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Sphingosine 1-phosphate receptor activation enhances BMP-2-induced osteoblast differentiation

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ABSTRACT

We previously demonstrated that sphingosine 1-phosphate (S1P) receptor-mediated signaling induced proliferation and prostaglandin productions by synovial cells from rheumatoid arthritis (RA) patients. In the present study we investigated the role of S1P receptor-mediated signaling for osteoblast differentiation. We investigated osteoblast differentiation using C2C12 myoblasts, a cell line derived from murine satellite cells. Osteoblast differentiation was induced by the treatment of bone morphogenic protein (BMP)-2 in the presence or absence of either S1P or FTY720 (FTY), a high-affinity agonist of S1P receptors. Osteoblast differentiation was determined by osteoblast-specific transcription factor, Runx2 mRNA expression, alkaline phosphatase (ALP) activity and osteocalcin production by the cells. Smad1/5/8 and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation was examined by Western blotting. Osteocalcin production by C2C12 cells were determined by ELISA. Runx2 expression and ALP activity by BMP-2-stimulated C2C12 cells were enhanced by addition of either S1P or FTY. Both S1P and FTY enhanced BMP-2-induced ERK1/2 and Smad1/5/8 phosphorylation. The effect of FTY was stronger than that of S1P. S1P receptor-mediated signaling on osteoblast differentiation was inhibited by addition of mitogen-activated protein kinase/ERK kinase (MEK) 1/2 inhibitor, indicating that the S1P receptor-mediated MEK1/2-ERK1/2 signaling pathway enhanced BMP-2-Smad signaling. These results indicate that S1P receptor-mediated signaling plays a crucial role for osteoblast differentiation.

1. Introduction

Bone formation requires coordination of osteoblasts and osteoclasts, mediated by multiple growth factors and cytokines [1]. The bone morphogenic proteins (BMPs) are members of the TGF-β superfamily, and play a central role in bone formation. BMPs are expressed preferentially in mesenchymal tissues prefiguring the future skeleton, developing bones, and differentiated chondrocytes and osteoblasts [2,3]. To date, angiogenic growth factors such as fibroblast growth factor (FGF)-2 and FGF-4 [4,5] and vascular endothelial growth factor (VEGF) [6] have been reported to act synergistically with BMP-2 to promote osteoblast differentiation. In contrast, the inhibitory effect of hepatocyte growth factor (HGF), one of the angiogenic growth factors, has also been reported [7,8].

Sphingosine 1-phosphate (S1P) is one of the cell-derived lysophospholipid growth factors that signal diverse cellular functions such as proliferation, angiogenesis, and inflammation [9]. We previously demonstrated that S1P receptor-mediated signaling induced proliferation and prostaglandin (PG) production by synovial cells from rheumatoid arthritis (RA) patients [10]. It also has been reported that S1P exerts a novel and physiologically important biological activity in myoblasts acting simultaneously as antiproliferative and differentiating agents [11,12]. In the present study we investigated the role of S1P receptor-mediated signaling for osteoblast differentiation using C2C12 myoblasts, a cell line derived from murine satellite cells, which provide a useful model to study osteoblast differentiation in vitro [13].

2. Materials and methods

2.1. Cell cultures

The C2C12 mouse myoblast cell line was purchased from American Type Culture Collection (Manassas, VA, USA) [13]. C2C12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA) containing 10% fetal calf serum (FCS) and

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenic protein; ERK, extracellular signal-regulated kinase; HGF, hepatocyte growth factor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PG, prostaglandin; S1P, sphingosine 1-phosphate.

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antibiotics (100 units/ml penicillin, and 100 μg/ml streptomycin) at 37 °C under a humid atmosphere of 95% air/5% CO_2.

2.2. Alkaline phosphatase (ALP) assay

C2C12 cells were seeded in 24-well tissue culture plates at a density of 1×10^5/ml/well. Cells were cultured for 7 days with BMP-2 (300 ng/ml) (R&D systems, Minneapolis, MN, USA) in the presence or absence of either S1P (0.01–0.1 μM) (Sigma) or FTY720 (FTY) (0.01–0.1 μM) (Cayman, Ann Arbor, MI, USA). ALP activity in the cells was determined using an ALP staining kit (Takara Bio, Shiga, Japan). The ALP staining area was measured quantitatively using Image J ver. 1.37 software.

2.3. Enzyme-linked immunosorbent assay (ELISA)

C2C12 cells were seeded in 24-well tissue culture plates at a density of 1×10^5/ml/well. After 2, 4 and 7 days culture with BMP-2 (300 ng/ml) in the presence or absence of either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM), concentrations of HGF in culture media were assayed using a mouse HGF ELISA kit (RayBio, Norcross, GA, USA). Concentrations of osteocalcin in culture media were determined after 7 and 10 days culture using a mouse osteocalcin ELISA kit (Biomedical Technologies, Inc., Stoughton, MA, USA).

2.4. Quantitative real time RT-PCR

Runx2 mRNA expression was determined using quantitative real time RT-PCR. C2C12 cells were seeded in 24-well tissue culture plates at a density of 1×10^6 cells/ml/well. The cells were stimulated with BMP-2 (300 ng/ml) with or without either S1P (0.01–0.1 μM) or FTY(0.01–0.1 μM). After 24 and 48 h of culture, RNA was extracted and quantitative real time RT-PCR was performed using a TaKaRa PCR kit (Takara).

2.5. Western blot analysis

C2C12 cells were seeded in 12-well tissue culture plates at an initial density of 2×10^6 cells/ml/well and were stimulated with BMP-2 (300 ng/ml) in the presence or absence of either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM). After 10 min stimulation, Western blot analysis was performed. Briefly, cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, CA, USA), and protein content was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard. Each sample (20 μg) was resolved on 10% polyacrylamide gels under denaturing conditions and then transferred to 0.45-μm nitrocellulose membranes. After blocking overnight at 4 °C with 5% nonfat milk in Tris-buffered saline – 0.01% Tween 20 (Santa Cruz Biotechnology), membranes were incubated overnight at 4 °C with primary antibody against mouse anti-phospho-smad1/5/8 antibody (1:1000 dilution in PBS; Santa Cruz Biotechnology) or mouse anti-β-actin antibody (Cell Signaling Technology, Beverly, MA, USA). After washing the membranes with Tris buffered saline – 0.05% Tween 20 (washing buffer), HRP-conjugated secondary antibody (1:1000 dilution in PBS; Santa Cruz Biotechnology) was added, followed by incubation for 45 min. After further washing, the color was similarly developed with luminol reagent (Santa Cruz Biotechnology), and HRP activity of blots was analyzed using a LAS1000 imager (Fuji film, Tokyo, Japan). C2C12 cells were also blotted with either anti-phospho-extracellular signal-regulated kinase (ERK) 1/2 antibodies or t-ERK1/2 antibodies (1:1000 dilution in PBS; Santa Cruz Biotechnology) after 5 min stimulation.

2.6. Effect of mitogen-activated protein kinase(MAPK)/ERK kinase (MEK) 1/2 inhibitor or pertussis toxin (PTX) on S1P-enhanced osteoblast differentiation

C2C12 cells were preincubated for 72 h in the presence of MEK1/2 inhibitor, U0126 (10 μM) (Promega, Madison, WI, USA). After rigorous washing, cells were stimulated with BMP-2 (300 ng/ml) in the presence or absence of either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM) at a density of 1×10^5/ml/well for an additional 7 days and ALP activity in the cells was determined using an ALP staining kit (Takara Bio). C2C12 cells were also preincubated for 16 h in the presence or absence of 100 ng/ml PTX (Sigma). After rigorous washing, cells were stimulated with BMP-2 (300 ng/ml) in the presence or absence of either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM) at a density of 1×10^5/ml/well for additional 10 days. Osteocalcin production by the cells was determined using an osteocalcin ELISA kit (Biomedical Technologies, Inc.).

2.7. Effect of S1P receptor antagonist on S1P-enhanced osteoblast differentiation

C2C12 cells were preincubated for 30 min in the presence or absence of either 10 μM $S1P_1$ antagonist: (W146) [14], $S1P_2$ antagonist: (JTE-013) [15], or $S1P_3$ antagonist: (CAY10444) [16] (Cayman Chemical, Ann Arbor, MI, USA). After rigorous washing, cells were stimulated with BMP-2 (300 ng/ml) in the presence or absence of either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM) at a density of 1×10^5/ml/well for additional 10 days. Osteocalcin production by the cells was determined using an osteocalcin ELISA kit (Biomedical Technologies, Inc.).

2.8. Statistical analysis

Results are expressed as the mean ± SE. The significance of the difference between the experimental results and control values was determined by Student's *t*-test. *P* values less than 0.05 were considered significant.

3. Results

3.1. S1P receptor-mediated signaling enhances osteoblast differentiation

We observed enhancing effects of S1P and FTY on ALP activity of BMP-2-stimulated C2C12 cells after 7 days of culture (Fig. 1A and B). Osteocalcin is a late osteoblast differentiation marker, and we next examined the effects of S1P receptor-mediated signaling on osteocalcin production by the cells. We observed enhancing effects of S1P and FTY on osteocalcin production of BMP-2-stimulated C2C12 cells after 10 days of culture (Fig. 1C). These enhancing effects of FTY were stronger than those of S1P. These results indicate that S1P receptor-mediated signaling enhances BMP-2 induced osteoblast differentiation.

3.2. S1P receptor-mediated signaling enhances Runx2 expression

Runx2 is an osteoblast-specific transcription factor, which is essential for the differentiation of osteoblasts from mesenchymal precursors [17,18]. Therefore, we examined whether Runx2 expression of BMP-2-stimulated C2C12 cells was enhanced by S1P receptor-mediated signaling. We examined Runx2 mRNA expression of BMP-2-stimulated C2C12 cells in the presence or absence of either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM). After 24

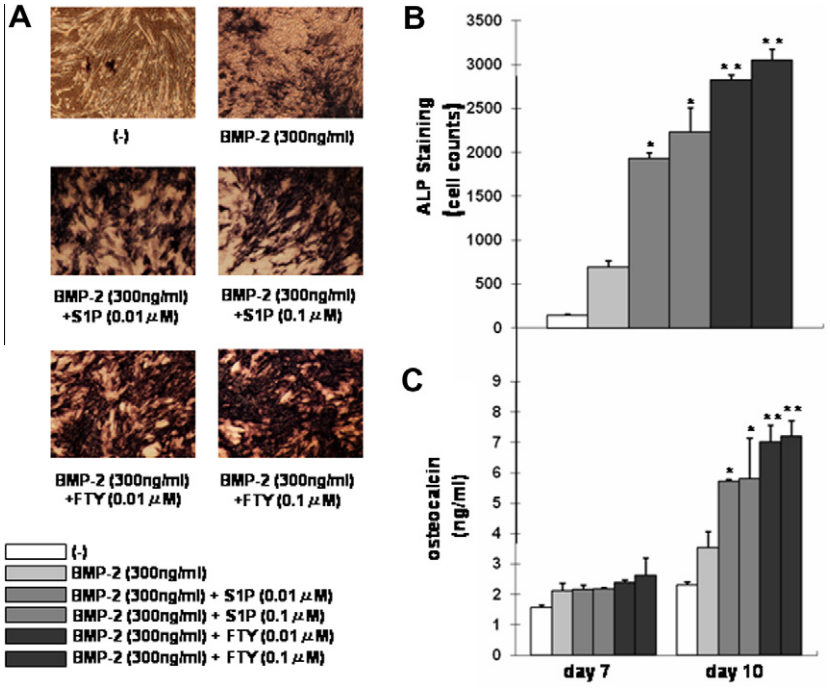

Fig. 1. S1P receptor-mediated signaling enhances BMP-2-induced osteoblast differentiation by C2C12 cells. C2C12 cells were treated with BMP-2 (300 ng/ml) with or without either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM). ALP histochemical staining was performed after 7 days of culture. (A) Representative images of staining for each experimental group. (B) Data represent mean ± SE (n = 3) for ALP staining cell counts for each experimental group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs BMP-2 treatment alone. (C) Osteocalcin production was examined after 7 and 10 days of culture. Data represent mean ± SE (n = 3) for each experimental group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs BMP-2 treatment alone.

and 48 h of culture, Runx2 mRNA levels by BMP-2-stimulated C2C12 cells were enhanced by both S1P and FTY (Fig. 2A).

3.3. S1P receptor-mediated signaling enhances BMP-Smad signaling

The osteogenic activity of BMP-2 is partly mediated by nuclear phosphorylation and nuclear translocation of Smads, which interact directly with DNA and associate with other transcription factors to regulate osteogenesis [19,20]. Therefore, we investigated whether S1P receptor-mediated signaling enhanced osteoblast differentiation by altering Smad phosphorylation. FTY significantly enhanced BMP-2-stimulated Smad1/5/8 phosphorylation by C2C12 cells, suggesting that S1P receptor-mediated signaling enhanced BMP-Smad signaling in C2C12 cells (Fig. 2B and C).

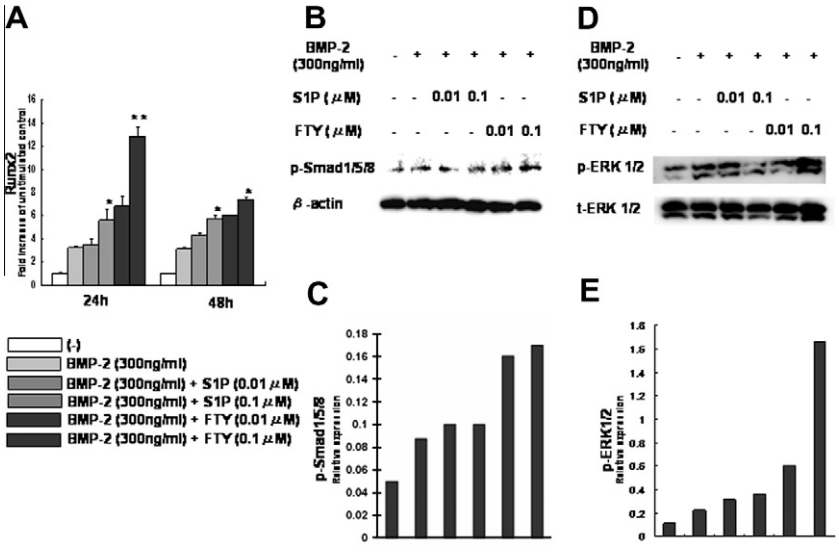

Fig. 2. S1P receptor-mediated signaling enhances BMP-2-induced Runx2 mRNA expression, Smads, and ERK1/2 phosphorylation by C2C12 cells. (A) C2C12 cells were treated with BMP-2 (300 ng/ml) with or without either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM) for 24 h and 48 h and Runx2 mRNA expression was determined by real time RT-PCR analysis. Data represent mean ± SE (n = 3) for each experimental group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs BMP-2 treatment alone. (B) C2C12 cells were treated with BMP-2 (300 ng/ml) with or without either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM) for 10 min and p-Smad1/5/8 expression was determined by Western blotting. Representative data of p-Smad1/5/8 and β-actin expression by C2C12 cells. (C) Relative p-Smad1/5/8 expression (p-Smad1/5/8/β-actin). (D) C2C12 cells were treated with BMP-2 (300 ng/ml) with or without either S1P (0.01–1 μM) or FTY (0.01–1 μM) for 5 min and p-ERK1/2 and t-ERK1/2 expression was determined by Western blotting. Representative data of p-ERK1/2 and t-ERK1/2 expression by C2C12 cells. (E) Relative p-ERK1/2 expression (p-ERK1/2/t-ERK1/2).

3.4. Signaling pathways involved in the biologic responses to S1P and FTY

To gain insight into the signaling pathways responsible for the observed biological effect of S1P receptor-mediated signaling, ERK1/2 was examined to assess the ability of S1P and FTY to activate protein kinases. BMP-2 enhanced ERK1/2 phosphorylation. In addition, both S1P and FTY enhanced BMP-2 stimulated ERK1/2 phosphorylation by C2C12 cells. These enhancing effects of FTY were stronger than those of S1P (Fig. 2D and E). These results suggest that BMP-2 activates ERK kinase and S1P receptor-mediated signaling augments BMP-2-induced ERK1/2 phosphorylation by C2C12 cells. We next examined the effect of a MEK1/2 inhibitor (U0126) (10 μM) on C2C12 cells stimulated with BMP-2 (300 ng/ml) with or without either S1P (0.1 μM) or FTY(0.1 μM). The MEK1/2 inhibitor significantly prevented osteoblast differentiation of BMP-2-stimulated C2C12 cells (Fig. 3).

3.5. Gi protein-dependent signaling pathways for osteoblast differentiation

In view of the S1P receptors' ability to couple to various G-proteins, the possibility that the biological effects of S1P and FTY were mediated by Gi was explored by employing PTX, which ADP-ribosylates and inactivates Gi. The enhancing effect of FTY on BMP-2-stimulated osteocalcin production was significantly affected by cell treatment with PTX (100 ng/ml, 16 h) (Fig. 4A). Since $S1P_1$ couples only to Gi, most of its effects are PTX sensitive [21]. However, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$ binds to PTX-sensitive as well as to PTX-insensitive Gq and G13 [22]. Therefore, we further evaluated possible involvement of these receptors in the S1P-induced osteoblast differentiation. Results show that cell treatment with $S1P_1$ but not $S1P_3$ antagonist significantly affected the enhancing effect of FTY on BMP-2-stimulated osteocalcin production. $S1P_2$ antagonist moderately affected the enhancing effect of FTY (Fig. 4B–D). Since phosphorylated FTY (FTY-P) does not bind to $S1P_2$ [23], these results suggest that S1P-MEK/ERK signaling mediated via $S1P_1$ mainly play a role for the modulation of osteoblast differentiation by FTY.

4. Discussion

FTY is a high-affinity agonist of S1P receptors which induces internalization of S1P receptors, rendering the cells unresponsive to S1P. We recently reported that FTY could inhibit arthritis and bone destruction in an RA model of SKG mice by inhibition of the inflammatory cell infiltration into the synovium and the production of inflammatory mediators such as IFN-γ and PG [24]. In the present study, we demonstrated that S1P receptor-mediated signaling enhanced BMP-induced osteoblast differentiation.

We observed that S1P receptor-mediated signaling enhanced expression of markers for osteoblast differentiation, such as ALP and osteocalcin by C2C12 cells stimulated with BMP-2. We also found that S1P receptor-mediated signaling enhanced Smad phosphorylation and Runx2 mRNA expression in BMP-2-stimulated C2C12 cells, suggesting that the effects of S1P receptor-mediated signaling on BMP-2 induced osteoblast differentiation may be due to the enhancement of early steps of the BMP-2 signaling pathway.

The activation of S1P receptors by S1P has been reported to cause phosphorylation of ERK in astrocytes [25,26]. Other cell types including endothelial cells and renal mesangial cells also show an increase in activation of ERK/MAPK in response to treatment with FTY at nanomolar concentrations [27,28]. We also observed that both S1P and FTY caused phosphorylation of ERK (data not shown). To gain insight into the molecular mechanisms responsible for the observed biological effects of S1P receptor-mediated signaling on osteoblast differentiation, we examined the ability of the S1P receptor-mediated signaling to enhance ERK1/2 phosphorylation. Both S1P and FTY enhanced ERK1/2 phos-

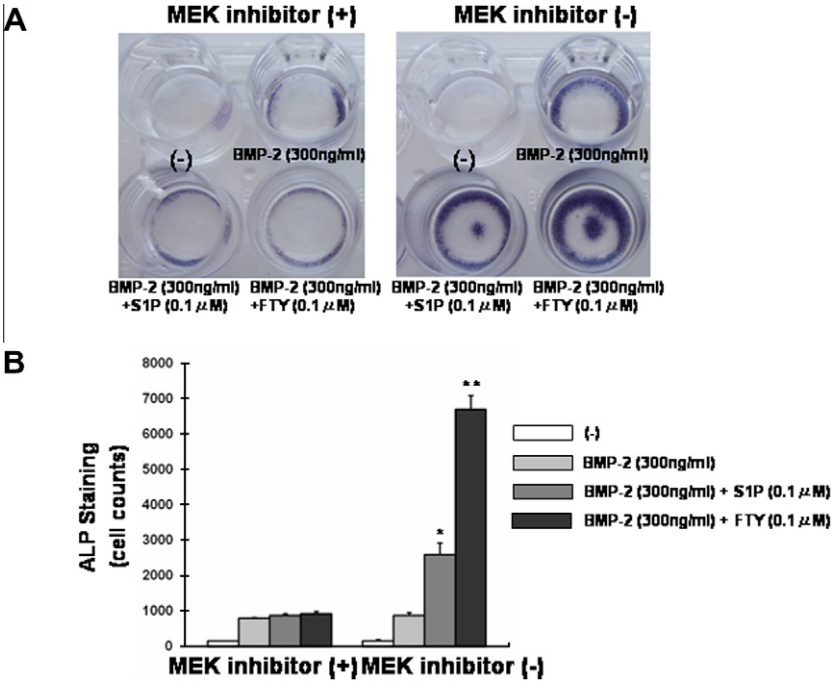

Fig. 3. MEK inhibition by U0126 suppresses BMP-2-induced ALP expression. C2C12 cells were preincubated for 72 h in the presence or absence of the MEK1/2 inhibitor, U0126 (10 μM). Cells were then stimulated with BMP-2 (300 ng/ml) in the presence or absence of either S1P (0.1 μM) or FTY (0.1 μM) at a density of 1×10^5/ml/well for an additional 7 days and ALP activity in the cells was determined. (A) Representative images for each experimental group. (B) Data represent mean ± SE ($n = 3$) for each experimental group. $^*p < 0.05$, $^{**}p < 0.01$ vs MEK1/2 inhibitor treatment.

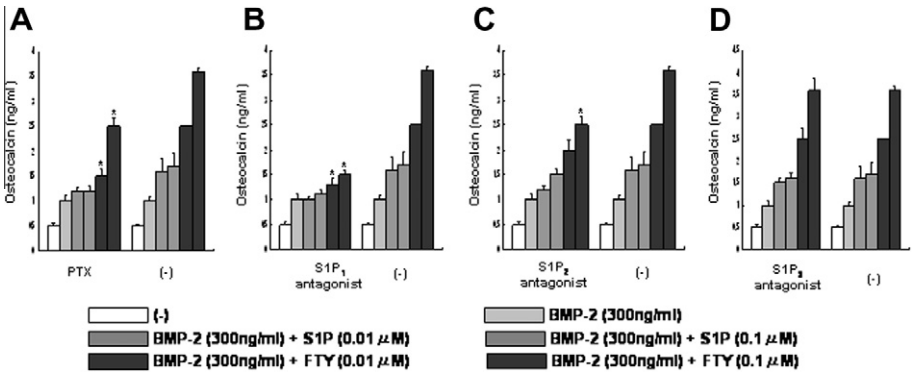

Fig. 4. $S1P_1$ inhibition significantly suppresses S1P-induced osteocalcin production by C2C12 cells. (A)C2C12 cells were preincubated for 16 h in the presence or absence of 100 ng/ml PTX. Cells were then stimulated with BMP-2 (300 ng/ml) in the presence or absence of either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM) at a density of 1×10^5/ml/well for additional 10 days and osteocalcin production by the cells was determined. (B–D) C2C12 cells were preincubated for 30 min in the presence or absence of either 10 μM $S1P_1$ antagonist: (W146), $S1P_2$ antagonist: (JTE-013), or $S1P_3$ antagonist: (CAY10444). Cells were then stimulated with BMP-2 (300 ng/ml) in the presence or absence of either S1P (0.01–0.1 μM) or FTY (0.01–0.1 μM) at a density of 1×10^5/ml/well for an additional 10 days and osteocalcin production by the cells was determined. $^*p < 0.05$ vs untreated control.

phorylation by BMP-2 in C2C12 cells. These enhancing effects of FTY were stronger than those of S1P. Furthermore, U0126, a MEK1/2 inhibitor, significantly suppressed osteoblast differentiation as determined by ALP staining induced by BMP-2 in C2C12 cells. These results suggest that S1P receptor-mediated signaling enhances BMP-2 induced osteoblast differentiation via activation of an ERK/ MAPK pathway. We also observed that the enhancement of osteoblast differentiation by S1P receptor-mediated signaling was significantly inhibited by PTX-pretreatment as well as $S1P_1$ antagonist. The result indicates that $S1P_1$ receptor mediated signaling pathways play a role for osteoblast differentiation.

We observed that BMP-2 stimulation induced ERK1/2 phosphorylation by C2C12 cells. It has been reported that in addition to the Smad signaling pathway, diverse intracellular molecules such as ERK, P38 MAPK, and c-jun also participate in BMP-2-induced osteoblast differentiation [29–31]. A recent study conclusively established a pivotal function for ERK signaling in osteoblast differentiation and skeletal development using transgenic mice expressing dominant negative or constitutively active MEK1 protein [32]. Furthermore, BMP-induced ERK signaling cooperatively regulates osteoblast differentiation partly via increasing the stability and transcriptional activity of Runx2 [33].

Although both S1P and FTY enhanced BMP-2-induced ERK1/2 phosphorylation and osteoblast differentiation by C2C12 cells, these enhancing effects of FTY were stronger than those of S1P. One explanation for these differential enhancing effects between S1P and FTY is the stronger affinity of FTY-P to $S1P_1$ than that of S1P [34]. Another explanation is that a novel FTY-mediated pathway for modulating ERK1/2 phosphorylation and osteoblast differentiation may exist as reported previously. The G proteins coupled receptors (GPCRs) family contains dozens of receptors with varying homologies to the S1P receptors that could potentially participate in transduction of FTY responses [35]. For example, a recent study described FTY interaction with the cannabinoid family of GPCRs [36]. We observed that $S1P_2$ antagonist, JTE013, moderately affected the enhancing effects of FTY on osteoblast differentiation in contrast to the results that FTY-P does not bind to $S1P_2$ [23]. One possible explanation for these conflicting results is that $S1P_4$-mediated FTY responses may enhanced osteoblast differentiation and JTE013 antagonized these responses since JTE013 can also function as an $S1P_4$ antagonist, as evidenced by results showing that JTE013 potently reduced S1P-stimulated calcium mobilization in HTC4 cells overexpressing $S1P_4$ [37]. Another explanation is that JTE013 can antagonize to another GPCRs with homologies to the S1P receptors that could potentially participate in transduction of FTY responses for osteoblast differentiation [36].

Ryu et al. first reported that S1P produced by osteoclasts induced RANKL expression by osteoblasts, thereby inducing osteoclast differentiation [38]. Ishii et al. reported that treatment of an $S1P_1$ agonist such as FTY mobilized osteoclast precursors from the bone surface, leading to inhibition of osteoclastogenesis [39]. However, direct effects of S1P receptor-mediated signaling on osteoblast differentiation have not yet been reported. This is the first report to demonstrate that S1P receptor-mediated signaling enhances osteoblast differentiation. FTY may be useful for the treatment of RA by enhancing osteoblast differentiation in the synovium.

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