Sphingosine-1-phosphate (S1P) stimulates signaling pathways via G-protein–coupled receptors and triggers diverse cellular processes, including growth, survival, and migration. In S1P1 receptor-deficient embryos, blood vessels were incompletely covered by vascular smooth muscle cells (VSMCs), indicating the S1P1 receptor regulates vascular maturation. Because S1P1 receptor expression is not restricted to a particular cell type, it was not known whether the S1P1 receptor controlled VSMC coverage of vessels in a cell-autonomous fashion by functioning directly in VSMCs or indirectly through its activity in endothelial cells (ECs). By using the Cre/loxP system, we disrupted the S1P1 gene solely in ECs. The phenotype of the conditional mutant embryos mimicked the one obtained in the embryos globally deficient in S1P1. Thus, vessel coverage by VSMCs is directed by the activity of the S1P1 receptor in ECs.

**Introduction**

Embryonic blood vessel development occurs via vasculogenesis, angiogenesis, and maturation.1-3 In vasculogenesis, endothelial cells (ECs) differentiate de novo and form the primary vascular plexus. In angiogenesis, the pre-existing network is remodeled by splitting and sprouting, producing a complex vascular tree of capillaries and vessels. In angiogenesis, cell-to-cell communication and interactions between ECs and VSMCs.4,5 To address this issue we have established mice with the S1P1 gene deleted specifically in ECs. Our results demonstrate that vessel coverage by VSMCs is directed by the activity of the S1P1 receptor in ECs.

**Study design**

**Generation of S1P1<sup>loxP</sup> mice**

The structure of the targeting vector is shown in Figure 1. Gene targeting in TC1 ES cells and generation of chimeric and heterozygous mice were done as described.15 Mice were genotyped by PCR analyses using DNA from yolk sacs or tail biopsies.

**Generation and analysis of S1P1<sup>conditional mutant mice**

To determine Cre-expression specificity. To detect the LacZ gene, primers LacZ-F (5’GGATCCCGCTGCTACCCG) and LacZ-R (5’GGATCCGCTGCTACCCG) were used. Embryos were obtained from timed-pregnant females with the morning of vaginal plug considered E0.5. Immunohistochemistry and LacZ expression analysis were done as described previously.15 The S1P1 antibody was obtained from Suzanne Mandala (Mercer, Whitehouse Station, NJ).

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Results and discussion

Generation and characterization of mice with EC-specific S1P\(_1\) deletion

The S1P\(_1\) gene consists of 2 exons and 1 intron; the second exon contains the entire coding region\(^{21}\) (Figure 1A). To produce an S1P\(_1\) allele that could be conditionally disrupted, a gene-targeting vector was constructed that contained loxP sequences flanking exon 2. One loxP site was placed within the intron, and 2 others, associated with the neomycin selection cassette, were placed 3' to the end of the gene (Figure 1A). This targeting vector was used to introduce these changes into the endogenous S1P\(_1\) gene in embryonic stem cells by homologous recombination. A clone that contained the S1P\(_1\)\(^{loxP}\) allele was injected into blastocysts to establish chimeric and, subsequently, heterozygous mice. Interbreeding the heterozygous mice yielded viable, homozygous S1P\(_1\)\(^{loxP/loxP}\) mice at the expected Mendelian frequency. The homozygous S1P\(_1\)\(^{loxP/loxP}\) mice appeared to be without abnormalities, indicating that the changes introduced into the S1P\(_1\) gene did not disrupt the locus. A null S1P\(_1\) allele (S1P\(_1\)\(^{KO}\)) produced embryonic lethality when in homozygous form.\(^{15}\)

To delete the S1P\(_1\) gene specifically in ECs we used a previously characterized transgenic mouse line that expresses the Cre recombinase under the control of the promoter and enhancer regions of the characterized transgenic mouse line that expresses the Cre recombinase (bottom; S1P\(_1\)\(^{loxP}\)). For detecting the wild-type (S1P\(_1\)\(^{WT}\)), knock-out (S1P\(_1\)\(^{KO}\)), and conditional alleles (S1P\(_1\)\(^{P2}\)) by polymerase chain reaction (PCR), the following primers were used: P1, 5'-GAGCGGAGGAAGTTAGGCCTCCTAAGAGATTGCAGCAA-3', P2, 5'-CTCTCAAGAGATTGCAGCAA. P1 and P2 amplify an approximately 250-bp fragment for the S1P\(_1\)\(^{P2}\) allele. For detecting the wild-type (S1P\(_1\)\(^{WT}\)), knock-out (S1P\(_1\)\(^{KO}\)), and conditional alleles (S1P\(_1\)\(^{P2}\)) by polymerase chain reaction (PCR), the following primers were used: P1, 5'-GAGCGGAGGAAGTTAGGCCTCCTAAGAGATTGCAGCAA-3', P2, 5'-CTCTCAAGAGATTGCAGCAA. P1 and P2 amplify an approximately 250-bp fragment for the S1P\(_1\)\(^{P2}\) allele and a 200-bp fragment for the S1P\(_1\)\(^{WT}\) and S1P\(_1\)\(^{P2}\) alleles. To detect the S1P\(_1\)\(^{loxP}\) allele, P1 and P2 (5'-GATCCTAAAGGATGCTCTGAGATGGCAACAA') were used. P1 and P2 amplify a 200-bp fragment. When primers P1, P2, and P3 were used in the same PCR reaction, the PCR products were digested with SacI prior to the electrophoresis, which converted the S1P\(_1\)\(^{loxP}\) fragment to 180 bp.

Figure 1. Generation and characterization of EC-specific S1P\(_1\), knock-out mice. (A) Targeting strategy to introduce the loxP sites into S1P\(_1\) gene. The S1P\(_1\) gene consists of 2 exons and 1 intron; the second exon (E2) contains the entire coding region. A 5.2-kilobase (kb) BamHI-SacI fragment, including exons 1 and 2 of the S1P\(_1\) gene, was subcloned into the XhoI site of the plloxPneo vector\(^{14}\) upstream of the neo cassette to place one loxP site (\(\gamma\)) within the intron. A 3.8-kb SacI-BamHI fragment containing the 3' untranslated region was subcloned downstream of the neo cassette which has associated 2 other loxP sites. BglII restriction site was inactivated during construction of the targeting vector. (B) The loxed S1P\(_1\) gene (top; S1P\(_1\)\(^{loxP}\)) and the structure of the gene after Cre-mediated recombination (bottom; S1P\(_1\)\(^{loxP}\)). For detecting the wild-type (S1P\(_1\)\(^{WT}\)), knock-out (S1P\(_1\)\(^{KO}\)), and conditional alleles (S1P\(_1\)\(^{P2}\)) by polymerase chain reaction (PCR), the following primers were used: P1, 5'-GAGCGGAGGAAGTTAGGCCTCCTAAGAGATTGCAGCAA-3', P2, 5'-CTCTCAAGAGATTGCAGCAA. P1 and P2 amplify an approximately 250-bp fragment for the S1P\(_1\)\(^{P2}\) allele and a 200-bp fragment for the S1P\(_1\)\(^{WT}\) and S1P\(_1\)\(^{P2}\) alleles. To detect the S1P\(_1\)\(^{loxP}\) allele, P1 and P2 (5'-GATCCTAAAGGATGCTCTGAGATGGCAACAA') were used. P1 and P2 amplify a 200-bp fragment. When primers P1, P2, and P3 were used in the same PCR reaction, the PCR products were digested with SacI prior to the electrophoresis, which converted the S1P\(_1\)\(^{loxP}\) fragment to 180 bp.

Figure 2. Characterization of mice with EC-specific S1P\(_1\), deletion. (A) Section of X-gal stained Tie2-Cre;ROSA26 E10.5 brain vessel showing lacZ staining specifically in ECs. (B) Tissue specificity of Tie2-Cre–mediated recombination of S1P\(_1\)\(^{WT}\) using E12.5 embryo tissue DNA samples and PCR primers as shown in Figure 1B. In embryos carrying the Tie2-Cre gene, substantially more recombination of the S1P\(_1\)\(^{WT}\) allele to produce the S1P\(_1\)\(^{loxP}\) allele was found in yolk sac and aorta as compared with embryonic neural tissue. No evidence of recombination was found in embryos without the Tie2-Cre gene. S1P\(_1\)\(^{WT}\), wild-type S1P\(_1\) allele; S1P\(_1\)\(^{P2}\), null S1P\(_1\) allele.\(^{15}\) (C) S1P\(_1\) expression in ECs in control and S1P\(_1\)\(^{loxP}\) Tie2-Cre embryonic brains at E12.5. Sections were stained with both isoelectric B4-fluorescein isothiocyanate (FITC; Vector Laboratories, Burlingame, CA), a specific marker for mouse ECs, and the anti-S1P\(_1\) antibody. Note that in control sections both isolectin B4–FITC and the anti-S1P\(_1\) antibody stain essentially all ECs that are stained with isolectin B4 also stain with the antibody to S1P\(_1\). In the section from the S1P\(_1\)\(^{loxP}\) Tie2-Cre conditional mutant, only a few ECs stain strongly with the S1P\(_1\) antibody. Midbrain neuroepithelia from control and S1P\(_1\)–conditional E12.5 embryos shown as positive control for S1P\(_1\) expression; both show about equal staining. (D) Phenotype of S1P\(_1\)–conditional mutant embryos. At E13.5, the yolk sacs of S1P\(_1\)\(^{loxP}\) Tie2-Cre conditional mutant embryos show normal vasculature but less blood compared with control embryos (top, arrowhead). The mutant embryos display an enlarged pericardial cavity (PCC, right), undeveloped limbs, and intraembryonic hemorrhages (bottom; FL, front limb; HL, hind limb). (E) Vascular maturation in S1P\(_1\)–conditional mutant embryos. Transverse sections of aorta (top) from an S1P\(_1\)\(^{loxP}\) Tie2-Cre embryo and a control embryo stained with anti-SM\(_\alpha\)-actin (DAKO, Carpinteria, CA). Note VSMCs are clustered to the ventral side of the aorta and fail to surround the vessel completely in the mutant. Also, ECs on the dorsal side of the aorta appear discontinuous (arrows). Cranial arteries (bottom) from S1P\(_1\)\(^{loxP}\) Tie2-Cre and control embryos stained with anti-SM\(_\alpha\)-actin. Note that VSMCs cluster on one side of the vessel of the S1P\(_1\)–conditional mutant embryo. Scale bars \(= 50 \mu m\).
EC-specific deletion of S1P1 impairs vascular maturation

When S1P1<sup>loxP/loxP</sup> mice were bred with S1P1<sup>WTKO</sup> mice carrying the Tie2-Cre transgene, no S1P1<sup>loxP/loxP;</sup>Tie2-Cre mice were found among 74 offspring, suggesting embryonic lethality. To determine the time of lethality in utero, embryos were analyzed at different days after coitum. At E12.5, the S1P1-conditional mutant embryos (S1P1<sup>loxP/Cre</sup>)(n = 34) appeared phenotypically similar to the global S1P1 knockout embryos (S1P1<sup>Ko/Ko</sup>). The S1P1-conditional mutant embryos showed an enlarged pericardial cavity, undeveloped and rounded limbs, and spots of bleeding along the body. Yolk sacs from S1P1-conditional mutant embryos were edematous and with less blood in vessels than in control embryos. At E13.5, the appearance of the S1P1-conditional embryos worsened with massive bleeding (n = 9) (Figure 2D). All S1P1-conditional mutant embryos examined were without a heartbeat at E14.5 (n = 13). Thus, these conditional mutant embryos died by E14.5 similar to the S1P1<sup>Ko/Ko</sup> embryos, which died between E12.5 and E14.5.

Maturation of vessels in S1P1-conditional mutant embryos was studied using smooth muscle (SM) α-actin as a marker for VSMCs<sup>15</sup> (Figure 2E). In transverse sections of aorta from control embryos, VSMCs were found to completely surround the vessels. In contrast, the aorta in S1P1-conditional knockout embryos were incompletely covered by VSMCs. Cells positive for SM-α-actin were found clustered on the ventral side of the vessel. The endothelial cell layer on the uncovered dorsal side of aorta appeared discontinuous, possibly because of the lack of VSMC support<sup>23</sup>. Similarly, vessels in the brains of S1P1-conditional mutant embryos were incompletely surrounded by VSMCs. These vessel abnormalities in the S1P1-conditional mutants resembled those described for the global S1P1 knockout embryos.<sup>15</sup>

Because the S1P1 receptor is expressed in both ECs and VSMCs, as well as in other cell types, it had been uncertain whether S1P1 functioned in ECs during vascular maturation. The results shown here indicate that vessel coverage by VSMCs is indeed directed by the activity of the S1P1 receptor in ECs. In addition, we have found that S1P1<sup>loxP/loxP</sup> mice carrying a Cre transgene under the control of the smooth muscle myosin heavy chain (SM-MHC) promoter<sup>24</sup> were viable as adults with an S1P1 gene deletion in smooth muscle. These smooth muscle–specific S1P1 KO mice did not show any evidence of hemorrhage or vascular maturation defects (M.L.A., G.K. Owens, R.L.P., unpublished results, 2003). These data suggest that S1P1 receptor expression in VSMCs may not be essential for the process of vessel coverage during development. However, because the expression of Cre recombinase driven by the SM-MHC promoter begins at about the time that VSMCs start to migrate around the vessels, we cannot be certain that the S1P1 deletion was timely enough to affect significantly the vascular maturation process. In any case, the evidence indicates that the expression of S1P1 receptor within ECs has an essential function to regulate the coverage of vessels by VSMCs. At this time an additional function of S1P1 within VSMCs during this process cannot be totally ruled out.

The promotion of functional EC-VSMC interactions by the S1P1 receptor may be incorporated into strategies for therapeutic angiogenesis: the formation of new blood vessels into ischemic tissues.<sup>25,26</sup> Endothelial growth factors such as vascular endothelial growth factor (VEGF) promote nascent vessel formation; however, these vessels are leaky and will regress unless stabilized by VSMC interactions.<sup>27</sup> Highly effective angiogenic therapies may ultimately require stimulation of multiple signaling pathways, including the S1P1 receptor to produce mature, stable vessels.

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References

G-protein-coupled receptor S1P₁ acts within endothelial cells to regulate vascular maturation

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