterization based on the effect of nonselective and selective S1P$_1$ receptor agonists on forskolin-induced cAMP accumulation demonstrates that the binding properties of the cloned *zS1P$_1$* receptor expressed in mammalian cells are similar to those shown by its human counterpart, thus confirming that *zS1P$_1$* encodes for the bona fide zebrafish ortholog of the human *S1P$_1$* gene. Consistent with a vascular pattern of expression of *S1P$_1$* in mouse embryo,[5] *zS1P$_1$* is expressed in the axial vasculature of the trunk of zebrafish embryos, whole-mount ISH showing that *zS1P$_1$* transiently marks the endothelium of DA and PCV at 24 to 30 hpf, being lost at 48 hpf. The *shh* signaling inhibitor cyclopamine or *vegf* antisense MO both abrogate expression of *zS1P$_1$* in the axial vasculature of the trunk, thus indicating that endothelial *zS1P$_1$* expression is under the control of the endothelial differentiation *shh/vegf* genetic pathway.[30] These data extend previous observations about the ability of vascular endothelial growth factor to upregulate *S1P$_1$* expression in mammalian endothelium.[41]

Here, we show that *zS1P$_1$* morphants are characterized by minor defects in the vascular development of intersomitic vessels, duct of Cuvier, and subintestinal vein basket. This was paralleled by early pericardial edema, lack of blood circulation, alterations of PCV structure, and a dramatic late generalized edema. Interestingly, several of these phenotypic features were observed also in *S1P$_1$* null mice,[5] as well as in *VE-cadherin* zebrafish morphants (C. Tobia, our unpublished observations, 2010).[32] However, *S1P$_1$*-null mice exhibit massive embryonic hemorrhage and deficient smooth muscle cell/pericyte recruitment in embryonic vasculature,[5] defects that were not observed in *zS1P$_1$* morphants. These species differences may be, at least in part, a result of the fact that zebrafish is characterized by a delay in smooth muscle cell/pericyte maturation with a very limited PCV coverage with respect to mammals.[42] Indeed, *transgelin*-positive mural cells are not yet detectable in PCV of zebrafish embryos at 80 hpf

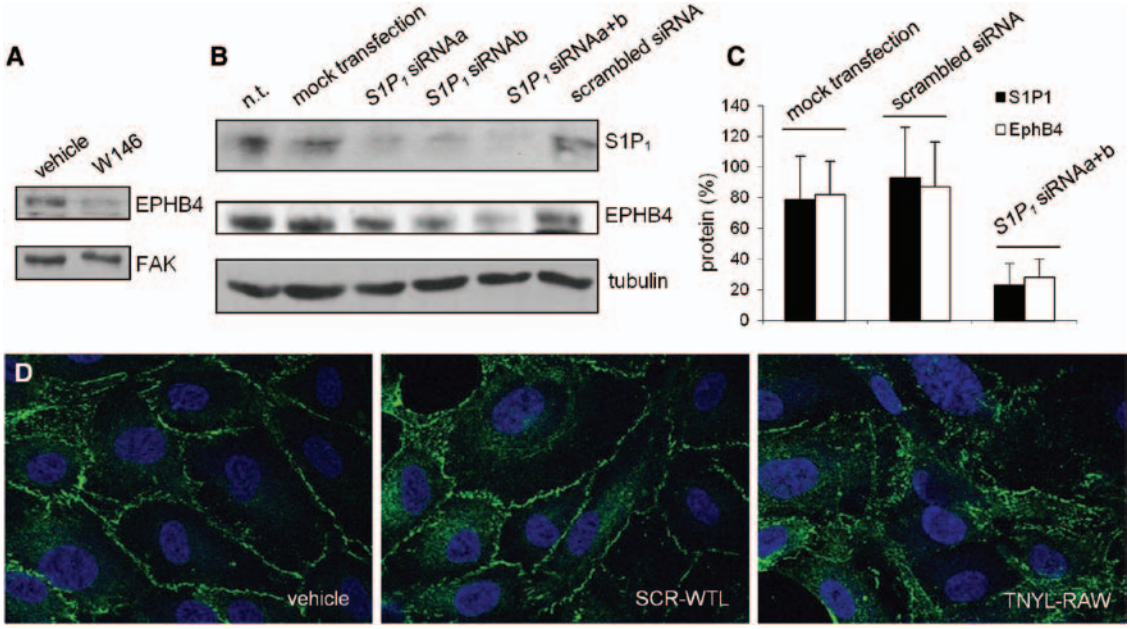

Figure 12. EPHB4 controls barrier integrity in human umbilical vein endothelial cells (HUVECs). **A**, HUVECs were incubated with vehicle or with 30-µmol/L W146 for 48 hours and cells lysates were probed with anti-EPHB4 antibody in a Western blot. **B**, HUVECs were left untreated (n.t.), mock transfected, or transfected with control-scrambled short interfering RNA (siRNA), 2 different *S1P*$_1$ siRNAs (a or b) or the pool of the 2 *S1P*$_1$ siRNAs (a+b). After 96 hours, cell lysates were probed by Western blotting with anti-S1P$_1$ and anti-EPHB4 antibodies. Uniform loading of the gels was assessed with anti-focal adhesion kinase (FAK) (**A**) or anti-tubulin (**B**) antibodies. **C**, The cell lysates of untreated, mock transfected, scrambled siRNA–transfected, and *endothelial sphingosine-1-phosphate receptor-1* (*S1P*$_1$) siRNAs (a+b)–transfected HUVECs were probed by Western blotting with anti-S1P$_1$ and anti-EPHB4 antibodies, and the corresponding immunoreactive bands were quantified by computerized image analysis and normalized for tubulin levels. The data are the mean±SD of the 3 independent transfection experiments and are expressed as percentage of immunoreactive protein levels in untreated cells. **D**, Confluent HUVECs were treated with vehicle or with 100 µmol/L control SCR-WTL peptide or TNYL-RAW peptide for 24 hours in complete cell culture medium. Then, cells were immunostained with anti–zonula occludens 1 (ZO1) antibody. Note that ZO1 localization in cell–cell contact regions is lost in TNYL-RAW–treated cells.

or 120 hpf,[42] when the vascular defects in *zS1P*$_1$ morphants are already established.

Lack of blood circulation and pericardial edema have been observed also in S1P$_2$ receptor *mil* zebrafish mutants as a consequence of profound defects in heart organogenesis.[14] At variance with *mil* mutants, we did not observe major defects in the developing heart of the *zS1P*$_1$ morphants, as indicated by the normal pattern of expression of the cardiac myogenesis genes *ventricular myosin heavy chain*, *atrial myosin heavy chain,* and *cardiac myosin light chain 2* (see Figure 5), demonstrating that the 2 S1P receptors play different roles in zebrafish development.

Edema formation may result from cardiovascular, lymphatic vascular or excretory system defects. To this respect, *zS1P*$_1$ morphants showed an apparently normal lymphatic thoracic duct at 4 dpf (C. Tobia, unpublished observations, 2010). Also, high molecular weight proteins (≥80 000) were present in the extravascular transudate we collected from edematous *zS1P*$_1$ morphants at 6 dpf but not in the transudate collected from *prox-1* morphants showing lack of lymphatic vessel development[43] as observed by SDS-PAGE (C. Tobia, unpublished observations, 2010). Thus, even though the effect of *zS1P*$_1$ knowdown on cardiac, lymphatic and excretory system development and function will deserve further investigation, our observations suggest that alterations of endothelial barrier integrity may contribute, at least in part, to the edematous phenotype observed in *zS1P*$_1$ morphants.

Actually, in keeping with a role for S1P$_1$ in vascular barrier integrity, *zS1P*$_1$ morphants showed significant alterations of vessel morphology, downregulation of *VE-cadherin* expression, and ZO1 disorganization in PCV but not in DA. It must be pointed out that *zS1P*$_1$ knockdown did not affect arterial/venous differentiation of endothelial cells in zebrafish. Indeed, *zS1P*$_1$ morphants did not show any change in the levels of expression of *shh*, *vegf*, *kdr,* and *fli-1*, as well as of the arterial markers *ephrinB2*[30] and *crlr*[34] and of the venous markers *flt4*[35] and *dab2*,[36] thus confirming previous observations in *S1P*$_1^{-/-}$ mice.[5] Nevertheless, the expression of the Eph receptor *EphB4a* was significantly downregulated in *zS1P*$_1$-MO–injected embryos. To this respect, it is interesting to note that *EphB4a* knockdown after MO injection at 1- to 4-cell stage has a dramatic impact on early vascular development and arterial/venous segregation in zebrafish.[39] In contrast, as stated above, *EphB4a* downregulation in *zS1P*$_1$ morphants, which occurs at 28 hpf in the already differentiated PCV, is paralleled only by minor defects in late vascular development, mostly affecting venous microcirculation (ie, the duct of Cuvier and the subintestinal vein basket) with no apparent effect on arterial/venous differentiation. Taken together, these data suggest that *EphB4a* may play a dual role in the circulatory system of zebrafish: an earlier effect on arterial/venous differentiation and a later effect on venous vascular integrity. In keeping with this hypothesis, *EphB4a* has been shown to regulate venous remodelling in the adult murine vasculature.[44]

Eph receptors have been implicated in the permeability of epithelial and endothelial barriers[45] and EphA receptors have been suggested to act downstream of E-cadherin to mediate epithelial cell-to-cell contacts.[37] Our data strongly suggest that a cadherin/Eph receptor cross talk may exist also in the endothelial cells, genetic *VE-cadherin*/*EphB4a* interactions mediating venous vascular integrity downstream of *S1P$_1$*. Several experimental evidences support this hypothesis. (1) The S1P/S1P$_1$ receptor system modulates *VE-cadherin* expression and the localization of this adherens junction component at intercellular junctions in mammalian endothelium.[10] (2) *zS1P$_1$* knockdown causes *VE-cadherin* and *EphB4a* downregulation in PCV of zebrafish morphants. (3) Both *zS1P$_1$* morphants and *VE-cadherin* morphants show early pericardial edema, lack of blood circulation, late generalized edema, disorganization of endothelial ZO1$^+$ junctions, and *EphB4a* downregulation. (4) Exogenous *VE-cadherin* is sufficient to rescue *EphB4a* expression and vascular integrity in the absence of *zS1P$_1$* activity. (5) Stable *S1P$_1$* knockdown downregulates *VE-cadherin* expression in different human endothelial cells lines.[46] (6) In keeping with the data obtained in *zS1P$_1$* zebrafish morphants, *S1P$_1$* siRNA transfection and the selective S1P$_1$ antagonist W146[15] downregulate EPHB4 receptor expression in mammalian HUVECs. (7) Similar to W146, the selective EPHB4 receptor antagonist TNYL-RAW peptide[18,19] prevents ZO1$^+$ intercellular contact organization in the same cells. Together, the data point to the existence of a *S1P$_1$*/*VE-cadherin*/*EphB4a* genetic pathway modulating vascular barrier organization in venous endothelium.

At present, the molecular mechanism(s) orchestrating this genetic pathway remain unravelled. To this respect, the capacity of *zS1P$_1$* in modulating intercellular barrier appears to be restricted to venous endothelium. Indeed, even though *zS1P$_1$* receptor is expressed in both DA and PCV endothelial cells, its knockdown results in *VE-cadherin* downregulation with consequent alterations of vessel morphology and disorganization of ZO1$^+$ endothelial junctions only in PCV, with no apparent alterations of arterial endothelium. This indicates that *VE-cadherin* expression and endothelial barrier integrity are controlled by different mechanisms in venous and arterial endothelium of zebrafish embryo.

Endothelium demonstrates remarkable heterogeneity in structure and function.[47] Within the microvasculature, junctions are tighter in arterioles compared with capillaries and are quite loose in venules, likely reflecting the role of postcapillary venules in mediating inflammation-induced extravasation of leukocytes and plasma constituents.[48] Accordingly, differences in the expression of adherens junctional proteins have been reported in arterial versus venous endothelial cells.[49,50] Several transcription factors, including members of the E-26 and Twist/Slug/Snail families, and Wnt/β-catenin signaling regulate *VE-cadherin* expression.[51] Further experiments are required to elucidate the transcriptional and signaling mechanisms responsible for the observed modulation of *VE-cadherin* expression in human[46] and zebrafish (present work) endothelium by *S1P$_1$* knockdown and the molecular bases responsible for the different responses of venous and arterial vascular beds to *zS1P$_1$* downregulation in zebrafish embryo.

Alterations of the endothelial barrier integrity may occur in different pathological conditions, including acute respiratory distress syndrome in the lung, ischemia-reperfusion stresses in the kidney and myocardium, and experimental autoimmune encephalomyelitis in the brain. In all these conditions, S1P receptor activation may favour vascular integrity, synthetic chemical agonists/antagonists of S1P receptors representing therapeutic modulators of the endothelial barrier.[4] Zebrafish is suitable for high-throughput screening of chemical compounds using robotic platforms.[52,53] Also, zebrafish has been proposed as a suitable animal model of human vascular malformation disorders.[6] Our findings indicate that this animal model may provide useful information about the molecular mechanisms regulating vascular barrier integrity and may be used for the screening of novel endothelial barrier-targeting therapeutics.

Acknowledgments

We thank Dr Franco Cotelli (University of Milan, Italy) for the helpful discussions and criticisms.

Sources of Funding

This work was supported, in part, by grants from Ministero dell'Istruzione, Università e Ricerca (Centro IDET, FIRB project RBAP11H2R9 2011), Lombardy Innate Immunity Network (LIIN), and Associazione Italiana per la Ricerca sul Cancro (AIRC grant no. 10396) to M. Presta and AIRC MFAG grant no. 9161 to S. Mitola.

Disclosures

None.

References

1. Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat Rev Immunol*. 2005;5:560–570.
2. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol*. 2003;4:397–407.
3. Rosen H, Sanna MG, Cahalan SM, Gonzalez-Cabrera PJ. Tipping the gatekeeper: S1P regulation of endothelial barrier function. *Trends Immunol*. 2007;28:102–107.
4. Marsolais D, Rosen H. Chemical modulators of sphingosine-1-phosphate receptors as barrier-oriented therapeutic molecules. *Nat Rev Drug Discov*. 2009;8:297–307.
5. Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest*. 2000;106:951–961.
6. Dejana E, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell*. 2009;16:209–221.
7. Komarova YA, Mehta D, Malik AB. Dual regulation of endothelial junctional permeability. *Sci STKE*. 2007;2007:re8.
8. Hla T. Signaling and biological actions of sphingosine 1-phosphate. *Pharmacol Res*. 2003;47:401–407.
9. Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, Hla T. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell*. 1999;99:301–312.
10. Wang L, Dudek SM. Regulation of vascular permeability by sphingosine 1-phosphate. *Microvasc Res*. 2009;77:39–45.
11. Thisse C, Zon LI. Organogenesis–heart and blood formation from the zebrafish point of view. *Science*. 2002;295:457–462.
12. Weinstein B. Vascular cell biology in vivo: a new piscine paradigm? *Trends Cell Biol*. 2002;12:439–445.
13. Im DS, Ungar AR, Lynch KR. Characterization of a zebrafish (Danio rerio) sphingosine 1-phosphate receptor expressed in the embryonic brain. *Biochem Biophys Res Commun*. 2000;279:139–143.

14. Kupperman E, An S, Osborne N, Waldron S, Stainier DY. A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development. *Nature*. 2000;406:192–195.
15. Sanna MG, Wang SK, Gonzalez-Cabrera PJ, Don A, Marsolais D, Matheu MP, Wei SH, Parker I, Jo E, Cheng WC, Cahalan MD, Wong CH, Rosen H. Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral $S1P_1$ antagonist in vivo. *Nat Chem Biol*. 2006;2:434–441.
16. Albert R, Hinterding K, Brinkmann V, Guerini D, Müller-Hartwieg C, Knecht H, Simeon C, Streiff M, Wagner T, Welzenbach K, Zécri F, Zollinger M, Cooke N, Francotte E. Novel immunomodulator FTY720 is phosphorylated in rats and humans to form a single stereoisomer. Identification, chemical proof, and biological characterization of the biologically active species and its enantiomer. *J Med Chem*. 2005;48:5373–5377.
17. Gonzalez-Cabrera PJ, Jo E, Sanna MG, Brown S, Leaf N, Marsolais D, Schaeffer MT, Chapman J, Cameron M, Guerrero M, Roberts E, Rosen H. Full pharmacological efficacy of a novel $S1P_1$ agonist that does not require S1P-like headgroup interactions. *Mol Pharmacol*. 2008;74:1308–1318.
18. Koolpe M, Burgess R, Dail M, Pasquale EB. EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity. *J Biol Chem*. 2005;280:17301–17311.
19. Salvucci O, de la Luz Sierra M, Martina JA, McCormick PJ, Tosato G. EphB2 and EphB4 receptors forward signaling promotes SDF-1-induced endothelial cell chemotaxis and branching remodeling. *Blood*. 2006;108:2914–2922.
20. Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol*. 2002;248:307–318.
21. Westerfield M. *The Zebrafish Book*. Eugene, OR: University of Pregon Press; 1995.
22. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn*. 1995;203:253–310.
23. Paffett-Lugassy NN, Zon LI. Analysis of hematopoietic development in the zebrafish. *Methods Mol Med*. 2005;105:171–198.
24. Isogai S, Horiguchi M, Weinstein BM. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol*. 2001;230:278–301.
25. Blum Y, Belting HG, Ellertsdottir E, Herwig L, Lüders F, Affolter M. Complex cell rearrangements during intersegmental vessel sprouting and vessel fusion in the zebrafish embryo. *Dev Biol*. 2008;316:312–322.
26. Hale JJ, Lynch CL, Neway W, et al. A rational utilization of high-throughput screening affords selective, orally bioavailable 1-benzyl-3-carboxyazetidine sphingosine-1-phosphate-1 receptor agonists. *J Med Chem*. 2004;47:6662–6665.
27. Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, Bruns C, Prieschl E, Baumruker T, Hiestand P, Foster CA, Zollinger M, Lynch KR. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem*. 2002;277:21453–21457.
28. Lawson ND, Vogel AM, Weinstein BM. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell*. 2002;3:127–136.
29. Cooper MK, Porter JA, Young KE, Beachy PA. Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science*. 1998;280:1603–1607.
30. Torres-Vázquez J, Kamei M, Weinstein BM. Molecular distinction between arteries and veins. *Cell Tissue Res*. 2003;314:43–59.
31. Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet*. 2000;26:216–220.
32. Montero-Balaguer M, Swirsding K, Orsenigo F, Cotelli F, Mione M, Dejana E. Stable vascular connections and remodeling require full expression of VE-cadherin in zebrafish embryos. *PLoS ONE*. 2009;4:e5772.
33. Lee JF, Zeng Q, Ozaki H, Wang L, Hand AR, Hla T, Wang E, Lee MJ. Dual roles of tight junction-associated protein, zonula occludens-1, in sphingosine 1-phosphate-mediated endothelial chemotaxis and barrier integrity. *J Biol Chem*. 2006;281:29190–29200.
34. Nicoli S, Tobia C, Gualandi L, De Sena G, Presta M. Calcitonin receptor-like receptor guides arterial differentiation in zebrafish. *Blood*. 2008;111:4965–4972.
35. Thompson MA, Ransom DG, Pratt SJ, et al. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev Biol*. 1998;197:248–269.
36. Song HD, Sun XJ, Deng M, et al. Hematopoietic gene expression profile in zebrafish kidney marrow. *Proc Natl Acad Sci USA*. 2004;101:16240–16245.
37. Cheng N, Brantley DM, Chen J. The ephrins and Eph receptors in angiogenesis. *Cytokine Growth Factor Rev*. 2002;13:75–85.
38. Mitchell IC, Brown TS, Terada LS, Amatruda JF, Nwariaku FE. Effect of vascular cadherin knockdown on zebrafish vasculature during development. *PLoS ONE*. 2010;5:e8807.
39. Herbert SP, Huisken J, Kim TN, Feldman ME, Houseman BT, Wang RA, Shokat KM, Stainier DY. Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. *Science*. 2009;326:294–298.
40. Hla T, Lee MJ, Ancellin N, Liu CH, Thangada S, Thompson BD, Kluk M. Sphingosine-1-phosphate: extracellular mediator or intracellular second messenger? *Biochem Pharmacol*. 1999;58:201–207.
41. Igarashi J, Erwin PA, Dantas AP, Chen H, Michel T. VEGF induces $S1P_1$ receptors in endothelial cells: Implications for cross-talk between sphingolipid and growth factor receptors. *Proc Natl Acad Sci USA*. 2003;100:10664–10669.
42. Santoro MM, Pesce G, Stainier DY. Characterization of vascular mural cells during zebrafish development. *Mech Dev*. 2009;126:638–649.
43. Yaniv K, Isogai S, Castranova D, Dye L, Hitomi J, Weinstein BM. Live imaging of lymphatic development in the zebrafish. *Nat Med*. 2006;12:711–716.
44. Muto A, Yi T, Harrison KD, Dávalos A, Fancher TT, Ziegler KR, Feigel A, Kondo Y, Nishibe T, Sessa WC, Dardik A. Eph-B4 prevents venous adaptive remodeling in the adult arterial environment. *J Exp Med*. 2011;208:561–575.
45. Ivanov AI, Romanovsky AA. Putative dual role of ephrin-Eph receptor interactions in inflammation. *IUBMB Life*. 2006;58:389–394.
46. Krump-Konvalinkova V, Yasuda S, Rubic T, Makarova N, Mages J, Erl W, Vosseler C, Kirkpatrick CJ, Tigyi G, Siess W. Stable knock-down of the sphingosine 1-phosphate receptor $S1P_1$ influences multiple functions of human endothelial cells. *Arterioscler Thromb Vasc Biol*. 2005;25:546–552.
47. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res*. 2007;100:158–173.
48. Curry FR, Adamson RH. Vascular permeability modulation at the cell, microvessel, or whole organ level: towards closing gaps in our knowledge. *Cardiovasc Res*. 2010;87:218–229.
49. Herwig MC, Müller KM, Müller AM. Endothelial VE-cadherin expression in human lungs. *Pathol Res Pract*. 2008;204:725–730.
50. Kevil CG, Okayama N, Trocha SD, Kalogeris TJ, Coe LL, Specian RD, Davis CP, Alexander JS. Expression of zonula occludens and adherens junctional proteins in human venous and arterial endothelial cells: role of occludin in endothelial solute barriers. *Microcirculation*. 1998;5:197–210.
51. Harris ES, Nelson WJ. VE-cadherin: at the front, center, and sides of endothelial cell organization and function. *Curr Opin Cell Biol*. 2010;22:651–658.
52. Pichler FB, Laurenson S, Williams LC, Dodd A, Copp BR, Love DR. Chemical discovery and global gene expression analysis in zebrafish. *Nat Biotechnol*. 2003;21:879–883.
53. Ny A, Autiero M, Carmeliet P. Zebrafish and Xenopus tadpoles: small animal models to study angiogenesis and lymphangiogenesis. *Exp Cell Res*. 2006;312:684–693.