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

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Objective—Endothelial sphingosine-1-phosphate (SIP) receptor-1 (SIP₁) affects different vascular functions, including blood vessel maturation and permeability. Here, we characterized the role of the SIP₁ ortholog in vascular development in zebrafish.

Methods and Results—zSIP₁ is expressed in dorsal aorta and posterior cardinal vein of zebrafish embryos at 24 to 30 hours postfertilization. zSIP₁ 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, zSIP₁ 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 zSIP₁ morphants. Finally, SIP₁ small interfering RNA transfection and the S1P 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 zSIP₁ in the regulation of venous endothelial barrier in zebrafish and identify a S1P/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

Endothelial barrier integrity is dependent upon a complex network of molecular interactions involving adherens and tight junctions. 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. However, the molecular and functional bases of the impact of the S1P/S1P₁ 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. 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. 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...
functioning circulation make the zebrafish embryo amenable for vascular biology studies. Expression of the zebrafish ortholog of the S1P1 receptor (zS1P1) has been observed in the zebrafish embryonic brain, whereas zebrafish mutants of the S1P1 receptor ortholog miles apart (mil) show organogenesis defects of the heart, demonstrating that bioactive lipid receptors play different roles in zebrafish development. In the present work, we performed the characterization and functional analysis of S1P1 receptor during vascular development in zebrafish. S1P1 is transiently expressed in the axial vasculature of the trunk of zebrafish embryos. S1P1 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, S1P1 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 S1P1-regulated element that controls venous endothelial barrier integrity by acting upstream of EphB4a. Finally, S1P1 small interfering RNA (siRNA) transfection and the specific S1P1 antagonist W1465 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 S1P1 in the regulation of blood–barrier integrity via a novel S1P1/VE-cadherin/EphB4a cascade that controls venous endothelial cell–cell junction assembly.

**Materials and Methods**

**Reagents**

SIP1, 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 (dihydropyran phosphate) ester (FTY720-P) and [2-(4-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-yl] amino) ethanol (CYM544) were synthesized as described. TNYL-RAW peptide (YNYLFSPNGIARAW) and scrambled peptide SCR-WTL (WTLAFARNYNGPS) were kindly provided by Dr O. Salvucci (Bethesda, MD).

**Zebrafish Stocks**

Wild-type AB and transgenic fg(tg:fl1:EGFP)10 embryos were fixed at 24 hpf as described. 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. For sectioning, fixed embryos were dehydrated in ethanol series, cleared in xilol, and paraffin embedded overnight.

**Morpholino-Mediated Knockdown of zS1P1 and VE-cadherin**

Antisense morpholino oligonucleotides (MO) (Gene Tools, Corvallis, OR) were directed against the 5' untranslated region spanning the S1P1, ATG start codon (zS1P1-MO: 5'-AGTGTCTGGCGATTGTT CATC(CA)-3') or were designed to complement exon–intron boundaries of the zebrafish VE-cadherin gene (VE-cadherin-MO: 5'-TTT ACAAGACGCTACCTTTCCAAA-3'). Standard MO (Std-MO: 5'-CCTCTTACCTAGTACATTTATA-3') was used as control. Routinely, MOs were microinjected in 4.0 mL volume into 1- to 4-cell stage embryos at the concentration of 0.4 pmoles/embryo for zS1P1-MO or 1.0 pmoles/embryo for VE-cadherin-MO. A subset of embryos was co-injected with 0.25 to 0.4 pmoles/embryo of either zS1P1-MO and human S1P1 mRNA (70–100 pg/embryo), murine VE-cadherin mRNA (40–60 pg/embryo), or human VEGF-A mRNA (100 pg/embryo). zS1P1-MO shows no significant homology with human S1P1 mRNA and murine VE-cadherin mRNA sequences.

**Microangiography**

Tetramethylrhodamine isothiocyanate–dextran (molecular weight 2.0×106; Invitrogen, Carlsbad, CA) was dissolved in double distilled water at 20 mg/mL and microinjected into the sinuses venosus of zebrafish embryos at 50 hpf as described.

**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 600B microscope and photographed with a digital camera.

**ZO1 Immunofluorescence Analysis of Zebrafish Embryos**

Control and MO-treated fg(tg:fl1:EGFP)10 embryos were fixed at 30 hpf in 4% paraformaldehyde for 2 hours at room temperature. Whole-mount immunofluorescence analysis was performed as described15 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 Transfection in Mammalian Cells and cAMP Assay**

The polymerase chain reaction (PCR) fragment with the full-length coding sequence of zS1P was cloned from the total RNA isolated from zebrafish embryos at 24 hpf (primer set: forward, 5'-TCCATGATGCAGTTTTTGGA-3'; reverse, 5'-AGTGTCTGGCGATTGTT CATC(CA)-3') into the pcRII-TOPO vector (Invitrogen). Next, the zS1P coding region was subcloned from pcRII-TOPO into the pcDNAS/FRT/TO vector (Invitrogen) using BamHI and Xbal (Fermentas Life Sciences, St. Leon-Rot, Germany). Chinese hamster ovary–FpIIn 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–FpIIn 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 Hank's balanced salt solution, and resuspended in 5.0 mM Hepes buffer, containing Hank's balanced salt solution with 0.05% fatty acid–free BSA (Sigma). Stimulation mixtures consisted of stimulation buffer with 3 μM forskolin, 0.5 mM ATP, 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 (PerkinElmer, 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 Victor2 (PerkinElmer). Data are expressed as the potency of the indicated compound to inhibit forskolin–induced cAMP accumulation (calculated as EC_{so} value, here defined as the negative logarithm of the EC_{so} 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 Biologic iCycler iQ Real-Time PCR Detection System using SYBR Green Supermix (Biorad, Hercules, CA) according to the manufacturer’s instructions. Primers sequence were as follows: Ephb4 (forward: 5’-GGGGGCTGCAGGAGAGCCA-3’; reverse: 5’-GATCCGGCTTCTTCTGCCA-3’); VCADherin (forward: 5’-TGAGCAAGGAGGGCGGAGGAG-3’; reverse: 5’-AGTGCCCACA CTGGGCGGA-3’); β-actin (forward: 5’-CAGAGCAGGAGAGT GGAACCA-3’; reverse: 5’-CACAAGGAACAGCCTACATGC-3’).

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

**HUEVC Cultures**

HUEVCs 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). HUEVCs were used at early passages (I–V) and grown on plastic surfaces coated with porcine gelatin (Sigma). When indicated, HUEVCs were transfected with control-scrambled siRNA or with specific S1P1 siRNAs (TAGCATTTGCAAGCTTCTAATA, S1P, siRNAa; and CTGGCTTCTGTACCTGCTCAA, S1P, 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 HUEVCs**

HUEVCs 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, S1P1 siRNA-transfected HUEVCs were analyzed 96 hours after transfection by Western blotting with anti-EphB4 and anti-S1P 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 HUEVCs**

Cells were seeded on gelatin-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 x63/1.4 NA oil objective.

## Results

### Vascular Expression of zS1P1

The complete coding sequence of zS1P1 (GenBank accession no. NM_131691) was cloned from the total RNA isolated from zebrafish embryos at 24 hpf. In keeping with previous observations,13 zS1P1 encodes for a putative 362 amino acid protein highly homologous to human and murine S1P_{R} receptors (70% and 71% similarity, respectively). Accordingly, both the selective S1P_{R} receptor agonists SEW287116 and CYM544217 and the nonselective S1P_{R} receptor agonists S1P, dhS1P, and synthetic FTY720-P27 potently inhibit forskolin-induced cAMP accumulation in Chinese hamster ovary–FpIn cells transiently transfected with the zS1P1 cDNA with the following ranking order: FTY720-P>dhS1P=S1P>CYM5442=SEW2871 (Table). Similar results were obtained for Chinese hamster ovary–FpIn cells transiently transfected with the human S1P_{R} cDNA (Table), thus, confirming that zS1P1 encodes for the bona fide zebrafish ortholog of the human S1P_{R} gene.

On this basis, we analyzed the expression pattern of the zS1P1 gene in zebrafish embryos by whole-mount ISH. In agreement with previous observations,13 zS1P1 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 zS1P1 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, zS1P1 marks the

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<td>CYM-5442</td>
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zS1P indicates zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1; dhS1P, dihydro-S1P.

*Chinese hamster ovary–FpIn cells were transiently transfected with zebrafish zS1P1 or human hS1P1 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_{so} value, here defined as the negative logarithm of the EC_{so} value) and represent the mean±SEM of 2 to 3 independent experiments. No effect was observed in mock-transfected cells (data not shown).
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 zS1P1 in the axial vasculature of the trunk of 26 hpf embryos was abrogated by treatment with cyclopamine, a potent inhibitor of shh signaling29 (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 zS1P1, thus, indicating that endothelial zS1P1 expression is under the control of the shh/vegf cascade.30

MO Knockdown of zS1P1 Function Results in Venous Vascular Barrier Defects
To assess the functional role of zS1P1 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 zS1P1 ATG start codon to inhibit protein translation (zS1P1-MO). Next, tg(fli1:EGFP)y1 transgenic zebrafish embryos in which EGFP expression is driven by the pan-endothelial marker fli-1 were injected at the 1– to 4-cell stage with different doses of zS1P1-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 1. Vascular expression of the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (zS1P1). A, D, and G, zS1P1 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. zS1P1 is expressed by the axial vasculature of the trunk (white arrow indicates dorsal aorta [DA]; yellow arrow, posterior cardinal vein [PCV]). Transverse sections confirm zS1P1 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.

Figure 2. Vascular zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (zS1P1) expression is mediated by vascular endothelial growth factor (vegf). zS1P1 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 zS1P1 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 zS1P1-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 zS1P1 morphants (Figure 4). At 2.5 days postfertilization (dpf), intersomitic vessels of all zS1P1-MO–injected embryos (n=181) appear slightly thinner than in control Std-MO–injected embryos (Figure 3J). Also, zS1P1 downregulation was associated with minor defects in the development of the duct of Cuvier and of the subintestinal vein basket in all morphants (Figure 3K and 3L). In keeping with the modest impact of zS1P1 downregulation on blood vessel development, no differences in the expression of shh, vegf, kdr, and fli-1 genes were observed in zS1P1-MO–injected versus Std-MO–injected embryos (data not shown). These data support previous observations in S1P1-null mice indicating that this S1P receptor is not essential for vasculogenic and angiogenic events.
S1P<sub>1</sub> receptor and its ligand S1P play an important role in the control of vascular barrier by affecting endothelial cell–cell junctions, including the expression of the adherens junction component VE-cadherin. The phenotype observed in zS1P<sub>1</sub> morphants prompted us to assess the possibility that zS1P<sub>1</sub> may control endothelial barrier integrity in zebrafish. On this basis, the effect of zS1P<sub>1</sub> downregulation on the organization of endothelial cell–cell junctions was investigated by whole-mount ISH of VE-cadherin 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. As shown in Figure 7B, zS1P<sub>1</sub> knockdown caused the selective downregulation of VE-cadherin in the PCV but not in the DA of zS1P<sub>1</sub> morphants. In keeping with the ISH data, the RT-PCR analysis showed a decrease of steady state VE-cadherin mRNA levels in zS1P<sub>1</sub> morphants when compared with control embryos (31%±12%, average of the 2 independent experiments).
Also, confocal microscopy analysis of the vasculature of the trunk of control and zS1P morphants at 30 hpf revealed a complete disorganization of the ZO1+ endothelial junctions in the PCV of all the zS1P morphants examined (n=5) (Figure 7D). Instead, no effect on ZO1 immunolocalization was observed in the DA of zS1P morphants and in both axial vessels of std-MO–injected embryos (Figure 7C). Together, the data indicate a nonredundant

Figure 6. Human hS1P1 overexpression rescues vascular defects in the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (zS1P1) morphants. zS1P1 morphants were injected with hS1P1 mRNA and lack of blood flow (A) and generalized edema (B) were analyzed at 30 hours postfertilization and 6 days postfertilization (dpf), respectively. hS1P1 overexpression caused a significant increase in the percentage of embryos with normal blood flow (122/161 [76%] vs 42/117 [36%] zS1P1 morphants injected or not with hS1P1 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%] zS1P1 morphants injected or not with hS1P1 mRNA, respectively; P<0.001, Fisher exact test). Western blot analysis confirmed the increased expression of S1P1 protein in 24 hpf zebrafish embryos 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 zS1P1 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 S1P1 mRNA (P<0.001, Fisher exact test). White arrow indicates DA; yellow arrow, PCV.

Figure 7. The zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (zS1P1) knockdown affects vascular endothelial cadherin (VE-cadherin) expression and zonula occludens 1 (ZO1) immunolocalization in posterior cardinal vein (PCV). Standard-MO (std-MO)– and zS1P1-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. zS1P1 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 zS1P1 morphants when compared with controls (insets in A and B).
vascular function for zS1P1 that affects venous endothelial cell–cell barrier in zebrafish.

Downregulation of Venous Endothelial EphB4a Expression in zS1P1 Morphants

The selective effect of zS1P1 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 zS1P1-MO–injected embryos. As shown in Figure 8, zS1P1 downregulation does not affect the expression of the specific arterial markers ephrinB230 and cris34 in the DA of zS1P1 morphants. Also, zS1P1-MO injection does not alter the expression of the venous markers flt435 and dab236 in the PCV. Thus, in keeping with previous observations in S1P1 null mice, the data indicate that zS1P1 knockdown does not affect arterial/venous differentiation of endothelial cells in zebrafish. However, zS1P1 downregulation caused the loss of the expression of the Eph receptor EphB4a in the PCV of zS1P1-MO–injected embryos (Figure 8J), as confirmed by the decrease of steady state EphB4a mRNA levels in zS1P1-null embryos when compared with controls (38%±9%, average of the 2 independent quantitative RT-PCR experiments). Of note, zS1P1 downregulation did not affect the expression of EphB4a in the gut of zebrafish embryos, thus demonstrating the specificity of the effect of zS1P1-MO on EphB4a expression in PCV (asterisk in Figure 8I and 8J).

The loss of EphB4a and VE-cadherin expression in venous endothelium of zS1P1 morphants (72% and 75% of zS1P1 morphants, respectively) was significantly reduced to 24% and 25% for the 2 genes in embryos coinjected with the human S1P1 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 zS1P1 morphants is not the direct consequence of the lack of blood flow (Figure 9). Finally, coinjection of the human S1P1 mRNA fully rescued ZO1 organization in the PCV of zS1P1 morphants (data not shown). These observations point to a role for S1P1 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. On this basis, we addressed the possibility that the venous endothelial barrier defects in zebrafish embryos lacking zS1P1 activity might be the consequence of defects in a putative zS1P1/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 pmol/embryo) showed downregulation of EphB4a expression (24/24 embryos), pericardial edema and lack of blood circulation (28/30 embryos, data not shown), and late generalized edema (19/22 embryos) with no changes in vascular zS1P1 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. On this basis, to assess whether exogenous VE-cadherin is sufficient to rescue EphB4a expression and vascular integrity in the absence of zS1P1 activity, zS1P1
morphants were injected with the mRNA encoding for murine VE-cadherin. As shown in Figure 11B and 11C, the loss of EphB4a expression in venous endothelium of zS1P1 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 zS1P1 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 zS1P1 morphants (Figure 11E). At variance, no rescue was observed after injection of zS1P1 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 zS1P1 morphants is a result of the VE-cadherin downregulation in PCV endothelium downstream of zS1P1, pointing to the existence of a zS1P1/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, 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 S1P1 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 S1P1 and the Eph receptor EPHB4. As anticipated, a 1-hour pretreatment of confluent HUVECs with 30 μmol/L of the selective S1P1 antagonist W146 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 S1P1 siRNAs, each causing a partial downregulation of S1P1 expression (Figure 12B). However, the simultaneous
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 $\approx$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 $zsS1P_1$, 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.

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, 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 $zsS1P_1$, receptor expressed in mammalian cells are similar to those shown by its human counterpart, thus confirming that $zsS1P_1$, 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, $zsS1P_1$, is expressed in the axial vasculature of the trunk of zebrafish embryos, whole-mount ISH showing that $zsS1P_1$, 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 S1P$_1$, in the axial vasculature of the trunk, thus indicating that endothelial $zsS1P_1$, expression is under the control of the endothelial differentiation shh/vegf genetic pathway. These data extend previous observations about the ability of vascular endothelial growth factor to upregulate S1P$_1$, expression in mammalian endothelium.

Here, we show that $zsS1P_1$, 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, as well as in VE-cadherin zebrafish morphants (C. Tobia, our unpublished observations, 2010). However, S1P$_1$-null mice exhibit massive embryonic hemorrhage and deficient smooth muscle cell/pericyte recruitment in embryonic vasculature, defects that were not observed in $zsS1P_1$, 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. Indeed, transgelin-positive mural cells are not yet detectable in PCV of zebrafish embryos at 80 hpf.

On this basis, we investigated the effect of the selective EPHB4 receptor antagonist TNYL-RAW peptide and of the control-scrambled peptide SCR-WTL 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.

Figure 11. Murine vascular endothelial cadherin (VE-cadherin) overexpression rescues vascular defects in the zebrafish ortholog of the endothelial sphingosine-1-phosphate receptor-1 (S1P$_1$) morphants. A, $zsS1P_1$, 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 $zsS1P_1$, morphants. B–E, Control and mVE-cadherin mRNA–injected $zsS1P_1$, 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 $zsS1P_1$, 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 $zsS1P_1$, morphants (E).
or 120 hpf, when the vascular defects in zSIP1 morphants are already established.

Lack of blood circulation and pericardial edema have been observed also in S1P2 receptor mil zebrafish mutants as a consequence of profound defects in heart organogenesis. At variance with mil mutants, we did not observe major defects in the developing heart of the zSIP1 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, zSIP1 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 zSIP1 morphants at 6 dpf but not in the transudate collected from prox-1 morphants showing lack of lymphatic vessel development as observed by SDS-PAGE (C. Tobia, unpublished observations, 2010). Thus, even though the effect of zSIP1 knockdown 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 zSIP1 morphants. Actually, in keeping with a role for S1P1 in vascular barrier integrity, zSIP1 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 zSIP1 knockdown did not affect arterial/venous differentiation of endothelial cells in zebrafish. Indeed, zSIP1 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 and cflr and of the venous markers flt4 and dab2, thus confirming previous observations in S1P−/− mice. Nevertheless, the expression of the Eph receptor EphB4a was significantly downregulated in zSIP1−/− 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. In contrast, as stated above, EphB4a downregulation in zSIP1 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.
Eph receptors have been implicated in the permeability of epithelial and endothelial barriers and EphA receptors have been suggested to act downstream of E-cadherin to mediate epithelial cell-to-cell contacts. 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 SIP. Several experimental evidences support this hypothesis. (1) The SIP/SIP receptor system modulates VE-cadherin expression and the localization of this adherens junction component at intercellular junctions in mammalian endothelium. (2) zSIP, a knockdown causes VE-cadherin and EphB4a downregulation in PCV of zebrafish morphants. (3) Both zSIP 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 zSIP. activity. (5) Stable SIP, knockdown downregulates VE-cadherin expression in different human endothelial cells lines. (6) In keeping with the data obtained in zSIP, zebrafish morphants, SIP, siRNA transfection and the selective SIP, antagonist W146 downregulate EphB4 receptor expression in mammalian HUVECs. (7) Similar to W146, the selective EphB4 receptor antagonist TNYL-RAW peptide prevents ZO1+ intercellular contact organization in the same cells. Together, the data point to the existence of a VE-cadherin 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 zSIP, in modulating intercellular barrier appears to be restricted to venous endothelium. Indeed, even though zSIP, 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. 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. Accordingly, differences in the expression of adherens junctional proteins have been reported in arterial versus venous endothelial cells. Several transcription factors, including members of the E-26 and Twist/Slug/Snail families, and Wnt/β-catenin signaling regulate VE-cadherin expression. Further experiments are required to elucidate the transcriptional and signaling mechanisms responsible for the observed modulation of VE-cadherin expression in human and zebrafish (present work) endothelium by SIP, knockdown and the molecular bases responsible for the different responses of venous and arterial vascular beds to zSIP, 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, SIP receptor activation may favour vascular integrity, synthetic chemical agonists/antagonists of SIP receptors representing therapeutic modulators of the endothelial barrier. Zebrafish is suitable for high-throughput screening of chemical compounds using robotic platforms. Also, zebrafish has been proposed as a suitable animal model of human vascular malformation disorders. 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.

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Disclosures
None.

References


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

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