

# Sphingosine 1-phosphate as a regulator of osteoclast differentiation and osteoclast–osteoblast coupling

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**Sphingosine 1-phosphate (S1P), produced by sphingosine kinase (SPHK), acts both by intracellular and extracellular modes. We evaluated the role of SPHK1 and S1P in osteoclastogenesis using bone marrow-derived macrophage (BMM) single and BMM/osteoblast coculture systems. In BMM single cultures, the osteoclastogenic factor receptor activator of NF- $\kappa$ B ligand (RANKL) upregulated SPHK1 and increased S1P production and secretion. SPHK1 siRNA enhanced and SPHK1 overexpression attenuated osteoclastogenesis via modulation of p38 and ERK activities, and NFATc1 and c-Fos levels. Extracellular S1P had no effect in these cultures. These data suggest that intracellular S1P produced in response to RANKL forms a negative feedback loop in BMM single cultures. In contrast, S1P addition to BMM/osteoblast cocultures greatly increased osteoclastogenesis by increasing RANKL in osteoblasts via cyclooxygenase-2 and PGE<sub>2</sub> regulation. S1P also stimulated osteoblast migration and survival. The RANKL elevation and chemotactic effects were also observed with T cells. These results indicate that secreted S1P attracts and acts on osteoblasts and T cells to augment osteoclastogenesis. Taken together, S1P plays an important role in osteoclastogenesis regulation and in communication between osteoclasts and osteoblasts or T cells.**

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## Introduction

Bone is a tissue continually regenerated through a remodeling process. In response to biochemical or mechanical signal, the basic multicellular unit (BMU) is organized at discrete sites in bone to perform remodeling. Once the BMU is

formed, osteoclasts are activated to resorb old bone. Osteoblasts are recruited to the resorption area and new bone matrix is produced to fill the resorption pits. Subsequent mineralization of the matrix completes the remodeling process. Therefore, the coordinated action of osteoclasts and osteoblasts is important for proper bone remodeling, and an imbalance in the activity of osteoclasts and osteoblasts leads to metabolic bone disorders. However, molecular mechanisms for the intricate interplay between the bone resorbing and forming cells have not been well characterized. Other cells present in bone such as vascular endothelial cells, bone stromal cells, and immune cells may also communicate with osteoclasts and osteoblasts, influencing bone metabolism under both physiological and pathological conditions.

Osteoclasts are specialized polykaryons that differentiate from monocyte–macrophage lineage precursors of hematopoietic cells. Receptor activator of NF- $\kappa$ B ligand (RANKL), a tumor necrosis factor (TNF) family member, plays an essential role in the commitment of precursors to osteoclastic differentiation (Suda *et al*, 1999; Boyle *et al*, 2003; Teitelbaum and Ross, 2003). It is also important for activation and survival of differentiated osteoclasts. Macrophage-colony stimulating factor (M-CSF) supports proliferation and survival of osteoclast precursors during differentiation. Early studies with coculture system of bone marrow osteoclast precursor cells and osteoblasts revealed that direct cell-to-cell interaction was needed for osteoclast generation. The cell surface molecular interaction for osteoclastic differentiation was later found to be between RANKL expressed on osteoblasts and RANK present on osteoclast precursors (Suda *et al*, 1999). Osteoprotegerin (OPG) is a soluble decoy receptor that binds RANKL, but unlike RANK, has no intracellular signaling capacity (Boyle *et al*, 2003). Therefore, the RANKL–RANK–OPG axis is critical to determine osteoclastogenesis and bone homeostasis.

RANKL binding to its cognate receptor RANK leads to recruitment of adaptor proteins TRAFs (TNF receptor-associated factors) (Lee and Kim, 2003; Boyle *et al*, 2003; Teitelbaum and Ross, 2003). Many *in vitro* and *in vivo* studies have provided evidence that TRAF6 may be the most critical TRAF protein in RANK signaling in osteoclasts (Lomaga *et al*, 1999; Naito *et al*, 1999; Kobayashi *et al*, 2001). The downstream signaling events ensuing TRAF6 recruitment to RANK include activation of kinases such as PI3K/Akt, IKKs, and MAPKs (Wong *et al*, 1999). The activation of signaling pathways mediated by these kinases leads to cytoskeletal organization necessary for migration and bone resorption activities, and induction of an array of genes required for differentiation and survival (Boyle *et al*, 2003; Lee and Kim, 2003; Teitelbaum and Ross, 2003). Recent studies have demonstrated that NFATc1 is the transcription factor profoundly increased by RANKL and required for osteoclastogenesis (Takayanagi *et al*, 2002). In turn, NFATc1 induction by

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RANKL is dependent on c-Fos and positive feedback by NFATc1 itself (Takayanagi *et al*, 2002; Matsuo *et al*, 2004).

Sphingosine kinase (SPHK) is a lipid kinase that phosphorylates sphingosine to generate sphingosine 1-phosphate (S1P). Two mammalian isoforms, SPHK1 and SPHK2, have been identified. S1P has been implicated in a variety of cellular processes, including cell differentiation, apoptosis, proliferation, and motility (Spiegel and Milstien, 2003; Futerman and Hannun, 2004). The diversity in cell responses elicited by S1P may be attained in part by its dual characteristics of being both an intracellular second messenger and an extracellular signal. In addition, the presence of multiple cell surface receptors may contribute to the complexity of S1P effects. Five mammalian S1P receptors have been identified so far—S1P<sub>1</sub>/endothelial differentiation gene 1 (EDG1), S1P<sub>2</sub>/EDG5, S1P<sub>3</sub>/EDG3, S1P<sub>4</sub>/EDG6, and S1P<sub>5</sub>/EDG8. Upon engagement with extracellular S1P, each of these receptors couples to a specific set of heterotrimeric G proteins to trigger plethora of intracellular signaling pathways (Spiegel and Milstien, 2003). Although the direct targets of intracellular S1P are not clear, the amount of intracellular S1P relative to ceramide and sphingosine has been suggested to be important in the regulation of cell cycle, apoptosis, and calcium homeostasis (Spiegel and Milstien, 2003). FTY-720, a structural analog of sphingosine and a novel immunosuppressant, can be converted to phosphate ester form by SPHK and phospho-FTY-720 binds with higher affinity than S1P to all S1P receptors except S1P<sub>2</sub> (Brinkmann *et al*, 2002; Yopp *et al*, 2003). In contrast to S1P-mimicking effects, functional antagonism against certain S1P-stimulated responses, such as T-cell chemotaxis and angiogenesis, have also been demonstrated for FTY-720 (Graeler and Goetzl, 2002; LaMontagne *et al*, 2006). Although the antagonistic function of FTY-720

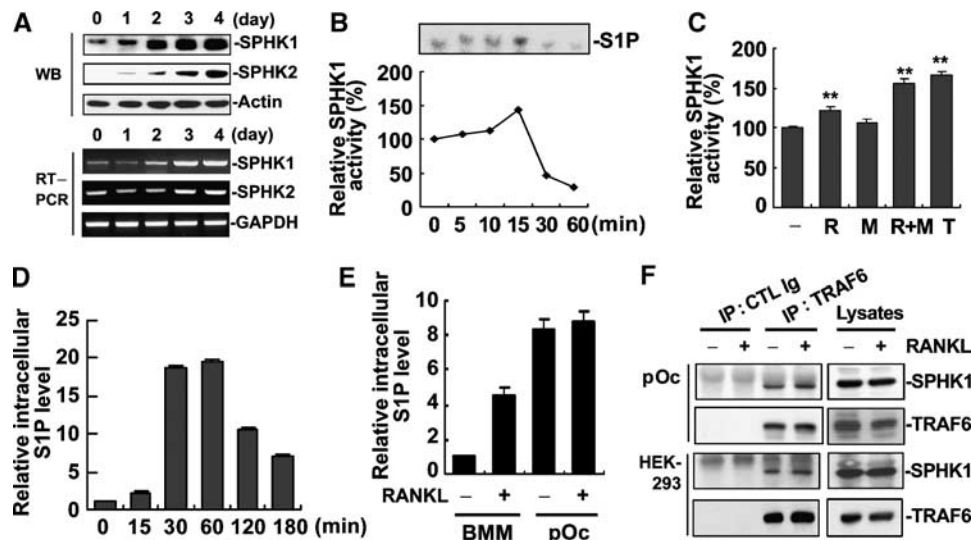
has been suggested to be due to internalization and partial degradation of S1P receptors (Kaneider *et al*, 2004), the precise mechanism of action of FTY-720 as well as that of S1P is far from comprehensive understanding.

In this study, we investigated the possibility of involvement of SPHK1 and S1P in osteoclast differentiation. We found that SPHK1 activated by RANKL plays dichotomous function for osteoclast differentiation: intracellular S1P attenuates osteoclast differentiation program, whereas S1P secreted from stimulated osteoclast precursor cells acts on osteoblasts to augment osteoclastogenesis by increasing RANKL expression. Secreted S1P also chemoattracts osteoblasts and enhances their survival. Our studies identify the SPHK1-S1P system as a novel player in osteoclastogenesis regulation and in communication between osteoclasts and osteoblasts in bone metabolism.

## Results

### SPHK1 expression and activity increase during RANKL-induced osteoclastogenesis

We analyzed expression levels of SPHK during osteoclast differentiation. Bone marrow-derived macrophages (BMMs) were cultured in the presence of RANKL plus M-CSF (RANKL/M-CSF) for 4 days to generate osteoclasts. Both mRNA and protein levels of SPHK1 and SPHK2 increased during the osteoclastogenesis from BMMs (Figure 1A). The increased gene expression of SPHK1 and SPHK2 was also detected in microarray experiments (data not shown). We next examined whether the activity of SPHK1 is regulated by osteoclastogenic stimuli. SPHK1 activity was found to be stimulated by RANKL/M-CSF (Figure 1B) when an *in vitro* assay was performed in the presence of Triton X-100, which



**Figure 1** SPHK1 expression and activity is stimulated by RANKL during osteoclastogenesis from BMMs. (A) BMMs were cultured in the presence of RANKL (100 ng/ml) and M-CSF (30 ng/ml). Cells were harvested at the indicated days and protein (upper three panels) and mRNA (lower three panels) levels of SPHK1 and SPHK2 were examined by Western blotting and RT-PCR. (B) BMMs were stimulated with RANKL (100 ng/ml) and M-CSF (30 ng/ml) and SPHK1 activity was measured from 80  $\mu$ g of cell lysates. (C) BMMs were stimulated with RANKL (100 ng/ml), M-CSF (30 ng/ml), RANKL plus M-CSF, or TNF- $\alpha$  (10 ng/ml) for 15 min and SPHK1 activity in 80  $\mu$ g cell lysates was measured. \*\* $P < 0.005$  versus control. (D) BMMs were labeled with <sup>32</sup>Pi and stimulated with RANKL (1  $\mu$ g/ml). Intracellular S1P levels were detected from 1  $\times 10^6$  cells as described in Materials and methods. (E) <sup>32</sup>Pi-labeled BMMs or pOc were stimulated with RANKL (1  $\mu$ g/ml) for 15 min. Intracellular S1P levels from 1  $\times 10^6$  cells were measured. (F) HEK293 cells were transfected with RANK, Flag-TRAF6, and HA-SPHK1. The transfected HEK293 cells and untransfected pOc were starved for 3 h and stimulated with RANKL (1  $\mu$ g/ml) for 15 min. Cell lysates were immunoprecipitated with anti-TRAF6 or control Ig, and the precipitates were immunoblotted with anti-SPHK1 followed by reprobing with anti-TRAF6.

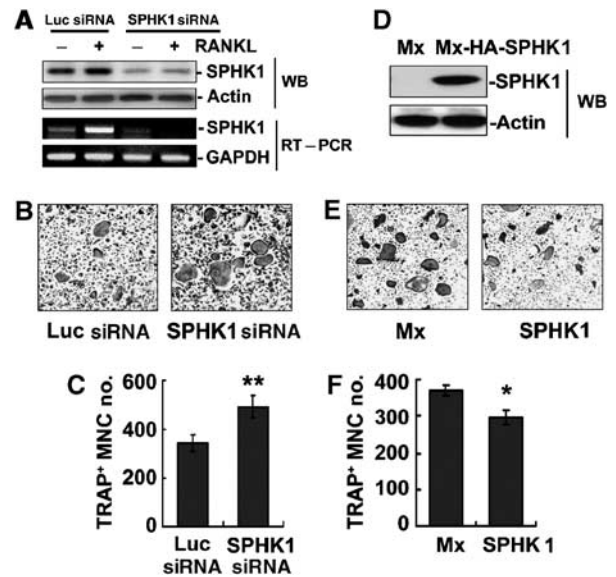
inhibits SPHK2 activity (Liu *et al*, 2000). RANKL alone could also stimulate SPHK1 activity; however, a synergy was observed when M-CSF was also present (Figure 1C). The intracellular level of S1P was also elevated in BMMs stimulated with RANKL (Figure 1D). Consistent with the increased SPHK levels during differentiation, the intracellular S1P levels were higher in differentiating pre-fusion osteoclasts (pOc) than in BMMs (Figure 1E). It has been reported that TRAF2 interacts with and thereby activates SPHK1 in response to TNF- $\alpha$  (Xia *et al*, 2002). As TRAF6 is a crucial adaptor molecule for RANK signaling, we next determined whether TRAF6 associates with SPHK1. Both in HEK293 cells transfected with TRAF6 and SPHK1 and in untransfected pOc, the complex of TRAF6 and SPHK1 was detected (Figure 1F). The complex formation was weakly stimulated by RANKL (Figure 1F). These results indicate that SPHK1 is upregulated and its activity is stimulated by RANKL in BMMs undergoing osteoclastic differentiation.

### SPHK1 negatively regulates osteoclast differentiation in BMM single culture

Unlike SPHK1, the role of SPHK2 in S1P-mediated cellular responses is elusive and the intracellular location of SPHK2 has not been conclusive (Igarashi *et al*, 2003; Maceyka *et al*, 2005). For this reason, we focused on the role of SPHK1 for osteoclastogenesis. First, we investigated whether downregulation of SPHK1 by siRNA would reduce osteoclastogenesis. Infection of BMMs with SPHK1 siRNA retroviruses resulted in a prominent decrease in the SPHK1 mRNA and protein levels (Figure 2A). Infected BMMs were induced to differentiate to osteoclasts by culturing with RANKL/M-CSF. During differentiation, BMMs convert to tartrate-resistant acid phosphatase-positive (TRAP<sup>+</sup>) cells and these TRAP<sup>+</sup> mononuclear cells fuse to generate TRAP<sup>+</sup> multinuclear cells (MNCs; osteoclasts). In contrast to our expectation, the formation of TRAP<sup>+</sup> MNCs was not reduced, rather modestly increased by SPHK1 siRNA compared with the control luciferase siRNA (Figure 2B and C). The number of generated TRAP<sup>+</sup> mononuclear cells was also higher in cells infected with SPHK1 siRNA than in the control cells (data not shown). In experiments with RAW264.7 cells, a model cell line for osteoclastogenesis, using oligocassette siRNA for SPHK1, similar results were observed (Supplementary Figure 1). We next evaluated effects of increased SPHK1 expression. BMMs were infected with retroviruses harboring HA-tagged SPHK1 and expression of HA-SPHK1 was confirmed (Figure 2D). The overexpression of SPHK1 weakly reduced the generation of mature osteoclasts (Figure 2E and F). These data indicate that SPHK1 activity negatively regulates osteoclastogenesis in this BMM culture system.

### SPHK1 reciprocally regulates RANKL activation of p38 and ERK MAPKs in BMMs

To gain insights into the mechanism by which SPHK1 siRNA increases and SPHK1 overexpression decreases osteoclastogenesis, we examined the effects of the SPHK1 modulations on RANK signaling. In BMMs with reduced SPHK1 expression by siRNA, RANKL stimulation of p38 MAPK was enhanced compared with that in the control luciferase siRNA infected cells (Figure 3A and B). On the other hand, ERK activation by RANKL was suppressed by SPHK1 siRNA retrovirus infection (Figure 3A and B). Overexpression of SPHK1 resulted in the

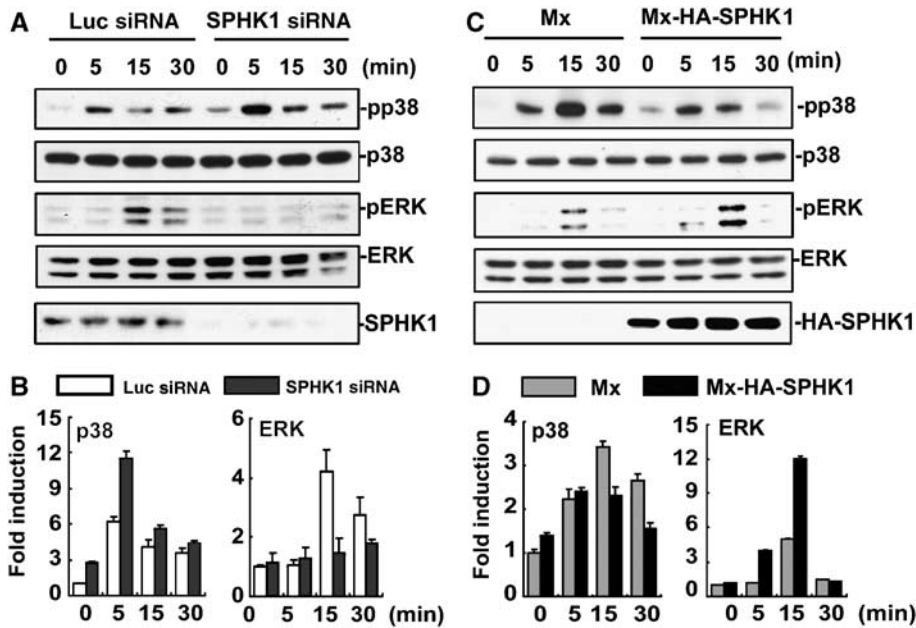


**Figure 2** SPHK1 inhibits RANKL-induced osteoclastogenesis in BMM single culture. (A) BMMs were infected with retroviruses encoding SPHK1 siRNA or luciferase (Luc) siRNA. SPHK1 protein and mRNA levels were evaluated by Western blotting and RT-PCR. (B, C) Infected BMMs were cultured for 4 days in the presence of RANKL (100 ng/ml) and M-CSF (30 ng/ml). Cells were TRAP-stained and the number of TRAP<sup>+</sup> MNC was counted. \*\**P*<0.005 versus Luc siRNA control. (D) BMMs were infected with retroviruses harboring HA-SPHK1 or the control viruses. The expression of SPHK1 was tested by Western blotting with anti-HA. (E, F) Infected BMMs were induced to differentiate to osteoclasts. The number of TRAP<sup>+</sup> MNC was scored. \**P*<0.01 versus control.

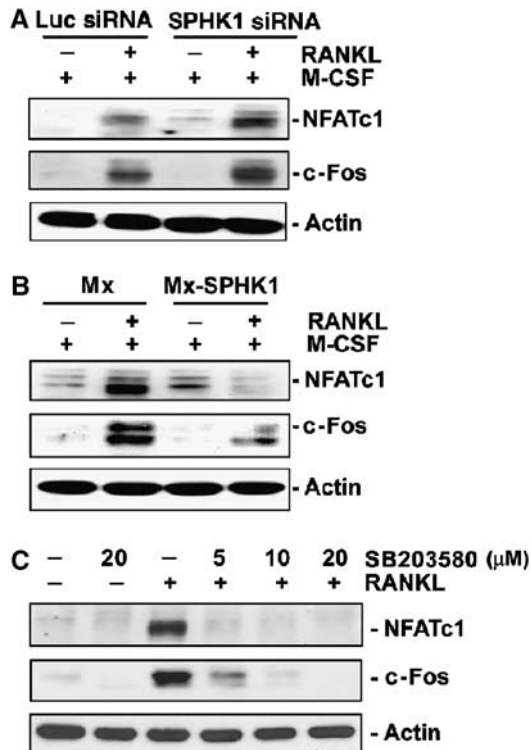
opposite effect; reduced p38 and augmented ERK activation by RANKL (Figure 3C and D). Therefore, the RANKL signaling to p38 and ERK MAPKs was reciprocally modulated by the regulation of SPHK1 expression in BMMs.

### SPHK1 inhibits NFATc1 and c-Fos induction by RANKL in BMMs

Recent studies have indicated the importance of NFATc1 transcription factor for differentiation of osteoclasts (Ishida *et al*, 2002; Takayanagi *et al*, 2002). Thus, we investigated whether the effect of SPHK1 on RANKL-induced osteoclastogenesis might be related to NFATc1. SPHK1 downregulation in BMMs by siRNA infection increased the induction of NFATc1 by RANKL (Figure 4A, top panel). On the contrary, overexpression of SPHK1 suppressed NFATc1 induction (Figure 4B, top panel). c-Fos is required for NFATc1 induction by RANKL (Takayanagi *et al*, 2002; Matsuo *et al*, 2004). SPHK1 siRNA enhanced RANKL-stimulated increase in c-Fos expression (Figure 4A, middle panel), whereas overexpression of SPHK1 attenuated c-Fos induction (Figure 4B, middle panel). As both the p38 activation (Figure 3B) and NFATc1 and c-Fos induction (Figure 4B) were suppressed by SPHK1 overexpression, the potential link between p38 and these transcription factors was tested. BMMs were incubated with RANKL in the presence of the p38-specific inhibitor SB203580. SB203580 treatment led to a strong inhibition in NFATc1 and c-Fos induction (Figure 4C). Therefore, p38-dependent regulation of c-Fos and NFATc1 mediates the suppressive role of SPHK1 in osteoclast differentiation from this culture system involving only BMMs.



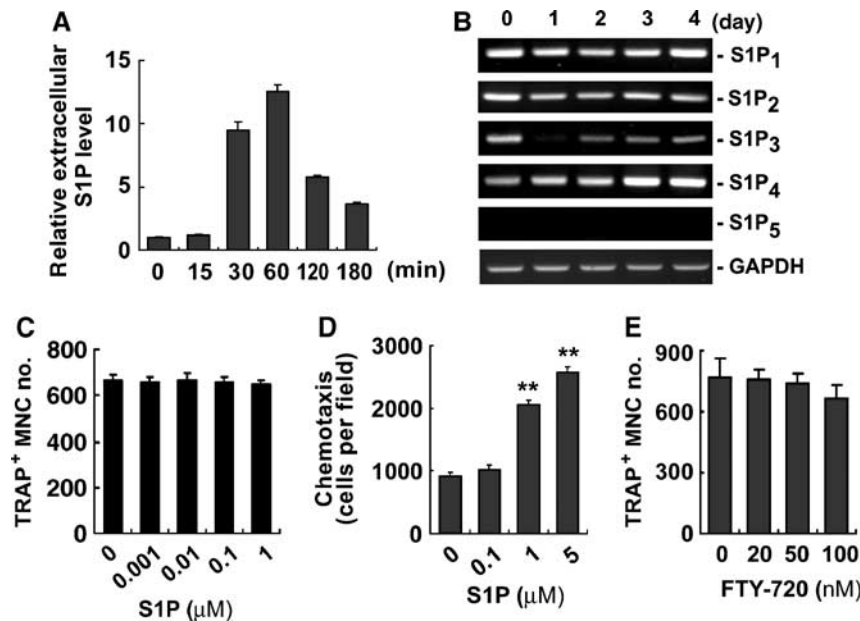
**Figure 3** SPHK1 reciprocally modulates RANKL-induced activation of p38 and ERK. (A) BMMs infected with SPHK1 or luciferase siRNA retroviruses were stimulated with RANKL (1  $\mu$ g/ml). The phosphorylated and total levels of p38 and ERK were examined by Western blotting. SPHK1 protein levels were also examined. (B) Quantification data for the phosphorylated p38 or ERK normalized to the level of total p38 or ERK are presented. (C) BMMs infected with HA-SPHK1 or the control retroviruses were stimulated with RANKL and cell extracts were Western blotted as above. (D) The levels of phosphorylated p38 or ERK were normalized to those of total p38 or ERK.



**Figure 4** SPHK1 suppresses NFATc1 and c-Fos induction by RANKL. (A) BMMs were infected with SPHK1 or Luc siRNA retroviruses. Cells were cultured for 24 h with RANKL (100 ng/ml) and M-CSF (30 ng/ml) and protein levels of NFATc1 and c-Fos were examined. (B) BMMs were infected with HA-SPHK1 or the control retroviruses and analyzed as in (A). (C) BMMs were stimulated with RANKL (100 ng/ml) in the presence of SB203580 for 24 h. The NFATc1 and c-Fos protein levels were detected.

#### Extracellular S1P does not affect osteoclast differentiation in BMM single culture

S1P generated by SPHK1 can function as both an extracellular ligand and an intracellular second messenger. To examine the potential role of extracellular S1P in osteoclastogenesis, we first determined whether RANKL increases S1P secretion from BMMs as a result of SPHK activation. As shown in Figure 5A, extracellular S1P level increased by RANKL treatment. Next, we explored the possibility of autocrine function of S1P. For this, the presence of S1P receptors in BMMs and osteoclasts was analyzed. BMMs and differentiating osteoclasts expressed all known S1P receptors except S1P<sub>5</sub> (Figure 5B). S1P<sub>5</sub> was detected in T cells, indicating that no detection of this receptor in BMMs was not due to an inadequate assay condition (Supplementary Figure 2). As the overexpression of SPHK1 reduced osteoclastogenesis by RANKL (Figure 2F), whereas RANKL stimulated extracellular S1P production (Figure 5A), we next tested whether addition of exogenous S1P would negatively regulate osteoclast differentiation through cell surface S1P receptors. S1P (1 nM–1  $\mu$ M) did not have any effect on RANKL-induced osteoclastogenesis from BMMs (Figure 5C). Neither S1P addition to the culture with lower RANKL concentrations nor S1P itself in the absence of RANKL could cause any difference in osteoclast differentiation (data not shown). In addition, NFATc1 induction by RANKL was not modulated by exogenous S1P (data not shown). The lack of effects of exogenous S1P was not due to malfunction of the receptors as S1P treatment caused increased migration of BMMs (Figure 5D) and p38 activation (Supplementary Figure 3). Moreover, FTY-720, a potential functional antagonist of S1P receptors, had no effect (Figure 5E). These data point that the apparent inhibition of osteoclastic differentiation of BMMs by increased SPHK1 activity (Figure 2) is not owing to the extracellular action of



**Figure 5** Extracellular S1P has no effect on osteoclastogenesis in BMM single culture. (A) BMMs were labeled with  $^{32}\text{P}_i$  and treated with RANKL (1  $\mu\text{g}/\text{ml}$ ). S1P levels in the culture supernatants from  $1 \times 10^6$  cells were determined. (B) BMMs were cultured in the presence of RANKL (100 ng/ml) and M-CSF (30 ng/ml). S1P receptor mRNA levels were analyzed. (C) BMMs were cultured for 5 days in medium supplemented with 1% FBS, RANKL (100 ng/ml), M-CSF (30 ng/ml), and S1P. The number of TRAP<sup>+</sup> MNCs was determined. (D) Migration of BMMs towards S1P was examined using transwell plates. \*\* $P < 0.005$  versus control. (E) BMMs were cultured for 4 days in the presence of FTY-720, RANKL (100 ng/ml), and M-CSF (30 ng/ml). The number of TRAP<sup>+</sup> MNCs was scored.

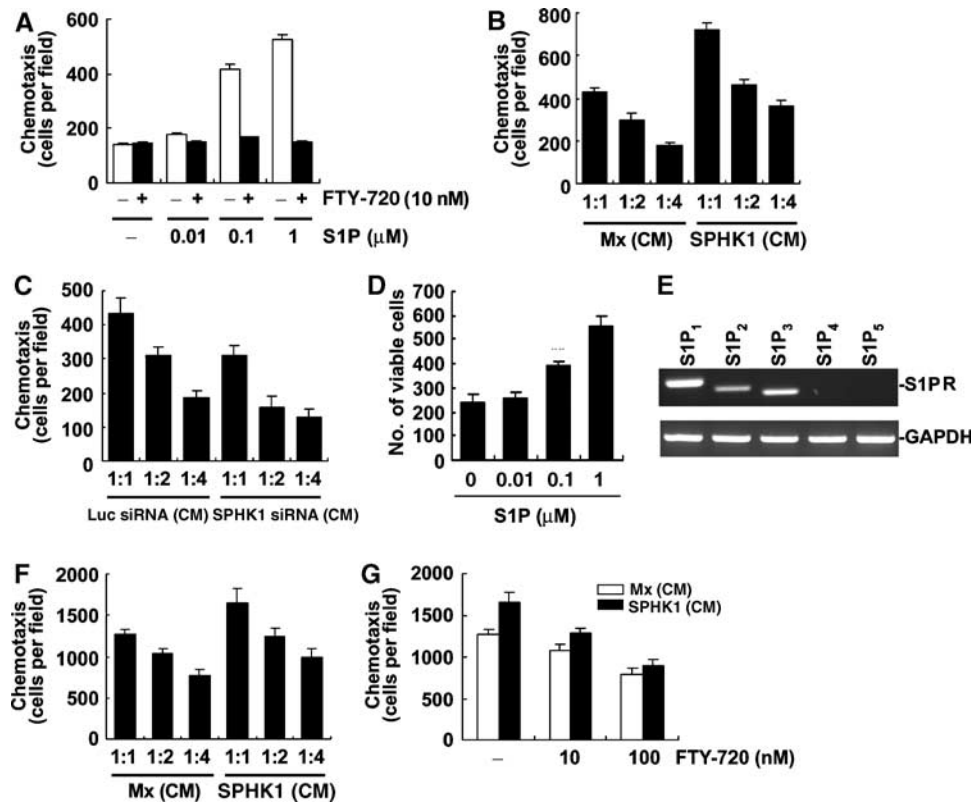
S1P secreted in response to RANKL. Rather, RANKL stimulation of SPHK1 exerts a moderate osteoclastogenesis inhibitory function through the action of intracellular S1P in BMMs.

#### **S1P increases chemotaxis and survival of osteoblasts and activated T cells**

Osteoblasts are recruited to resorption sites to fill the eroded cavity with new bone. For osteoblast attraction, factors released by osteoclasts may play an important role. S1P produced by osteoclast precursors during differentiation may function in a paracrine manner to osteoblasts. To explore this possibility, we examined the chemotactic effects of S1P on primary mouse osteoblasts. S1P increased osteoblast chemotaxis at the range of 0.01–1  $\mu\text{M}$  (Figure 6A). The chemotactic effect was abolished by FTY-720 (Figure 6A). In addition, the chemotaxis of osteoblasts was higher toward the conditioned medium (CM) from differentiating BMMs transduced to overexpress SPHK1 (SPHK1-CM) as compared with that from control cells (Figure 6B). Consistently, the CM from differentiating BMMs infected with SPHK1 siRNA retroviruses showed lower chemotactic activity for osteoblasts (Figure 6C). The elevated and reduced S1P production by SPHK1 overexpression and SPHK1 siRNA, respectively, was confirmed (Supplementary Figure 4). S1P also enhanced osteoblast survival in serum-deprived condition (Figure 6D). In support of the responses to S1P, mouse osteoblasts expressed S1P receptors S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> (Figure 6E). Osteoclast-derived factors may also have effects on T cells in inflammatory bone erosion diseases such as rheumatoid arthritis (RA). We examined whether S1P from osteoclastic cells has chemotactic effects on T cells. The SPHK1-CM increased T-cell chemotaxis (Figure 6F), which was suppressed by FTY-720 (Figure 6G).

#### **S1P potentiates osteoclast differentiation in coculture of BMMs and osteoblasts by increasing RANKL expression**

Osteoclasts are generated when BMMs and osteoblasts are cocultured in the presence of  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> (VtD<sub>3</sub>) and/or prostaglandin E<sub>2</sub>. As S1P released during osteoclastic differentiation can act in a paracrine manner on osteoblasts, we examined the effects of S1P on osteoclastogenesis in the coculture system. Addition of S1P (1  $\mu\text{M}$ ) greatly increased VtD<sub>3</sub>-induced osteoclast formation in the coculture (Figure 7A). This stimulatory effect of S1P on osteoclastogenesis was also observed when the coculture cells were treated with a low concentration of PGE<sub>2</sub> ( $10^{-8}$  M), but not with higher concentrations of PGE<sub>2</sub> (Figure 7B). In cocultures treated with PGE<sub>2</sub> ( $10^{-8}$  M) and VtD<sub>3</sub> ( $10^{-10}$  M), S1P also enhanced osteoclast generation (Figure 7C). The lack of S1P effect in the presence of high-dose PGE<sub>2</sub> suggests the possibility that the osteoclastogenesis-stimulatory effect of S1P is associated with S1P-dependent PGE<sub>2</sub> elevation, which will increase RANKL expression (Li *et al*, 2002a) and subsequently stimulate osteoclast differentiation. In fact, a prominent increase in RANKL expression was observed in S1P-treated osteoblasts (Figure 7D). When both S1P and VtD<sub>3</sub> were present, RANKL expression was further increased, whereas OPG level was decreased, resulting in a greater rise in the ratio of RANKL to OPG. Moreover, osteoblasts treated with SPHK1-CM exhibited higher RANKL expression than those treated with the control CM (Figure 7E). Similar RANKL-elevating effect by S1P and SPHK1-CM was observed in activated T cells (Figure 7F and G). To further characterize the relationship of S1P with osteoclastogenesis, we included FTY-720 in cocultures of BMMs and calvarial osteoblasts. As shown in Figure 7H, FTY-720 dramatically inhibited osteoclastogenesis at nano-



**Figure 6** Extracellular S1P stimulates osteoblast chemotaxis and survival. (A) Mouse calvarial osteoblasts were treated with 10 nM FTY-720 or vehicle, and allowed to migrate for 24 h towards 0.01–1 μM S1P using transwell plates. (B) Osteoblasts were allowed to migrate for 24 h towards the indicated CM diluted at the indicated ratio. (C) Osteoblasts were allowed to migrate for 24 h towards the indicated CM. (D) Osteoblasts were incubated with S1P in serum-free media for 72 h. The number of surviving cells was counted after hematoxylin staining. (E) Osteoblasts were collected and S1P receptor mRNA was detected by RT-PCR. (F) T cells were allowed to migrate for 3 h towards the indicated CM. (G) T cells were allowed to migrate for 3 h in the presence of FTY-720 towards the indicated CM diluted at 1:1 ratio.

molar concentrations. In accordance with the anti-osteoclastogenic effects, FTY-720 significantly suppressed the S1P- and VtD<sub>3</sub>-stimulated RANKL expression in osteoblasts (Figure 7I). These results indicate that S1P induces RANKL expression in osteoblasts, and thereby facilitates osteoclastogenesis in co-cultures of BMMs and osteoblasts. It is interesting that FTY-720 reduced the osteoclastogenesis and RANKL induction responses by VtD<sub>3</sub> alone (Figure 7H and I). These findings suggest that S1P receptors may also be involved in the signaling by VtD<sub>3</sub> alone.

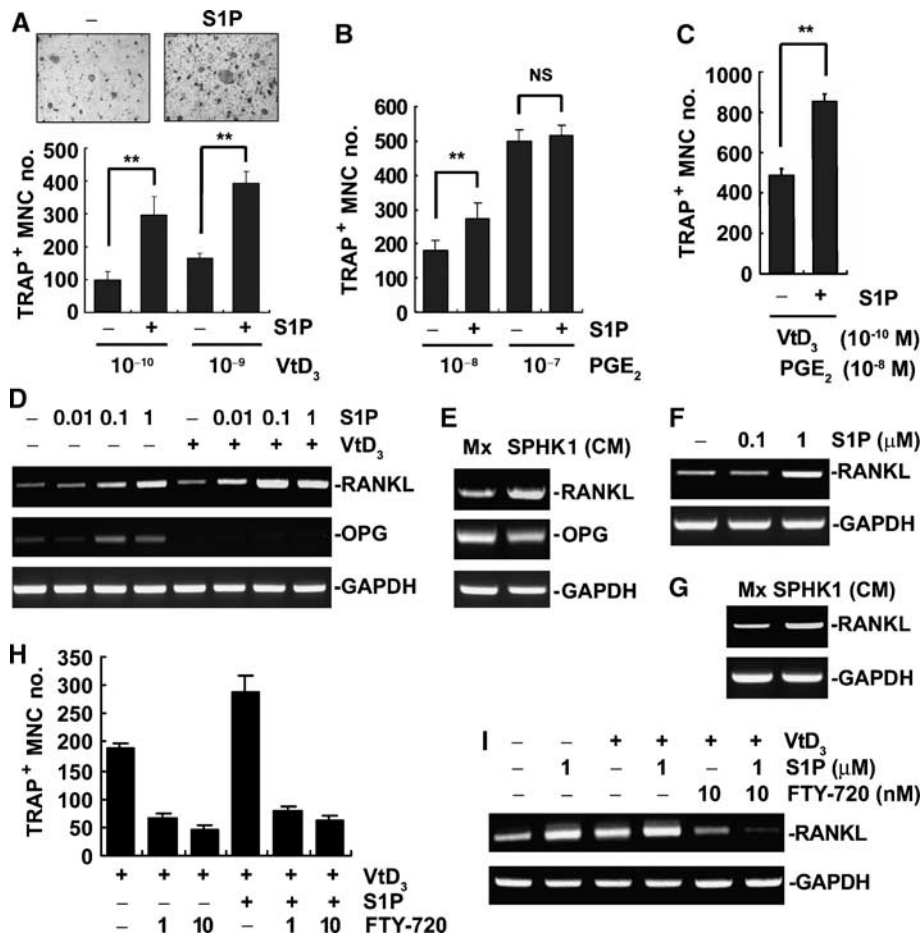
#### **S1P increases RANKL expression in osteoblasts by inducing PGE<sub>2</sub> secretion through COX2 regulation**

We next investigated whether PGE<sub>2</sub> is involved in the S1P-dependent regulation of RANKL expression. First, we determined whether S1P stimulates PGE<sub>2</sub> production. EIA assays revealed that S1P alone or in combination with VtD<sub>3</sub> increased PGE<sub>2</sub> production in osteoblasts (Figure 8A). As cyclooxygenase-2 (COX2) and microsomal prostaglandin E synthase-1 (mPGES1) are key enzymes involved in PGE<sub>2</sub> production, we next examined the levels of these enzymes. Figure 8B shows that S1P increased protein levels of COX2 in osteoblasts. S1P effect on COX2 mRNA levels was inconsistent (data not shown). S1P had only minimal effect on mPGES1 protein level, whereas VtD<sub>3</sub> increased it (Figure 8B). The CM from pOc that overexpressed SPHK1 also increased COX2 compared with the control CM (Figure 8C). The SPHK1 CM had no effect on mPGES1 level

(Figure 8C). Whether the catalytic activity itself is stimulated by S1P was also tested by treating the cells with S1P for only 10 min during which no protein level change occurred (Supplementary Figure 5). S1P increased the enzymatic activity of both COX2 and mPGES1, whereas VtD<sub>3</sub> alone did not have any effect (Figure 8D and E). Treatment with both S1P and VtD<sub>3</sub> showed some synergy (Figure 8D and E). The COX2 inhibitor NS398 attenuated the induction of RANKL mRNA expression (Figure 8F) and the stimulation of osteoclastogenesis by S1P (Figure 8G). Taken together, S1P stimulates RANKL expression in osteoblasts via PGE<sub>2</sub> production by upregulating the COX2 protein level and the catalytic activity of COX2 and mPGES1.

#### **S1P regulates COX2 expression via ERK and p38 pathways in osteoblasts**

S1P binding to its cell surface receptors has been reported to regulate the activity of MAPKs via G-protein-mediated signaling pathways (Spiegel and Milstien, 2003). Therefore, we sought to investigate the potential participation of MAPKs in the upregulation of COX2 and RANKL by S1P in osteoblasts. First, whether S1P can activate ERK and p38 MAPKs in osteoblasts was examined. S1P stimulated ERK and p38, and less effectively JNK, activation in a dose-dependent manner in the range of 0.1–10 μM (Figure 9A and data not shown). Next, the effect of PD98059, SB203580, and SP600125, specific inhibitors of the ERK, p38, and JNK pathways, respectively, on the S1P-stimulated induction of



**Figure 7** S1P potentiates osteoclast differentiation in coculture of BMMs and osteoblasts. (A) Coculture cells were treated with VtD<sub>3</sub> and S1P (1 μM) for 6 days and TRAP<sup>+</sup> MNCs generated were scored. \*\**P* < 0.005 compared with S1P-untreated controls. (B) Cocultures were treated with PGE<sub>2</sub> and S1P (1 μM) for 6 days. TRAP<sup>+</sup> MNCs were counted. \*\**P* < 0.005 versus S1P-untreated controls. NS, not significant. (C) Cocultures were carried out in the presence of PGE<sub>2</sub> (10<sup>-8</sup> M), VtD<sub>3</sub> (10<sup>-10</sup> M), and S1P (1 μM). TRAP<sup>+</sup> MNC formation was assessed. \*\**P* < 0.005 versus S1P-untreated controls. (D) Primary osteoblasts were treated with S1P and VtD<sub>3</sub> (10<sup>-8</sup> M) for 24 h. RANKL and OPG mRNA levels were analyzed by RT-PCR. (E) Osteoblasts were incubated for 24 h with indicated CM and RANKL mRNA levels were examined by RT-PCR. (F) T cells were activated with ionomycin and PMA and treated with S1P for 24 h. RANKL mRNA levels were determined by RT-PCR. (G) T cells were activated and treated with SPHK1-CM for 24 h. RT-PCR was performed to detect RANKL mRNA. (H) Coculture was carried out in the presence of FTY-720, VtD<sub>3</sub> (10<sup>-9</sup> M), and S1P (1 μM). TRAP<sup>+</sup> MNCs were scored. (I) Osteoblasts were incubated with VtD<sub>3</sub> (10<sup>-9</sup> M), S1P, and FTY-720 for 24 h. RANKL mRNA was analyzed by RT-PCR.

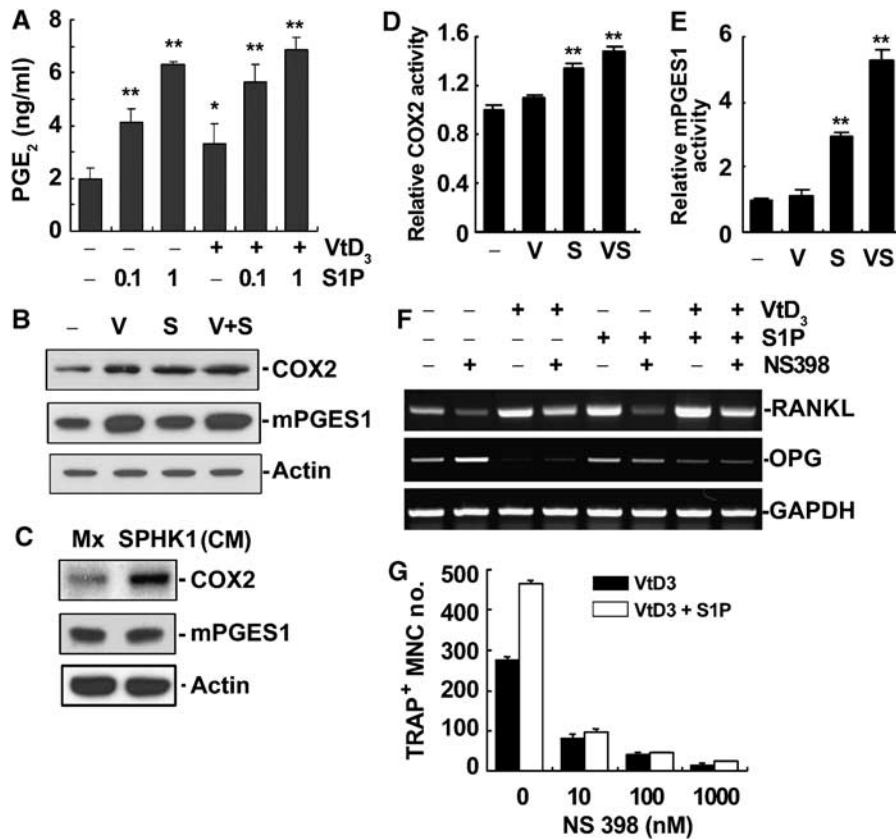
RANKL was assessed. As shown in Figure 9B, all the inhibitors suppressed RANKL induction by S1P. These inhibitors also blocked upregulation of COX2 by S1P in osteoblasts (Figure 9C). Effects of the inhibitors on COX2 mRNA were inconsistent (data not shown). In line with the suppressive effect on COX2, the inhibitors suppressed PGE<sub>2</sub> production in osteoblasts (Figure 9D). Interestingly, S1P most strongly stimulated ERK and PD98059 was most potent in the inhibition of PGE<sub>2</sub> production. Taken together, S1P activates ERK and p38, and less prominently JNK, to upregulate COX2 level, which leads to increased PGE<sub>2</sub> production and subsequent RANKL induction.

## Discussion

In the present study, we demonstrate that SPHK1 has a dichotomous role in osteoclast differentiation, depending on the microenvironment where osteoclast precursors are present. SPHK1 has a negative role in osteoclastogenesis when a single population of osteoclast precursor cells (BMMs) is

induced to differentiate by the soluble osteoclastogenic factor, RANKL. On the other hand, in coculture conditions, where cell surface RANKL on osteoblasts supports osteoclastic differentiation of BMMs, SPHK1 and its product, S1P, function positively in osteoclastogenesis. RANKL stimulates SPHK1 activity and S1P production in the precursor and differentiating BMMs during osteoclastogenesis. However, gene manipulation experiments show that suppression of SPHK1 expression by siRNA introduction enhances, whereas overexpression by SPHK1 retroviral transduction decreases osteoclast formation when BMMs are challenged with RANKL in the absence of osteoblasts. These observations suggest that SPHK1 activation and subsequent S1P production in response to the RANKL differentiation signal comprises a negative feedback loop in osteoclastogenesis. This BMM-autonomous negative feedback is likely to be mediated by intracellular S1P as exogenous addition of S1P does not have any effect on BMM osteoclastogenesis.

RANKL triggers the activation of MAPK family kinases in osteoclast precursors (Lee and Kim, 2003). Evidence for the



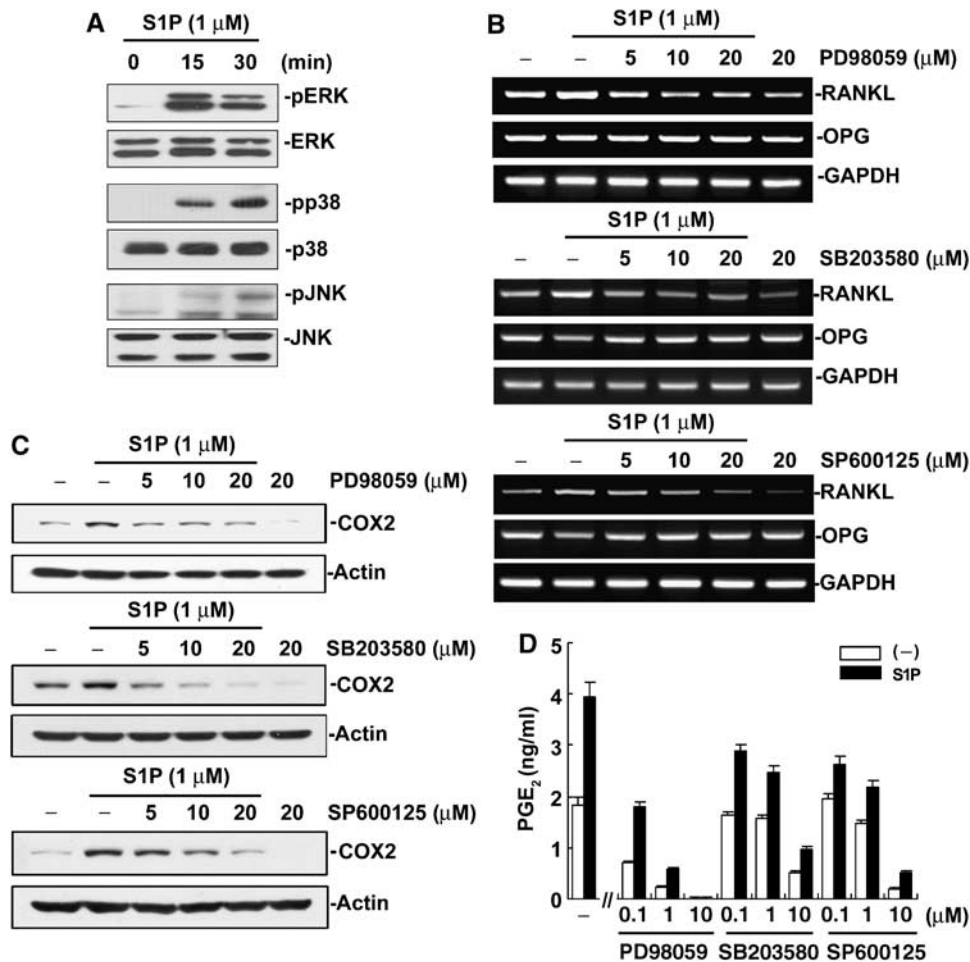
**Figure 8** S1P increased RANKL expression by inducing PGE<sub>2</sub> secretion through COX2 regulation. (A) Calvarial osteoblasts were treated for 24 h with S1P and VtD<sub>3</sub> (10<sup>-8</sup> M). PGE<sub>2</sub> levels in the culture supernatants were determined by EIA. \*P<0.01; \*\*P<0.005 versus control. (B) Osteoblasts were treated with S1P (1 μM) and/or VtD<sub>3</sub> (10<sup>-8</sup> M) for 24 h. COX2 and mPGES1 protein levels were determined. (C) Osteoblasts were incubated for 24 h with the indicated CM. COX2 and mPGES1 proteins were detected. (D, E) Osteoblasts were stimulated with S1P (1 μM) and/or VtD<sub>3</sub> (10<sup>-8</sup> M) for 10 min. The COX2 and mPGES1 activities were determined as described in Materials and methods. (F) Osteoblasts were treated with S1P (1 μM), VtD<sub>3</sub> (10<sup>-8</sup> M), and NS398 (1 μM). RANKL and OPG mRNA levels were examined by RT-PCR. (G) Cocultures of BMMs and osteoblasts were carried out in the presence of VtD<sub>3</sub> (10<sup>-9</sup> M), S1P (1 μM), and NS398. TRAP<sup>+</sup> MNCs were scored.

requirement of p38 MAPKs for osteoclastogenesis has been accumulated by studies with pharmacological inhibitors and dominant-negative forms of kinases (Matsumoto *et al*, 2000; Lee *et al*, 2002; Li *et al*, 2002b; Huang *et al*, 2006). In contrast, conflicting results have been reported on the role of ERKs in osteoclast differentiation. The ERK pathway inhibitor PD98059 displayed no, suppressive, or augmenting effects on osteoclastogenesis depending on the cell types of osteoclast precursors, culture conditions, and concentrations of the inhibitor (Matsumoto *et al*, 2000; Hotokezaka *et al*, 2002; Lee *et al*, 2002). The contribution of ERKs to osteoclastogenesis may depend on the context of balance with other MAPK activities. In line with this notion, the balance in the p38 and ERK activation appears critical for the function of SPHK1 in osteoclast differentiation. The negative effect of SPHK1 overexpression and the positive effect of SPHK1 knockdown by siRNA on BMM osteoclastogenesis well correlate with the changes in the extents of ERK and p38 activation by RANKL; the increased p38 and decreased ERK activity parallel with enhanced osteoclastogenesis. Whether S1P modulates these MAPKs directly or whether S1P targets other upstream components in the signaling pathways to these MAPKs is an intriguing question to be addressed. Our study also indicates that the influence of SPHK1 on osteoclastogenesis is associated with RANKL regulation of NFATc1 and c-Fos, key

transcription factors required for osteoclast differentiation. The induction of NFATc1 and c-Fos by RANKL in BMMs was dependent on p38 activity. Thus, p38 is an important mediator for the modulation of osteoclastogenesis by SPHK1.

In contrast to BMM single cultures, SPHK1 activity positively regulates osteoclastogenesis in cocultures of BMMs and osteoblasts. S1P significantly increases osteoclast formation in coculture treated with VtD<sub>3</sub>. This effect of S1P appears to be due to stimulation of PGE<sub>2</sub> synthesis, which leads to an increase in RANKL expression in osteoblasts (Li *et al*, 2002a). As RANKL stimulation of BMMs results in S1P production and secretion, the induction of RANKL by S1P in osteoblasts constitutes a positive feedback loop for osteoclastogenesis in the coculture system. Thus, SPHK1 activity can generate both a negative and a positive feedback, depending on the culture system. In normal bone remodeling, osteoclast precursors and differentiating osteoclasts are likely to be in the vicinity of preosteoblasts, osteoblasts, and marrow stromal cells. Therefore, it is more likely that SPHK1 activity functions positively in osteoclast development under this condition. Under conditions where osteoblasts forming new bone matrix are not around, the negative feedback mechanism shown in BMM single culture may have a role in holding massive osteoclastogenesis in check to prevent unbalanced excessive bone resorption.



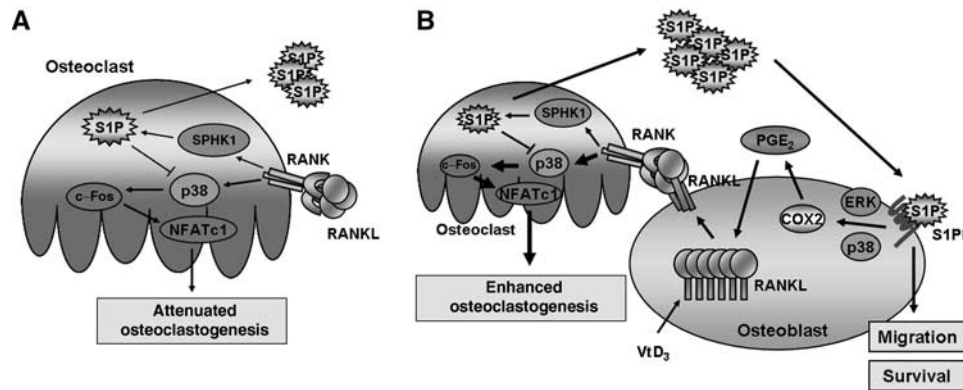


**Figure 9** S1P regulates COX2 expression via MAPK pathways in osteoblasts. (A) Primary osteoblasts were stimulated with S1P. The phosphorylated and total levels of ERK, p38, and JNK were examined by Western blotting. (B) Osteoblasts were stimulated with S1P in the presence of PD98059, SB203580, and SP600125 for 24 h. The RANKL and OPG mRNA levels were examined by RT-PCR. (C) Cells were stimulated as in (B) and COX2 protein levels were examined. (D) Osteoblasts were treated with S1P (1 μM) and indicated inhibitor for 24 h. PGE<sub>2</sub> in the culture supernatant was determined by EIA.

S1P has been shown to play important roles in cell migration for vascular and cardiac development and lymphocyte trafficking (Spiegel and Milstien, 2003). It also functions as an autocrine chemotactic factor to mast cells (Jolly *et al*, 2004). S1P produced by RANKL stimulation during osteoclastogenesis may attract, in both autocrine and paracrine manners, osteoclast precursors and other types of cells in bone. In fact, S1P exhibited chemotactic effect on the migration of BMM osteoclast precursors and osteoblasts. Attraction of osteoclast precursors will facilitate cell fusion with differentiating cells to generate multinucleated mature osteoclasts. However, this stimulatory effect on osteoclastogenesis may be compromised by the intrinsic action of intracellular S1P that negatively regulates osteoclast differentiation. Paracrine action of S1P to osteoblasts will attract the bone-forming cells to the vicinity of osteoclasts. In our study, S1P and the CM of BMMs transduced to overexpress SPHK1 stimulated migration and survival of osteoblasts. This paracrine mode of S1P function must be beneficial to coupling between bone resorption and new bone formation in normal bone remodeling.

COX2 catalyzes the rate-limiting step in prostaglandin biosynthesis (Dixon, 2004). The regulation of COX2 levels has been described at both transcriptional and post-transcrip-

tional levels (Dixon *et al*, 2003; Ramsay *et al*, 2003). Several transcription factors including AP-1 (Guo *et al*, 2001) and CREB (Park *et al*, 2005) have been shown to be involved in the transcriptional induction of COX2. COX2 expression is also regulated by control of mRNA stability and translation efficiency, which is mediated through sequences in the 3'-untranslated region (Mukhopadhyay *et al*, 2003). In addition, protein degradation has been implicated in COX2 regulation (Zaric and Ruegg, 2005). These diverse aspects lend regulation mechanisms for COX2 complex, and the relative contribution of each element of COX2 regulation appears different in different cell types in response to different stimuli. MAPKs of the ERK, JNK, and p38 families have been implicated in both transcriptional and post-transcriptional control of COX2 expression (Xu *et al*, 2000; Rousseau *et al*, 2002; Park *et al*, 2005; Wu *et al*, 2006). However, the type of MAPKs involved and the regulation aspect targeted have been differentially described for different experimental conditions. We for the first time demonstrate that S1P increases COX2 in osteoblasts. Our study is also the first to show the involvement of p38 in S1P-induced COX2 upregulation in any cell type. The participation of ERK in S1P-stimulated COX2 increase has been documented in other cell types (Hsieh *et al*,



**Figure 10** Schematic diagrams for osteoclastogenesis modulation by SPHK1 and S1P. (A) When RANKL binds RANK on BMMs, osteoclastogenesis is started by the activation of downstream signals such as p38, c-Fos, and NFATc1. S1P is also produced by the activation of SPHK1 in response to RANKL. Intracellular S1P constitutes a negative feedback loop in osteoclastogenesis by suppressing p38. (B) S1P produced in BMMs in response to RANKL is exported to extracellular environment and binds the S1P receptor in osteoblasts. The S1P–S1P receptor interaction stimulates MAPKs to upregulate COX2 and PGE<sub>2</sub> in osteoblasts. PGE<sub>2</sub> then induces RANKL expression, which augments osteoclastogenesis. In addition, extracellular S1P stimulates migration and survival of osteoblasts. In this situation, SPHK1 and S1P comprise a positive feedback loop for osteoclastogenesis.

2006). The identification of precise targets of ERKs and p38 MAPKs in the COX2 increase by S1P in osteoblasts requires further investigation.

Our data indicate that S1P-stimulated COX2 induction and PGE<sub>2</sub> production increase RANKL in osteoblasts, which consequently stimulates osteoclast formation. COX2, PGE<sub>2</sub>, and RANKL may comprise an important axis in pathogenic conditions involving bone erosion. In ovariectomized animal models of osteoporosis, increased COX2 activity, PGE<sub>2</sub> production, and RANKL induction have been suggested to participate in osteoclast generation and bone resorption (Kanematsu *et al*, 2000). Recently, in synoviocytes from RA patients, S1P was shown to stimulate COX2 expression and PGE<sub>2</sub> production (Kitano *et al*, 2006). RANKL is expressed by synovial fibroblasts and activated T cells from RA tissues (Gravallese *et al*, 2000; Takayanagi *et al*, 2000). Although direct evidence is lacking, the S1P–COX2–PGE<sub>2</sub> axis is likely to participate in RANKL induction in RA synovium. In RA synovium, SPHK activation and S1P production during osteoclast differentiation will provoke more RANKL expression through COX2–PGE<sub>2</sub> production in surrounding cells, such as preosteoblasts, synovial fibroblasts, and T cells. This will compose a vicious cycle of osteoclast generation and bone resorption, exacerbating the disease condition. S1P can also function to recruit activated T cells and other immune cells to the site of osteoclastogenesis in pathologic conditions. We observed that the CM from SPHK1-overexpressing pOC-stimulated T-cell migration. Furthermore, extracellular S1P enhanced RANKL expression in activated T cells. Therefore, S1P produced during osteoclastogenesis under inflammatory conditions, such as RA and periodontal diseases, may play a detrimental role through combined effects on T-cell chemotaxis and RANKL expression.

Based on our data presented here, a schematic diagram of osteoclastogenesis modulation by the SPHK1–S1P signaling is shown in Figure 10. When RANKL binds to RANK on BMMs, osteoclastic differentiation is triggered by activation of downstream signals such as p38, c-Fos, and NFATc1. S1P is also generated upon the activation of SPHK1 in response to RANKL. Intracellular production of S1P comprises a negative

feedback loop in osteoclastogenesis owing to its inhibitory effect on p38 activation in BMMs. S1P exported to extracellular environment can bind to S1P receptors in osteoblasts. Binding of S1P to S1P receptors on osteoblasts leads to an increase in COX2 level through a mechanism dependent on ERK and p38. COX2 induction stimulates PGE<sub>2</sub> production, which induces RANKL expression. The increased RANKL molecules in osteoblasts in turn bind to more RANK on BMMs and facilitate osteoclastic differentiation. Extracellular S1P also stimulates osteoblast migration and survival, contributing to the coupling between osteoclast and osteoblast activity.

In conclusion, our studies reveal that SPHK1 and S1P play important roles in osteoclastogenesis regulation. To our knowledge, this is the first study showing involvement of SPHK1 and S1P in osteoclast differentiation. Our results may provide a basis for exploring the therapeutic potential of S1P receptor antagonists for inflammatory disease-associated bone destruction.

## Materials and methods

### Reagents

See Supplementary data.

### Plasmid preparation

The full-length human SPHK1 cDNA was reverse-transcribed amplified from HeLa cell mRNA and cloned into pSR $\alpha$ -HA vector. To generate retroviral vectors harboring HA-tagged SPHK1 (pMX-HA-SPHK1), DNA fragments encoding HA-SPHK1 were sub-cloned into the pMX retroviral vector. For construction of retroviral vectors for siRNA, targeting oligonucleotides were annealed and ligated into the pSuper-retro vector (Oligoengine, Seattle, WA) using *Bam*HI and *Hind*III sites. The sequences of used oligonucleotides are provided in Supplementary Table 1.

### BMM and osteoclast cultures

BMM culture and osteoclast generation were performed as described (Huang *et al*, 2006; Supplementary data).

### Coculture

Mouse calvarial osteoblasts were isolated from 2-day-old ICR mice. The calvarias were digested in HBSS containing 0.02% type I collagenase (GIBCO BRL), 0.05% trypsin, and 0.53 mM EDTA for

20 min at 37°C with vigorous shaking. The digestion procedure was repeated six times, and the cells isolated by the last three digestions were combined. The isolated cells were seeded at  $5 \times 10^5$  cells per 10-cm culture dish and grown to confluence. The cells of passage 2 were used for coculture.  $1 \times 10^4$  osteoblasts and  $1 \times 10^5$  BMMs were seeded in each well of 96-well plates in  $\alpha$ -MEM/10% FBS and cultured for 6 days.

#### Retroviral gene transfer and conditioned medium

Generation of retroviruses and BMM infection were performed as described (Huang *et al*, 2006; Supplementary data). The CM used in Figures 6–8 was collected from pOc generated by culturing BMMs infected with indicated retroviruses for 2–3 days with 100 ng/ml RANKL and 30 ng/ml M-CSF.

#### Sphingosine kinase activity assay

Sphingosine kinase activity was measured as previously described (Olivera and Spiegel, 1998). Cells were scraped in SPHK assay buffer (20 mM Tris (pH 7.4), 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM  $\beta$ -glycerophosphate, 15 mM NaF, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxyypyridoxine) and disrupted by freeze-thawing. An 80  $\mu$ g portion of cell extracts in 185  $\mu$ l volume was mixed with 5  $\mu$ l of [ $\gamma$ - $^{32}$ P]ATP (5  $\mu$ Ci) containing  $\text{MgCl}_2$  (0.2 M) and 10  $\mu$ l of 1 mM sphingosine (dissolved in 5% Triton X-100) and then incubated for 30 min at 37°C. The reaction was terminated with 10  $\mu$ l of 1 N HCl. A 400  $\mu$ l portion of chloroform/methanol/HCl (100:200:1, v/v) mixture was added and mixed. Then, 120  $\mu$ l of chloroform and 120  $\mu$ l of 2 M KCl were added and phases separated by centrifugation. The organic phase was dried and resolved by thin-layer chromatography (TLC) on silica gel G60 with SPHK1-butanol/methanol/acetic acid/water (80:20:10:20, v/v). The radioactive spots corresponding to S1P were detected using phosphorimager, BAS-1500 (Fujifilm Co. Ltd).

#### S1P level determination

Cells were seeded at  $1 \times 10^6$  cells/well in six-well plates and incubated for 4 h with phosphate-free DMEM and metabolically labeled with [ $^{32}$ P]orthophosphate for 4 h. Cells were washed with phosphate-free DMEM and treated with 1  $\mu$ g/ml RANKL in 1 ml/well volume for indicated times. To determine extracellular S1P, the whole culture medium was collected and centrifuged. One milliliter of the supernatant was mixed with 1 ml chloroform, 1 ml methanol, and 9  $\mu$ l HCl. The organic phase was dried under vacuum, and resolved by TLC as described above. To determine intracellular S1P levels, cells were scraped in 400  $\mu$ l methanol and 25  $\mu$ l HCl was added. Lipids were extracted by the addition of 400  $\mu$ l chloroform, 400  $\mu$ l KCl, and 40  $\mu$ l 3 M NaCl. The aqueous phase was mixed with 50  $\mu$ l HCl and then with 400  $\mu$ l chloroform, and lipid phase was extracted. The lipid phase extracts were combined, dried under vacuum, and resolved by TLC as above.

#### Western blotting, co-immunoprecipitation, and RT-PCR analyses

Western blot, co-immunoprecipitation, and RT-PCR experiments were conducted as previously described (Shin *et al*, 2002; Ha *et al*, 2003). For Western blotting shown in Figure 1A, boiling sample

buffer was used to lyse the cells. For co-immunoprecipitation shown in Figure 1F, a light chain-specific secondary antibody was used and experimental details are described in Supplementary data. The sequences of PCR primers are provided in Supplementary data. The number of amplification cycles was determined to be in a linear range of amplification. Twenty-two cycles were run for GAPDH and 30–35 cycles for the others.

#### Migration assay

Migration assay was conducted using a 24-well Transwell plate (Corning) possessing 8- $\mu$ m pores. Cells were serum-starved for 3 h and  $5 \times 10^5$  osteoblasts or  $1 \times 10^6$  T cells were applied to the upper well and incubated with various doses of S1P or CM in the lower well. After the indicated time, the side of membranes facing the upper well was wiped of cells and stained with hematoxylin. The number of migrated cells was counted.

#### T-cell preparation

Total spleen cells were separated from 6-week-old ICR mouse and processed with CD4<sup>+</sup> T-cell isolation kits using magnetic cell separation (MACS) (Miltenyi Biotech) as per the manufacturer's instructions. To activate T cells, 750 ng/ml ionomycin and 10 ng/ml PMA were added and cultured for 24 h.

#### PGE<sub>2</sub> production assay

Osteoblasts were incubated with S1P and VtD<sub>3</sub> for 24 h. PGE<sub>2</sub> secreted into culture supernatants was measured by enzyme-linked immunosorbent assay using a commercial kit (Amersham).

#### COX2 and mPGES1 activity assay

Osteoblasts were incubated in the absence or presence of 10 nM NS398 (COX2 inhibitor) or 1  $\mu$ M MKK886 (mPGES1 inhibitor) for 30 min. The medium was changed with one supplemented with 30  $\mu$ M arachidonic acid and cells were stimulated with S1P and/or VtD<sub>3</sub> for 10 min at 37°C. The released PGE<sub>2</sub> was measured as above. The PGE<sub>2</sub> level difference between inhibitor-treated and untreated samples was considered as the corresponding enzyme activity.

#### Statistical analysis

All experiments except ones involving  $^{32}$ Pi-labeling were performed 3–4 times. Each quantitative experiment was performed in 3–5 replicates, except  $^{32}$ Pi-labeling experiments performed in duplicate. The data are expressed as means  $\pm$  s.d. from the representative experiments. Statistical significance of differences between experimental and control groups was evaluated by Student's *t* test.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

## Acknowledgements

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