Modulation of the expression of sphingosine 1-phosphate 2 receptors regulates the differentiation of pre-adipocytes

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Received September 18, 2014; Accepted June 5, 2015

DOI: 10.3892/mmr.2015.4388

Abstract. Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator that regulates multiple signals through S1P receptors responsible for biological responses. In particular, the $S1P_2$ receptor has distinct roles in the S1P-mediated differentiation of certain cell types. The present study was the first, to the best of our knowledge, to report the role of the $S1P_2$ receptor in the adipocyte differentiation of 3T3-L1 pre-adipocytes. In order to investigate the influence of $S1P_2$ receptors in the anti-adipogenic effects of S1P, $S1P_2$ receptor silencing and overexpression of were used. $S1P_2$ overexpression with adenoviral vectors inhibited adipogenesis and inhibited the expression of peroxisome proliferator-activated receptor γ (PPARγ), adiponectin and CCAAT/enhancer binding protein-α, which were upregulated following incubation in differentiation media. Furthermore, S1P completely lost its ability to impair adipogenic differentiation following silencing of $S1P_2$. Silencing of the $S1P_2$ receptor additionally blocked the downregulation of PPARγ protein and phospho-c-Jun N-terminal kinase protein induced by S1P treatment. In conclusion, the present study demonstrated that the $S1P_2$ receptor is a key signaling molecule in the S1P-dependent inhibition of adipogenic differentiation and additionally suggested that selective targeting of $S1P_2$ receptors may have clinical applications for the treatment of obesity.

Introduction

Obesity is an increasingly prevalent metabolic disorder and has become an epidemic condition in developed countries in recent decades (1). In addition, the prevalence of metabolic syndromes and various chronic diseases, including diabetes, fatty liver, coronary artery disease and hypertension, are increasing due to the rise of obesity (2). Increases in body fat mass result from an increase in the number and size of adipocytes (3,4). Previous studies have demonstrated that the induction of obesity, resulting in alterations in the number of adipocytes (adipogenic differentiations) and adipocyte size (lipid accumulations) can be initiated by dietary factors (3,4). A previous study demonstrated that increases in the number of adipocytes during the aging process may influence the development of obesity observed in older individuals (5). Thus, adipogenesis may be an important factor in the development of obesity.

Adipocytes differentiate from mesenchymal stem cells, which have the capacity for differentiation into myoblasts, chondroblasts, osteoblasts or adipocytes (6). Tissue-specific differentiation is regulated by a variety of differentiation factors in accordance with the cells' conditions. Among the differentiation factors influencing adipogenesis, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBP-α, C/EBP-β and C/EBP-γ) are considered the key factors for the induction of adipogenesis in mesenchymal stem cell-mediated differentiation. These two factors are required for the expression of adipocyte-specific genes such as adiponectin (7).

Sphingosine-1-phosphate (S1P) is part of a key group of signaling sphingolipids recognized to serve diverse roles in a variety of cellular processes, including apoptosis, migration, differentiation and proliferation in a variety of cell types, including endothelial cells, smooth muscle cells, mesenchymal stem cells and macrophages (8-10). S1P has been demonstrated to act as a ligand of G-protein-coupled receptors, namely S1P receptors (11). Five members of the S1P receptor group ($S1P_{1-5}$) have been identified in mammals, which possess distinct expression profiles and affinities toward S1P (12,13). In particular, $S1P_2$ receptors are widely expressed throughout the body, including in the brain, heart, lung, thymus, kidney, spleen and adipose tissues (14,15). $S1P_2$ receptors are associated with differentiation of various tissue types, including those associated with the central nervous system (CNS), as well as the differentiation of mesenchymal stem cells and osteoblasts (10,16,17). During early stages of CNS development,

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Key words: sphingosine-1-phosphate, sphingosine 1-phosphate 2 receptor, 3T3-L1, adipocyte, differentiation

$S1P_2$ receptors have been identified to be expressed in young animals and differentiate into neuronal cells (16). In addition, $S1P_2$ receptors upregulate myogenic differentiation of myoblast cells and adipose-derived mesenchymal stem cells (10,17,18). However, to date, the effect of S1P-mediated $S1P_2$ receptors on adipogenic differentiation has remained to be fully elucidated.

The present study investigated the hypothesis that S1P inhibits adipocyte differentiation via the regulation of $S1P_2$ receptors. Therefore the effects of up- or downregulation of $S1P_2$ receptors on differentiation of adipocytes were examined. Furthermore, the levels of the adipogenic differentiation markers PPARγ, C/EBP-α and adiponectin were assessed.

Materials and methods

Reagents. S1P was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). S1P was prepared as a 2-mM solution in 0.3 M NaOH and then further diluted in cell culture medium. 3-isobutyl-1-methylxanthine, dexamethasone and insulins were purchased from Sigma-Aldrich (St. Louis, MO, USA). $S1P_2$ antibody and normal goat immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture and differentiation. Pre-adipocyte cell line 3T3-L1 cells and human embryonic kidney cell line HEK 293 cells were obtained from the American Type Culture collection (Rockville, MD, USA). The 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics: 100 μg/ml gentamycin (Invitrogen Life Technologies, Carlsbad, CA, USA) and 100 μg/ml penicillin-streptomycin (HyClone, Logan, UT, USA). To induce differentiation, 2 days post-confluent 3T3-L1 cells were incubated in MDI induction media (DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μm dexamethasone and 1 μg/ml insulin) for 2 days. In certain experiments, S1P (10 μM) was added at the time of the induction of differentiation. Two days subsequent to the addition of MDI (day 2), the media was replaced with insulin media. The AdipoRed assay and detection of glycerol release contents were performed on day 7.

Construction of recombinant adenoviruses. Mouse $S1P_2$-expressing adenoviruses and empty vector adenoviruses were purchased from Genenmed, Inc. (Seoul, Korea; Gen-E008-001). Recombinant adenoviruses were amplified in human embryonic kidney HEK-293 cells and purified using the Vivapure AdenoPACK kit (Sartorius AG, Göttingen, Germany) according to the manufacturer's instructions (19).

$S1P_2$ RNA interference. 3T3-L1 cells were transfected with validated Stealth™ small interfering (si)RNAs (Invitrogen Life Technologies) directed against $S1P_2$ using Lipofectamine 2000 (Invitrogen Life Technologies) RNA interference transfection protocol. Sequences of the $S1P_2$ siRNAs used were as follows: Sense, 5'-AGAAGAUUC UCCACCACGAUGGCGC-3' and anti-sense, 5'-GCGCCA UCGUGGUGGAGAAUCUUCU-3'. The Stealth™ RNA interference negative control (medium G/C) were also obtained from Invitrogen Life Technologies.

Quantification of lipid content. Lipid content was quantified using the commercially available AdipoRed Assay Reagent (Lonza, Verviers, Belgium) in accordance with the manufacturer's instructions. In brief, pre-adipocytes were grown in 24-well plates and then incubated with MDI media with or without S1P during the adipogenic phase. On day 7, the culture supernatant was removed and the cells were carefully washed with 500 μl phosphate-buffered saline (PBS). The wells were then filled with 300 μl PBS and 30 μl AdipoRed reagent, followed by incubation for 10 min at 37˚C. Fluorescence was measured with excitation at 485 nm and emission at 572 nm.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from 3T3-L1 cells treated with S1P using the Easy-spin™ total RNA extraction kit (Intron Biotechnology, Inc., Seongnam, Korea). cDNA synthesis was performed following the instructions of the Takara Prime Script™ 1st Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan). For RT-qPCR, 1 μl gene primers with iTaq SYBR Green supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a 20-μl reaction volume was used. The sequences of the primers used for RT-qPCR were as follows: $S1P_2$ forward, 5'AACAGCAAGTTCCACTCA GCAATG3' and reverse, 5'GGCGGAGAGCGTGATGAA GG3'; PPARγ forward, 5'CGGAAGCCCTTTGGTGACTTT ATG3' and reverse, 5'GCAGCAGGTTGTCTTGGATGT C3'; C/EBP-α forward, 5'CGGGAACGCAACAACATC GC3' and reverse, 5'TGTCCAGTTCACGGCTCAGC3'; adiponectin forward, 5'TGACGGCAGCACTGGCAAG3' and reverse, 5'TGATACTGGTCGTAGGTGAAGAGAAC3'; and β-actin forward, 5'TGAGAGGGAAATCGTGCGTGAC3' and reverse, 5'GCTCGTTGCCAATAGTGATGACC3'. All primers were purchased from Bioneer Inc. (Daejeon, Korea).

All reactions with iTaq SYBR Green Supermix were performed on the CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). The PCR program was as follows: Denaturation (95˚C for 10 min), amplification and quantification for 40 cycles (95˚C for 10 sec, 55-60˚C for 30 sec, and 72˚C for 30 sec with a single fluorescent measurement), melting curve analysis (65-95˚C, with a heating rate 0.2˚C/sec and continuous fluorescence measurement), and final cooling to 12˚C.

The amplification of specific RT-qPCR products was confirmed by performing a melting-curve step at the end of each run. Across all the assays, none of the quantification cycle (Cq) values were higher than 40. No-template and no-reverse transcription controls were run to determine any contamination or the generation of primer dimers. All amplifications were run in triplicate.

Western blot analysis. The 3T3-L1 cells were lysed in lysis buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM dithiothreitol and protease inhibitor mixture]. All reagents for cell lysis were purchased from Sigma-Aldrich. Proteins were electrophoretically resolved by 8-15% SDS-PAGE and immunoblotting was performed as previously described (20). Images were captured using the Fusion FX7 Acquisition system (Vilbert Lourmat GmbH,

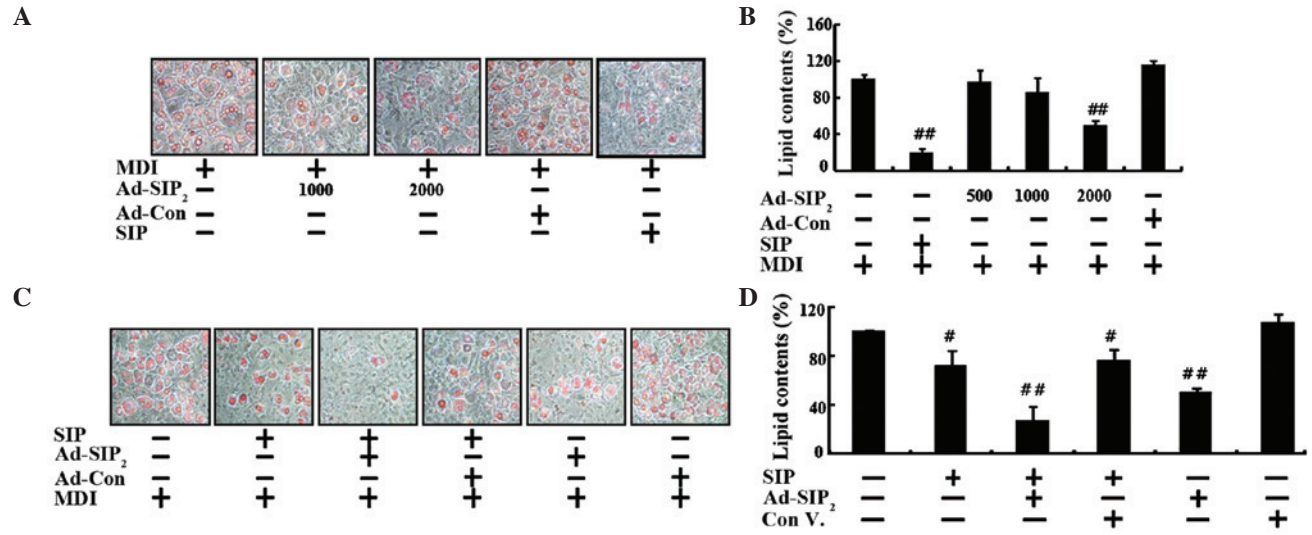

Figure 1. Overexpression of the $S1P_2$ receptor suppresses adipogenesis and enhances anti-adipogenic effects resulting from S1P treatment. (A) 3T3-L1 cells infected with adenoviruses expressing empty vector (Ad-Con; MOI, 2,000) or mouse $S1P_2$ receptor (Ad-$S1P_2$) for 48 h were induced to differentiate for 6 days. AdipoRed assays were performed. Adipocytes are indicated by red staining (magnification, x200). (B) Quantified fluorescence intensities. (C) 3T3-L1 cells infected with adenoviruses expressing empty vector (Ad-Con; MOI, 2,000) or mouse $S1P_2$ (Ad-$S1P_2$) for 48 h were treated with S1P (0.1 μM) and induced to differentiate for 6 days. AdipoRed assays were performed as described (magnification, x200). (D) Quantified fluorescence intensities. Values are expressed as the mean ± standard error (n=3). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. MDI control. The experiments were performed in triplicate and data represent three independent experiments. $S1P_2$, sphingosine 1-phosphate 2; Ad, adenovirus; Con, control; MOI, multiplicity of infection; MDI, media.

Eberhardzell, Germany). The immunoreactive bands were detected with an enhanced chemiluminescence detection system (Thermo Fisher Scientific). The antibodies used for immunoblotting were PPARγ (cat. no. sc-7273; Santa Cruz Biotechnology, Inc.), $S1P_2$ (cat. no. sc-31577; Santa Cruz Biotechnology), phosphorylated-c-Jun N-terminal kinase (p-JNK) (cat. no. 9255; Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (cat. no. A5441; Sigma-Aldrich). Goat, (cat. no. sc-3887) mouse (cat. no. sc-2025) and rabbit (cat. no. sc-2027) secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.

Statistical evaluation. All values are expressed as the mean ± standard error and were compared using Student's t-test and analysis of variance with Duncan's test. The SAS statistical package version 8.1 (SAS Institute, Inc., Cary, NC, USA) was used for analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

$S1P_2$ receptor overexpression suppresses adipogenesis and enhances the anti-adipogenic effects of S1P. To verify the differentiative role of $S1P_2$ receptors in adipogenesis, 3T3-L1 cells were infected with adenovirus for the expression of either the $S1P_2$ receptor or an adenovirus carrying an empty vector at multiplicity of infection values of 500, 1,000 and 2,000, followed by incubation in MDI. Overexpression of $S1P_2$ receptor proteins reduced the triglyceride accumulation induced by MDI-mediated adipocyte differentiation, whereas empty vector-transfected cells showed similar amounts of lipid accumulation to those in the control group incubated in MDI only (Fig. 1A and B). To confirm the anti-adipogenic function of the $S1P_2$ receptor, $S1P_2$ receptor-overexpressing cells were co-treated with S1P. The results showed that S1P treatment inhibited MDI-mediated adipogenesis and that simultaneous $S1P_2$ receptor overexpression markedly enhanced the inhibition of MDI-mediated adipogenesis of 3T3-L1 adipocytes (Fig. 1C and D). These results provided further evidence for the anti-adipogenic effects of S1P being mediated via the activation of $S1P_2$ receptor signaling.

$S1P_2$ receptor overexpression suppresses mRNA expression of adipogenic factors. The present study further investigated whether the anti-adipogenic effects of $S1P_2$ receptors are involved in mediating the mRNA and protein expression levels of adipogenic transcriptional factors (Fig. 2A-C). The expression levels of PPARγ, C/EBP-α and adiponectin mRNA were observed to be increased in MDI-treated adipocytes, and the elevated mRNA expression levels were suppressed by $S1P_2$ receptor overexpression. Western blot analysis confirmed that the $S1P_2$ receptor was overexpressed in the adipocytes treated with the adenoviral $S1P_2$ overexpression vector; furthermore, $S1P_2$ receptor overexpression downregulated the phosphorylation of JNK and expression of PPARγ protein (Fig. 2D). These results indicated that the activation of the $S1P_2$ receptor caused by $S1P_2$ receptor overexpression suppressed mRNA and protein expression levels of adipogenic factors, which exerted anti-adipogenic effects on 3T3-L1 adipocytes.

Silencing of the $S1P_2$ receptor abolishes the inhibition of lipid accumulation by S1P. siRNA was used to eliminate the $S1P_2$ in pre-adipocytes and determine the effects of $S1P_2$ knockdown on the S1P-mediated adipocyte differentiation. 3T3-L1 cells were transfected with either $S1P_2$ siRNA or negative control siRNA, and 3T3-L1 cells at 2 days post-confluence were then incubated in MDI induction media with or without S1P (10 μM) for 2 days. The media was then replaced with insulin media to further induce adipogenesis. Following $S1P_2$ knockdown, S1P

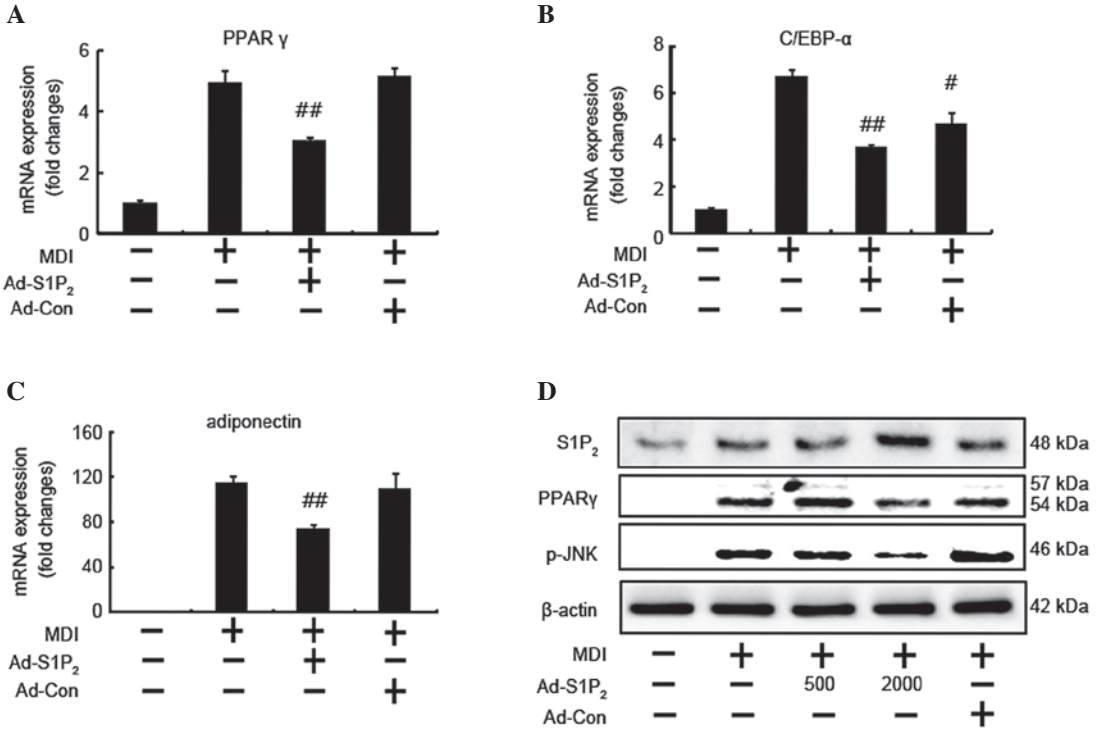

Figure 2. Overexpression of the S1P$_2$ receptor suppresses adipogenic factors. (A-C) 3T3-L1 cells infected with adenoviruses expressing empty vector (Ad-Con; multiplicity of infection, 2,000), or mouse S1P$_2$ (Ad-S1P$_2$) for 48 h were induced to differentiate for 2 days and harvested at day 2 during the differentiation period. The mRNA expression of (A) PPARγ, (B) C/EBP-α and (C) adiponectin was analyzed by reverse transcription-quantitative polymerase chain reaction. Values are expressed as the mean ± standard deviation of data from three separate experiments; each experiment was performed in triplicate. (D) Pre-adipocytes were treated as described. At day 2, the protein levels of S1P$_2$, PPARγ and p-JNK were analyzed by western blot analysis. The experiments were repeatedly performed to confirm the results. The data were analyzed using Student's t-test. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. MDI control. S1P$_2$, sphingosine 1-phosphate 2; Ad, adenovirus; Con, control; PPARγ, peroxisome proliferator-activated receptor γ; p-JNK, phosphorylated c-Jun N-terminal kinase; MDI, media.

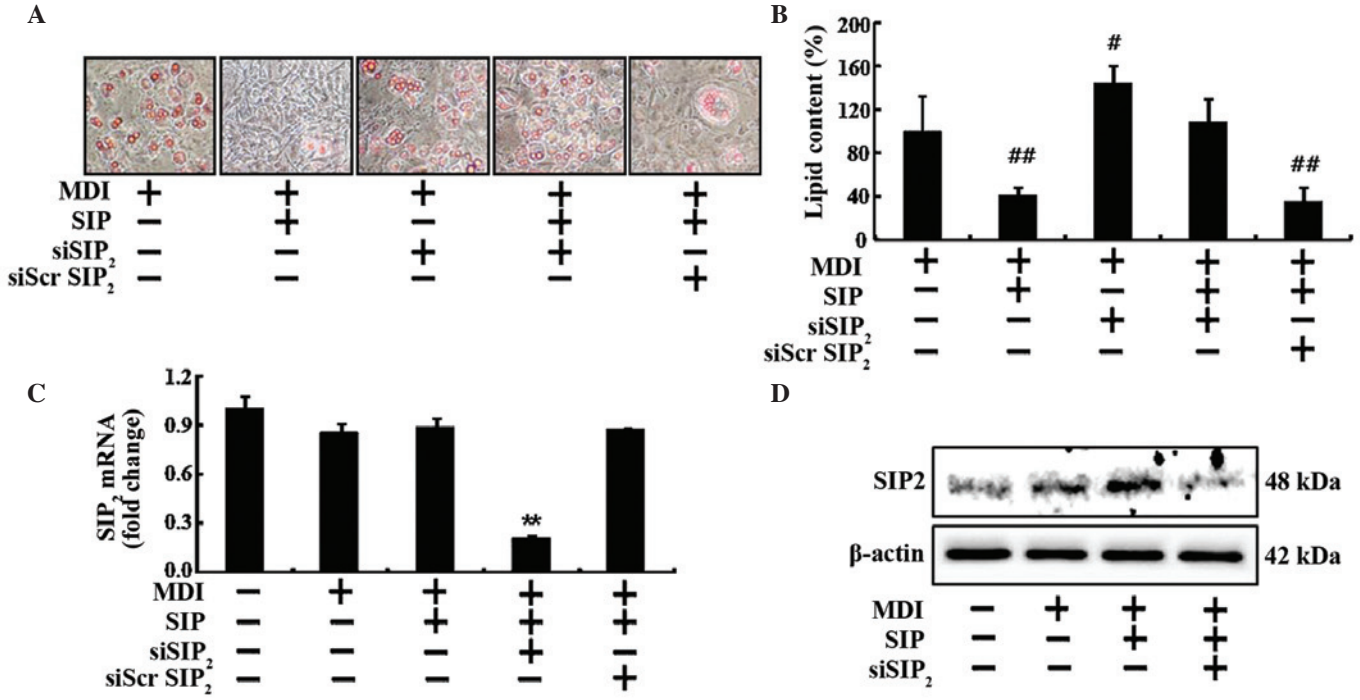

Figure 3. Knockdown of the S1P$_2$ receptor inhibited adipogenesis and lipid accumulation by S1P. (A) 3T3-L1 cells were transfected with either S1P$_2$ siRNA or negative control siRNA for 24 h, then cells were induced to differentiate for 6 days and AdipoRed assays were performed. (B) Quantified fluorescence intensities. (C and D) 3T3-L1 cells were transfected with either S1P$_2$ siRNA or negative control siRNA for 24 h, and then cells were induced to differentiate for 2 days. S1P$_2$ expression levels were evaluated by real-time reverse transcription-quantitative polymerase chain reaction and western blotting. $^{\#}$P<0.05, $^{\#\#}$P<0.01; vs. MDI control. Values are expressed as the mean ± standard error (n=3). The experiments were performed in triplicate and data represent three independent experiments. S1P$_2$, sphingosine 1-phosphate 2; siRNA, small interfering RNA; MDI, media; Scr, scrambled.

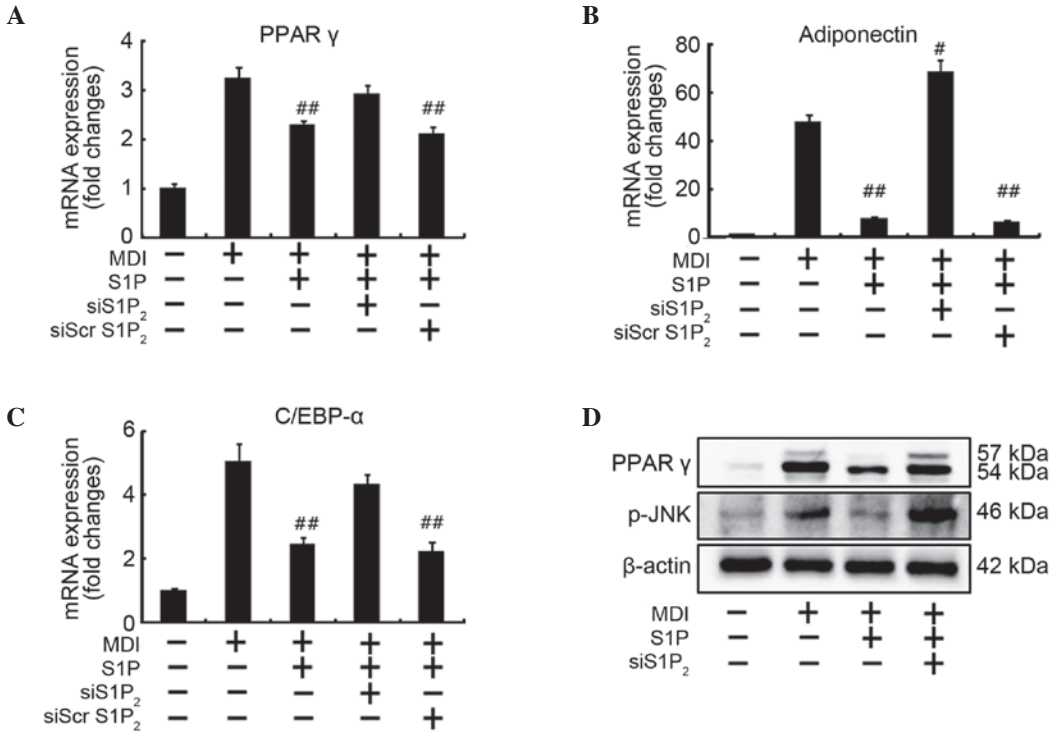

Figure 4. Silencing of the $S1P_2$ receptor abolishes the downregulation of PPARγ, C/EBP-α and adiponectin expression induced by S1P. (A-C) 3T3-L1 cells were transfected with either $S1P_2$ siRNA or negative control siRNA for 24 h; then the transfected pre-adipocytes were induced to differentiate with S1P (10 μM) and harvested at day 2 during the differentiation period. The mRNA expression of (A) PPARγ, (B) C/EBP-α and (C) adiponectin was analyzed by reverse transcription-quantitative polymerase chain reaction. Values are expressed as the mean ± standard deviation of data from three separate experiments; each experiment was performed in triplicate. (D) Pre-adipocytes were treated as described. At day 2, the protein levels of PPARγ and p-JNK were analyzed by western blotting. The experiments were repeatedly performed to confirm the results. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. MDI control. $S1P_2$, sphingosine 1-phosphate 2; PPAR, peroxisome proliferator-activated receptor γ; p-JNK, phosphorylated c-Jun N-terminal kinase; MDI, media.

completely lost its ability to impair adipogenic differentiation (Fig. 3A and B). $S1P_2$ mRNA and protein expression levels were markedly reduced in cells treated with $S1P_2$ siRNA, relative to those in cells treated with control siRNA (Fig. 3C and D). Of note, $S1P_2$ protein expression was upregulated following treatment with S1P, indicating that S1P may inhibit adipogenic differentiation via upregulation of $S1P_2$ (Fig. 3D).

Silencing of the $S1P_2$ receptor abolishes the S1P-induced downregulation of PPARγ, C/EBP-α and adiponectin expression. The present study further investigated whether the downregulation of the expression of major adipogenic transcriptional factors by S1P was a $S1P_2$ receptor-mediated process. Silencing of the $S1P_2$ receptor resulted in restoration of the mRNA expression levels of PPARγ, C/EBP-α to levels similar to those in the control group incubated with MDI only, and an increased adiponectin expression compared to that in the MDI-non-treated group (Fig. 4A-C). The results indicated that S1P inhibits adipogenic differentiation by downregulation of the major adipogenic transcriptional factors, which were involved in $S1P_2$ receptor-mediated signaling. Silencing of the $S1P_2$ receptor additionally blocked the downregulation of PPARγ protein and phospho-JNK protein induced by S1P treatment (Fig. 4D). These results demonstrated that silencing of the $S1P_2$ receptor using $S1P_2$ siRNA abolishes the inhibitory effect of S1P on adipogenesis, indicating that the $S1P_2$ receptor may serve a pivotal role in the regulation of adipogenic differentiation.

Discussion

Adipocytes are generated by differentiation of mesenchymal stem cells (6). Mesenchymal stem cells possess the ability to differentiate into numerous cell types, including adipocytes, osteoblasts, chondrocytes and smooth muscle cells. Previous studies have suggested that myogenesis, adipogenesis and fibrogenesis are competitive processes in the differentiation of mesenchymal stem cells (6,10). In addition, Nincheri *et al* (10) demonstrated that adipose tissue-derived mesenchymal stem cells differentiated into smooth muscle cells via the upregulation of $S1P_2$ receptors. Thus, it was hypothesized that upregulation of $S1P_2$ receptors may inhibit the differentiation of progenitor adipocytes into adipocytes. The results of previous studies are in agreement with those of the present study, indicating that overexpression of $S1P_2$ receptors inhibits the differentiation of 3T3-L1 pre-adipocytes into adipocytes.

The recruitment of fat cells in adipose tissue requires the differentiation of pre-adipocytes into adipocytes (adipogenesis), a process tightly controlled by the transcription factors PPARγ and C/EBP-α (21,22). In particular, PPARγ is regarded as the key regulator of adipogenesis. Forced expression of PPARγ is sufficient to induce adipocyte differentiation in fibroblasts, and no factor is known that promotes adipogenesis in the absence of PPARγ (21-23). In the present study, overexpression of $S1P_2$ had inhibitory effects on PPARγ and C/EBP-α expression in 3T3-L1 adipocytes. In addition, $S1P_2$

knockdown abrogated the downregulation of PPARγ and C/EBP-α. These observations suggested that $S1P_2$ activation may have anti-adipogenic effects in adipogenic differentiation.

Studies have been conducted on the effects of S1P on cell differentiation. S1P has been reported to act as a regulator of osteoclast differentiation (24) in addition to myogenic differentiation (18,25). It is widely accepted that S1P and the $S1P_2$ receptor are associated with myogenic differentiation of mesenchymal stem cells through G(i)-coupled S1P receptor interactions. In addition, S1P interferes with the differentiation of human monocytes into competent dendritic cells (26). Numerous signaling pathways that are activated in response to stimulation of cells by S1P are initiated by activation of S1P-specific receptors (10,27,28). However, it had yet to be clarified whether direct treatment with S1P is able to influence adipogenic differentiation (10,17,18). Therefore, the direct effect of S1P was examined in the present study, and the results demonstrated that S1P exerted anti-adipogenic effects via upregulation of $S1P_2$ protein levels.

The extracellular signal-regulated kinase, p38 and JNK pathways are intracellular mitogen-activated protein kinase (MAPK) signaling pathways that serve pivotal roles in numerous essential cellular processes, including proliferation and differentiation (3,18,29). MAPKs are activated by a large variety of stimuli and one of their major functions is to connect cell surface receptors to transcription factors in the nucleus, which consequently triggers long-term cellular responses (29). Overexpression of $S1P_2$ was observed to inhibit MDI-induced phosphorylation of JNK in the present study. When induced to differentiate, growth-arrested 3T3-L1 pre-adipocytes synchronously re-enter the cell cycle and undergo mitotic clonal expansion (MCE). MCE is a pre-requisite for differentiation of 3T3-L1 pre-adipocytes into adipocytes (30).

In conclusion, the results of the present study suggested that the anti-adipogenic activity of S1P is mediated via $S1P_2$. The present study identified for the first time, to the best of our knowledge, that the inhibitory effect of S1P on adipogenic differentiation proceeded via the upregulation of $S1P_2$ and additionally suggested that $S1P_2$ activation may be a therapeutic target for obesity. Therefore, the development of $S1P_2$ receptor sub-type-specific ligands may be beneficial for potential medical interventions.

Acknowledgements

The present study was supported by a grant from the National Research Foundation of Korea, funded by the Korean government (grant no. 2013R1A1A2063931).

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