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**Changes in S1P<sub>1</sub> and S1P<sub>2</sub> expression during embryonal development  
and primitive endoderm differentiation of F9 cells**

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## **Abstract**

Sphingosine 1-phosphate (S1P) is a ligand for S1P family receptors (S1P<sub>1</sub>- S1P<sub>5</sub>). Of these receptors, S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> are ubiquitously expressed in adult mice, while S1P<sub>4</sub> and S1P<sub>5</sub> are tissue-specific. However, little is known of their expression during embryonal development. We performed Northern blot analyses in mouse embryonal tissue and found that such expression is developmentally regulated. We also examined the expression of these receptors during primitive endoderm (PrE) differentiation of mouse F9 embryonal carcinoma (EC) cells, a well-known *in vitro* endoderm differentiation system. *SIP*<sub>2</sub> mRNA was abundantly expressed in F9 EC cells, but little *SIP*<sub>1</sub> and no *SIP*<sub>3</sub>, *SIP*<sub>4</sub>, or *SIP*<sub>5</sub> mRNA was detectable. However, *SIP*<sub>1</sub> mRNA expression was induced during EC-to-PrE differentiation. Studies using small interference RNA of *SIP*<sub>1</sub> indicated that increased S1P<sub>1</sub> expression is required for PrE differentiation. Thus, S1P<sub>1</sub> may play an important function in PrE differentiation that is not substituted for by S1P<sub>2</sub>.

**Keywords:** Sphingolipid; Sphingosine 1-phosphate; S1P receptor; S1P<sub>1</sub>; Embryonal development; Differentiation; Endoderm; F9 cells

## Introduction

As an extracellular signaling molecule, sphingosine 1-phosphate (S1P) affects several cellular processes including proliferation, migration, differentiation, actin cytoskeleton reorganization, and adherens junction assembly [1]. Through these cellular responses, S1P plays important functions in the vascular and immune systems. S1P is a ligand for S1P/Edg family receptors, which are seven-span membrane proteins coupled to heterotrimeric G proteins. To date, five members of this family have been identified in mammals, S1P<sub>1</sub>/Edg1, S1P<sub>2</sub>/Edg5/H218, S1P<sub>3</sub>/Edg3, S1P<sub>4</sub>/Edg6, and S1P<sub>5</sub>/Edg8 [2-5]. Of the five, S1P<sub>1</sub> is arguably the most important, since only disruption of the *S1P<sub>1</sub>* gene causes embryonic lethality, which is due to severe hemorrhage resulting from a defect in vascular stabilization [6]. In contrast, *S1P<sub>2</sub>* and *S1P<sub>3</sub>* knockout mice are viable and exhibit only mild phenotypes [7-9]. In adult mice, S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> are known to be ubiquitously expressed, whereas S1P<sub>4</sub> and S1P<sub>5</sub> have been detected only in specific tissues [4,7]. However, the expression of the receptors has not been well studied during embryonal stages.

Each S1P receptor specifically couples to certain G proteins. S1P<sub>1</sub> couples only with G<sub>i</sub> proteins [10,11], whereas S1P<sub>2</sub> and S1P<sub>3</sub> couple with G<sub>i</sub>, G<sub>q</sub> and G<sub>12/13</sub> [11]. Although S1P<sub>2</sub> and S1P<sub>3</sub> couple with the same set of G proteins, their affinities differ. S1P<sub>2</sub> exhibits the highest affinity toward G<sub>12/13</sub>, whereas S1P<sub>3</sub> appears to primarily couple with G<sub>q</sub>, then G<sub>i</sub> [9,12]. Reflecting their distinct preferences and affinities for specific G-proteins, S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> can induce different, sometimes opposite, cellular responses. For example, S1P<sub>1</sub> and S1P<sub>3</sub> stimulation by S1P induces cell

migration, but  $S1P_2$  stimulation inhibits it [12-14]. Thus, regulating the expression of S1P receptors to provide a cellular balance may determine the effect of S1P on cellular processes.

Differentiation during embryogenesis is accompanied by dynamic changes in protein expression that promote cell and tissue-specific development. To date, information regarding the expression of S1P receptors during embryogenesis is limited. Changes in  $S1P_1$  expression during mouse embryonic development have been observed in the embryo proper and in two extra-embryonic tissues (the allantois and the yolk sac) [15].  $S1P_1$  expression patterns during mice embryogenesis have also been examined [6]. In the present study we performed Northern blot analyses in mouse embryonal tissues and found that  $SIP_1$ ,  $SIP_2$ , and  $SIP_3$  mRNA expression is developmentally regulated. In addition, we examined the expression and regulation of these receptors using an F9 *in vitro* differentiation model. F9 embryonal carcinoma (EC) cells are a well-known system for extra-endoderm differentiation in early mouse embryogenesis. In the presence of retinoic acid (RA), F9 EC cells differentiate toward primitive endoderm (PrE) [16], and if dibutyryl cyclic AMP ( $bt_2cAMP$ ) is added together with the RA, the F9 EC cells differentiate to parietal endoderm (PE) via PrE [17]. We examined the expression profile for each S1P receptor throughout this differentiation. Little  $S1P_1$  is expressed in F9 EC cells, but expression is induced during PrE differentiation. This expression is in direct contrast to  $S1P_2$  expression, which is down-regulated during the EC-to-PE (via PrE) differentiation [18]. The other S1P receptors are not detectable at either stage. Thus, the expression profile of the S1P receptors is altered during

differentiation. We also report here that *SIP<sub>1</sub>* small interference RNA (siRNA) inhibited PrE differentiation, suggesting that the induction of *SIP<sub>1</sub>* is required for this differentiation.

## Materials and methods

*Cell culture and transfection.* Mouse F9 cells were grown on 0.1% gelatin-coated dishes in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO; D6429) containing 10% fetal bovine serum and supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. For differentiation experiments, 1 µM all-*trans*-RA (Sigma) with or without 250 µM bt<sub>2</sub>cAMP (Sigma) was added to the medium. Transfections were performed using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA).

*Plasmids.* The pCE-puro plasmid, a mammalian expression vector, contains a puromycin-resistant gene used for selecting stable transformants [19]. The pCE-puro 3xFLAG-4 plasmid is a derivative of the pCE-puro plasmid and was designed to produce a C-terminal triple FLAG (3xFLAG)-tagged protein. The pCE-puro SIP<sub>1</sub>-3xFLAG and pCE-puro SIP<sub>2</sub>-3xFLAG plasmids encoding *SIP<sub>1</sub>-3xFLAG* and *SIP<sub>2</sub>-3xFLAG*, respectively, were constructed by cloning *SIP<sub>1</sub>* or *SIP<sub>2</sub>* into the pCE-puro 3xFLAG-4 plasmid.

*Preparing stable transformants of F9 cells.* F9 derivatives that stably express SIP<sub>1</sub>-3xFLAG (F9-18 and F9-19) or SIP<sub>2</sub>-3xFLAG (F9-14 and F9-15) were prepared by transfection of the pCE-puro SIP<sub>1</sub>-3xFLAG or pCE-puro SIP<sub>2</sub>-3xFLAG plasmid, respectively, into F9 cells and by subsequent puromycin selection at 0.5 µg/ml for 1 week. F9-17 cells are control cells that were generated by transfection of pCE-puro 3xFLAG-4 vector and subsequent puromycin-selection.

*Northern blot analysis and reverse transcription (RT)-PCR.* To examine the embryonal development-specific expression of *SIP*<sub>1</sub>, *SIP*<sub>2</sub>, and *SIP*<sub>3</sub> mRNA, blots containing 20 µg total RNA from mouse embryos at several stages of development were purchased from Seegene (Seoul, Korea). To likewise examine mRNA expression in F9 cells, blots were prepared using total RNA isolated from F9 EC, PrE, and PE cells with Trizol Reagent (Invitrogen) as instructed by the manufacturer. Fixed amounts of total RNA (20 µg) were separated on a 1% agarose/2.2 M formaldehyde gel and transferred to positively charged nylon membranes (Roche Diagnostics, Indianapolis, IN).

<sup>32</sup>P-labeled probes were prepared from cDNA for each SIP receptor, which had been amplified using specific primers (for *SIP*<sub>1</sub>, 5'-ATGGTGTCCACTAGCATCCCGG-3' and 5'-TTAGGAAGAAGAATTGACGTTTCCAGACG-3'; for *SIP*<sub>2</sub>, 5'-CCACCATGGGCGGCTTATACTCAGAGTACC-3' and 5'-TCAGACCACTGTGTTACCCTCC-3'; for *SIP*<sub>3</sub>, 5'-AAGCCATGGCAACCACGCATGCGCAGG-3' and 5'-TCACTTGCAGAGGACCCCGTTCTG-3'; for *SIP*<sub>4</sub>, 5'-GGCCATGAACATCAGTACCTGG-3' and 5'-CTAGGTGCTGCGGACGCTGGAAATGC-3'; and for *SIP*<sub>5</sub>, 5'-GTGCTGGTCCGCCACCCTCGCTTCC-3' and 5'-TCAGTCTGTAGCAGTAGGCACCAGG-3') then labeled with [<sup>32</sup>P] using the TAKARA Bio (Shiga, Japan) random primer DNA labeling kit, version 2.

Hybridization of the probes to the mRNA blots was carried out in ExpressHyb Hybridization Solution (BD Biosciences Clontech, Palo Alto, CA) at 68°C for 2 h. Radioactivities were quantified using a Bio-Imaging Analyzer BAS2500 (Fuji Photo Film, Tokyo, Japan).

RT-PCR was performed using total RNAs prepared from untreated or differentiated F9 cells as templates and SuperScript™ One-Step RT-PCR System with Platinum Taq (Invitrogen). The primers used included for *SIP<sub>1</sub>*, 5'-ATGGTGTCCACTAGCATCCCGG-3' and 5'-TTAGGAAGAAGAATTGACGTTTCCAGACG-3'; for *SIP<sub>2</sub>*, 5'-CCACCATGGGCGGCTTATACTCAGAGTACC-3' and 5'-TCAGACCACTGTGTTACCCTCC-3'; for *actin*, 5'-ATGGATGACGATATCGCTGCGCTGG-3' and 5'-CTAGAAGCACTTGCGGTGCACGATG-3'; for *GATA-4*, 5'-ATGTACCAAAGCCTGGCCATGGCCGCCAACC-3' and 5'-TTACGCGGTGATTATGTCCCCATGACTGTC-3'; and for *GATA-6*, 5'-ATGTACCAGACCCTCGCCGCCCTGTCCAGC-3' and 5'-TCAGACCAGGGCCAGAGCACACCAAGAATCC-3'.

*Phalloidin staining.* Phalloidin staining of F-actin was performed on F9 EC, PrE, and PE cells as described previously using a fluorescence microscopy AxioSkop 2 plus (Carl Zeiss, Oberkochen, Germany) [19].

*Immunoblotting.* Cells were washed twice with PBS, suspended in buffer A (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol), and sonicated. After removal of cell debris by centrifugation, protein concentrations of the resulting lysates were quantified using BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Samples were mixed with 1X SDS sample buffer (buffer A containing a trace amount of bromophenol blue and 10% 2-mercaptoethanol) and boiled at 100°C for 3 min. Proteins were separated by SDS-PAGE and transferred to Immobilon™ polyvinylidene difluoride membrane (Millipore, Billerica, MA). After it was blocked with 5% skim milk in TBST (Tris-buffered-saline with 0.05% Tween 20), the membrane was incubated for 1 h with an anti-Dab2 (BD Biosciences Clontech) or anti-actin (Sigma) antibody, each diluted 1/1000. The membranes were washed three times with TBST, incubated for 1 h with an HRP-conjugated anti-mouse IgG F(ab')<sub>2</sub> fragment (1/7500 dilution; Amersham Biosciences, Piscataway, NJ), then washed three times with TBST three times and once with TBS. Labeling was detected by the ECL detection method (Amersham Biosciences).

*RNA interference.* The siRNAs were purchased from Greiner Bio-One (Kremsmünster, Austria). The nucleotide sequences of the respective RNAs targeted by the siRNAs are as follows: *SIP<sub>1</sub>* siRNA, 5'-AACTGACTTCAGTGGTGTTCA-3' (nucleotides 137-157 of mouse *SIP<sub>1</sub>* ORF); *SIP<sub>2</sub>* siRNA, 5'-AAGGTCAAGCTCTACGGCAGT-3' (nucleotides 406-426 of mouse *SIP<sub>2</sub>* ORF); and scrambled *SIP<sub>1</sub>* siRNA, 5'-AATTGATGATCGTTCCGGACT-3'. F9 cells were

transfected with the appropriate siRNA together with the pCE-puro vector, which carries a puromycin resistant gene. Sixteen h after transfection on day 0, 1  $\mu$ M RA and 0.5  $\mu$ g/ml puromycin were added to the culture medium to initiate differentiation and to kill the untransfected cells, respectively. Medium was changed every 2 days, and puromycin was used for 48 h. Cells were recovered on days 0 through 4, and total cell lysates were prepared. Dab2 and actin were detected in the cell lysates by immunoblotting. Total RNA was prepared from cells on day 1 (40 h after transfection), and subjected to RT-PCR as described above.

## Results

### *Regulation of $SIP_1$ , $SIP_2$ , and $SIP_3$ expression during embryonal development*

$SIP_1$ ,  $SIP_2$ , and  $SIP_3$  are expressed ubiquitously among tissues in adult mice [7]. However, their developmental stage-specific expression patterns have not been studied in depth. Therefore, we performed Northern blot analyses using a mouse embryo full stage blot. As shown in Fig. 1,  $SIP_1$  mRNA is abundantly expressed throughout mouse embryogenesis with a transient peak around embryonic day (E) 8.5. The expression of  $SIP_2$  mRNA is high in earlier developmental stages but low in later stages. The highest  $SIP_2$  mRNA expression was observed at E7.5; the expression gradually decreased toward E18.5. Conversely, the  $SIP_3$  mRNA is low in earlier developmental stages (E4.5 to E9.5) but rapidly increase at E10.5, reaching a maximal level at E11.5. The expression then gradually decreases. These results indicate that  $SIP_1$ ,  $SIP_2$ , and  $SIP_3$  each exhibits a characteristic, developmental stage-specific expression pattern.

### *Induction of $SIP_1$ during PrE differentiation of F9 cells*

Having determined that the expression of SIP receptors changes during development, we set out to explore the molecular mechanisms responsible. However, a whole animal model is not suitable for such analysis. Therefore, we used F9 cells, a well-known model system for extra-endoderm differentiation in early mouse embryogenesis. Reportedly,  $SIP_2$  mRNA is gradually down-regulated during the EC-to-PE (via PrE) differentiation of F9 cells [18]. However, in that study expression of the other SIP receptors was not examined. Therefore, we investigated the expression

levels of all S1P receptors in F9 cells at each differentiation stage. Treatment with 1  $\mu$ M RA for 3-4 days induced F9 EC cells to differentiate into PrE cells, so that the cells became enlarged, and numerous, F-actin-dense projections appeared (Fig. 2). Combined treatment with 1  $\mu$ M RA and 250  $\mu$ M bt<sub>2</sub>cAMP induced further differentiation of PrE cells into PE cells. PE cells were readily distinguished from PrE cells by their morphology, which is characterized as round shapes with long cell processes, usually two (Fig. 2). Most of the EC cells had differentiated to PE cells by 6 days post-treatment.

We prepared total RNA from F9 cells treated with RA alone or with RA/bt<sub>2</sub>cAMP for 0, 3, or 6 days, and performed Northern blot analyses using specific probes for each S1P receptor. Untreated, F9 EC cells expressed a significant amount of *SIP*<sub>2</sub> mRNA (Fig. 3). As reported [18], the *SIP*<sub>2</sub> mRNA levels decreased by day 3 of treatment with RA/bt<sub>2</sub>cAMP, and were further decreased at day 6 (Fig. 3). Treatment with RA alone did not cause a reduction in *SIP*<sub>2</sub> at day 3, but the levels were slightly decreased by day 6. In contrast, *SIP*<sub>7</sub> mRNA was barely detected in F9 EC cells, however its expression was induced by a 3 day treatment with RA or with RA/bt<sub>2</sub>cAMP (Fig. 3). Overall, the *SIP*<sub>7</sub> levels were higher in the presence of bt<sub>2</sub>cAMP than in its absence, but the expression decreased in cells treated for 6 days with either treatment (Fig. 3). These results indicate that *SIP*<sub>7</sub> mRNA is induced during EC-to-PrE differentiation. No *SIP*<sub>3</sub>, *SIP*<sub>4</sub>, or *SIP*<sub>5</sub> mRNA was detected at any differentiation stage (Fig. 3).

We further investigated the *SIP*<sub>7</sub> mRNA induction over time. When F9 EC cells were treated with RA alone, the *SIP*<sub>7</sub> mRNA levels gradually increased, reaching

maximum at day 3 (Fig. 4A). Maximum expression was maintained through day 5, but the levels slightly decreased at day 6. Thus,  $SIP_1$  was induced during the EC-to-PrE differentiation. Treatment with RA/bt<sub>2</sub>cAMP induced more  $SIP_1$  mRNA expression than treatment with RA alone did, however, after reaching maximum level at day 3, the  $SIP_1$  levels gradually decreased (Fig. 4A). These results suggest that in the course of successive differentiation from EC-to-PrE-to-PE,  $SIP_1$  mRNA transiently peaks at the PrE stage.

Consistent with the previous report [18], during treatment with RA/bt<sub>2</sub>cAMP  $SIP_2$  mRNA levels slightly increased at day 1 then gradually decreased, reaching minimal levels at day 4 (Fig. 4B). In contrast, RA treatment caused a large increase at day 1, followed by only a slight decrease. At days 3 and 4, around which PrE differentiation was complete, the  $SIP_2$  levels were equivalent to those observed in the untreated EC cells. These results indicate that  $SIP_2$  levels are high at the EC and PrE stages but are nearly absent at the PE stage. Thus, SIP may function only through  $SIP_1$  at the PE stage in contrast to the EC stage, at which  $SIP_2$  is predominant.

#### *Requirement of $SIP_1$ in the PrE differentiation*

To investigate whether  $SIP_1$  or  $SIP_2$  is involved in the differentiation of EC cells into PrE cells, we prepared siRNAs specific to each of their sequences, as well as  $SIP_1$  scrambled siRNA. RT-PCR using total RNAs prepared from F9 cells transfected with the  $SIP_1$ ,  $SIP_2$ , or the scrambled siRNA confirmed a reduction in the  $SIP_1$  and  $SIP_2$  mRNAs by their specific siRNAs (Fig. 5A). F9 cells were transfected with each of these

siRNAs and treated with RA to induce PrE differentiation. Differentiation to PrE was monitored by investigating the expression of the PrE marker Dab2 [20]. As shown in Fig. 5B, F9 cells transfected with the scrambled siRNA expressed two spliced isoforms of Dab2, p96 and p67, around days 3 and 4 of treatment, much like the expression in untransfected F9 cells (data not shown). Similar bands were also observed in cells transfected with the *SIP<sub>2</sub>* siRNA, indicating that it had no effect. However, the *SIP<sub>1</sub>* siRNA significantly inhibited the induction of Dab2 expression. Further RT-PCR analysis revealed that similar induction of two other PrE markers, the transcription factors GATA-4 [21] and GATA-6 [22], was also reduced by the *SIP<sub>1</sub>* siRNA but not by the *SIP<sub>2</sub>* siRNA (Fig. 5C). These results suggest that the induction of *SIP<sub>1</sub>* is required for the progression of PrE differentiation.

#### *Overexpression of SIP<sub>1</sub> has no effect on PrE differentiation of F9 cells*

We next examined whether overexpression of *SIP<sub>1</sub>* in F9 EC cells could stimulate PrE differentiation. Two independent F9 cells stably expressing *SIP<sub>1</sub>-3xFLAG* or *SIP<sub>2</sub>-3xFLAG* were incubated with RA/bt<sub>2</sub>cAMP, and the expression of Dab2 was examined. As shown in Fig. 6, neither overexpression of *SIP<sub>1</sub>-3xFLAG* nor that of *SIP<sub>2</sub>-3xFLAG* stimulated PrE differentiation. Thus, *SIP<sub>1</sub>* expression is necessary but not sufficient for the PrE differentiation.

## Discussion

In the present study, we have demonstrated that *SIP<sub>1</sub>*, *SIP<sub>2</sub>*, and *SIP<sub>3</sub>* mRNA each exhibit a characteristic, developmental stage-specific expression pattern. *SIP<sub>1</sub>* mRNA transiently increases around E8.5, soon after the beginning of vasculogenesis. Vasculogenesis is initiated by the birth of angioblasts (endothelial cell precursors), followed by angioblast aggregation in extraembryonic regions. High levels *SIP<sub>1</sub>* expression have been observed in dorsal aorta, intersomitic arteries, and capillaries at E9.5 [6]. Thus, the high expression of *SIP<sub>1</sub>* at E8.5 and E9.5 may be related to the active vasculogenesis.

Although *SIP<sub>1</sub>* is the most important S1P receptor in the development of a stable and mature vascular system, *SIP<sub>2</sub>* and *SIP<sub>3</sub>* also function coordinately. *SIP<sub>1</sub>*-null mice appeared normal up to E11.5, but bleeding was evident at E12.5 [6]. Wide-spread hemorrhage and severe edema were observed at E13.5, and the mice could not survive beyond E14.5 [6]. Although both *SIP<sub>2</sub>*- and *SIP<sub>3</sub>*-null mice developed normally vascular systems, *SIP<sub>1</sub>* *SIP<sub>2</sub>* double knockout mice and *SIP<sub>1</sub>* *SIP<sub>2</sub>* *SIP<sub>3</sub>* triple knockout mice exhibited more severe vascular defects than those of the *SIP<sub>1</sub>* single knockout mice [23]. About half of the triple null mice exhibited bleeding at E10.5, and most of those died at E11.5. This timing coincides with a rapid induction of *SIP<sub>3</sub>* at E10.5 (Fig. 1), suggesting that *SIP<sub>3</sub>* has some function in the development of vascular system around these embryonic stages.

We have also demonstrated here that the expression patterns of *SIP<sub>1</sub>* and *SIP<sub>2</sub>* mRNAs change during the differentiation of F9 cells, with EC expressing little *SIP<sub>1</sub>* and

high levels of *SIP*<sub>2</sub>; PrE, high levels of *SIP*<sub>1</sub> and high levels of *SIP*<sub>2</sub>; and PE, intermediate levels of *SIP*<sub>1</sub> and little *SIP*<sub>2</sub> (Figs 3 and 4). In addition, analysis using siRNA revealed that *SIP*<sub>1</sub> expression is required for PrE differentiation (Fig. 5). Considering that PrE cells contain a high amount of *SIP*<sub>2</sub>, *SIP*<sub>1</sub> might have an important function in PrE differentiation that is not substituted for by *SIP*<sub>2</sub>. Such differences in function are well established in regards to cell migration [12-14].

Differentiation is a complicated process, in which many transcriptional factors, and thus many genes, are temporally regulated. During F9 differentiation, the most immediate gene response is likely mediated by RA receptors bound to RA. Two classes of such receptors are known, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each of which has three isotypes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). These receptors form homodimers or heterodimers and activate the transcription of genes containing the retinoic acid response element (RARE). The RXR $\alpha$ /RAR $\gamma$  heterodimer has been shown to be important for the PrE and PE differentiation of F9 cells [24].

Genes exhibiting changes in their levels during RA-mediated PrE differentiation are classified mainly into two groups, those whose expression changes within the first 24 h and those peaking around day 3 [25,26]. The first group includes genes directly activated by RA, but also those activated indirectly. Several transcriptional factors, including AP-1, GATA-4, and GATA-6, are induced around day 3 [21,27-29] and may be involved in the expression of genes in the second group. We found that *SIP*<sub>1</sub> mRNA expression reaches maximum at day 3, whereas *SIP*<sub>2</sub> mRNA transiently increases on day 1. These results suggest that *SIP*<sub>1</sub> and *SIP*<sub>2</sub> belong to the second and first groups,

respectively, although the transcription factors involved in their increases are presently unknown. Using the transcription factor database TRANSFAC and accompanying programs (<http://transfac.gbf.de/TRANSFAC>), we found two putative AP-1 binding sites in the *SIP<sub>1</sub>* promoter region. Thus, it is possible that AP-1 is responsible for the induction of *SIP<sub>1</sub>* during EC-to-PrE differentiation.

*SIP* is abundant in blood [30] and, therefore, in extracellular spaces. However, *SIP* also exists intracellularly in most cells, although the levels are quite low. Intracellular *SIP* is proposed to function as a second messenger of various stimuli such as growth factors and cytokines [31]. We recently reported that sphingosine kinase and *SIP* lyase, both of which are involved in *SIP* metabolism, were up-regulated during F9 PrE differentiation [19]. Moreover, *SIP* accumulation, resulting from the disruption of the *SIP* lyase gene or the overproduction of sphingosine kinase, resulted in accelerated of the PrE differentiation [19], suggesting that intracellular *SIP* is also involved in this process. Thus, it appears that extracellular and intracellular *SIP* cooperatively regulate PrE differentiation.

Expression of *SIP<sub>1</sub>* is known to be altered during several types of development and differentiation stages. For example, in human endothelial cells the expression of *SIP<sub>1</sub>* was rapidly increased by treatment with phorbol 12-myristate 13-acetate, which induces differentiation of these cells into capillary-like, tubular structures [32]. Changes in *SIP<sub>1</sub>* expression during embryonic development has also been shown in the embryo proper and in two extra-embryonic tissues (the allantois and the yolk sac) [15].

Treatment of endothelial cells with vascular endothelial growth factor (VEGF) rapidly

induces expression of S1P<sub>1</sub>, which is attenuated by the tyrosine kinase inhibitor genistein and by the protein kinase C inhibitor calphostin C [33]. Moreover, S1P<sub>1</sub> levels change during T cell maturation and activation. In the course of T cell maturation in the thymus, S1P<sub>1</sub> is strongly upregulated [34,35]. Later, when mature naïve T cells encounter their specific antigens in the lymph node, S1P<sub>1</sub> is downregulated to prevent S1P gradient-induced exit from the lymph node [34]. After activation and clonal expansion, T cells restore their S1P<sub>1</sub> levels and exit [34]. The molecular mechanism regulating such S1P<sub>1</sub> expression is unclear, however, and further analysis is needed. For this purpose, the well-examined F9 system will be useful.

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## Figure legends

Fig. 1. Expression of *SIP*<sub>1</sub>, *SIP*<sub>2</sub>, and *SIP*<sub>3</sub> mRNA during mouse embryonal development. (A) A <sup>32</sup>P-labeled *SIP*<sub>1</sub>, *SIP*<sub>2</sub>, or *SIP*<sub>3</sub> probe was hybridized to 20 μg of total RNA from each stage of a mouse embryo (upper three panels). To demonstrate uniform RNA loading, 28S and 18S ribosomal RNAs were stained with ethidium bromide (bottom panel). Embryo samples from E4.5 to E6.5 included extra-embryonic tissues and maternal uterus, while the samples from E7.5 to E9.5 were conceptuses, including embryo and extraembryonic tissues. The samples from E10.5 to E18.5 were solely embryos. (B) Radioactivity associated with each SIP receptor in (A) was quantified using a BAS-2500 bioimaging analyzer and expressed as a percentage relative to the value at E4.5.

Fig. 2. Differentiation of F9 EC cells to PrE and PE cells. F9 EC cells are differentiated to PrE cells by treatment with 1 μM RA over 3 days. Co-incubation with 1 μM RA and 250 μM bt<sub>2</sub>cAMP causes differentiation of F9 EC cells to PrE around day 3 and further differentiation to PE at days 5 to 6. All cells were fixed, permeabilized, stained with phalloidin to visualize F-actin, and observed under a fluorescence microscope.

Fig. 3. Only *SIP*<sub>1</sub> and *SIP*<sub>2</sub> are expressed and regulated during F9 differentiation. Total RNA was prepared from F9 cells incubated with 1 μM RA or with 1 μM RA/250 μM bt<sub>2</sub>cAMP for the indicated times. Fixed amounts of RNA (20 μg) were separated by agarose gel electrophoresis and subjected to Northern blotting using a <sup>32</sup>P-labeled probe

specific for the indicated *SIP* receptor or for *actin* to demonstrate uniform RNA loading.

Fig. 4. Kinetics of *SIP*<sub>1</sub> and *SIP*<sub>2</sub> expression during F9 differentiation. F9 cells were incubated with 1 μM RA or with 1 μM RA/250 μM bt<sub>2</sub>cAMP for the indicated times. Total RNA was prepared from each culture and equivalent amounts (20 μg) were subjected to Northern blotting using a <sup>32</sup>P-labeled probe for *SIP*<sub>1</sub> (A) or *SIP*<sub>2</sub> (B). Radioactivities associated with the *SIP*<sub>1</sub> and *SIP*<sub>2</sub> mRNAs were quantified using a Bio-Imaging Analyzer BAS2500. Values are illustrated relative to the amount of the *SIP*<sub>1</sub> mRNA at day 1 or the *SIP*<sub>2</sub> mRNA at day 0, and represent the mean ± SD from three independent experiments.

Fig. 5. Involvement of *SIP*<sub>1</sub> in PrE differentiation. F9 cells were transfected with *SIP*<sub>1</sub> siRNA, *SIP*<sub>2</sub> siRNA, or *SIP*<sub>1</sub> scrambled (sc) siRNA and incubated for 16 h. PrE differentiation was then initiated by the addition of 1 μM RA. (A) Total RNA was prepared from F9 cells at day 1 following RA treatment and subjected to RT-PCR analysis using primers specific for *SIP*<sub>1</sub>, *SIP*<sub>2</sub>, or *actin*. (B) At the indicated times following RA treatment, total lysates were prepared. Proteins (10 μg) were separated by SDS-PAGE, followed by immunoblotting with an anti-Dab2 antibody, or, to demonstrate uniform protein loading, an anti-actin antibody. (C) Total RNA was prepared at the indicated times following RA treatment and was subjected to RT-PCR analysis using primers specific for *GATA-4*, *GATA-6*, or *actin*.

Fig. 6. Forced expression of S1P<sub>1</sub> or S1P<sub>2</sub> has no effect on PrE differentiation. F9 cells stably expressing vector only (F9-17), S1P<sub>1</sub>-3xFLAG (F9-18 and F9-19), or S1P<sub>2</sub>-3xFLAG (F9-14 and F9-15) were incubated with 1  $\mu$ M RA and 250  $\mu$ M bt<sub>2</sub>cAMP for the indicated times. Total proteins (15  $\mu$ g) were separated by SDS-PAGE, followed by immunoblotting with an anti-Dab2 or anti-actin antibody.











