Sphingosine-1-phosphate Signaling Promotes Critical Migratory Events in Vasculogenesis*

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Here we have investigated the role of sphingosine-1-phosphate (SIP) signaling in the process of vasculogenesis in the mouse embryo. At stages preceding the formation of blood vessels (7.5–8 dpc) in the embryo proper, yolk sac, and allantois, the SIP receptor SIP2 is expressed in conjunction with SIP1 and/or SIP3. Additionally, sphingosine kinase-2 (SK2), an enzyme that catalyzes the formation of SIP, is expressed in these tissues throughout periods of vasculogenesis. Using the cultured mouse allantois explant model of blood vessel formation, we found that vasculogenesis was dependent on SIP signaling. We showed that SIP could replace the ability of serum to promote vasculogenesis in cultured allantois explants. Instead of small poorly reticulated clusters of rounded endothelial cells that formed under serum-free conditions, SIP promoted the formation of elongated endothelial cells that arranged into expansive branched networks of capillary-like vessels. These effects could not be reproduced by vascular endothelial growth factor or basic fibroblast growth factor administration. The ability of SIP to promote blood vessel formation was not due to effects on cell survival or on changes in numbers of endothelial cells (Flk1/PECAM), angioblasts (Flk1/PECAM), or undifferentiated mesodermal cells (Flk1/PECAM). The SIP effect on blood vessel formation was attributed to it promoting migratory activities of angioblasts and early endothelial cells required for the expansion of vascular networks. Together, our findings suggest that migratory events critical to the de novo formation of blood vessels are under the influence of SIP, possibly synthesized via the action of SK2, with signaling mediated by SIP receptors that include SIP1, SIP2, and SIP3.

Sphingosine-1-phosphate (SIP) is a bioactive sphingolipid generated by the action of sphingosine kinase on sphingosine.

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"...in accordance with 18 U.S.C. Section 1734 solely to indicate this fact."
for S1P signaling in angiogenic and maturation phases of neo-
vascularization, little is known as to whether S1P signaling is
important for vasculogenesis, the process of de novo formation
of blood vessels from mesodermal progenitor cells. Herein, we
establish that S1P receptors and sphingosine kinase are ex-
pressed in prevascularized embryonic tissues and throughout
the stages of vasculogenesis, thus supporting a role for S1P
signaling in de novo blood vessel formation. Furthermore, us-
ing the murine allantois explant culture model of vasculogenesis
(22) we demonstrated a requirement for S1P signaling in
vasculogenesis.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Other Reagents—** Rat anti-mouse-PECAM (CD31) and -Flk1 (VEGFR-2) were purchased from BD Biosciences. Donkey anti-rat IgG conjugated to either fluorescein isothiocyanate or indi-
docyanine were provided by the MUSC Lipidomics Core (Department of Biochemistry, MUSC, Charleston, SC). Lysophosphatidic acid (LPA, chain 18:1; oleoyl acyl group) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL).

**Reverse Transcription and PCR of RNA Isolated from Mouse Embryos, Yolk Sacs, and Allantoides—** Embryos (7.5–9.5 days post coitum (dpc)) were removed from timed pregnant CD1 mice (Harlan, Indianapolis, IN) and placed into 4 °C Dulbecco’s phosphate-buffered saline (dPBS, Cellgro, Herndon, VA). Yolk sacs and allantoides were dissected away from embryos, and each was placed into RNA Later (Ambion, Inc., Austin, TX). Total RNA was extracted using RNA Stat-60 (Tel-Test, Inc. College Station, TX) from 60 7.8–8.0 dpc embryos, 15 8–8.4 dpc embryos, 30 8.5–9.0 dpc embryos, 30 7.8–8.0 dpc yolk sacs, 15 8–8.4 dpc yolk sacs, 30 8.5–9.0 dpc yolk sacs, 30 9.5–10.0 dpc allantoides, 60 7.8–8.0 dpc allantoides, 15 8–8.4 dpc allantoides, 30 8.5–9.0 dpc allantoides, and 30 9.5–10.0 dpc allantoides (all of embryos were free of yolk sacs) from 110 7.5–8.0 dpc allantoides, 50 8–8.4 dpc allantoides, 130 8.5–9.0 dpc allantoides, 30 9.5–10.0 allantoides, 60 7.8–8.0 dpc yolk sacs, 15 8–8.4 dpc yolk sacs, 30 8.5–9.0 dpc yolk sacs, 30 9.5–10.0 dpc allantoides, 60 7.8–8.0 dpc allantoides, 15 8–8.4 dpc allantoides, 30 8.5–9.0 dpc allantoides, and 30 9.5–10.0 dpc allantoides (all of embryos were free of extraembryonic membranes). Reverse transcription of total mRNA using random hexamer oligodeoxynucleotide primers and cDNA synthesis was performed using a SuperScript First-Strand synthesis System from Life Technologies, Inc. (Gaithersburg, MD). PCR primers (Table I) were used to amplify the cDNA sequence. Annealing temps and cycles of amplification for each primer pair are indicated in Table 1. PCR was per-
formed in 0.2 ml of thin wall polypropylene tubes in a MJ Research Dyad thermal cycler (Waltham, MA). PCR products were separated on 1% Agarose 1000 (Invitrogen) gels with 0.5× Tris borate-EDTA buffer in the presence of 5 μg/ml ethidium bromide.

**Flow Cytometry—** Allantoides from 8.5 dpc embryos were cultured for 18 h in Dulbecco’s modified Eagle’s medium or in this medium containing either 10% serum or 1 μg/ml S1P. Cultured allantois explants were dissociated in 0.5 ml of 1× trypsin-EbDTA at 37 °C for 5 min. Trypsin was inactivated by the addition of 1 ml of serum-containing medium. Cells were pelleted by centrifugation at 80–100 × g for 10 min and then resuspended in 0.5 ml of serum-containing medium and incubated at 37 °C for 40 min to allow cells to recover. The cells were pelleted by centrifugation at 80–100 × g and resuspended in 300 μl of dPBS containing 1% bovine serum albumin, 0.01% sodium azide, and 10 ng/ml DNase (FACS buffer) and kept on ice for the remainder of the experiment. The cell suspension was filtered through a cell strainer (BD Biosciences), and cells were counted on a hemocytometer. Aliquots (100 μl) of the cells at 1×10^6 cells/μl were incubated with anti-Flk1 IgG conjugated to phycoerythrin and anti-CD31/PECAM IgG conjugated to fluorochrome-conjugated anti-IgGs (Jackson ImmunoResearch Laborato-
ries, Inc.) were added at 10 μg/ml in 3% bovine serum albumin and dPBS. Fluorochrome-conjugated anti-IgGs (Jackson ImmunoResearch Laborato-
ries, Inc.) were added at 10 μg/ml and incubated for 1.5 h. In some explants, the cells were counterstained with either 0.5 ng/ml Hoescht 33342 dye or Propidium Iodide (Eugene, OR) or 5 μg/ml 4′,6-diamidino-2-phenylindole (Biotest, Hayward, CA) was used to produce montages of images of allantoic explants captured on the Leica DMR microscope and to measure the diameters of mesothelial layers and vascular networks.

**Immunostaining of Cultured Allantois Explants—** Explants were fixed in 2% paraformaldehyde by adding 0.8 ml of 3% paraformalde-
hyde, dPBS, 0.01% sodium azide (dPBSA) (Cellgro, Herndon, VA) to culture medium and incubating for 20 min at room temperature. The fixed explants were washed in dPBSA and then permeabilized in dPBSA/0.5% Triton X-100 for 15 min at room temperature. Permeabi-
ized explants were treated with blocking solution (3% bovine serum albumin, dPBSA) and then incubated for 1.5 h with antibodies to PECAM or Flk1, diluted to 20 μg/ml in 3% bovine serum albumin and dPBSA. Fluorochrome-conjugated anti-IgGs (Jackson ImmunoResearch Laborato-
ries, Inc.) were added at 10 μg/ml and incubated for 1.5 h. In some explants, the cells were counterstained with either 0.5 ng/ml Hoescht 33342 dye or Propidium Iodide (Eugene, OR) or 5 μg/ml 4′,6-diamidino-2-phenylindole (Biotest, Hayward, CA) was used to produce montages of images of allantoic explants captured on the Leica DMR microscope and to measure the diameters of mesothelial layers and vascular networks.

**Confocal Microscopy and Image Processing—** Immunolabeled allan-
toic explants were examined using either a Bio-Rad MRC-1024 laser-scanning confocal microscope or a Leica DMR light/eclipse fluorescence microscope equipped with a SPOT RT color camera (Vashaw Scien-
tific, Norcross, GA). For laser-scanning confocal microscopy, optical sections of the cultured explants were collected along the Z axis and collapsed into a single focal plane using the manufacturer’s software to produce a single virtual image. Adobe Photoshop 7 (Adobe Systems, San Jose, CA) was used to compile montages of images of allantoic explants captured on the Leica DMR microscope and to measure the diameters of mesothelial layers and vascular networks.

| GenBank™ Accession no. | Forward primer 5′-3′ | Reverse primer 3′-5′ | cDNA segment amplified | Annealing temp °C | Cycle no. |
|------------------------|---------------------|----------------------|------------------------|------------------|----------|
| S1P1 (Edg-1) NM_007901 | CACGCCCACCACTCTAAGC | CACGCCCACCACTCTAAGC | 740–1357               | 54               | 35       |
| S1P2 (Edg-5) NM_114581 | CCTGGGCATGCTCACTCTTG | CCTGGGCATGCTCACTCTTG | 670–1659               | 59               | 35       |
| S1P3 (Edg-6) NM_010101 | CGGCCACCACTCTAAGTCA | CGGCCACCACTCTAAGTCA | 637–1351               | 60               | 40       |
| S1P5 (Edg-8) NM_015380 | GCCCCACCACTCTAAGTAAAT | GCCCCACCACTCTAAGTAAAT | 430–870                 | 59               | 40       |
| SK1 NM_114581 | GGGAGATTCTTCTTACAGTCA | GGGAGATTCTTCTTACAGTCA | 661–1151                | 59               | 40       |
| SK2 AK04951 | GGAGGCCTTTGTCCTCTCTCTT | GGAGGCCTTTGTCCTCTCTCTT | 725–1324                | 60               | 35       |
| V-Cadherin XM_038019 | CCGCTGACGCTCCATAGA | CCGCTGACGCTCCATAGA | 1702–1990               | 54               | 40       |
| β-Actin X03672 | CGGGACCTGACAGATAACAA | CGGGACCTGACAGATAACAA | 627–844                 | 57               | 30       |
| GAPDH NM_008084 | CCTGCTGACGCTCTCCTT | CCTGCTGACGCTCTCCTT | 310–1008                | 54               | 26       |
Statistical Analysis—Microsoft Excel (Redmond, WA) was used to perform a one-way ANOVA to identify statistical differences in allantois explant measurements as well as in flow cytometry data. Kaleidagraph (version 3.6.2, Synergy Software, Reading PA) was used to determine the statistical significance of differences using Tukey’s post hoc comparisons. The /H9251 alpha/H9251 was set to 0.05 for all of the statistical analyses. All of the charts were plotted using Kaleidagraph, and the error bars represent confidence intervals at 95%.

RESULTS

S1P1, S1P2, and S1P3 Expression Coincide with the Process of Vasculogenesis—RT-PCR was used to determine the temporal pattern of expression S1P receptors (S1P1–S1P5) during mouse embryonic development. S1P receptor expression was evaluated in the embryo proper and two extraembryonic tissues (the allantois and the yolk sac) at stages during which these tissues were undergoing vasculogenesis. The level of VE-cadherin mRNA expression was used as a measure of blood vessel formation in each tissue. VE-cadherin expression was first detected at 8.5–9.0 dpc in the embryo proper, yolk sac, and allantois (Fig. 1). In the prevascularized allantois (i.e. 7.5–8.0 dpc prior to the expression of VE-cadherin mRNA), S1P1, S1P2, and S1P3 transcripts were expressed (Fig. 1). The expression of these three receptors persisted in the allantois during stages when nascent blood vessels first appeared (8.25 dpc) (22, 23) and through periods in which angiogenesis and blood vessel maturation occurred (8.5–10 dpc). S1P1 and S1P2 mRNA expression showed a marked increase in expression during this later period. S1P4 and S1P5 transcripts were not detected in the allantois at any stage examined, suggesting that these receptors are not involved in vasculogenesis. In the yolk sac, prior to the expression of VE-cadherin (7.8–8.4 dpc), S1P1, S1P2, S1P3, and S1P5 transcripts were expressed (Fig. 1). In the prevascularized embryo proper (7.8–8.4 dpc), the expression of S1P2, S1P3, S1P4, and S1P5 was evident but little or no S1P1 was detectable (Fig. 1). Fig. 2 summarizes the temporal pattern of expression of each of the S1P receptor mRNAs in the tissues examined. Based on the collective findings, S1P1, S1P2, and S1P3 but not S1P4, and S1P5 are expressed during periods of vasculogenesis (i.e. <8.4 dpc). Only S1P2 appeared to be expressed during earliest stages of vasculogenesis, irrespective of the site. However, S1P2 expression in prevascularized tissues was accompanied by the expression of either S1P1 or S1P3 or both. The expression of S1P1, S1P2, and S1P3 receptors was also found to persist through later developmental stages during periods in which existing blood vessels become further reticulated and mature (8.5–10 dpc).

Sphingosine Kinase-2 Is Expressed during the Earliest Stages in Allantois Vasculogenesis—RT-PCR was also used to investigate the expression of sphingosine kinase-1 and -2 in the developing mouse embryo, yolk sac, and the allantois. In the prevascularized embryo proper, yolk sac, and allantois, sphingosine kinase-2 (SK2) was expressed from the earliest stage examined (7.5–8 dpc) and the expression persisted through to the latest stages examined (9.5–10 dpc) (Fig. 1). The level of the SK2 amplicon appeared to increase coincident with the expression of VE-cadherin in each of the three tissues (Fig. 1). By contrast, the expression of SK1 transcripts was not detected in the allantois at any of the stages examined. In the yolk sac, SK1 expression was not detected prior to the expression of

![Fig. 1. RT-PCR analysis of the expression of S1P receptors and sphingosine kinase mRNAs in the embryo proper, yolk sac, and allantois at different stages of development. PCR was performed using cDNA templates prepared from 7.5–9.5 dpc mouse allantoids, yolk sacs, and embryos free of extraembryonic membranes (Embryo Proper). Primers were based on mouse sequences encoding the indicated S1P receptors, SK1, SK2, VE-cadherin (VE-cad), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) beta-actin and collagen type I, alpha (Col1a1). Col1a1 primers were designed from sequences on either side of a short intronic sequence and thus yielded a larger amplicon (488 bp) in the event of contamination by genomic DNA. Each cDNA template was tested for genomic contamination via 40 cycles of PCR with Col1a1 primers. Conditions for S1P1 primers were optimized using cDNA prepared from adult mouse lung RNA.](https://www.jcb.org/doi/fig/10.1083/jcb.200703110/fig1)
VE-cadherin. In the embryo proper, SK1 was expressed at the earliest stages (7.8–8 dpc) but its expression was absent at 8–8.4 dpc and then reappeared at 8.5–10 dpc (Fig. 1). Since control reactions indicate that there was genomic DNA contamination of the 7.8–8 dpc embryo proper cDNA template, the apparent expression of SK1 at this early stage is equivocal. Taken together, the findings indicate that SK2 but not SK1 expression coincides with the earliest stages of vasculogenesis, irrespective of the site. The absence of expression of SK1 in the developing allantois, a tissue in which vasculogenesis is a key event, suggests that SK1 is not required for vasculogenesis. Furthermore, the findings also suggest that S1P expressed in embryonic tissues via the action of SK2 may be influencing the early events in the process of vasculogenesis.

**S1P Promotes Vasculogenesis in Cultured 7.8 dpc Allantois Explants**—To investigate the role of S1P signaling in vasculogenesis, we evaluated the effects of exogenously added S1P on the process of de novo blood vessel formation in the cultured allantois explant model (22, 24). Because serum is a rich source of S1P (25), we first evaluated the effect of culturing the explants in serum-free conditions. As shown in Fig. 3, 7.8 dpc allantois explants cultured in the absence of serum failed to form an expansive network of blood vessels as detected using antibodies to PECAM, a protein expressed by endothelial cells (Fig. 3D). Instead of the branched network of capillary-like vessels typically observed when the explants are cultured in serum-containing medium (Fig. 3, A–C), only small poorly reticulated clusters of rounded PECAM-positive cells formed in the absence of serum (Fig. 3, D–F). Often clusters of PECAM-positive cells were observed with few cell protrusions evident (Fig. 3, E and F, arrows). By contrast, in 7.8 dpc allantois explants cultured in serum-free medium containing S1P, there was a marked difference in the pattern of PECAM-positive cells (Fig. 3, G–I) as compared with explants cultured in the absence of serum. Networks of interconnected endothelial cells (i.e., reactive with PECAM antibodies) were evident in explants cultured in the presence of S1P (Fig. 3, G–I). Morphologically, PECAM-positive cells formed in the presence of S1P appeared less well spread than the ones formed in the presence of serum (Fig. 3, C versus I). Because endothelial cells (PECAM-positive cells) were formed in the 7.8 dpc allantois cultured in the absence of serum, the formation of the endothelial cell lineage does not appear to be dependent on S1P signaling.

**S1P Promotes Expansion of Vascular Networks in Cultured 8.5 dpc Allantois Explants**—We next evaluated the influence of exogenous S1P on blood vessel formation in 8.5 dpc allantois...
explants. At 8.5 dpc, the allantois was vascularized and undergoing extensive neovascularization (22). When explanted, 8.5 dpc allantois explants were cultured in the presence of 10% fetal bovine serum and a highly branched network of PECAM-positive blood vessels was apparent after 24 h (Fig. 4A). However, in the absence of serum, PECAM-positive endothelial cells remained in the central portion of the explant and displayed extremely limited degree of reticulated network formation (Fig. 4B). By contrast, in serum-free medium containing S1P (Fig. 4C), vascular network formation was comparable with that observed in explants cultured in serum-containing medium. Not only did S1P promote network branching to a level equivalent to that observed in serum (Fig. 4A), serum-free medium containing S1P (1 μM) was able to improve the extent of vascular network branching over that observed in explants cultured in the absence of serum. However, LPA at a concentration of 1 μM elicited modest improvement in the qualitative aspects of the vascular networks (Fig. 5D), but the degree of vascular network formation was always less than that observed using 1 μM S1P (Fig. 5C). Because LPA is known to bind to the S1P receptor, S1P1, with low affinity (Kd = 2.3 μM) (26), we evaluated the effect of LPA at 6 μM and observed a greater degree of blood vessel formation as compared with that achieved using 1 μM LPA with clearly more blood vessels present than in explants cultured in the absence of serum (data not shown). Whether the observed micromolar dosage LPA effects are mediated by S1P1, or some other LPA-binding receptor remains to be elucidated.

VEGF and bFGF Do Not Influence Allantois Vasculogenesis

Because VEGF is known to be important in the assembly of angioblasts into cordlike structures during vasculogenesis (22), we next evaluated the ability of VEGF to influence blood vessel assembly in
8.5 dpc allantois explants cultured in the absence of serum. We observed that blood vessel networks within explants cultured in serum-free medium containing VEGF (Fig. 6D) were not as expansive as those present in explants cultured in serum (Fig. 6A) or S1P-containing medium (Fig. 6E). Furthermore, the vascular networks formed in the serum-free medium containing VEGF had less avascular spaces and were much more densely packed with PECAM-positive cells (i.e. endothelial cells) (Fig. 6D) as compared with the networks formed in serum-free medium (Fig. 6C). When VEGF was combined with S1P, there was a marked expansion of the vascular network (Fig. 6F) as compared with explants cultured in serum-free medium containing VEGF (Fig. 6D). Furthermore, the vasculature that formed in the presence of both VEGF and S1P (Fig. 6F) displayed a reduction in the amount of avascular space as compared with networks formed in medium containing serum (Fig. 6A) or serum-free medium containing S1P (Fig. 6E). The findings indicate that S1P and VEGF have distinct effects on 8.5-dpc allantois vascular network formation. S1P appears to promote expansion of allantois vascular networks, whereas VEGF appears to increase the density of endothelial cells and promote vascular fusion. The ability of exogenously added VEGF to promote an increase in endothelial cell numbers and lead to the formation of fused sinusoidal vessels is well established (27–30).

Recent findings suggest that S1P signaling might be involved with bFGF signaling (31, 32). Therefore, we tested the effect of bFGF on the formation of blood vessels in allantois explants. We found that blood vessels formed in serum-free medium containing bFGF (Fig. 7D) were not discernibly different from those observed in 8.5 dpc explants cultured in serum-free medium alone (Fig. 7B). Overall, bFGF did not promote the vascular network expansion that was observed in S1P-treated explants (Fig. 7C).

S1P Does Not Influence Cell Survival in Cultured 8.5 dpc Allantois Explants—The aforementioned findings indicated that S1P elicited qualitative effects on the blood vessels of cultured allantois explants. Specifically, it appeared that the S1P alone was capable of supporting the expansion of vascular networks in cultured 8.5 dpc explants to a similar extent as serum. Therefore, we assessed the extent to which S1P effects
on vascular network expansion might be due to it influencing allantois cell growth. We found that 8.5 dpc allantois explants cultured in serum-free medium containing S1P did not have significantly different numbers of total cells as compared with explants cultured in serum-free medium (p = 0.19) (Fig. 8A). In addition, S1P, which is known to be survival factor for certain types of cells (33), did not significantly alter the level of total cell death occurring in cultured explants as compared with the level observed in explants cultured in serum-containing medium or serum-free medium (p = 0.84) (Fig. 8B).

**SIP Does Not Influence Allantois Endothelial Cell Numbers or Differentiation of Angioblasts to Form Endothelial Cells**—Flow cytometry was also used to evaluate the effect of SIP on the growth of specific subpopulations of 8.5 dpc allantois cells (i.e. undifferentiated mesodermal cells, angioblasts, and endothelial cells). As shown in Fig. 8C, the explants cultured in the absence of serum or in serum-free medium containing S1P had ~2-fold lower percentage of endothelial cells (i.e. Flk-1+/PECAM− population) as compared with explants cultured in the presence of serum (p < 0.0001). Importantly, there was no significant difference in the number of endothelial cells in explants cultured in the absence of serum versus explants cultured in serum-free medium containing S1P (p = 0.71) (Fig. 8C). Thus, the ability of SIP to promote blood vessel formation was not due to it acting to increase the number of endothelial cells over the levels observed in explants cultured in serum-free medium. Similarly, there was not a significant effect of SIP on the numbers of angioblasts (i.e. Flk-1+/PECAM− cells) (ANOVA p = 0.24) (Fig. 8D) or undifferentiated mesodermal cells (i.e. Flk-1−/PECAM− cells) (ANOVA, p = 0.29) as compared with angioblasts explants cultured in serum-free medium or in the presence of serum (Fig. 8, D and E, respectively). These findings indicate that SIP does not influence the differentiation of allantois mesodermal cells to form angioblasts or angioblasts to form endothelial cells.

**Quantification of the Effects of SIP on the Expansion of Cultured Allantois Vascular Networks and Mesothelial Layers**—To quantitatively measure the diameters of PECAM-positive vascular networks in 7.5–7.8 dpc allantois explants cultured in serum-containing medium, serum-free medium, and serum-free medium containing S1P. As shown in Fig. 9G, the average diameters of the vascular networks formed in 7.5–7.8 dpc allantois explants cultured in serum-containing medium were 2.1-fold greater than those that formed in serum-free medium (p = 0.0001). Additionally, we measured the influence of S1P on the expansion of the mesothelial layer, the epithelial layer that forms the surrounding sheath of the allantois. After using the Hoechst 33342 stain to label the nuclei (Fig. 9, A–C), the average diameter of the mesothelial discs formed in serum-free medium containing S1P was found to be only 1.36-fold greater than the diameter for those explants grown in serum-free medium (p = 0.004) (Fig. 9H). Finally, we measured the influence of SIP on the percentage of the mesothelial disc area that was occupied by the vascular network (i.e. (vascular network area/mesothelial disc area) × 100). As a result, it was found that the vascular networks formed in 7.5–7.8 dpc allantois explants cultured in the presence of S1P occupied 61% mesothelial disc area (Fig. 9D). By contrast, the vascular networks formed in the explants cultured in serum-free medium occupied only 26% mesothelial area (Fig. 9F). In addition, vascular networks formed in the presence of serum occupied 65% mesothelial area (Fig. 9F). Thus, S1P promoted a 2.3-fold greater degree (p < 0.0001) of vascular network expansion relative to mesothelial disc expansion as compared with what occurred in serum-free medium. The effect of S1P on the ratio of vascular network area/mesothelial disc area was similar to that observed in serum-containing medium.

We next quantified the effects of SIP on vascular network and mesothelium expansion in 8.5 dpc allantois explants cultured in serum-containing medium, serum-free medium, or serum-free...
medium containing S1P. As shown in Fig. 9J, the average diameters of the vascular networks formed in 8.5 dpc allantois explants cultured in S1P-containing medium were ~2-fold greater than those that formed in serum-free medium ($p < 0.001$). Furthermore, the average vascular network diameter of the S1P-treated explants was not significantly different from that of explants cultured in serum-containing medium ($p = 0.92$). The average diameter of the mesothelial discs formed in serum-free medium containing S1P was found to be ~1.4-fold greater than for those explants grown in serum-free medium ($p = <0.0001$) and only slightly less ($p = 0.044$) than for those explants cultured in serum-containing medium (Fig. 9K). As for the influence of S1P on the percentage of the mesothelial disc area that was occupied by the vascular network, it was found that vascular networks formed in the presence of S1P occupied 72.5% mesothelial disc area (Fig. 9L). By contrast, vascular networks formed in the explants cultured in serum-free medium occupied 40% mesothelial area (Fig. 9L). In addition, the vascular networks formed in the presence of serum occupied 59% mesothelial area (Fig. 9L). Thus, S1P increased the ratio of vascular network area/mesothelial disc area by nearly 50% over that observed in serum-free medium ($p = 0.0003$) and 20% greater than serum-containing medium ($p = 0.016$).

Upon visual inspection of Hoescht 33342-stained explants, it appeared that the number of nuclei in explants cultured in the absence of serum was less than the number of nuclei cultured in the presence of S1P (Fig. 9, B and C). Because the total numbers of cells in explants cultured in the presence or absence

**Fig. 9.** S1P promotes expansion of allantoic vascular networks to a greater extent than expansion of the mesothelium. A–C, epifluorescent microscopy images of 8.5 dpc allantois explants cultured for 24 h in serum-containing medium (A), serum-free medium (B), or serum-free medium containing 1 μM S1P (C) and stained with Hoescht 33342 stain (red nuclei) and labeled with PECAM antibodies and fluorescein isothiocyanate-conjugated secondary IgG. Panels D–F are laser-scanning confocal microscope images of central regions of 8.5 dpc allantois explants cultured in serum-containing medium (D), serum-free medium (E), and serum-free medium containing S1P (F) stained with Draq5 to reveal nuclei. For D–F, optical sectioning of entire allantois explants was performed and the complete Z series collapsed. To derive quantitative data pertaining to the effects of culture conditions on vascular network and mesothelium expansion, measurements (double arrowheaded lines) were made as indicated in panel B on the diameter of the Hoescht 33342-stained mesothelial disc and the diameter of the PECAM-stained vascular network. For each explant evaluated, a pair of diameter measurements were taken for the vascular network and averaged and the same was done for the mesothelium. G, H, and I represent data from 7.5–7.8 dpc allantois explant cultures. J, K, and L represent data from 8.5 dpc allantois explant cultures. For the values plotted in G–L, the experimental number ($n$) for each was 8–12.
## Table II

| Allantois stage at culture | Vascular network diameter | Vascular network area | Mesothelium diameter | Mesothelial area | Percentage of mesothelial area |
|---------------------------|--------------------------|----------------------|----------------------|------------------|--------------------------------|
| 7.5–7.8 dpc Serum         | 0.27 ± 0.07 mm           | 0.57 ± 0.36 mm²      | 3.06 ± 0.22 mm       | 1.36 ± 0.83 mm²  | 65%                            |
| 8.5–8.0 dpc Serum         | 0.20 ± 0.03 mm           | 0.83 ± 0.17 mm²      | 2.48 ± 0.45 mm       | 0.96 ± 0.33 mm²  | 26%                            |
| 7.5–7.8 dpc No serum      | 0.21 ± 0.04 mm           | 0.75 ± 0.09 mm²      | 2.53 ± 0.12 mm       | 1.21 ± 0.43 mm²  | 78%                            |
| 8.5–8.0 dpc No serum      | 0.17 ± 0.02 mm           | 0.43 ± 0.05 mm²      | 1.69 ± 0.08 mm       | 0.57 ± 0.09 mm²  | 43%                            |

**DISCUSSION**

The findings presented herein indicated that S1P signaling contributes to the process of murine vasculogenesis. First, it was shown that the S1P receptor S1P2 was expressed in conjunction with S1P1 and/or S1P3 at stages preceding the formation of blood vessels (i.e. 7.5–8 dpc) in the embryo proper, yolk sac, and allantois. Furthermore, SK2, an enzyme that catalyzes the formation of S1P, was also expressed in the allantois, yolk sac, and embryo proper prior to the vascularization. Finally, we showed that exogenous S1P or sphingosine, but not VEGF or bFGF, could effectively replace the requirement of serum for promoting vasculogenesis in cultured allantois explants.

The process of de novo blood vessel assembly involves several key steps (22) that are diagrammatically depicted in Fig. 10. Major steps in the process include the birth of angioblasts from mesodermal progenitors and their differentiation into endothelial cells. Considering the fact that endothelial cells indeed formed in 7.5 dpc allantois explants cultured in serum-free medium, it is evident that the formation of both angioblasts and endothelial cells is not dependent on exogenously added S1P. Because SK2 is expressed in the 7.5–8.0 dpc allantois, we cannot rule out that, in the absence of exogenous S1P, endogenously expressed S1P contributes to the differentiation events required to generate endothelial cells. However, the fact that S1P treatment of cultured allantois explants did not alter angioblast or endothelial cell numbers supports the possibility that the lineage process is not driven by S1P signaling. By contrast, endothelial cell numbers increase in allantois explants treated with VEGF (data not shown), consistent with the well characterized mitogenic effects of VEGF on endothelial cells (27). Our observation that exogenous S1P did not lead to an increase in endothelial cell number is in contrast to the report that S1P can stimulate human umbilical vein and bovine aortic endothelial cell proliferation (15) and may highlight a difference in S1P responsiveness between early endothelial cells and cultured endothelial cells derived from mature blood vessels.

Based on our morphometric analyses, we concluded that exogenously added S1P mediates the expansion of nascent blood vessel networks in cultured allantois explants. In 7.5–7.8 dpc allantois explants cultured in the absence of S1P, it appears that there is a failure of either angioblasts or early endothelial cells to migrate. As a result, the vascular networks that form remain confined to a limited area with constituent cells displaying an abnormally high packing density. A role for S1P in promoting motility of angioblasts and early endothelial cells is consistent with its reported effects on the motility of
cultured endothelial cells (16) and other types of cells (34). The importance of angioblast/early endothelial cell migration in the context of vasculogenesis has not been established. As a result of gastrulation, angioblasts first appear randomly distributed throughout the mesoderm (35). One model for the formation of vascular networks is that angioblasts merely extend processes to connect with other angioblasts and thus form polygonal arrangements. Another model, which is not necessarily mutually exclusive of the former model, is that angioblasts movement is required for the formation of a primary network. Our findings support the later model and indicate a role for S1P signaling in mediating angioblast/endothelial cell motility and extension. We believe that the observed expansion of vascular networks in response to S1P stimuli was the result of the outward migration of pioneering angioblasts/early endothelial cells that subsequently organize into a nascent vascular network. The fact that we observed isolated clusters of endothelial cells at the outer margins of the S1P-treated explants is evidence that the S1P-stimulated vascular network expansion was not due to angiogenic sprouting (Fig. 3G).

A remarkable finding from our studies was the fact that VEGF was not able to mimic the effects of S1P on angioblast/early endothelial cell motility and produce an expansive network. Indeed when explants were treated with VEGF alone, endothelial cells formed into a fused mass of small diameter. These findings demonstrated that S1P and VEGF have distinct effects on angioblast/early endothelial cell behaviors. Apparently, in the absence of S1P signaling, VEGF signaling was not sufficient to drive the migration of angioblasts/early endothelial cells. As a consequence of the failure of the cells to move and the increase in cell number due to the mitogenic effects of VEGF, the density-dependent phenomenon of vascular fusion occurred (36). Supporting the distinct roles of S1P and VEGF was the fact that when explants were cultured in the presence of both S1P and VEGF, the expected combinatorial phenotype was observed (i.e. expansion of the area of the vascular network and fusion of endothelial cells) (Fig. 6F).

Whereas our findings point to a role for S1P signaling in the process of vasculogenesis, the question is which of the S1P receptors acts as mediator(s)? Based on RT-PCR analysis, we have found that S1P1, S1P2, and S1P3, but not S1P4 and S1P5, are expressed at sites of mouse embryo vasculogenesis at stages early enough for them to participate in vasculogenesis. However, evidence from targeted inactivation of S1P1, S1P2, and S1P3 genes does not support a critical role for these recep-

tors individually in the process of vasculogenesis (20, 37–39).

Recently, double knock-out mice lacking S1P1 and S1P2 and triple knock-out (TKO) mice lacking S1P1, S1P2, and S1P3 have been generated (40). Both double knock-out and TKO embryos showed a 30% lethality with associated bleeding at 10.5 dpc. The TKO condition was more severe than that of the double knock-out as indicated by the fact that 100% lethality was reached at an earlier stage by TKO embryos (i.e. double knock-out, 100% lethality at 14.5 dpc; TKO, 100% lethality at 11.5 dpc) (40). The vascular defects observed in these knock-out mice have been attributed to faulty angiogenesis (40). However, considering that vasculogenesis precedes angiogenesis, it is possible that defective vasculogenesis may be the underlying basis for observed vascular abnormalities in these knockouts. Additional support for the involvement of S1P signaling in vasculogenesis comes from targeted deletion of Gα13, a G protein that couples to S1P2, S1P3, and S1P4 (14). Gα13 deficiency results in embryonic lethality with embryos having defective vascular development (e.g. absence of yolk sac blood vessels) (21). Importantly, PECAM-positive endothelial cells formed in the yolk sac of Gα13-null embryos but failed to assemble into vascular networks, suggestive of a defect in vasculogenesis. Cranial blood vessels that form via the process of vasculogenesis appeared elaborated and disorganized. Furthermore, the finding that fibroblasts from Gα13-deficient embryos exhibit impaired motility (21) makes it plausible that defective angioblast and endothelial cell motility may contribute to the observed vascular abnormalities.

Recently, five orphan G protein-coupled receptors (i.e. Gpr3, Gpr6, Gpr12, Gpr61, and Gpr63) have been identified as S1P receptors (41–43). Rather little is known regarding these receptors. Pertussis toxin inhibits Gpr6, indicating that it couples to inhibitory G proteins (Gi) (41). Gpr6 also activates sphingosine kinase (41). Gpr3, Gpr6, Gpr12, and Gpr63 have been shown to be expressed by endothelial cells (i.e. human umbilical vein endothelial cells, human coronary artery endothelial cells, human pulmonary microvascular endothelial cells, and human pulmonary artery endothelial cells) as well as by smooth muscle cells (42, 44). It remains to be determined whether any of these receptors might also be additional candidates for mediators of S1P signaling required for angioblast motility.

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