S1P1 inhibits sprouting angiogenesis during vascular development

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SUMMARY
Coordination between the vascular system and forming organs is essential for proper embryonic development. The vasculature expands by sprouting angiogenesis, during which tip cells form filopodia that incorporate into capillary loops. Although several molecules, such as vascular endothelial growth factor A (Vegfa), are known to induce sprouting, the mechanism that terminates this process to ensure neovessel stability is still unknown. Sphingosine-1-phosphate receptor 1 (S1P1) has been shown to mediate interaction between endothelial and mural cells during vascular maturation. In vitro studies have identified S1P as a pro-angiogenic factor. Here, we show that S1P1 acts as an endothelial cell (EC)-autonomous negative regulator of sprouting angiogenesis during vascular development. Severe aberrations in vessel size and excessive sprouting found in limbs of S1P1-null mouse embryos before vessel maturation imply a previously unknown, mural cell-independent role for S1P1 as an anti-angiogenic factor. A similar phenotype observed when S1P1 expression was blocked specifically in ECs indicates that the effect of S1P1 on sprouting is EC-autonomous. Comparable vascular abnormalities in S1P1-null zebrafish embryos suggest cross-species evolutionary conservation of this mechanism. Finally, genetic interaction between S1P1 and Vegfa suggests that these factors interplay to regulate vascular development, as Vegfa promotes sprouting whereas S1P1 inhibits it to prevent excessive sprouting and fusion of neovessels. More broadly, because S1P, the ligand of S1P1, is blood-borne, our findings suggest a new mode of regulation of angiogenesis, whereby blood flow closes a negative feedback loop that inhibits sprouting angiogenesis once the vascular bed is established and functional.

KEY WORDS: Sphingosine-1-phosphate receptor 1 (S1P1), Endothelial cell, Mouse, Limb vasculature, Zebrafish, Angiogenesis, Vascular remodeling, Sprouting, Filopodia, Intersegmental vessels, Caudal vein plexus

INTRODUCTION
The embryonic vascular system is initiated when endothelial precursors called angioblasts form a primitive network of tubular endothelial structures in a process known as vasculogenesis (Risau, 1991). Next, the uniform primary plexus undergoes remodeling to generate a more complex, hierarchical network of vessels of varying sizes. This occurs by sprouting, branching and pruning of pre-existing vessels in a process called angiogenesis (Risau, 1997). The remodeling process is tightly coordinated in order to supply the increasing demands of the developing embryo for nutrients and oxygen.

Sprouting angiogenesis forms new vessels by creating loops between neighboring sprouts through anastomosis (reviewed by Potente et al., 2011). During this process, endothelial cells (ECs) are specified into either tip or stalk cells by lateral inhibition, through activation of the Dll4/Notch signaling pathway by vascular endothelial growth factor A (Vegfa) (Hellstrom et al., 2007; Lobov et al., 2007; Suchting et al., 2007). Tip cells are found at the edge of the sprout and lead the way by extending numerous long filopodia and migrating in response to angiogenic cues. These include Vegfa signaling, which promotes the sprouting process (Gerhardt, 2008; Gerhardt et al., 2003), and the ephrin-Eph, Slit-Robo, netrin-Unc5b and semaphorin-plexin families, which mediate tip cell guidance (Adams and Eichmann, 2010; De Smet et al., 2009; Larrivee et al., 2009; Ruhrberg et al., 2002; Weinstein, 2005). Adjacent stalk cells then proliferate towards the migrating tip cell and form the vascular lumen (Gerhardt et al., 2003).

Once a vascular network achieves adequate size and configuration, sprouting angiogenesis has to cease to allow neovessel stabilization. In contrast to the wealth of information on the mechanism that promotes angiogenesis and vessel growth, little is known on the mechanism that terminates sprouting angiogenesis to prevent excessive sprouting and stabilize the vasculature. A mechanism that was implicated in vascular stabilization is the maturation process, during which vessels are invested with mural cells, namely vascular smooth muscle cells (VSMCs) and pericytes (Benjamin et al., 1998; Carmeliet, 2000; Hungerford and Little, 1999; Jain, 2003). Mural cells provide structural support for increased blood pressure and control EC proliferation, vascular permeability and vessel diameter (Nehls and Drenckhahn, 1993). Several molecules were identified to control the recruitment of these cells to the developing vessels, including S1P1 (S1pr1 – Mouse Genome Informatics) (Allende and Proia, 2002; Allende et al., 2003), a G protein-coupled receptor for sphingosin-1-phosphate (S1P; also known as Mbtps1) (Lee et al., 1998).

S1P is a bioactive sphingolipid metabolite produced by several cellular sources, such as platelets, erythrocytes and ECs (Hla et al., 2008; Pappu et al., 2007; Venkataraman et al., 2008), and is found in high concentration in the blood serum (Hla et al., 2008; Yatomi et al., 2000). The vital role of S1P1 in vascular development was demonstrated by knockout of the S1P1 gene in mice (Liu et al., 2000). S1P1-null embryos die in utero between E12.5 and E14.5 as...
a result of severe bleeding throughout their bodies. Vasculogenesis and angiogenesis in those mice appeared normal and the vascular abnormalities were attributed to a defect in the association of mural cells with nascent vessels, which exhibited incomplete coverage by these cells (Allende and Proia, 2002; Liu et al., 2000).

Interestingly, S1P1+/− embryos also exhibit defective vasculature in the limb bud (Chae et al., 2004). Previous studies by ourselves and others have shown that the limb vasculature undergoes extensive remodeling during its development (Ambler et al., 2001; Eshkar-Oren et al., 2009; Seichert and Rychter, 1972a; Seichert and Rychter, 1972b). The primary vasculature of the limb bud originates from the dorsal aorta. During limb development, Vegfa expression in the limb mesenchyme drives the rearrangement of the vascular plexus into a highly patterned network. This includes a single axial artery that splits into a network of small capillaries, which drain into a thicker marginal vein (Eshkar-Oren et al., 2009).

In this study, we revisit the role of S1P1 in angiogenesis based on our finding that at early stages of limb development the vasculature lacks mural cell coating. We show that S1P1 acts independently of mural cells and EC-autonomously to inhibit sprouting angiogenesis, thereby promoting vessel stability. We then demonstrate in zebrafish the evolutionary conservation of this mechanism. Finally, we propose a model for sprouting angiogenesis whereby regulatory interaction between Vegfa and S1P1 maintain balance between induction and restriction of this process.

MATERIALS AND METHODS

Mice
SIP1−/− and SIP1loxP/loxP embryos were genotyped as previously described (Chae et al., 2004). To obtain endothelial-specific SIP1 knockout, SIP1loxP/loxP mice were crossed with mice expressing Cre recombinase under the control of the EC-specific promoter VE-Cadherin-Cre (Jackson Laboratories). The offspring was crossed with SIP1loxP/loxP to obtain SIP1loxP/VE-CadCre embryos. The generation of floxed-Vegfa (Gerber et al., 1999), floxed-Hif1α (Ryan et al., 2000) and Prx1 (also known as Prrxl – Mouse Genome Informatics)-Cre mice (Logan et al., 2002) have been described previously.

Inducible Vegfa overexpression in the limb mesenchyme was carried out by the reverse tetracycline transactivator (rtTA)/tetracycline-responsive element (tetO)-driven transgene system (Belteki et al., 2005; Gossen et al., 1995), with Prrxl-Cre mouse (Logan et al., 2002) as an inducer. Briefly, tetO-Vegfa mice were crossed with rtTA mice. Mice heterozygous for rtTA and tetO-Vegfa (rtTA-tetO-Vegfa) were crossed with mice heterozygous for Prx1-Cre transgene as an inducer. To induce Vegfa expression, doxycycline was administered to pregnant females starting at embryonic day (E) 8.5 and embryos heterozygous for Prx1-Cre, rtTA and tetO-Vegfa (Prx1-Cre rtTA-peg3-Vegfa) were compared with embryos heterozygous for rtTA and Prx1-Cre alleles (control).

In all timed pregnancies, plug date was defined as E0.5. For harvesting of embryos, timed-pregnant female mice were sacrificed by CO2 intoxication. The gravid uterus was dissected out and suspended in a bath of cold PBS, and the embryos were harvested after amnionectomy and removal of the placenta. Tail genomic DNA was used for genotyping.

Zebrafish husbandry and morpholino oligonucleotide (MO) injection
Fish were bred and raised under standard conditions (Kimmel et al., 1995). Tg(fli1:egfp) embryos were produced and staged as previously described (Yaniv et al., 2006). sIP1, antisense MOs (GeneTools) were designed against the start codon/5’ UTR to block translation (ATGGGATTGCATCACTCACTGTGG3’). MOs were injected into Tg(fli1:egfp) embryos at the one-cell stage and analyzed morphologically at 2-3 days post fertilization (dpf).

mRNA rescue experiment
The primers were used to amplify the full-length coding sequences of zebrafish slp: forward, 5’-ATGGGATTGATCGACTGGATTTTACACTCTGAGATGGA-3’; reverse, 5’-TCTAGAATCGTCCCTTTAAG-3’. To prevent slp, MO binding to injected mRNA, the sequence of the MO binding site was modified such that it would not affect amino acid sequence (represented by lower case letters in the forward primer). After TOPo cloning and sequencing (Invitrogen, Carlsbad, CA, USA), a Gateway-compatible middle entry clone (Invitrogen) was generated using Gateway BP clonase (Invitrogen)-mediated recombination. The slp coding sequences was then transferred into a pCS1V-GFP vector using Gateway LR clonase (Invitrogen)-mediated reaction to produce the pCS1V/GFP plasmid. After linearization with NotI, plasmids were used as templates for mRNA synthesis. Capped sense RNA was synthesized using SP6 RNA polymerase and the mMESSAGE mMACHINE system (Ambion, Austin, TX, USA). One-cell-stage embryos were microinjected with slp mRNA and antisense MOs.

In situ hybridization
Zebrafish whole-mount in situ hybridization was performed as previously described (Thissie and Thissie, 2008). Antisense probes were generated using the following primers: sIP1 forward, GCACCTCCTCTGTGGCCTTG; reverse, GCGGGCGATGAACAGCCG.

Inhibition of Flk1
SUS4516 (Sigma) dissolved in DMSO was added to the culture media solution at a final concentration of 5 μM. Dechorionated zebrafish embryos [20 hours post fertilization (hpf)] were subjected to SUS4516 treatment for 1 hour, then the chemical was washed and embryos were allowed to develop until 48 hpf.

Microscopy and imaging
Confocal imaging was performed using a Zeiss LSM 780 upright confocal microscope (Carl Zeiss, Jena, Germany) with a W-Plan Apochromat ×20 objective, NA 1.0. eGFP was excited with a 488 nm argon laser. Long-term, time-lapse in vivo imaging of zebrafish embryos was performed as previously described (Yaniv et al., 2006). z-stacks were acquired at 2.5-μm increments every 12 minutes. Images were processed off-line with ImageJ (NIH).

Quantitative RT-PCR (qRT-PCR)
For qRT-PCR analysis, 1 μg total RNA was used to produce first-strand cDNA. Reverse transcription was performed with SuperScriptII (Invitrogen) according to the manufacturer’s protocol. qRT-PCR was performed using SYBR Green (Roche). Values were calculated using the second derivative method and normalized to Gapdh RNA expression. For Vegfa, the primers used were: 5’-ACAGAAAGGAAGACGAGTGCT-3’ (forward) and 5’-CACACAGGACCGCTTGAGATGTA-3’ (reverse).

Whole-mount and section immunofluorescence
For whole-mount immunofluorescence, freshly dissected tissues were fixed overnight in 4% paraformaldehyde (PFA), transferred to PBS, then dehydrated to methanol and stored at −20°C until use. Samples were rehydrated to PBS and incubated for 2 hours in blocking solution (PBS containing 10% normal goat serum and 1% Triton X-100) and then incubated overnight at 4°C with primary antibody anti-PECAM (CD31; BD Pharmingen, San Diego, CA, USA) diluted 1:50 in blocking solution. Samples were washed in PBS containing 1% Triton X-100 at room temperature for 4 hours, and then incubated overnight at 4°C with biotinylated anti-rat secondary antibody (diluted 1:100; Vector Laboratories) and Cy2-conjugated streptavidin antibody (1:100; Jackson ImmunoResearch, West Grove, PA, USA) diluted in 1% BSA in PBS.

For section immunofluorescence, embryo limbs were embedded in OCT (Tissue-Tek) after 2-6 hours fixation in 4% PFA and 10 μm-thick cryostat sections were made. Cryosections were postfixed for 30 minutes in 4% PFA and permeabilized with 0.2% Triton in PBS. In order to block nonspecific binding of immunoglobulin, sections were incubated with 7% goat serum in PBS. Following blockage, cryosections were incubated
overnight at 4°C with primary antibodies: rat anti-CD31 (BD Pharmingen; diluted 1:100), rat anti-NG2 (Millipore; 1:200), rabbit anti-desmin (Dako Cytomation; 1:100) rabbit anti-β-galactosidase (Immunology Consultants Laboratory; 1:200). Sections were then washed in PBS and incubated with secondary fluorescent antibodies: Cy2 anti-rabbit (1:100; Jackson Laboratories) or Alexa Fluor 488-labeled goat anti-rat IgG (Molecular Probes). Samples were then washed and mounted on glass slides. For immunofluorescence staining of mural cells, samples were sectioned by vibrotome at 70 μm. Whole-mounts and sections were examined with an LSM 510 laser-scanning confocal microscope (Carl Zeiss).

Statistical methods
Variables are presented as mean ± s.e.m. In all measured variables but one, comparison between wild-type (WT) and mutant embryos was carried out using Student’s t-test and statistical significance was defined as P ≤ 0.05. Comparison of blood vessel diameter between WT and S1P1−/− mouse embryos was carried out using the Mann-Whitney U test (also known as Wilcoxon rank-sum test).

RESULTS

Increased blood vessel diameter in limbs of S1P1−/− mouse embryos
To elucidate the possible role of S1P1 in vascular remodeling, we revisited the vascular phenotype in the developing limb of S1P1−/− mice. To study the spatial organization of the limb vasculature, we performed whole-mount immunostaining of E9.5 and E11.5 wild-type (WT) and S1P1−/− mouse forelimbs using an anti-CD31 antibody, which marks ECs. Whereas the vasculature in WT limbs comprised a uniform capillary network of small tubes that were distributed throughout the limb bud (Fig. 1A,B), the S1P1−/− limb vasculature lacked organization and consisted of vessels with increased lumen diameter (Fig. 1A′/B′). The effect of S1P1 loss of function could be detected as early as E9.5, the onset of limb formation. At that stage, an increase of ~30% in vessel diameter was observed in S1P1−/− forelimbs, relative to control limbs (Fig. 1C). At E11.5, the phenotype deteriorated and resulted in extremely enlarged vessels (Fig. 1B′). Hematoxylin and Eosin (H&E) staining of E11.5 limb cross-sections further validated the formation of enlarged vessels in the S1P1−/− limbs (Fig. 1D,D′). Quantitative analysis of cross-sections from WT and S1P1−/− limbs also showed a significant reduction in vessel number and a significant increment in total vessel density in S1P1−/− embryos (Fig. 1E,F).

Together, these results suggest that S1P1 plays a central role in limb vascular remodeling.

S1P1 regulates vascular remodeling independently of mural cells
As mentioned, S1P1 has been implicated in vascular stabilization by mediating the interaction between ECs and mural cells (Allende et al., 2003; Liu et al., 2000; Paik et al., 2004). As mural cells were previously suggested to promote vascular stability (Benjamin et al., 1998; von Tell et al., 2006), this mediating role of S1P1 could explain the defects in vascular remodeling observed in its absence. In order to explore this hypothesis, we examined the maturation
state of limb vessels by immunostaining sections from E11.5-14.5 WT mouse embryos with three different markers for smooth muscle cells and pericytes, namely NG2 (Cspg4 – Mouse Genome Informatics), \( \alpha \)-smooth muscle actin and desmin (Fig. 2).

Examination showed that mural cells were absent from the vasculature at E11.5 (Fig. 2B-B\(^{/H11033}\)) and were observed only between E13.5 and E14.5 (Fig. 2E-F\(^{/H11033}\)).

Our finding that during the initial stages of limb development the vasculature consists of an EC network that has not undergone maturation, combined with the vascular aberrations observed in \( \text{S1P1}^{-/-} \) limbs as early as E9.5, strongly suggest a new, mural cell-independent role for \( \text{S1P1} \) in vascular remodeling.

**S1P1 regulates vascular remodeling EC-autonomously**

Our finding that \( \text{S1P1} \) is necessary for vascular remodeling independently of mural cells raised the question of whether its activity is autonomously required by ECs. To address this question, we first analyzed \( \text{S1P1} \)-expressing cells in E11.5 limbs by using mice with a \( \text{lacZ} \) cassette in the \( \text{S1P1} \) locus (Liu et al., 2000). Cross sections of \( \text{S1P1}^{+/+}\text{lacZ} \) forelimbs were immunostained with antibodies for \( \beta \)-galactosidase and CD31, as markers for \( \text{S1P1} \) and ECs, respectively. As seen in Fig. 3A-C, \( \text{S1P1} \) expression was restricted to ECs in the developing limb. We next deleted \( \text{S1P1} \) specifically in ECs by using the VE-cadherin-Cre mouse (\( \text{S1P1}^{loxP/loxP} \text{-VEcad-Cre} \)). Cross and longitudinal sections of E13.5 control and conditional knockout (cKO) forelimbs were stained with H&E (Fig. 3D-G\(^/3\)) or immunostained with anti-CD31 (Fig. 3H-L\(^/3\)). Notably, \( \text{EC-specific S1P1 cKO mice exhibited similar vascular phenotypes to those observed in limbs of S1P1-null embryos, namely vessels with increased lumen diameter. Together, these results imply that S1P1 regulates vascular remodeling in an EC-autonomous manner.**

**Regulatory role of S1P1 in vascular remodeling is evolutionarily conserved**

Our finding of a new, mural cell-independent role for \( \text{S1P1} \) in vascular development prompted us to study its involvement in this process in zebrafish embryos, in which the vasculature consists of an EC network until 72 hpf (Santoro et al., 2009). Because the expression profile of \( \text{s1p1} \) (\( \text{s1pr1} \) – Zebrafish Information Network) has not been previously characterized in zebrafish, we first performed in situ hybridization (ISH) for \( \text{s1p1} \) in developing embryos. As can be seen in Fig. 4, ISH results demonstrated a dynamic expression pattern during 24-52 hpf, which was similar to the pattern observed in mouse embryos (Liu et al., 2000; Meng and Lee, 2009). Notably, \( \text{s1p1} \) expression was prominent in the zebrafish neural tube and vasculature.

Next, to study the role of \( \text{s1p1} \) in vascular development, we knocked down \( \text{s1p1} \) by translational blocking antisense morpholino (\( \text{s1p1} \) MO) in \( \text{Tg(fli:egfp)}^{y1} \) embryos (Isogai et al., 2003). The morphant embryos showed several developmental defects at 3 dpf. These included hindbrain malformations, pericardial edema and heart defects (Fig. 5A,B). These malformations were similar to the defects observed in \( \text{S1P1}^{-/-} \) mice (Allende and Proia, 2002). To demonstrate the specificity of the knockdown effect, we rescued the phenotype by injection of \( \text{s1p1} \) RNA to \( \text{s1p1} \) MO embryos (supplementary material Fig. S1).

Examination of the vasculature of \( \text{s1p1} \) MO embryos revealed several vascular defects. In zebrafish, the onset of intersegmental vessel (ISV) formation is at 24 hpf, as ECs from the dorsal aorta...
in s1p1 MO embryos these vessels remained below the midline (Fig. 5E). Eventually, the migration and formation of these vessels was accomplished (supplementary material Fig. S2).

These results further support the notion that S1P1 regulates vascular remodeling by affecting EC behavior prior to maturation.

S1P1 negatively regulates sprouting angiogenesis

The abnormal vessel size observed in mice could be the consequence of excessive sprouting. To study the possible role of S1p1 in sprouting angiogenesis, we used the mutant zebrafish model. We focused on the ISVs and on the caudal vein plexus (CVP) (Fig. 6A), which form by this process in zebrafish (Choi et al., 2011; Zhong, 2005). In control embryos, the ISV sprouted from the dorsal aorta at 24 hpf by extending numerous long filopodia (Fig. 6B). These sprouts stretched between each pair of somites and anastomosed to give rise to the dorsal longitudinal anastomotic vessel (DLAV). At 48 hpf, when ISV formation was completed, the filopodia that had been instrumental for this process stopped forming. Examination of the ISV sprouting process in s1p1 MO embryos (Fig. 6B) revealed comparable filopodial numbers with control embryos at 24 hpf. However, at 48 hpf, the morphant’s ISV continued to extend numerous filopodia in all directions.

Unlike the ISV, the CVP forms as a very dense capillary network (Choi et al., 2011; Wiley et al., 2011). To maintain this structure and prevent fusion of forming vessels, inhibition of excessive filopodia formation is crucial. Analysis of the axial
vessel at the caudal part of the s1p1 MO embryo at 48 hpf showed failure of the CVP to remodel into a capillary network (Fig. 6C). The CVP appeared to be fused in s1p1 MO embryos, whereas WT siblings displayed a clear network with spaces between capillaries (Fig. 6C/H11032).

In order to analyze these differences in more detail, we followed the formation of the CVP in vivo by long term, time-lapse imaging. WT embryos exhibited ECs that extended filopodia, which eventually met and formed capillary loops. Once these loops formed, the number of filopodia decreased, preventing the capillaries from further fusion and thereby preserving their structure and size (Fig. 7A; supplementary material Movie 1). In contrast to this tightly regulated process, ECs in s1p1 MO embryos continued to sprout and to extend numerous filopodia even when capillary loops were fully established. This resulted in the formation of supernumerary connections between adjacent sprouts, with a concomitant reduction in the space between capillary loops. Ultimately, the hypersprouting capillaries fused to form one thick vessel instead of a well-defined plexus (Fig. 7A; supplementary material Movie 2).

Next, we analyzed EC sprouting in mouse forelimbs at E9.5. WT limbs exhibited low numbers of filopodial extensions (Fig. 7Ba,Bb). By comparison, the S1P1−/− limb vasculature exhibited an increase in EC filopodia (Fig. 7Bc,Bd). In addition, whereas most of the filopodia in the WT made no connection with adjacent vessels, the filopodia in the S1P1−/− limb extended from one vessel to another, establishing ectopic interconnections (Fig. 7Bd). Those interconnections might have caused fusion of adjacent capillaries, which would explain the reduction in vessel number observed at E11.5 (Fig. 1F).

Together, these results suggest a crucial role for S1P1 in the termination of sprouting angiogenesis during vascular remodeling.

S1P1 inhibits both Vegfa-dependent and -independent sprouting angiogenesis

Vegfa is a key regulator of sprouting angiogenesis that was previously shown to control vascular development in the limb (Eshkar-Oren et al., 2009). One way by which S1P1 can inhibit sprouting is by negatively regulating Vegfa activity. This would imply that elevation of Vegfa signaling should lead to a similar vascular phenotype as observed in the S1P1−/− limb. To overexpress Vegfa specifically in the forming limb, we used a triple transgenic mouse system, in which the expression of the reverse tetracycline
transactivator (rtTA) and the tetracycline-responsive element (tetO-Vegfa) was induced by Prxl-Cre (Belteki et al., 2005; Gossen et al., 1995). As can be seen in Fig. 8A,B, the vasculature in Vegfa-overexpressing limbs consisted of vessels with increased lumen diameter relative to the WT, as was observed for S1P1−/− limbs.

Previous studies also reported increased Vegfa expression in E11.5 S1P1−/− limbs (Chae et al., 2004). To address the possibility that the vascular phenotype in S1P1−/− limbs was a result of elevated Vegfa expression, we compared the progression of the vascular phenotype with the expression profile of Vegfa. As mentioned, abnormal vasculature was evident in S1P1−/− limbs as early as E9.5, at the onset of limb formation (Fig. 1A–B’). However, quantification of Vegfa expression by qRT-PCR at that stage revealed comparable levels in S1P1−/− and WT limbs (Fig. 8C). This suggests that initially, vascular defects in S1P1−/− mutant are not a result of an increase in Vegfa levels. Yet, at a later stage (E11.5), Vegfa was upregulated in S1P1−/− limbs relative to the control (Fig. 8D), suggesting that Vegfa might contribute to the progression and severity of the phenotype.

Although we ruled out the possibility that Vegfa upregulation was the mechanism underlying hypersprouting in S1P1−/− limbs, S1P1 could still regulate sprouting angiogenesis by antagonizing Vegfa activity. To test this supposition, we either blocked or attenuated Vegfa expression in limbs of S1P1−/− embryos and examined the effect on the vascular phenotype. To block Vegfa expression in limb mesenchyme of S1P1−/− embryos, we used Prxl-Cre as a deleter (VegafloxP/loxPPrxl-Cre, S1P1−/−) (Logan et al., 2002). To reduce its levels, we deleted the expression of Hif1a (Hif1aloxP/loxPrxl-Cre), a well-documented transcriptional regulator of Vegfa (Forsythe et al., 1996; Liu et al., 1995). Previously, we showed that conditional knockout of Hif1a in mouse limb mesenchyme, using Prxl-Cre, results in a 30% reduction in Vegfa expression (Amarilio et al., 2007). Whole-mount forelimbs from WT, S1P1−/−, S1P1-Vegfa double knockout (dKO) and S1P1-Hif1a dKO embryos at E11.5 were stained with CD31 (Fig. 8E–R). As shown before, S1P1−/− forelimbs exhibited severe defects in limb vasculature that included enlarged vessels and an increase in vessel sprouting (Fig. 8G,H). In contrast to the S1P1−/− phenotype, Vegfa KO in limb mesenchyme led to a dramatic decrease in blood vessel density in the forelimb (Fig. 8I,J).

Interestingly, the phenotype of the dKO mice was similar to the phenotype of the Vegfa KO mice, with no trace of the hypersprouting observed in the S1P1−/− mice (Fig. 6K,L). Limbs of S1P1-Hif1a dKO embryos exhibited a partial rescue of the S1P1−/− phenotype, as there was a reduction in vessel density and diameter and only a few large vessels (Fig. 6Q,R).

Together, these results strongly imply that the effect of S1P1 on vascular development is Vegfa-dependent, suggesting that S1P1 negatively regulates induction of sprouting angiogenesis by Vegfa.

These findings in mice prompted us to investigate whether this genetic interaction between s1p1 and vegfa is conserved in zebrafish. In a previous study, vegfa overexpression in zebrafish resulted in two subintestinal vessel (SIV) phenotypes, namely dilated vessels and ectopic sprouting (Serbedzija et al., 1999). These phenotypes are similar to those observed in s1p1 MO embryos (supplementary material Fig. S2). To examine the interaction between s1p1 and vegfa, SU5416, an inhibitor of the Vegfa receptor Flk1 (Kdrl – Zebrafish Information Network), was administered to s1p1 MO and WT embryos. As shown previously (Serbedzija et al., 1999), blockade of the Vegfa pathway using SU5416 inhibits SIV development. As expected, s1p1 MO embryos treated with SU5416 showed a similar phenotype (Fig. 9), demonstrating the dependence of S1P1 activity on Vegfa.

Interestingly, a recent study showed that CVP development is independent of vegfa (Wiley et al., 2011). This finding allowed to explore the possibility that S1P1 can act independently of Vegfa. Indeed, blocking the Vegfa pathway using SU5416 in WT embryos had no effect on CVP development (Fig. 9); conversely, the
SU5416 inhibitor did not rescue the CVP phenotype in $s_{1p1}$ MO embryos. These intriguing results led us to conclude that in the CVP, $S1p\,1$ regulates sprouting angiogenesis independently of Vegfa.

**DISCUSSION**

During organogenesis, the vasculature accommodates growing demands for oxygen and nutrients by remodeling into a complex network composed of different-sized vessels (Coffin and Poole, 1988; Drake et al., 1998; Flamme et al., 1995; Folkman, 2003; Risau and Flamme, 1995). Sprouting angiogenesis plays a central role in the remodeling process by forming new vascular loops, which expand the vascular network. Over the years, several signaling pathways have been identified to induce angiogenic sprouting (Adams and Alitalo, 2007; Eilken and Adams, 2010; Jakobsson et al., 2010). By contrast, we know little about the equally important mechanism that terminates this process to allow vascular stability. Our finding that $S1P\,1$ negatively regulates the sprouting process, in both mouse and zebrafish embryos, provides an important molecular component in this mechanism. This finding provides an explanation for the cessation of filopodia extension once two tip cells have anastomosed and formed a new vascular loop. This is necessary to prevent excessive connections between tip cells, which would ultimately disrupt the organization of the vascular system. By restricting the sprouting process, $S1P\,1$ acts as a control mechanism that stabilizes the newly formed network and prevents further sprouting.

Another mechanism that may promote vascular stabilization by restricting sprouting is the maturation process (Benjamin et al., 1998; von Tell et al., 2006). Previous studies have attributed the effect of $S1P\,1$ on vascular development to its role in regulating interactions between ECs and mural cells during maturation (Allende and Proia, 2002; Liu et al., 2000). However, this mechanism cannot operate at developmental stages when vessels lack mural cell coverage. Our analysis shows that the limb bud vasculature is not invested by mural cells for several days of development. Delay in vascular maturation was also reported in the developing wing of chick embryos, in which the vasculature was not coated by VSMCs (Vargesson and Laufer, 2001). Our finding of abnormal vasculature prior to maturation in limbs in which $S1P\,1$ expression was blocked in ECs strongly suggests that $S1P\,1$ has a previously undescribed, mural cell-independent role in vascular development.

Previous studies identify $S1P$ as a pro-angiogenic factor (Schmid et al., 2007; Yonesu et al., 2009). By contrast, we show that $S1P\,1$ acts to block sprouting and restrict angiogenesis. There can be several explanations for this difference. The most likely is that the pro-angiogenic effect was demonstrated in vitro, whereas our work was carried out in vivo. With regard to the mode of action by which $S1P\,1$ negatively regulates sprouting, our study provides several pieces of evidence in support of the hypothesis that $S1P\,1$
restricts this process by antagonizing the activity of an angiogenic factor such as Vegfa. We show that increased expression of Vegfa in the limb led to vascular abnormalities similar to those observed upon S1P1 loss of function, whereas reduction in Vegfa expression rescued the S1P1 phenotype. That Vegfa levels were initially comparable between WT and S1P1−/− mouse embryos negates the possibility that S1P1 regulates Vegfa expression, leaving the point of interaction between these two pathways an open question. Nevertheless, S1P1 signaling may also be involved in vascular remodeling independently of Vegfa signaling. Our finding that in s1p1 MO zebrafish embryos CVP development, which is not dependent on vegfa (Wiley et al., 2011), was affected clearly supports this possibility. Such a Vegfa-independent mechanism might involve regulation of cytoskeletal rearrangement and filopodia formation. Previous studies demonstrate the involvement of S1P1 in the assembly of cortical actin by inducing redistribution of molecules such as cortactin and Arp2/3 (Lee et al., 2006). Disruption of this distribution might cause aberrant organization of actin in the cytoskeleton and disproportional filopodia formation. Further analysis is required to fully decipher the inhibitory effect of S1P1 on angiogenic sprouting.

Another interesting question regards the mechanism that allows the formation of vessels with different sizes. Indications for such a mechanism are the two main phenotypes we observed both in mice and in zebrafish upon S1P1 loss-of-function, namely hypersprouting and increased vessel diameter. We show at early developmental stages (E9.5 in mice and 24 hpf in zebrafish) that the S1P1 loss-of-function phenotype commences with hypersprouting. As development proceeds, excessive sprouting induces fusion of sprouts that form one overly thick vessel instead of a well-defined plexus. The outcome of this process is reduced vessel number combined with increased vessel diameter. In light of this finding, it is tempting to speculate that sprouting may also serve as a mechanism that controls vessel diameter. This mechanism operates by maintaining the activity of tip cells after a loop has formed, leading eventually to the merging of newly formed loops into one larger vessel. Such a model was previously suggested by Drake and Little (Drake and Little, 1999).

In mice, S1P1 is expressed in endothelial cells and S1P is found in the blood serum (Hla et al., 2008; Yatomi et al., 2000). Given that S1P is secreted into the blood, a physiological feedback loop mechanism could explain the involvement of S1P1 in sprouting angiogenesis (Fig. 10). Prior to the formation of a capillary loop, tip cells are poorly exposed to the blood stream and, as a
consequence, to S1P. Once a functional capillary loop is formed, these cells come into contact with the blood stream that carries S1P. This enables S1P to interact with its receptor to inhibit the Vegfa pathway and thereby to terminate the sprouting process. In zebrafish, blood circulation starts at ~25 hpf, which coincides with the emergence of the vascular phenotype in the morphant. This concurrence supports the notion of a physiological feedback loop.

This study identifies S1P as a major component in a mechanism that negatively regulates angiogenic sprouting. This mechanism acts EC-autonomously and independently of mural cell. Our findings provide a new module in the regulation of embryonic angiogenesis, which terminates sprouting to prevent excessive loop formation and stabilize the vascular network.

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