



# Requirement for sphingosine 1-phosphate receptor-1 in tumor angiogenesis demonstrated by in vivo RNA interference

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**Angiogenesis, or new blood vessel formation, is critical for the growth and spread of tumors. Multiple phases of this process, namely, migration, proliferation, morphogenesis, and vascular stabilization, are needed for optimal tumor growth beyond a diffusion-limited size. The sphingosine 1-phosphate (S1P) receptor-1 (S1P<sub>1</sub>) is required for stabilization of nascent blood vessels during embryonic development. Here we show that S1P<sub>1</sub> expression is strongly induced in tumor vessels. We developed a multiplex RNA interference technique to downregulate S1P<sub>1</sub> in mice. The small interfering RNA (siRNA) for S1P<sub>1</sub> specifically silenced the cognate transcript in endothelial cells and inhibited endothelial cell migration in vitro and the growth of neovessels into subcutaneous implants of Matrigel in vivo. Local injection of S1P<sub>1</sub> siRNA, but not a negative control siRNA, into established tumors inhibited the expression of S1P<sub>1</sub> polypeptide on neovessels while concomitantly suppressing vascular stabilization and angiogenesis, which resulted in dramatic suppression of tumor growth in vivo. These data suggest that S1P<sub>1</sub> is a critical component of the tumor angiogenic response and argue for the utility of siRNA technology in antiangiogenic therapeutics.**

## Introduction

Sphingosine 1-phosphate (S1P), a potent lipid mediator produced from the metabolism of sphingomyelin, acts on a family of S1P G protein-coupled receptors (S1P<sub>n</sub>) and transduces intracellular signals involved in numerous cellular processes (1, 2). In vascular endothelial cells, S1P binds to S1P receptors 1–3 (S1P<sub>1–3</sub>) to induce migration, proliferation, cell survival, and morphogenesis into capillary-like structures (3, 4). Such responses require the function of S1P<sub>1</sub>, which was originally isolated as an inducible gene from endothelial cells (4, 5). In addition to the well-characterized effects of S1P on endothelial cell migration and survival, it also induces the formation of cell-cell adherens junctions, thus inhibiting paracellular permeability of solutes and macromolecules (4, 6, 7). In vivo studies showed that S1P synergized with polypeptide angiogenic factors such as FGF-2 and VEGF to induce angiogenesis and vascular maturation in mouse models in which the extracellular matrix Matrigel was implanted subcutaneously (4). Moreover, S1P<sub>1</sub>-KO mice died in utero between embryonic days 13.5 and 14.5 due to a defect in vascular stabilization — entailing the reinforcement of nascent endothelial tubes with pericytes and vascular smooth muscle cells — which suggests that this receptor is required for vascular development (8). These studies form a basis for the emerging concept that S1P is a potent regulator of vascular growth and development, at least during embryogenesis.

Many regulators of embryonic vascular development also play important regulatory roles in pathologic angiogenesis in the adult. For example, the *VEGF* gene is not only critical for the

various phases of embryonic vascular development but is also important in tumor angiogenesis (9). Indeed, a neutralizing antibody against VEGF has recently shown efficacy in colorectal cancer treatment in a combination regimen with conventional chemotherapeutic agents (10, 11). Given that S1P acts via G protein-coupled receptors, a class of receptors that are amenable to pharmacologic inhibition, it is of interest to ascertain whether this bioactive mediator plays a role in angiogenesis in the adult mouse. Indeed, little is known about the role of S1P in the adult vasculature, particularly with respect to angiogenesis. S1P<sub>1</sub> is expressed in selected vascular beds; for example, in the cardiac ventricular microvessels of adult mice (12). Whether it is expressed in angiogenic vessels in vivo is not known. Here, we document that S1P<sub>1</sub> is induced in angiogenic vessels in vivo.

Loss-of-function genetic and/or pharmacologic approaches are required to critically determine the function of S1P receptors in vivo. Lack of specific pharmacologic tools, coupled with the fact that the *S1P<sub>1</sub><sup>-/-</sup>* mice die during mid-gestational stages of embryogenesis, have hampered efforts to decipher the postnatal functions of this receptor. We therefore utilized RNA interference (RNAi) technology, which was recently discovered to be a naturally occurring mechanism of posttranscriptional gene silencing (13). It was originally shown that long double-stranded RNA (dsRNA) species are cleaved into duplex RNAs of 21–23 nt with 2-bp overhangs, termed small interfering RNAs (siRNAs), which potently and specifically suppress gene expression by the induction of the RNA-induced silencing complex (14). RNAi occurs widely in nature and is implicated in embryonic development and normal adult physiology (15, 16). Administration of siRNAs into eukaryotic cells induces specific gene silencing in vitro and in vivo (17, 18). Thus, the potential of RNAi technology in therapeutic gene regulation has received significant interest (19).

The limitations of the synthetic siRNA technology include the empirical nature of target site selection and the expense associated with chemical synthesis of siRNA in the quantities required

**Nonstandard abbreviations used:** dsRNA, double-stranded RNA; MEEC, mouse embryonic EC; RNAi, RNA interference; S1P, sphingosine 1-phosphate; S1P<sub>1</sub>, S1P receptor-1; siRNA, small interfering RNA; 3'-UTR, 3'-untranslated region.

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for *in vivo* administration (15). Recent work has shown that the use of multiplex siRNA species can achieve synergistic suppression of genes (20, 21). Such multiplex species of siRNAs can be produced by the eukaryotic enzyme Dicer or the *E. coli* RNaseIII enzyme. To overcome the problem of off-target suppression of related gene sequences in the protein-coding regions, we developed a method to suppress genes based on the 3'-untranslated region (3'-UTR) sequences, which are not under the same degree of evolutionary conservation as the coding regions (22). In this study, we used the *E. coli* RNaseIII enzyme to generate 3'-UTR-directed multiplex siRNA species to effectively downregulate gene expression. We show the utility of this method in the suppression of S1P<sub>1</sub> expression *in vitro* and *in vivo*.

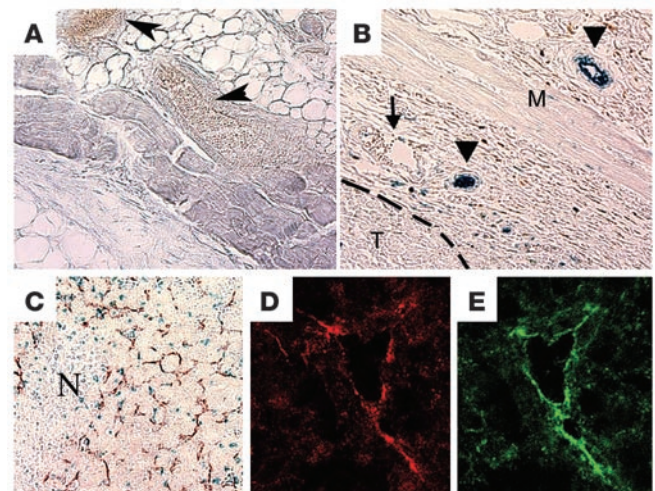
## Results

**Induction of S1P<sub>1</sub> expression during tumor angiogenesis.** S1P<sub>1</sub> mRNA is strongly induced in endothelial cells during embryonic angiogenesis (8). In the adult mouse, S1P<sub>1</sub> is expressed in both vascular and nonvascular cells under basal conditions in selected tissues (12). However, the expression of S1P<sub>1</sub> in angiogenic vessels in the adult has not been examined. We thus implanted Lewis lung carcinoma cells – which do not endogenously express the S1P<sub>1</sub> transcript (Supplemental Figure 1; supplemental material available at <http://www.jci.org/cgi/content/full/114/8/1082/DC1>) and are capable of establishing tumors in immunocompetent mice – subcutaneously into S1P<sub>1</sub><sup>-/-</sup> mice. We analyzed tissue sections for the expression of S1P<sub>1</sub> by examining the activity of β-gal, which is directed from the endogenous S1P<sub>1</sub> gene promoter (8, 12). In normal skin microvessels, S1P<sub>1</sub> expression was not detected (Figure 1, A and B). However, S1P<sub>1</sub> expression was induced in the vasculature upon the implantation of tumor cells. Endothelial cells from both small and large vessels surrounding the tumor expressed S1P<sub>1</sub>. Immunohistochemical analysis of X-gal-stained tumor sections with the endothelial cell marker CD31 and smooth muscle cell marker α-SMA showed that highest expression of S1P<sub>1</sub> is in the endothelial cell compartment (data not shown). However, the intensity of X-gal staining was generally much lower in vascular smooth muscle cells. S1P<sub>1</sub> expression was also noted in angiogenic vessels that had infiltrated the tumor. As shown in Figure 1C, many microvessel-like structures stained blue, which suggests that intratumoral vessels express S1P<sub>1</sub>. This was further confirmed by immunofluorescence staining with an antibody specific for the murine S1P<sub>1</sub> polypeptide (Figure 1, D and E) (23). It should also be noted that some CD31-negative cells, which have the morphology of infiltrating monocytic and/or immune cells, also are positive for X-gal staining in both growing and necrotic areas of the tumor (Figure 1C). This is consistent with the knowledge that many cells derived from the hematopoietic lineage (such as monocytes, T cells, and mast cells) also express S1P<sub>1</sub> (23, 24). These data suggest that S1P<sub>1</sub> is induced in intratumoral capillaries and juxtatumoral large vessels during tumor angiogenesis.

**Gene silencing by *E. coli* RNaseIII-generated siRNA.** To determine the function of S1P<sub>1</sub> during tumor angiogenesis, we developed a novel RNAi method to downregulate its expression. We hypothesized that utilization of the 3'-UTR sequences as a template to derive multiplex siRNA species might overcome some of the problems associated with siRNA technology. Indeed, sequence homology analysis using the BLAST algorithm did not reveal significant similarity between the 3'-UTR of S1P<sub>1</sub> and the 3'-UTR

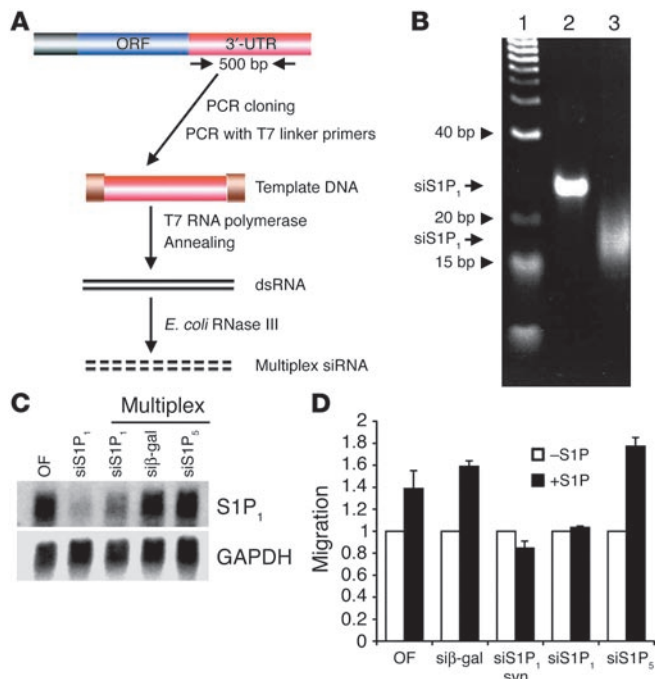
of the other four S1P receptors. In addition, we hypothesized that utilization of *E. coli* RNaseIII, a double strand-specific endoribonuclease, would produce duplex RNA species that can silence genes by an siRNA-like mechanism. In fact, recent studies published after the initiation of our work show that small duplex RNA produced by *E. coli* RNaseIII enzyme does indeed induce RNA interference in cultured cells (20).

Our procedure is shown schematically in Figure 2A. We amplified a 500-bp fragment of the 3'-UTR from mouse S1P<sub>1</sub> cDNA (25). Double-stranded RNA was produced by PCR with T7 RNA polymerase binding site-containing primers and *in vitro* transcription, followed by annealing of the sense and antisense RNA products. Extensive digestion with the *E. coli* RNaseIII resulted in the formation of multiplex RNA of approximately 15–20 bp (Figure 2B). RNaseIII did not digest single-stranded substrates of S1P<sub>1</sub> 3'-UTR. In addition, formation of smaller mol wt species was double strand-, RNaseIII-, and time-dependent. Complete digestion (500 nM enzyme, 4 hours treatment at 37°C) resulted in the formation of 15- to 20-bp double-stranded RNA species. This fraction had the RNA silencing activity, whereas we did not observe appreciable RNA silencing in larger or smaller regions of the gel (with much lower amounts of ethidium bromide-stained material). Similarly, we produced siRNAs for S1P<sub>5</sub> and β-gal, both of which are not expressed in endothelial cells.



**Figure 1**

Induction of S1P<sub>1</sub> expression in tumor xenografts. Normal skin tissue (A) and subcutaneous tumor nodules (B) from the back of S1P<sub>1</sub><sup>+/-</sup>LacZ mice were excised and stained with X-gal to detect the expression of β-gal marker as described. Sections were cut and imaged under a bright-field microscope using a ×20 objective lens. β-gal expression (blue) was detected in blood vessels only in mice with the growing tumor (arrowheads in B). However, blood vessels in normal skin tissues (arrowheads in A) and few blood vessels with veinlike morphology in tumor-bearing tissue (arrow in B) were S1P<sub>1</sub>-negative. T, tumor; M, skeletal muscle layer. Dashed line indicates margin of the tumor. (C) X-gal-stained, fixed tissues were counterstained with anti-CD31 antibody to detect blood vessels, and the intratumor region is shown. At least 5 animals were used in this study. N, necrotic center. (D and E) Frozen sections of the subcutaneous implants of the tumor were analyzed in an immunofluorescence assay with the anti-S1P<sub>1</sub> antibody (red) and anti-CD31 antibody (green) and imaged by a confocal microscope as described in Methods. Note that intratumoral blood vessels express S1P<sub>1</sub>, whereas tumor cells do not express this receptor.



**Figure 2**

Gene silencing by siRNA. **(A)** Scheme for the generation of multiplex siRNA. ORF, open reading frame. **(B)** Gel electrophoresis of synthetic and multiplex siRNA. Synthetic siRNA or multiplex siRNA for S1P<sub>1</sub> were analyzed by a non-denaturing gel electrophoresis and detected by ethidium bromide staining. Lane 1: DNA mol wt markers; lane 2: 21-bp synthetic siRNA for S1P<sub>1</sub> (siS1P<sub>1</sub>); lane 3: RNaseIII-digested siRNA for S1P<sub>1</sub>. **(C)** Mouse endothelial cells were transfected with various siRNAs and total RNA was isolated and analyzed by Northern blot analysis using S1P<sub>1</sub> or GAPDH probes as described. Data show the results of a representative experiment that was repeated at least 3 times. siβ-gal, siRNA for β-gal. **(D)** Mouse endothelial cells were transfected with various siRNAs and analyzed in a chemotaxis assay using S1P (100 nM) as a chemoattractant. Data represent mean ± SE from a representative experiment that was repeated twice. syn, synthetic; OF, oligofectamine alone.

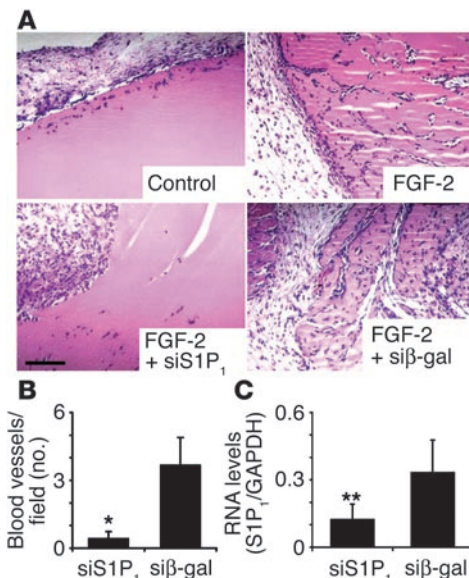
The efficacy and specificity of the siRNA for S1P<sub>1</sub> in the suppression of gene expression was tested next. We treated mouse embryonic ECs (MEECs) with either synthetic siRNA from the open reading frame region of the mouse S1P<sub>1</sub> mRNA or the RNaseIII-derived siRNA for S1P<sub>1</sub> at a concentration of 200 nM. The RNA was delivered into endothelial cells by lipid-mediated transfection as described (26). The effect of siRNA was assessed by measuring the steady-state levels of S1P<sub>1</sub> mRNA by Northern blot analysis 48 hours after transfection. As shown in Figure 2C, both multiplex and synthetic siRNA for S1P<sub>1</sub> strongly suppressed the expression of the endogenous S1P<sub>1</sub> mRNA. In separate experiments, siRNA for β-gal potently suppressed β-gal activity and protein levels in murine endothelial cells from heterozygous S1P<sub>1</sub> KO mice that had the β-gal gene knocked into the S1P<sub>1</sub> locus (S1P<sub>1</sub><sup>+/LacZ</sup> mice), whereas treatment with transfection reagent was without effect (Supplemental Figure 2). S1P<sub>5</sub> expression was not detected in these cells and therefore was used as an irrelevant siRNA. However, siRNA for S1P<sub>5</sub> or β-gal siRNA did not suppress the S1P<sub>1</sub> transcripts, which suggests that the siRNA species achieved sequence-specific suppression of gene expression.

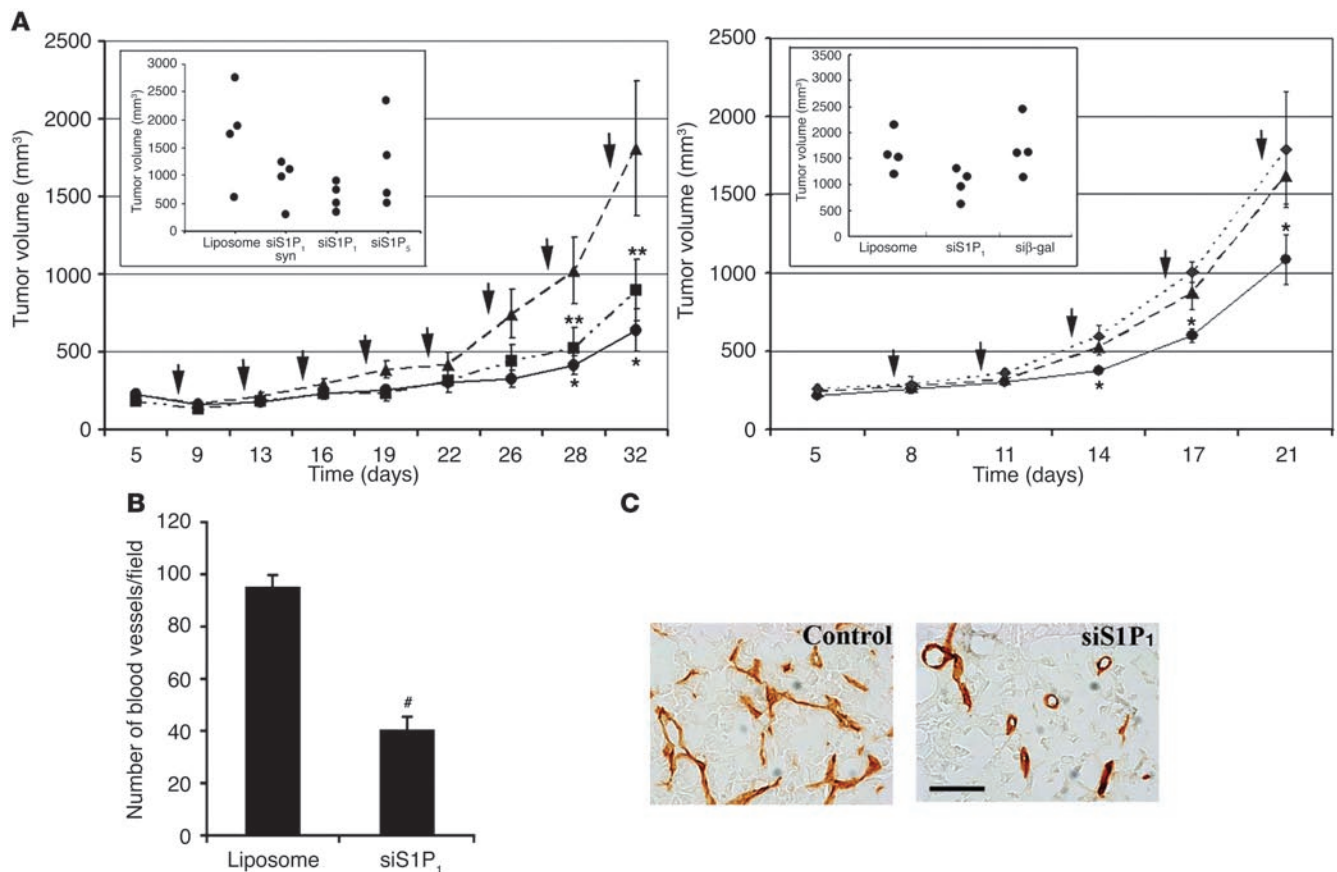
**Figure 3**

Inhibition of angiogenesis in vivo by S1P<sub>1</sub> siRNA. **(A)** Matrigel mixture containing multiplex siRNA with or without FGF-2 was injected subcutaneously into athymic nude mice as described. The Matrigel plugs were harvested 7 days after implantation, fixed, sectioned, and H&E-stained as described. Images are obtained from a representative field of an experiment (n = 3) that was repeated twice. Scale bar: 100 μm. **(B)** The number of CD31-positive blood vessels in the Matrigel plugs was determined by immunohistochemistry and plotted (n = 4). Results represent mean ± SE. \*P < 0.02. **(C)** Total RNA was isolated from the Matrigel plugs with siRNA for S1P<sub>1</sub> or β-gal in the presence of FGF-2. S1P<sub>1</sub> mRNA expression in the cells within Matrigel plugs was quantified by Northern blot analysis and normalized with respect to GAPDH expression (n = 3). \*\*P < 0.1. In **B** and **C**, multiplex siS1P<sub>1</sub> and siβ-gal indicate respective siRNAs in the presence of FGF-2 as described in **A**.

To determine whether the siRNA for S1P<sub>1</sub> is functional in endothelial cells, we tested its effect on endothelial cell migration in a Boyden chamber assay (3). As shown in Figure 2D, both synthetic siRNA and multiplex siRNA for S1P<sub>1</sub> but not the multiplex siRNA for S1P<sub>5</sub> or β-gal potently suppressed the S1P-induced migration of murine endothelial cells. These data suggest that multiplex siRNA for S1P<sub>1</sub> is likely to influence angiogenic response, of which endothelial cell migration is an early critical step.

*Inhibition of angiogenesis in vivo by S1P<sub>1</sub> siRNA.* In previous studies, we have shown that antisense oligonucleotides against S1P<sub>1</sub> suppressed FGF-2-induced angiogenesis in vivo (4). To test whether S1P<sub>1</sub> siRNA regulates angiogenesis in the adult mouse, we performed subcutaneous angiogenesis assay using Matrigel. FGF-2 strongly induced cell infiltration and blood vessel formation (Figure 3). siRNA for S1P<sub>1</sub> significantly inhibited FGF-2-induced angiogenesis and cell infiltration, whereas irrelevant siRNA (β-gal) did not have significant effect on angiogenesis (Figure 3A). Microvessel counting after immunohistochemical staining for CD31 showed that siRNA for S1P<sub>1</sub> potently suppressed angiogenesis (Figure 3B). RNA was isolated from Matrigel plugs and analyzed for the expression of S1P<sub>1</sub> transcript by Northern blot analysis. As shown in Figure 3C, siRNA for S1P<sub>1</sub> significantly suppressed the expression of the cognate transcript in a specific manner.



**Figure 4**

Inhibition of tumor growth and angiogenesis by S1P<sub>1</sub> siRNA. (A) Lewis lung carcinoma cells were implanted subcutaneously and allowed to establish as growing tumors, and various siRNA-liposome complexes were injected into the tumor every 3 days (where indicated by arrows) as described. Tumor volume was measured and plotted at various times following treatment with synthetic or multiplex siRNA for S1P<sub>1</sub> (synthetic siS1P<sub>1</sub>, squares; multiplex siS1P<sub>1</sub>, circles) or liposome alone (triangles). The inset shows the tumor volume at 32 days for additional controls. Data represent mean  $\pm$  SE of an experiment that was repeated twice. The right panel shows an independent experiment repeated with the inclusion of another control siRNA (multiplex  $\beta$ -gal siRNA, diamonds).  $n = 4$ ; \* $P < 0.05$ ; \*\* $P < 0.1$ . (B) Intratumoral microvessel density was quantified from multiple fields as described after CD31 staining. # $P < 0.0003$ . (C) Morphology of tumor vessels is shown from a representative field. Note that vessel morphology and density are altered by S1P<sub>1</sub>, but not control, siRNA. Scale bar: 20  $\mu$ m.

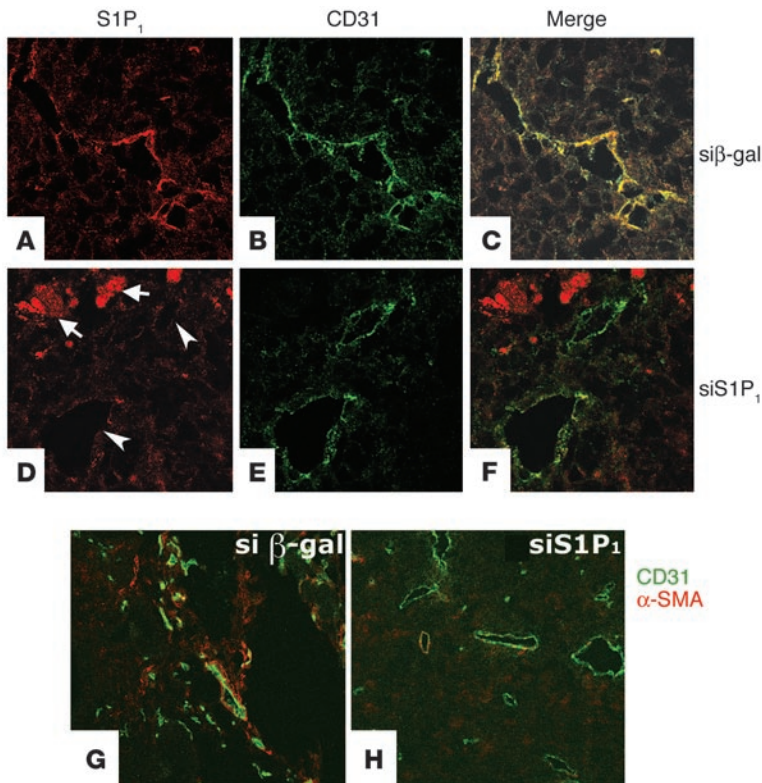
**Inhibition of tumor growth and angiogenesis by S1P<sub>1</sub> siRNA.** To determine the functional consequences of S1P<sub>1</sub> function in tumor growth and angiogenesis, we injected multiplex S1P<sub>1</sub> siRNA-liposome complexes locally into xenografts of Lewis lung carcinoma cells in nude mice. Synthetic siRNA-liposome complexes (positive control), liposome alone, and S1P<sub>5</sub> siRNA-liposome complexes (negative controls) were also injected. As shown in Figure 4A, both synthetic and multiplex siRNA for S1P<sub>1</sub> potently suppressed tumor growth. Liposome alone was not inhibitory, and siRNA for S1P<sub>5</sub> had a minor but statistically insignificant effect on tumor growth rate. Furthermore, siRNA for  $\beta$ -gal did not suppress tumor growth. These data suggest that the expression of S1P<sub>1</sub> is important for optimal tumor growth.

S1P<sub>1</sub> was not detected in Lewis lung carcinoma cells by Northern blot analysis (Supplemental Figure 1). In addition, immunofluorescence microscopy of tumor sections with an anti-S1P<sub>1</sub> antibody did not detect the expression of this receptor in implanted tumor cells in vivo (Figure 1 and below). Moreover, treatment of Lewis lung cancer cells in vitro with siRNA for S1P<sub>1</sub> did not suppress growth of these cells, whereas it suppressed

the growth of MEECs (Supplemental Figure 3). Thus, the effect of siRNA for S1P<sub>1</sub> is likely to be on the host, namely, angiogenic endothelial cells, which express this receptor.

To determine the potential antiangiogenic action of the S1P<sub>1</sub> siRNA, we quantified the microvessels in the tumor tissue. As shown in Figure 4B, microvessel density was significantly reduced by the local administration of siRNA for S1P<sub>1</sub>. Morphological analysis showed that branching and morphogenesis of tumor vasculature are profoundly affected (Figure 4C). These data suggest that S1P<sub>1</sub> function in the angiogenic vessels is critical for optimal tumor angiogenesis, an essential process for tumor growth.

The suppression of S1P<sub>1</sub> by intratumoral injection of siRNA was confirmed by immunostaining of the S1P<sub>1</sub> polypeptide using the murine S1P<sub>1</sub> antibody (23). Strong immunostaining of S1P<sub>1</sub> was detected in endothelial cells within the tumor (Figure 5A). The expression of S1P<sub>1</sub> in endothelial cells was significantly reduced by siRNA for S1P<sub>1</sub>, but not by the irrelevant siRNA, which suggests that downregulation of S1P<sub>1</sub> contributed to the suppression of angiogenesis and tumor growth. (Figure 5D). In



**Figure 5**

Inhibition of S1P<sub>1</sub> expression and vascular stabilization in siRNA-injected tumor xenografts. Tumor xenografts were injected with multiplex siRNAs for β-gal (A–C and G) or S1P<sub>1</sub> (D–F and H) as described. Tissue sections were harvested at 15 days and stained for S1P<sub>1</sub> (red, A and D) and CD31 (green, B and E). Overlays are shown in C and F. siRNA for S1P<sub>1</sub> potently suppressed the expression of S1P<sub>1</sub> in endothelial cells (arrowheads). Arrows indicate S1P<sub>1</sub> positivity in necrotic areas of the tumor. (G and H) Some sections were costained with anti-α-SMA and anti-CD31, and then α-SMA and CD31 were detected with secondary antibodies conjugated with alexa 568 (red, α-SMA) and alexa 488 (green, CD31) as described. Images were acquired using a confocal microscope. Note that α-SMA–positive cell association with angiogenic vessels were reduced after treatment with siRNA for S1P<sub>1</sub>.

the necrotic areas of the tumor, diffuse S1P<sub>1</sub> immunostaining was observed. Treatment with siRNA for S1P<sub>1</sub> did not suppress this signal. The identity of these structures is not known but is likely to be cell debris.

Since vascular stabilization contributes to tumor angiogenesis (27, 28) and since S1P<sub>1</sub> regulates vascular stabilization during development (8), we studied whether injection of siRNA for S1P<sub>1</sub> would influence vascular stabilization of tumor vessels. Stabilization of tumor vessels was assessed by immunofluorescence microscopy of tumor tissue sections with the α-SMA antibody (Figure 5, G and H). Injection of siRNA for S1P<sub>1</sub> strongly reduced mural cell coverage of tumor vessels (Figure 5H). These data suggest that siRNA for S1P<sub>1</sub> inhibits the stabilization of nascent blood vessels and that this might be a mechanism via which vascular growth is inhibited.

### Discussion

S1P is beginning to be recognized as a critical mediator in the growth and maintenance of the vascular system. In vitro studies clearly show that it is one of the most potent inducers of endothelial cell migration (3, 29, 30). In addition, endothelial cell survival, morphogenesis, NO synthesis, and proliferation are induced by S1P in cultured endothelial cells (1). These effects are attributed to signal transduction pathways regulated by S1P receptors, particularly S1P<sub>1</sub>. The relevance of S1P<sub>1</sub> in the vascular system is underscored by the fact that it is abundantly expressed in cultured endothelial cells and its expression is induced by tumor promoters, growth factors, and biomechanical shear stress (31, 32). Extension of these studies to in vivo models of angiogenesis also show the requirement for S1P<sub>1</sub> in adult angiogenesis; for example, downregulation of S1P<sub>1</sub> expression with antisense

phosphothioate oligonucleotides resulted in the suppression of angiogenesis in the Matrigel model of angiogenesis in the mouse (4). However, during embryonic development, the *S1p1* gene is not required for early developmental angiogenesis. Instead, this receptor is absolutely required for proper vascular stabilization, i.e., enforcement of nascent endothelial vessels with the mural cells, for example vascular smooth muscle cells (8, 27). Therefore it is important to assess the role of S1P<sub>1</sub> in adult angiogenesis, a process that occurs in many pathological conditions such as solid tumor growth (33).

We show here that S1P<sub>1</sub> is induced in endothelial cells during tumor angiogenesis. Using the Lewis lung carcinoma model of tumor growth, we show that microvessels within the tumor as well as juxtatumoral large vessels express S1P<sub>1</sub>, as indicated by the induction of β-gal activity, which is under the control of the endogenous S1P<sub>1</sub> promoter. Immunofluorescence analysis with the S1P<sub>1</sub> antibody also confirmed that S1P<sub>1</sub> expression is induced in tumor vessels. Angiogenic vessels in this model also undergo vascular stabilization, since many endothelial cells have a mural cell coat, as detected by α-SMA staining. Tumor infiltrating cells with a leukocytic morphology also express S1P<sub>1</sub>. These data suggest that S1P/S1P<sub>1</sub> signaling may be involved in the regulation of tumor angiogenesis.

To address the specific role of S1P<sub>1</sub> in tumor angiogenesis, we developed a novel method to silence *S1p1* gene expression using the siRNA method. This was essential because the null mutation of the *S1p1* gene induced embryonic lethality. Endothelial cell-specific deletion of the *S1p1* gene was also lethal and exhibited a very similar phenotype (34). Thus, epigenetic approaches are needed, especially in the absence of pharmacologic antagonists for S1P<sub>1</sub>.

RNAi has emerged as a potentially promising technology to analyze the function of specific genes by loss-of-func-



tion approaches. At present, RNAi technology is effective in downregulating specific gene products in tissue culture and in model organisms such as *Caenorhabditis elegans* (16). The use of RNAi to downregulate cellular and viral genes in vivo in mammals is limited at present (19). A recent report showed that local injection of synthetic siRNA against VEGF into established tumors suppressed angiogenesis and tumor growth (35).

We developed the multiplex siRNA method, which offers potential improvements over existing methodology. First, targeting the 3'-UTR of transcripts for gene silencing offers several advantages; for example, this strategy is anticipated to minimize the problem of off-target silencing, as the 3'-UTR sequences are not generally conserved among related transcripts in multigene families, including in the S1P receptors. Second, the use of the *E. coli* RNaseIII, a highly stable enzyme (36), facilitates the efficient production of siRNA species in amounts required for in vivo administration (20). Third, multiplex siRNA species may achieve a higher opportunity for efficient gene silencing in an unbiased manner (20).

Our data show that the siRNA for S1P<sub>1</sub> suppressed the S1P<sub>1</sub> transcript in endothelial cells. Irrelevant siRNAs, such as those for S1P<sub>5</sub> and  $\beta$ -gal, were ineffective, which suggests the specificity and efficacy of our procedure. Downregulation of S1P<sub>1</sub> in mouse endothelial cells resulted in the inhibition of S1P-induced chemotaxis, an important step in angiogenesis. These in vitro studies suggested that the siRNA for S1P<sub>1</sub> is likely to suppress angiogenesis in vivo.

To test the efficacy of the siRNA for S1P<sub>1</sub> in angiogenesis in vivo, we delivered it to the site of angiogenesis by mixing with the extracellular matrix Matrigel. It is known that extracellular matrix proteins such as Matrigel and atelocollagen facilitate the uptake and stability of plasmid DNA, antisense oligonucleotides, and siRNA in vivo (35, 37). We show that incorporation of siRNA into Matrigel followed by subcutaneous implantation results in the efficient suppression of S1P<sub>1</sub> transcript in infiltrating cells. This resulted in the potent suppression of neovessel formation in the Matrigel plug, which suggests that siRNA is capable of silencing the S1P<sub>1</sub> gene in vivo.

To evaluate the role of S1P<sub>1</sub> in tumor angiogenesis, we injected siRNA in the established tumor xenografts implanted subcutaneously in mice. Local delivery of S1P<sub>1</sub> siRNA inhibited tumor growth and angiogenesis concomitantly with the significant suppression of S1P<sub>1</sub> in the intratumoral endothelial cells. Control siRNAs for S1P<sub>5</sub> and  $\beta$ -gal did not significantly suppress tumor growth and angiogenesis. This tumor-suppressive effect of S1P<sub>1</sub> siRNA is unlikely to be due to direct effects on tumor cells, as Lewis lung carcinoma cells do not express the S1P<sub>1</sub>, as determined by Northern blot analysis. In addition, proliferation of Lewis lung carcinoma cells was not altered by in vitro treatment with siRNA. Rather, S1P<sub>1</sub> siRNA inhibited angiogenesis, as indicated by the suppression of microvessel density. In addition to the microvessel density within the tumor, vessel morphology is also different. Tumor vasculature treated with S1P<sub>1</sub> siRNA has fewer vascular branch points, which might be due to impaired migration of endothelial cells or its precursor cells. S1P is a potent chemoattractant for endothelial cells, and S1P<sub>1</sub> is a major S1P receptor regulating endothelial cell migration (3). It is therefore likely that S1P<sub>1</sub> contributes to tumor angiogenesis, in part, by regulating the migration of endothelial cells. It is also possible that S1P<sub>1</sub> regulates the homing/migration of monocytic cells into

the sites of angiogenesis and thereby modulates endothelial cell functions. In addition, S1P<sub>1</sub> in various cells regulates cell survival (4), and, therefore, downregulation of this receptor with specific siRNA could induce apoptosis within the tumor. Indeed, siRNA for S1P<sub>1</sub>-injected tumors contained numerous necrotic regions. Further, endothelial cell morphogenesis could be regulated by S1P<sub>1</sub>, which is consistent with the finding that the vessels in the siRNA for S1P<sub>1</sub>-treated tumors contain altered morphology with reduced branch points.

Deletion of the *S1p1* gene in the mouse resulted in embryonic vascular stabilization defect, leading to vascular hemorrhage and lethality (8). Whether S1P<sub>1</sub> is needed for vascular stabilization in the adult is not known. In fact, vascular stabilization is thought to be needed for optimal angiogenesis in tumors, since angiogenic vessels are ensheathed by mural cells (albeit abnormally) (27, 38). Secondly, inhibition of mural cell coverage of intratumoral vessels with PDGF receptor tyrosine kinase inhibitors either alone or together with VEGF inhibitors significantly attenuated tumor growth in vivo (28). Our data show that inhibition of S1P<sub>1</sub> expression with siRNA inhibited mural cell coverage of neovessels, which suggests that this may constitute an additional mechanism involved in the inhibition of tumor vascular growth.

In conclusion, we show that S1P<sub>1</sub> is induced in the tumor vasculature in vivo. We also show that downregulation of S1P<sub>1</sub> expression with siRNA is effective in inhibiting angiogenesis and tumor growth in vivo. Inhibition of S1P<sub>1</sub> expression in the tumor vasculature may be a novel therapeutic approach in the control of pathologic angiogenesis. Further, the siRNA approach may find general utility in the suppression of genes that are upregulated in pathologic conditions.

## Methods

**Preparation of multiplex siRNA.** *E. coli* RNaseIII plasmid was obtained from Allan Nicholson (Wayne State University, Detroit, Michigan, USA). The RNaseIII polypeptide was overexpressed in *E. coli* strain DH5 $\alpha$  and purified using Ni<sup>2+</sup> beads as previously described (36). Purified RNaseIII (>95% purity and comparable activity as described in ref. 23) was dialyzed and stored at -20°C in 50% glycerol, 0.5M NaCl, 30 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, and 0.5 mM DTT. Template DNAs with the length of 500 nt of 3'-UTR were prepared by PCR using hybrid primers containing the T7 promoter sequence. Sense and antisense transcripts produced by in vitro transcription with T7 RNA polymerase were heated in boiling water bath for 5 minutes and cooled to room temperature for annealing in 30 mM HEPES-KOH (pH 7.4), 100 mM potassium acetate, and 2 mM magnesium acetate. Short dsRNA fragments were obtained by complete digestion with *E. coli* RNaseIII (500 nM) to the substrate dsRNA in 30 mM Tris-HCl (pH 8.0), 160 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA, and 10 mM MgCl<sub>2</sub> at 37°C for 4 hours. siRNA was extracted with phenol/chloroform/isopropanol, followed by ethanol precipitation. Products were analyzed by non-denaturation polyacrylamide gel electrophoresis and stained with ethidium bromide. Concentration of siRNA was determined using a standard extinction coefficient of dsRNA of approximately 20 bp.

PCR primer pairs were as follows: sense S1P<sub>1</sub> 3'-UTR: forward 5'-TCCTAATACGACTCACTATAGGGCTGTTGATACTGAGGGAAGC-3', reverse 5'-TCCACAACCTCCTTCTGATGA-3'; antisense S1P<sub>1</sub> 3'-UTR: forward 5'-CTGTTGATACTGAGGGAAGC-3', reverse 5'-TCCTAATACGACTCACTATAGGGTCCACAACCTCCTTCTGATGA-3'; sense S1P<sub>5</sub> 3'-UTR: forward 5'-TCCTAATACGACTCACTATAGGGCCCTTTGCAAGAACAGACTGA-3', reverse 5'-



CCAGCCCTTAGCACACTCTTA-3'; antisense S1P<sub>5</sub> 3'-UTR: forward 5'-CCCTTTGCAAGAACAGACTGA-3', reverse 5'-TCCTAATACGACTCACTATAGGGCCAGCCCTTAGCACACTCTTA-3'; sense β-gal: forward 5'-TCCTAATACGACTCACTATAGGGACTGGCAGATGCACGGT-TAC-3', reverse 5'-CGTAGGTAGTCACGCAACTC-3'; antisense β-gal: forward 5'-ACTGGCAGATGCACGGTTAC-3', reverse 5'-TCCTAATACGACTCACTATAGGGCGTAGGTAGTCACGCAACTC-3'.

**Endothelial cell chemotaxis assay.** MEECs were isolated from S1P<sub>1</sub><sup>+/+</sup> and S1P<sub>1</sub><sup>-/-</sup> mice as described (39). MEECs were transfected with 200 nM of siRNAs using oligofectamine (Invitrogen) as described (26). After 48 hours, cell migration assay was performed as previously described (3).

**Preparation of cationic liposomes.** Cationic liposomes were prepared with an 11:9 molar ratio of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; Avanti Polar Lipids Inc.) and cholesterol (Sigma-Aldrich) in chloroform (10 mg/ml). Chloroform was evaporated using nitrogen stream, and residual solvent was removed by placing lipid mixtures under vacuum pump for 1 hour. Dried lipids were resuspended in 5% sucrose and sonicated for 20 minutes at 37°C. To generate unilamellar liposome vesicles, we passed the hydrated mixture through the Mini-Extruder (Avanti Polar Lipids Inc.).

**Northern blot analysis.** Total RNA from tissues or MEECs was isolated using RNA-STAT (Tel-Test Inc.) as described by the manufacturer. Total RNA (5 μg) was subjected to Northern blot analysis using cDNA probes specific to mouse S1P<sub>1</sub> or GAPDH as described (5).

**Tumor angiogenesis and growth.** Experiments using animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center. Lewis lung carcinoma cells (10<sup>6</sup>; American Type Culture Collection) were mixed with 100 μl of Matrigel solution (BD Biosciences – Discovery Labware) at 4°C and injected subcutaneously into the dorsal skin of nude mice and allowed to grow. After 5 days, siRNA-liposome complexes were locally administered into growing tumors by intratumoral injection on every 3rd day. siRNA-liposome complexes (60 μl) were obtained by incubating 15 μg of siRNA and 25 μl of liposomes (10 mg/ml) in 5% glucose for 20 minutes at room temperature. The complexes were injected into 4–6 sites on tumor. Tumor growth was determined using a caliper to measure tumor diameter. Tumor volume was calculated by the equation,  $V = (L \times W^2) \times 0.5$  ( $V$ , volume;  $L$ , length; and  $W$ , width). Angiogenesis in tumor sections was evaluated by counting the number of CD31-positive blood vessels in the 5 most densely vascularized areas within tumor as described previously (26). All CD31 positive vessels (regardless of shape, branch points, size, lumens) were considered as a single vascular unit and were counted. Tumor vascular stabilization was assessed by counting all positive cells that expressed the α-SMA antigen in the immunofluorescence assay.

**β-gal (LacZ) staining of tissues.** Tissues were dissected in cold PBS and fixed in 0.2% glutaraldehyde and 1.5% paraformaldehyde in PBS, pH 7.4, at room temperature for 90 minutes, then washed in PBS. Staining was performed at 37°C or room temperature in 0.02% X-gal, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, and 2 mM MgCl<sub>2</sub> in PBS overnight. The stained tissues were washed, postfixed in 4% paraformaldehyde, and embedded in paraffin; 5 μm-sections were then cut and imaged as described (40).

**In vivo Matrigel angiogenesis assay.** In vivo angiogenesis assay was performed as previously described (4). Briefly, female athymic nude mice were injected subcutaneously with 0.2 ml of Matrigel mixture containing 2 μM of siRNA with or without FGF-2 (4 μg/ml). After 7 days, Matrigel plugs were harvested, fixed with 10% formalin in PBS, and embedded in paraffin. Sections were stained with H&E or anti-CD31 antibody as described below.

**Immunohistochemistry.** Tissues sections were postfixed in 4% paraformaldehyde for 20 minutes, treated in 3% hydrogen peroxide/methanol for 30 minutes, blocked in 10% normal goat serum for 20 minutes, incubated with anti-CD31 antibody (BD Biosciences – Pharmingen) for 1 hour at room temperature, biotinylated goat-anti mouse IgG (DakoCytomation) for 30 minutes, and VECTASTAIN Elite ABC biotin-avidin-peroxidase complex (Vector Laboratories) for 30 minutes. Sections were then developed with diaminobenzidine and diaminobenzidine enhancer (Vector Laboratories). Smooth muscle cells were stained with anti-α-SMA antibody (Sigma-Aldrich). Rabbit polyclonal antibodies against murine S1P<sub>1</sub> were obtained from S. Mandala at Merck Research Laboratories. The S1P<sub>1</sub> staining was performed as previously described (23).

**Statistical analysis.** All results are expressed as means ± SE, and  $n$  represents the number of individual experiments. Statistical analyses were performed with Student's  $t$  test.

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