Sphingosine 1-Phosphate Receptor Signaling Regulates Proper Embryonic Vascular Patterning*

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Background: Sphingosine 1-phosphate (S1P) signaling in vascular development is not well understood. S1P is present in zebrafish plasma. Combined knockdown of S1P receptors 1 and 2 (s1pr1 and s1pr2) in the zebrafish results in synergistic perturbation of vascular patterning and development.

Conclusion: Cooperative signaling between s1pr1 and s1pr2 regulates zebrafish vascular development.

Significance: S1P receptor regulation of vascular development is conserved in evolution.

Sphingosine 1-phosphate (S1P) binds G-protein-coupled receptors (S1P1–5) to regulate a multitude of physiological effects, especially those in the vascular and immune systems. S1P receptors in the vascular system have been characterized primarily in mammals. Here, we report that the S1P receptors and metabolic enzymes are conserved in the genome of zebrafish Danio rerio. Bioinformatic analysis identified seven S1P receptor-like sequences in the zebrafish genome, including duplicated orthologs of receptors 3 and 5. Sphingolipidomic analysis detected erythrocyte and plasma S1P as well as high plasma ceramides and sphingosine. Morpholino-mediated knockdown of s1pr1 causes global and pericardial edema, loss of blood circulation, and vascular defects characterized by both reduced vascularization in intersegmental vessels, decreased proliferation of intersegmental and axial vessels, and hypersprouting in the caudal vein plexus. The s1pr2 gene was previously characterized as a regulator of cell migration and heart development, but its role in angiogenesis is not known. However, when expression of both s1pr1 and s1pr2 is suppressed, severely reduced vascular development of the intersegmental vessels was observed with doses of the s1pr1 morpholino that alone did not cause any discernible vascular defects, suggesting that s1pr1 and s1pr2 function cooperatively to regulate vascular development in zebrafish. Similarly, the S1P transporter, spns2, also cooperated with s1pr1. We propose that extracellular S1P acts through vascular S1P receptors to regulate vascular development.

Sphingosine 1-phosphate (S1P) is a lipid mediator that binds to five G-protein-coupled receptors (S1P1–5) to regulate cell proliferation, survival, migration, and differentiation. Sphingosine, a metabolite of sphingomyelin metabolism, is phosphorylated by sphingosine kinases (SphK1 and -2) to form S1P, which in turn is metabolized by S1P phosphohydrolases (SPP1 and -2) or S1P lyase (1). The prototypic receptor, S1P1, was cloned as a phorbol 12-myristate 13-acetate-inducible immediate early transcript from human umbilical vein endothelial cells (2). Ultimately, S1P as a high affinity ligand for S1P1 (originally known as the endothelial differentiation gene or EDG-1) was described previously (3). S1P1, S1P2, and S1P3 are expressed in many tissues, particularly in the cardiovascular, nervous, and immune systems (4, 5). The expression patterns of S1P3 and S1P6, in contrast, are more restricted. S1P5 is mainly localized to the lymphoid, hematopoietic tissue, and lung (6), whereas S1P6 is mainly expressed in the brain and spleen (7, 8).

Functions of S1P have been revealed by genetic loss of function studies in mice (9, 10). Knock-out of S1p1 in mice leads to intrauterine lethality between E12.5 to E14.5 due to severe hemorrhaging, which was presumed to result from a vascular maturation defect (9). Recent analysis of postnatal, conditional deletion of S1pr1 in endothelial cells results in hypersprouting of endothelial tip cells in the developing mouse retina independently of mural cell defects, suggesting its fundamental role in vascular development (11). In contrast, embryos lacking S1pr2 are viable; however, when challenged, they display decreased pathological neovascularization during the oxygen-induced retinopathy model (12). In addition, S1pr2 knock-out mice show defective vascular structures in the inner ear (stria vascularis), which leads to the degeneration of inner ear structures, deafness, and abnormal equilibrium phenotypes (13). Moreover, a combined knock-out of S1pr1 and S1pr2 results in bleeding, and lethality occurs about 2 days earlier (E10.5–12.5) than in S1pr1 single null embryos (10), thus indicating that S1pr1 functions as a supportive rather than essential receptor.

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S1P Receptors Regulate Vascular Development

for regulating murine vascular development. Because of this early lethality, functions for embryonic angiogenesis beyond hemorrhaging have not been clearly delineated for such loss of function studies in mice.

S1P receptor signaling is widely utilized in vertebrates (14). In the zebrafish it was shown that a point mutation in the miles apart gene (mil), which encodes S1pr2, prevents cardiac precursor cells from migrating to the midline, thus resulting in cardia bifida (15). During zebrafish gastrulation, mil was shown to regulate cell motility and directionality of the prechordal plate progenitor cells (16). Additionally, zebrafish carrying a mutation in the S1P transporter, spinster 2 (spns2), phenocopy the mutant mil fish (17). Despite the cell migratory defects previously characterized in the mil and spns2 zebrafish, no vascular defects were described. In addition, a cardia bifida phenotype was not observed in the mouse.

Developing zebrafish embryos are sufficiently permeable to oxygen and therefore often tolerate defects in the cardiovascular system, which can facilitate detailed evaluation of phenotypes that are more difficult to delineate in the mouse. Here, we report that the S1P receptors and enzymes crucial for S1P metabolism are conserved in the zebrafish genome. We demonstrate that knockdown of s1pr1 via morpholinos causes edema, loss of blood circulation, and vascular defects. In addition, we show that knockdown of s1pr2 along with s1pr1 results in a much more dramatic vascular phenotype. Similarly, the S1P transporter, spns2, also cooperates with s1pr1. Therefore, s1pr1 is an important regulator of zebrafish vascular development and cooperates with s1pr2 and spns2 to exert its functions in the vascular system.

**EXPERIMENTAL PROCEDURES**

Quantitative RT-PCR—Staged wild-type embryos or adult tissue specimens were homogenized with TRIzol LS (Ambion), and total RNA was isolated (Qiagen). Total RNA (1 μg) was used to generate cDNA using reverse transcriptase and random hexamers (Roche Applied Science). Primer sequences were designed using Primer3 and are listed in Table 1. LightCycler 480 SYB Green 1 Master Mix (Roche Applied Science) was used to analyze cDNA by quantitative RT-PCR using the Light Cycler 480II (Roche Applied Science). The PCR cycle conditions were 95 °C for 15 min followed by 40 cycles at 94 °C for 14 s, 54 °C for 30 s, and 72 °C for 30 s.

Maintenance of Zebrafish—Wild-type (AB/TUB) and transgenic zebrafish from the Zebrafish International Research Center (Eugene, OR) were maintained at 28.5 °C and staged as described previously (18). We used the following endothelial specific transgenic fluorescent reporter lines: Tg(ctl:EGFP)A41 (cytosolic EGFP) (19), Tg(jkras-mCherry)X198 (membrane-targeted mCherry) (20), and the nucleus-targeted EGFP reporter Tg(jk1:EGFP-NLS) (21). The Tg(gata1:dsRed) reporter line was used to examine red blood cell circulation (22).

**Morpholino Oligomer Injection—**Morpholino oligomers (MOs) were purchased from Gene Tools (Philomath, OR). The sequences of all MOs used in this study are listed in Table 2. Two distinct MOs were designed for s1pr1 that target the 5′-UTR around the start codon to block mRNA translation (translation blockers, designated s1pr1 MO1 and MO2). Blast analysis indicated the MOs were specific for s1pr1 (no overlap with other sequences). A previously validated translation-blocking MO was used against s1pr2 (s1pr2 MO1) (23), and a second nonoverlapping MO (s1pr2 MO2) was designed to validate the double knockdown experiment. A previously validated p53 MO was used to ensure that the gene-specific phenotype described here was not the result of p53-dependent apoptosis (24). The spns2 MO was validated for specificity in previously published work (17). All morphants were compared with stage-matched embryos that were injected with a control morpholino. Each MO was titrated by injection into 1–4-cell fertilized embryos to determine a minimal dose for the reproducible phenotype. Microinjection of MOs was performed using a PLI-100 Pico-Injector (Harvard Apparatus).

Cloning—The s1pr1 full-length clone was obtaining using cDNA generated from RNA of 24 h post-fertilization (hpf) wild-type zebrafish embryos. The open reading frame was PCR-amplified using the following forward and reverse primers, respectively: 5′-ATGGATGACCTAATCGGCCAGCAC and 5′-TAAAGAGAAGAAGGTGATATT. Following ligation into the pCR1-TOPO vector (Invitrogen), the insert was subcloned into pcDNA3. Each MO was titrated by injection into 1–4-cell fertilized embryos to determine a minimal dose for the reproducible phenotype. Microinjection of MOs was performed using a PLI-100 Pico-Injector (Harvard Apparatus).

**TABLE 1**

| Primer sequences for qRT-PCR |
|------------------------------|
| The abbreviations used are as follows: F, forward; R, reverse. |

- **s1pr1**
  - F: CAC AAC AAT GGC AAG ACC TG
  - R: TAT GCT GTC GAT GCA GTC TCC

- **s1pr2**
  - F: CAT CAT TCT GCT GTC CAT CG
  - R: AGA TGA TGA AGA CGCCAA GGG

- **s1pr3a**
  - F: ATG GAT GAC GAG CTT GAA CC
  - R: TCC TGG GTG TGC CCG ACT TG

- **s1pr3b**
  - F: CCA TTT GGA GGA ACC ACA AGA
  - R: TCG CTT GGG GAT AAA TGG AGG

- **s1pr4**
  - F: TCA GCC TCT TCA TCC TCC TG
  - R: TGT TCC GGT CAA GAT GAC

- **s1pr5a**
  - F: ATG CGC AGA GGC ATT CTA AGA
  - R: GCA TCG CAA ACT TCT TGA CC

- **s1pr5b**
  - F: TGC AGC GCC ACT ACA ACT AC
  - R: AGA CCT GCT CAG CTA GTG

- **spp1**
  - F: ATA CCC CTG ACT CGT TTT CTG
  - R: TAG ATG CGG CTC AAA CAC AC

- **spp2**
  - F: CAT AGC ACA GCA ACT CCA ACA
  - R: AGA CTT CAT GGC CCA GAG TG

- **splk1**
  - F: TGG AGA AGG CTT ACT TTG TG
  - R: GGA TAT AGT GAC AGA AGG AGC AG

- **splk2**
  - F: AAT TCT GCC TTT TGG CTC TG
  - R: GCA GAA AAC AGC AGT TGA GG

- **spl**
  - F: TCG GGG TAG TAT GTC TAA AAG
  - R: TCC AGA GCT TTG TGG AGC TG

**TABLE 2**

| Morpholino oligomer sequences |
|-------------------------------|
| s1pr1 MO1, 5′-AGTGTCCTGGCGGTTAGGCATCCTCC-3′ |
| s1pr1 MO2, 5′-GTTGCCACGTGGCTTGGCATTTTAT-3′ |
| s1pr2 MO1, 5′-CGGCAACAGACGGCAAGTACCTCAT-3′ |
| s1pr2 MO2, 5′-TCAAGGGCGGCGCTTTTTTTGAGGC-3′ |
| s1pr5a MO1, 5′-GCCCTTACCTCGAGTCAATTTATA-3′ |
| s1pr5a MO2, 5′-GAGCGGATTGCTTTCGAAAGATG-3′ |
ment, forward 5′-TCAGCGTTATTCATGGCC and reverse 5′-GATATTCCAGAAGACACTATGG. Following ligation into the pCRII-TOPO vector (Invitrogen), the vector was linearized, and SP6 or T7 RNA polymerase was used to synthesize the probes.

Whole-mount in Situ Hybridization—Whole-mount in situ hybridization was performed as described previously (25). Embryos were treated with 0.003% phenylthiourea (Sigma) to prevent pigmentation. Following fixation in 4% paraformaldehyde (Sigma), embryos were treated with 10 μg/ml proteinase K (Roche Applied Science). Hybridization was performed at 68 °C in 50% formamide buffer (Roche Applied Science) with digoxigenin-labeled RNA probes (Roche Applied Science).

Plasma Collection—Plasma was collected as described previously (26). Briefly, fish were anesthetized using ice water. A steel blade was used to make a diagonal incision between the anal fin and caudal fin. A low retention pipette tip attached to a P20 pipettor that had been coated with sodium heparin (Sigma) was used to collect the blood. Typically 5–15 μl of blood was obtained per fish, and samples from multiple fish (8) were pooled into a single Eppendorf tube. Samples were spun at 1000 rpm in an Eppendorf centrifuge (model 5424) for 15 min. The supernatant was removed into a clean Eppendorf tube. Packed blood cells and plasma were submitted to the analytical core facility at the Medical University of South Carolina for biochemical analysis of ceramide and sphingolipid levels by liquid chromatography mass spectrometry. For estimation of S1P concentrations, packed RBC volume, prepared as described above, was considered.

Preparation of Tissue Samples for Lipid Analysis—Brain, heart, liver, and tail tissue were dissected from adult zebrafish and snap-frozen in liquid nitrogen. The tissue samples were pulverized using a mortar and pestle; the resulting powder was and snap-frozen in liquid nitrogen. The tissue samples were packed RBC volume, prepared as described above, was considered.

Immunofluorescence Staining of Fixed Embryos—For confocal microscopy, embryos were fixed at the desired developmental stage and antibody stained to enhance the signal from the endogenous fluorescent transgene. Following overnight fixation in 4% paraformaldehyde (Sigma) at 4 °C, embryos were rinsed four times for 20 min each in PBS/Tween (Fisher) (0.1% Tween in 1× PBS), permeabilized for 25 min in cold acetone (Fisher), and then rinsed again four times for 2 min each in PBST. Embryos were blocked in 2% bovine serum albumin (Sigma) in PBST for 2 h and then incubated with the primary antibody diluted in blocking reagent overnight at 4 °C. Following four washes in PBST, the embryos were incubated in secondary antibody diluted in blocking reagent for 2 h at room temperature and then rinsed four times for 20 min each in PBST prior to imaging. The following primary antibodies were used at a concentration of 1:500: anti-green fluorescent protein mouse IgG₂a, monoclonal 3E6 (Invitrogen), and rabbit DsRed polyclonal (Clontech). The following secondary antibodies were used at a concentration of 1:500: Alexa 488 goat antimouse IgG₂a (Invitrogen) and Alexa 568 goat anti-rabbit IgG (Invitrogen).

Imaging Analysis—Fish were anesthetized using Tricaine (United States Biochemical) prior to imaging. Bright field images were taken using a Nikon SMZ1500 fluorescence microscope with an Insight Firewire 2 digital camera and SPOT advanced software. Fluorescent images were taken using a Zeiss Axio Observer.Z1 microscope and captured using a Zeiss AxioCam CCD camera. For confocal analysis, fixed and stained embryos were mounted in 1% low melt agarose (National Diagnostics) dissolved in water. Confocal images were taken with a Leica TCS SP5 microscope (Leipzig, Germany) using a water dipping ×40 lens with an NA of 0.8 and 1.3 digital zoom. Confocal images were also taken using an Olympus Fluoview Microscope with a ×60 lens and analyzed using Fluoview software. Images were processed with ImageJ (1.42q) Imaris (Bitplane Inc.) and Adobe PhotoshopCS4 software.

RESULTS

S1P Metabolic Enzymes and Receptor Family Are Conserved in the Zebrafish—Genomic database analysis identified the presence of seven S1P receptor sequences in the zebrafish. For two receptors, s1pr3 and s1pr5, the zebrafish genome contained duplicated copies of the mammalian orthologs (denoted s1pr3a, s1pr3b, s1pr5a, and s1pr5b) (Fig. 1A). The percent identity at the protein level between the zebrafish S1P receptors and the corresponding human receptors. C, percent identity at the protein level between the enzymes responsible for biosynthesis of S1P in the zebrafish and human.

FIGURE 1. S1P receptors and enzymes required for its biosynthesis are conserved in the zebrafish. A, seven S1P receptors were identified in the zebrafish with duplication of s1pr3 and s1pr5. B, percent identity at the protein level between the zebrafish S1P receptors and the corresponding human receptors. C, percent identity at the protein level between the enzymes responsible for biosynthesis of S1P in the zebrafish and human.
FIGURE 2. Expression levels quantified by quantitative RT-PCR of the S1P receptors and enzymes required for its biosynthesis. A, $s1pr1$, $s1pr2$, and $s1pr3$ are expressed during early embryonic development in the zebrafish. Expression levels are normalized to 24 hpf. B, $s1p1$, $s1pr2$, $s1pr3a$, $s1pr3b$, $s1pr4$, and $s1pr5a$ are expressed in the intestine, heart, brain, kidney, and eye of adult zebrafish. Expression levels are normalized to the intestine. C, $spp1$, $spp2$, $spl$, $sphk1$, and $sphk2$ are expressed during early embryonic development in the zebrafish. Expression levels are normalized to 24 hpf. D, $spp1$, $spl$, and $sphk2$ are expressed in the intestine, heart, brain, kidney, and eye of the adult zebrafish. Expression levels are normalized to the intestine. E, sorting of 24 hpf $Tg(fli:EGFP)^{+/y}$ embryos for GFP-positive endothelial cells and $Tg(Gata1:dsRed)$ embryos for dsRed positive blood cells followed by qRT-PCR analysis revealed that $s1pr1$, $s1pr2$, and $s1pr3$ are enriched in zebrafish endothelial cells, and $spp1$, $spl$, and $sphk2$ are enriched in both zebrafish endothelial and blood cells. Expression levels are normalized to the whole embryo.
conserved in the zebrafish. We identified two sphingo-
sine 1-phosphate phosphatases (spp1 and spp2), two sphin-
gosine kinases (sphk1 and sphk2), and one sphingosine lyase (spl). The percent identity between the human and zebrafish
enzymes ranges from 48 to 64% at the protein level
(Fig. 1C).

Bioinformatics analysis indicates synteny between the
zebrafish sphk2 gene and the human SPHK2 gene. Likewise,
synteny is also observed between the Xenopus sphk1 gene
and the zebrafish sphk1 as well as between the zebrafish spp genes
and the human spp genes.

Quantitative RT-PCR analysis was conducted to examine
relative expression levels of S1PRs during embryogenesis.
Transcripts for s1pr1 were first detected during somitogenesis
(Fig. 2A). In sharp contrast, s1pr2 is maternally expressed; those
transcripts are depleted during the cleavage phase, and the gene
is subsequently expressed during gastrulation (Fig. 2A). Tran-
scripts for s1pr3 were first detected at 24 hpf (Fig. 2A). Tran-
scripts encoding the other zebrafish S1P receptors examined
(s1pr3b, s1pr4, s1pr5a, and s1pr5b) were not detectable by
quantitative RT-PCR at the early developmental time points
analyzed.

Relative expression levels of the zebrafish s1prs were exam-
inied in select tissues (intestine, heart, brain, kidney, and eye)
from adult zebrafish. Enrichment of s1pr1 was seen in the brain,
as in mammalian species, whereas s1pr2, s1pr3a, s1pr3b, and
s1pr5a were ubiquitously expressed in the adult tissue types
examined (Fig. 2B). Expression of s1pr4 was enriched in the
kidney, the site of zebrafish hematopoiesis, which is consistent
with its expression in mammalian lymphoid and hematopoietic
tissues (6). Expression of s1pr5b was not detected in the tissues
examined.

Relative expression levels of the key enzymes important for
S1P biosynthesis were also examined by quantitative RT-PCR.
The two zebrafish spp genes displayed inverse expression pat-
terns (Fig. 2C). Although detectable at low levels during early
developmental stages, spp1 was most highly expressed from
somitogenesis to 3 days post-fertilization (dpf). In contrast,
abundant maternal transcripts for spp2 were found, although
its expression levels dropped precipitously by the cleavage
stage. Low levels of spl transcripts were detectable at the
early time points studied, although expression levels
increased by 3 days post-fertilization (Fig. 2C). Expression of
sphk1 was initially detected during somitogenesis and began
to decline by 3 days post-fertilization, whereas sphk2 was
maternally expressed. The sphk2 transcript levels decreased
slightly during the cleavage stage and then were maintained at a
relatively similar level for the remainder of the developmental
time points analyzed (Fig. 2C). In the adult zebrafish, only spp1,
spl, and sphk2 were detectable. Expression levels were highest
in the intestine for all three genes, and spp1 was also highly
expressed in the kidney (Fig. 2D).

We sorted GFP-positive endothelial cells or dsRed-positive
erythroid cells from 24 hpf Tg(fli:EGFP)Y1 and Tg(gata1:dsRed)
zyebrafish embryos, respectively. We found that transcripts for
s1pr1, s1pr2, and s1pr3a were enriched in endothelial cells (Fig.
2E). Upon examining the S1P enzymes that were highly

FIGURE 2—continued
expressed at 24 hpf, we found that *spp1*, *spl*, and *sphk2* were enriched in both endothelial and erythroid cells (Fig. 2E).

**Zebrafish Sphingolipid Levels Are Enriched in Blood Plasma**—Plasma and RBC samples were isolated from the adult zebrafish and analyzed by liquid chromatography/mass spectrometry. In comparison with mouse plasma, zebrafish plasma displayed significantly higher levels of ceramides, in particular long chain fatty acid-containing species (Fig. 3A and Table 3). This was not seen in packed blood cells, which are primarily RBCs (Fig. 3B and Table 4). Importantly, zebrafish plasma and packed blood cells contained significant levels of S1P (∼90 and ∼140 nM, respectively) (Fig. 3, C and D and Tables 3 and 4). This was not as high as the S1P levels in mouse plasma and packed blood cells (∼800 and 2400 nM, respectively). Interestingly, zebrafish plasma contained higher dihydrosphingosine, sphingosine, and S1P as compared with the zebrafish (Fig. 3B and D and Table 4). As significantly higher levels of S1P (∼90 nM) were found in zebrafish plasma than the affinity of S1P to its receptors, these data suggest that plasma-derived S1P is sufficiently abundant to activate its vascular receptors.

Sphingolipid levels were also analyzed in zebrafish brain, heart, liver, and tail tissue (Fig. 3, E–F, and Table 5). Among
these tissues, S1P levels were highest in the zebrafish brain (33.9 pmol/mg). These values coincide with previously published data showing enrichment of S1P levels in the mouse brain as compared with other tissues (27). S1P levels in the heart, liver, and tail were 5.8, 4.5, and 5.7 pmol/mg, respectively. These levels are similar to S1P levels measured in mouse thymus (27).

### Table 3

|       | Mouse plasma | Zebrafish plasma |
|-------|--------------|-----------------|
| C14-Cer | 4.4 ± 0.2 | 24.1 ± 2.8 |
| C16-Cer | 16.3 ± 1.8 | 165.3 ± 18.3 |
| C18-Cer | 17.8 ± 1.6 | 17.6 ± 0.5 |
| C18:1-Cer | 1.1 ± 0.8 | 3.9 ± 1.3 |
| C20-Cer | 34.2 ± 5.2 | 404.1 ± 120.1 |
| C20:1-Cer | 1 ± 0.6 | 22.8 ± 7.5 |
| C20:4-Cer | 1.2 ± 0.6 | 2.0 ± 0.6 |
| C22-Cer | 108.2 ± 13.3 | 472.1 ± 66.0 |
| C22:1-Cer | 11.2 ± 2.2 | 93.2 ± 22.0 |
| C24-Cer | 504.3 ± 108.9 | 2660.5 ± 361.5 |
| C24:1-Cer | 489.3 ± 84.6 | 3156.2 ± 558.0 |
| C26-Cer | 12.6 ± 1.0 | 1883.6 ± 375.5 |
| C26:1-Cer | 3.7 ± 0.0 | 1509.2 ± 291.5 |
| Dihydro-C16-Cer | 6.2 ± 2.1 | 78.3 ± 17.9 |
| Dihydro-C18-Cer | 41.9 ± 5.2 | 239.7 ± 45.7 |
| Dihydro-C18:1-Cer | 21.4 ± 4.9 | 138.0 ± 25.6 |
| Sphingosine | 253.6 ± 37.6 | 9.4 ± 1.5 |
| S1P | 789.4 ± 142.0 | 88.4 ± 6.1 |

### Table 4

|       | Mouse RBC | Zebrafish RBC |
|-------|----------|---------------|
| C14-Cer | 10.7 ± 0.1 | 69.8 ± 2.1 |
| C16-Cer | 582.1 ± 109.5 | 318.3 ± 31.7 |
| C18-Cer | 146.4 ± 0.2 | 24.4 ± 2.5 |
| C18:1-Cer | 12.3 ± 3.2 | 2.1 ± 2.2 |
| C20-Cer | 194.3 ± 1.6 | 234.6 ± 31.2 |
| C20:1-Cer | 6.9 ± 2.5 | 22.1 ± 11.5 |
| C20:4-Cer | 6.1 ± 1.0 | 30.1 ± 5.2 |
| C22-Cer | 1037.3 ± 21.4 | 239.7 ± 16.2 |
| C22:1-Cer | 83.6 ± 7.3 | 1346.6 ± 27.8 |
| C24-Cer | 4495.3 ± 370.6 | 6260.5 ± 81.8 |
| C24:1-Cer | 2084.1 ± 65.7 | 1657.1 ± 151.1 |
| C26-Cer | 706.1 ± 177.4 | 410.4 ± 115.7 |
| C26:1-Cer | 97.7 ± 20.1 | 368.8 ± 42.5 |
| Dihydro-C16-Cer | 149.7 ± 5.3 | 53.1 ± 8.0 |
| Dihydro-C18-Cer | 2397.9 ± 111.5 | 19.9 ± 2.0 |
| Dihydro-C18:1-Cer | 2397.9 ± 111.5 | 19.9 ± 2.0 |
| Dihydro-S1P | 2397.9 ± 111.5 | 19.9 ± 2.0 |
| Sphingosine | 1588.4 ± 388.7 | 11.6 ± 3.2 |
| S1P | 2424.6 ± 142.0 | 142.8 ± 16.3 |

### Table 5

|       | Brain (pmol/mg) ± S.D. | Heart (pmol/mg) ± S.D. | Liver (pmol/mg) ± S.D. | Tail (pmol/mg) ± S.D. |
|-------|------------------------|------------------------|------------------------|-----------------------|
| C14-Cer | 3.2 ± 0.1 | 7.2 ± 2.2 | 6.8 ± 5.1 | 4.2 ± 1.1 |
| C16-Cer | 20.4 ± 5.7 | 44.4 ± 10.1 | 34.9 ± 21.4 | 43.7 ± 10.8 |
| C18-Cer | 70.0 ± 7.1 | 5.7 ± 1.6 | 5.8 ± 3.2 | 3.0 ± 0.7 |
| C18:1-Cer | 0.4 ± 0.1 | 0.3 ± 0.3 | 0.7 ± 0.4 | 0.1 ± 0.0 |
| C20-Cer | 49.3 ± 3.5 | 10.0 ± 0.4 | 27.4 ± 18.9 | 9.6 ± 2.3 |
| C20:1-Cer | 0.8 ± 0.3 | 0.6 ± 0.1 | 2.3 ± 1.4 | 0.7 ± 0.1 |
| C20:4-Cer | 0.1 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.0 | 0.0 ± 0.0 |
| C22-Cer | 276.9 ± 77.6 | 28.1 ± 1.9 | 26.2 ± 16.5 | 12.5 ± 2.7 |
| C22:1-Cer | 41.9 ± 9.5 | 26.0 ± 7.8 | 18.5 ± 11.3 | 10.6 ± 3.7 |
| C24-Cer | 380.4 ± 112.1 | 69.9 ± 15.3 | 47.2 ± 17.3 | 20.5 ± 10.3 |
| C24:1-Cer | 449.6 ± 102.8 | 97.0 ± 20.5 | 80.8 ± 48.9 | 35.0 ± 8.9 |
| C26-Cer | 62.4 ± 15.4 | 29.1 ± 9.0 | 26.0 ± 9.4 | 4.7 ± 1.2 |
| C26:1-Cer | 86.1 ± 17.9 | 34.9 ± 10.5 | 23.2 ± 9.6 | 8.0 ± 1.8 |
| Dihydro-C16-Cer | 4.2 ± 0.4 | 4.1 ± 3.1 | 2.4 ± 1.4 | 1.5 ± 0.3 |
| Dihydro-C18-Cer | 16.6 ± 1.8 | 23.4 ± 18.2 | 3.8 ± 0.7 | 2.8 ± 0.4 |
| Dihydro-S1P | 35.7 ± 6.5 | 2.5 ± 1.4 | 0.4 ± 0.3 | 1.8 ± 0.5 |
| Sphingosine | 48.0 ± 4.4 | 29.7 ± 5.3 | 53.8 ± 34.1 | 6.4 ± 1.5 |
| S1P | 33.9 ± 4.6 | 5.8 ± 2.6 | 4.5 ± 2.5 | 5.7 ± 1.3 |
recover by 72 hpf (supplemental Fig. 1). To determine whether any of the phenotypes displayed by the s1pr1 morphants were the result of off-target induced p53-dependent apoptosis, zebrafish were co-injected with 4 ng of a morpholino that has previously been validated at this dose to deplete p53 (24, 29) along with 4 ng of the s1pr1 MO1. Co-injection of the s1pr1 morphants with the p53 MO did not lessen the severity of the edema and circulatory phenotypes or the vascular phenotype (supplemental Fig. 2, A–D). Thus, the phenotype characterized for s1pr1 knockdown is not the result of a p53-dependent off-target effect and is likely due to specific knockdown of the s1pr1 receptor.

To determine whether the knockdown of s1pr1 affected endothelial cell abundance, fertilized eggs derived from Tg(flk1: ras-mCherry)Ras741C and Tg(flk1:EGFP-NLS) double transgenic fish were injected with 4 ng of the control MO (Fig. 5G) or 4 ng of the s1pr1 MO1 (Fig. 5H) and imaged at 32 hpf, a time point by which the primary angiogenic vasculature, namely the ISVs and DLAV, should have formed. Control morphants had significantly more endothelial cells in both the angiogenic (ISV and DLAV) vessels (Fig. 5J) and the vasculogenic axial vessels (dorsal aorta and posterior cardinal vein) (Fig. 5F) as compared with the s1pr1 morphants, suggesting that s1pr1 could regulate cell abundance within both angiogenic and vasculogenic vessels by affecting endothelial cell specification, proliferation, and/or survival.

Several antibodies to mammalian S1pr1 did not cross-react with the zebrafish protein (data not shown). The S1P receptors consist of two exons, the second of which encodes the entire open reading frame for the receptor, thus making the use of splice-blocker morpholinos problematic. Therefore, to confirm specificity of the phenotype, a second translation-blocking morpholino (s1pr1 MO2) was generated to target a distinct sequence upstream of the ATG transcription start site. Injection of 8 ng of s1pr1 MO2 phenocopied the edema, cardiac, and blood phenotypes seen in the embryos injected with 4 ng of s1pr1 MO1 (supplemental Fig. 3, A and B) and resulted in embryos with mild vascular defects consisting of improper lateral connections of the ISVs (supplemental Fig. 3C, yellow asterisks). Co-injection of both translation-blocking morpholinos at sub-threshold doses (that do not generate a phenotype when injected alone; supplemental Fig. 3, D–G) yielded embryos that also displayed the edema, cardiac, and blood phenotypes seen in the embryos injected with 4 ng of MO1 (supplemental Fig. 3, H and I) suggesting that these two morpholinos have the same target and generate similar phenotypes.

In addition to the ISVs, development of the caudal vein plexus (CaVP) is a well studied model for developmental angiogenesis in the zebrafish (30). As compared with control-injected zebrafish (Fig. 6A), fish injected with 4 ng of s1pr1 MO1 (Fig. 6B) displayed a significant decrease in length, width, and total area of the CaVP. Strikingly, injection of 100 pg of s1pr1 mRNA (Fig. 6C) resulted in the opposite effect by increasing width and total area of the CaVP (Fig. 6D). Compared with control-injected fish (Fig. 6E), s1pr1 morphants phenocopied the mouse retinal defect as injection of s1pr1 MO1 resulted in significantly increased filopodial abundance (Fig. 6F), whereas injection of mRNA resulted in decreased filopodial abundance (Fig. 6, G and H). These findings suggest that s1pr1 is important for critical developmental events such as vascular sprouting, patterning, endothelial cell abundance, and barrier function.

**Knockdown of s1pr1 and s1pr2 Exacerbates the Phenotype seen Following Knockdown of s1pr1 Alone**—The s1pr2 gene is important for regulating the migration of cardiac precursor cells to the midline, as a point mutation in the gene yields cardiac bifida (15). Vascular defects were not described for these mil mutants, such that s1pr2 is not known to regulate angiogenesis. ISV sprouting is normal in embryos injected with the control MO (Fig. 7A), 15 ng of s1pr2 MO1 (a dose that recapitulates a cardiac and tail-blistering phenotype as described previously (17)) (Fig. 7B), or low doses of s1pr1 MO1 at 1 or 2 ng, respectively (Fig. 7, C and D). However, when expression of both s1pr1 and s1pr2 is knocked down, a cardiac and tail blisters phenotype is seen that does not affect the ISVs and DLAV (Fig. 7, E and F). Co-injection of s1pr1 and s1pr2 morpholinos at 2 ng each resulted in embryos with mild edema, dilated heart, and shorter axial vessels compared with control-injected embryos (Fig. 7, G and H).
and s1pr2 is blocked by co-injection of both morpholinos, severely reduced vascular development of the intersegmental vessels was observed with doses of the s1pr1 morpholino that alone did not cause any discernible vascular defects (Fig. 7, E and F). Co-injection of the s1pr1 and s1pr2 double morphants with the p53 MO did not lessen the severity of the phenotypes (supplemental Fig. 2, E–H). To confirm that the results obtained with these two morpholinos were specific, s1pr1 MO1 was injected with a second translation-blocking morpholino against s1pr2 (s1pr2 MO2). Results obtained with s1pr1 MO1 and s1pr2 MO2 phenocopied those observed with s1pr1 MO1 and s1pr2 MO1 (supplemental Fig. 4).

S1P Transporter spns2 Cooperates with s1pr1 to Regulate Angiogenesis—The spns2 gene functions as an S1P transporter in S1P secretion and regulates myocardial precursor migration (17). The phenotype of zebrafish with a point mutation in spns2 is identical to the phenotype exemplified by mil mutant fish; both display cardia bifida and epithelial tail blisters. Based on

FIGURE 5. Knockdown of s1pr1 causes edema, circulatory, and vascular phenotypes. Bright field images of control morphant (A) and s1pr1 morphant (B) are shown. The s1pr1 morphants have edema of the yolk and pericardium (548 affected/579 injected; n = 8 independent experiments) at 48 hpf. In Tg(gata1: dsRed) fish injected with control MO (C), there was normal blood cell circulation, whereas following injection of s1pr1 MO1 4 ng (D), there was failure of the red blood cells to migrate from the caudal hematopoietic tissue (548 affected/579 injected; n = 8 independent experiments) at 48 hpf. In Tg(fli1:EGFP) fish injected with control MO (E), there was normal vascular development, whereas embryos injected with s1pr1 MO1 4 ng (F) revealed that many injected fish displayed some ISVs that failed to sprout beyond the horizontal myoseptum to produce the DLAV (319 affected/361 injected; n = 6 independent experiments) at 48 hpf. Yellow asterisks indicate stunted ISVs. To determine whether the knockdown of s1pr1 affected endothelial cell abundance, Tg(flk1:red-mCherry)H896 and Tg(flk1:EGFP-NLS) double transgenic fish were injected with control MO 4 ng (G) or s1pr1 MO1 4 ng (H) and imaged at 32 hpf. Quantitation of nuclei number shows more nuclei in the angiogenic vessels (ISVs and DLAV) of control-injected fish (n = 14) compared with the s1pr1 morphant embryos (n = 15), p < 0.0001 (I) and more nuclei in the vasculogenic axial vessels (DA and PCV) in the control-injected fish compared with the s1pr1 morphant embryos p = 0.0242 (J). Nuclei were quantified in four consecutive ISVs or in axial vessels from a representative field of view.
these findings, we tested whether *spns2* played a cooperative role with *s1pr1* in regulating vascular sprouting. ISV sprouting is normal in embryos injected with the control MO (Fig. 8A) or 2 ng of *spns2* MO (a sub-threshold dose of the previously validated morpholino) (Fig. 8B). Knockdown of both *s1pr1* and *spns2* (at doses that by themselves did not affect ISV sprouting) resulted in stunted development of the ISVs (Fig. 8, C and D).

Therefore, the interaction between *s1pr1* and *spns2* is required for proper embryonic vascular patterning in the zebrafish.

**DISCUSSION**

Here, we show conservation of the key S1P receptors and enzymes important for S1P biosynthesis in the zebrafish. Database analysis revealed zebrafish nucleotide sequences similar to
all five mammalian S1P receptors. Additionally, two homologs were identified for s1pr3 and s1pr5, likely due to the whole genome duplication of the teleost fish lineage (31–33). Early embryonic development is dependent upon maternal gene transcripts that are generated during oogenesis. Some transcripts of the S1P receptors and enzymes important for S1P metabolism are maternally expressed (s1pr2, spp1, spp2, spl, and sphk2), suggesting that these function during early embryonic development. There is a decrease in transcript levels for these genes during the maternal-zygotic transition and a subsequent increase as the zygotic genes are activated.

Additionally, expression of the s1pr genes in adult fish tissue conforms at least generally conserved expression patterns with mice. Enrichment of s1pr1 was seen in the brain, as in mammals, and expression of s1pr4 was enriched in the kidney, the site of zebrafish hematopoiesis, which is consistent with its expression in mammalian lymphoid and hematopoietic tissues (6). The other receptors, s1pr2, s1pr3a, s1pr3b, and s1pr5a, were ubiquitously expressed in the adult tissue types examined. In the adult zebrafish, high expression levels of spp1, spl, and sphk2 were detectable in the intestine, a result consistent with findings that dietary sphingolipid hydrolysis and metabolism occur in the intestine of mice (34).

The ligand specificity of zebrafish s1pr1 has been shown to be similar to mammalian S1pr1. Im et al. (35) showed that 100 pm S1P was sufficient to inhibit intracellular cAMP production in s1pr1-transfected cells (IC50 = 1 nM). High levels of lysophosphatidic acid (as high as 10 μM) were unable to have the same effect. Structurally related agonists of S1P (such as dihydro-S1P) inhibit cAMP accumulation; however, other lysophospholipids had no effect.

S1P levels measured from adult zebrafish plasma samples (~90 nM) display sufficient levels to activate endothelial cells as bioactive S1P in the plasma is ~10 nM, which is near the Kd value of the ligand for the receptor (36). Interestingly, zebrafish plasma contains higher levels of ceramide, dihydro sphingosine, and sphingosine but lower levels of dihydro-S1P and S1P in comparison with mouse plasma. These findings suggest that in the zebrafish, high sphingosine-1-phosphate phosphatase activity may aid in the conversion of S1P to its precursors sphingosine and ultimately ceramide, whereas in the mouse the activity of sphingosine kinase may dominate to convert ceramide and sphingosine into S1P. In mammals a steep vascular S1P gradient, formed due to significantly higher levels of S1P in the plasma and lymph as compared with the tissue interstitium (37), promotes the egress of lymphocytes and hematopoietic
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stem cells (38, 39). In the zebrafish, the role of the S1P gradient has not been examined, but our data indicate that significant levels of S1P are enriched in blood cells (RBCs) and plasma. This could result in the activation of S1P receptors in vascular and hematopoietic systems.

A recent paper examining s1pr1 knockdown by morpholinos in zebrafish also showed edema (pericardial and general) and the circulation phenotype that we describe here (40). Although the authors did not characterize the ISV-sprouting phenotype, this might be due to the lower dose of morpholino that they used for their injections. The authors did describe thinner ISVs in zebrafish also showed edema (pericardial and general) and defects in the duct of Cuvier and subintestinal vein basket, used for their injections. The authors did not characterize the ISV-sprouting phenotype, this might be due to the lower dose of morpholino that they used for their injections. The authors did describe thinner ISVs and defects in the duct of Cuvier and subintestinal vein basket, which are vascular phenotypes that we also observed in our experiments (see supplemental Fig. 1).

In mice it has been shown that deletion of S1pr1 results in embryonic lethality because of its role within endothelial cells in regulating the coverage of blood vessels by vascular smooth muscle cells (9, 41). To understand whether this phenotype is conserved in other species, and to evaluate in more detail potential functions in embryonic angiogenesis, we examined knockdown of s1pr1 in the zebrafish. Here, we characterized vascular defects in two vascular beds, the ISVs and CaVP. Following morpholino knockdown of s1pr1, we saw stunted vascular sprouting of the ISVs resulting in incomplete formation of the DLAV. In contrast, we showed that morpholino knockdown of s1pr1 causes excessive filopodial sprouting in the CaVP, whereas injection of s1pr1 mRNA has the opposite effect and decreases filopodial sprouting as compared with control-injected fish. These two findings likely indicate heterogeneity in the function of s1pr1 in these two vascular beds. The ISV defects occur in sprouts from the axial artery, whereas the defects in the CaVP occur in a vein, thus suggesting that s1pr1 exerts different effects on arteries and veins.

Our finding of excessive sprouting angiogenesis following knockdown of s1pr1 in the zebrafish CaVP complements recent work (11, 42) showing that conditional knock-out of S1pr1 in murine endothelial cells results in alterations in the primary vascular plexus of the retina. More specifically, these mice display increased vascular density, excessive numbers of filopodia-containing tip cells, and increased branch points in the retinal vessels as compared with wild-type mice (11). Similarly, when we examine sprouting angiogenesis in the zebrafish CaVP following s1pr1 morpholino knockdown, we also saw increased filipodia sprouting in this vascular bed as compared with control morphants. In contrast, overexpression of S1pr1 results in suppression of tip cell formation in the developing mouse retina, a finding that is paralleled following injection of s1pr1 mRNA into zebrafish embryos. Additionally, S1pr1 was shown to be essential for flow-dependent endothelial shear stress sensing in endothelial cells in vitro and in vivo. As the zebrafish can allow for the examination of flow-dependent signaling in real time, it may be feasible to use the zebrafish as a tool for better understanding the mechanism of flow-mediated S1P$_1$ activation.

We believe that our knockdown phenotypes reflect specific targeting of s1pr1 for several reasons. First, the phenotypes are reproducible in essentially 100% of the embryos and are highly consistent using two distinct morpholinos. Importantly, co-injection of two s1pr1 morpholinos at concentrations that are individually sub-threshold recapitulates the phenotype of each when used above the threshold, arguing that they target the same transcript. Finally, the phenotypes are independent of

![Image](image-url)
p53, suggesting that they cannot be attributed to p53-dependent off-targeting artifacts.

To unmask collaborative functions between s1pr1 and s1pr2, we knocked down expression of both receptors and found a substantially more severe vascular phenotype than was seen in embryos following knockdown of only s1pr1. These results corroborate murine data showing that S1pr1 and S1pr2 double knock-out mice display a more severe vascular phenotype than do S1pr1 single knock-out mice. Formation of an immature vascular network in the S1pr1 and S1pr2 double knock-out mice results in embryonic lethality that is on average 2 days earlier (E10.5–12.5) than in the S1pr1 single null embryos (E12.5–14.5) (10). Because of this early hemorrhaging and subsequent lethality, the role of S1pr1 and S1pr2 in embryonic angiogenesis had not been clearly examined in mice. As knockdown of both receptors in the zebrafish did not result in early lethality but rather a distinct vascular phenotype, we showed that s1pr1 and s1pr2 have redundant or cooperative functions for the development of a stable and mature vascular system during embryonic development.

The S1P transporter spns2 functions in the same pathway as mil because both regulate myocardial precursor cell migration. As a consequence, zebrafish expressing mutant spns2 phenotype copy the mil phenotypes of cardio bifida and epithelial tail blistering. Knockdown of both s1pr1 and spns2 (at doses that themselves did not affect ISV sprouting) resulted in stunted development of the ISVs, showing a remarkably similar phenotype as full knockdown of s1pr1. Therefore, the interaction between s1pr1 and spns2 is required for proper vascular patterning in the zebrafish.

In conclusion, we show that the key receptors and enzymes important for sphingosine metabolism and signaling are conserved in the zebrafish. We also show the presence of significant levels of S1P in the zebrafish plasma that are sufficient to activate its receptors on endothelial cells. Morpholino knockdown of s1pr1 results in pericardial edema, lack of blood circulation, and distinct vascular defects in the arterial and venous beds, namely failure of arterial-derived ISVs to sprout completely and excessive sprouting of the venous-derived CaVP. Combined knockdown of s1pr1 and s1pr2 causes a stronger vascular phenotype in the ISVs indicating that s1pr1 and s1pr2 function cooperatively to regulate vascular formation in the zebrafish. Thus, sufficient S1P is present in the plasma to signal via multiple endothelial S1P receptors to regulate vascular development in vertebrates.

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