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Reversine increases the plasticity of lineage-committed preadipocytes to osteogenesis by inhibiting adipogenesis through induction of TGF-β pathway *in vitro*

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ABSTRACT

Reversine has been reported to reverse differentiation of lineage-committed cells to mesenchymal stem cells (MSCs), which then enables them to be differentiated into other various lineages. Both adipocytes and osteoblasts are known to originate from common MSCs, and the balance between adipogenesis and osteogenesis in MSCs is reported to modulate the progression of various human diseases, such as obesity and osteoporosis. However, the role of reversine in modulating the adipogenic potential of lineage-committed preadipocytes and their plasticity to osteogenesis is unclear. Here we report that reversine has an anti-adipogenic function in 3T3-L1 preadipocytes *in vitro* and alters cell morphology and viability. The transforming growth factor-β (TGF-β) pathway appears to be required for the anti-adipogenic effect of reversine, due to reversine-induced expression of genes involved in TGF-β pathway and reversal of reversine-inhibited adipogenesis by inhibition of TGF-β pathway. We show that treatment with reversine transformed 3T3-L1 preadipocytes into MSC-like cells, as evidenced by the expression of MSCs marker genes. This, in turn, allowed differentiation of lineage-committed 3T3-L1 preadipocytes to osteoblasts under the osteogenic condition *in vitro*. Collectively, these findings reveal a new function of reversine in reversing lineage-committed preadipocytes to osteogenesis *in vitro*, and provide new insights into adipose tissue-based regeneration of osteoblasts.

1. Introduction

Adipose tissue plays a key role in the development of various chronic diseases and excess adipose tissue is inversely correlated with bone and muscle generation [1]. Adipocytes, myocytes and osteoblasts are known to originate from common mesenchymal stem cells (MSCs) [2,3]. While the transcription programs and cellular signaling pathways that determine the progenitor cell fate to a specific lineage have been extensively studied, the molecular mechanisms of transdifferentiation of lineage-committed cells to other cell types have not yet been fully explored.

Previous studies have reported the transdifferentiation potential of preadipocytes to osteoblasts and myoblasts. Ectopic expression of runt-related transcription factor 2 (Runx2), a transcription factor essential for osteoblast differentiation and mitogen-activated protein kinase phosphatase-1 (MKP-1) in mouse 3T3-L1 preadipocytes have been reported to promote transdifferentiation of preadipocytes to bone-forming osteoblasts *in vitro* [4]. In addition, activated morphogenetic protein-2 (BMP-2) and BMP-2/BMP receptor axis in differentiated human adipocytes [5] and mouse 3T3-F442A preadipocytes [6] have been shown to promote osteoblast differentiation. On the other hand, overexpression of aortic carboxypeptidase-like protein (ACLP) has been reported to transdifferentiate preadipocytes and adipocyte-derived multipotent progenitor cells into smooth muscle-like cells [7] and skeletal myocytes [8], respectively.

Reversine, a synthetic 2, 6-disubstituted purine analog, has been reported to induce dedifferentiation of lineage-committed mouse myoblasts to multipotent progenitor cells that can differentiate into either osteoblasts or adipocytes under the proper condition [9,10]. Reversine was also found to transform primary murine and human dermal fibroblasts into myogenic-competent cells [11]. Reversine has also been shown to promote differentiation of porcine muscle derived stem cells to female germ-like cells [12],

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and dedifferentiation of mouse macrophages to mesenchymal progenitor-like cells [13]. Despite the knowledge of its potential role in regenerative medicine, the role of reversine in reprogramming of lineage-committed preadipocytes is unclear.

In the present study, we examine the role of reversine in modulating the differentiation ability of lineage-committed preadipocytes to adipocytes and osteoblasts *in vitro*. We present the evidence that reversine-treated 3T3-L1 preadipocytes exhibit impaired adipogenesis, at least in part, through induction of transforming growth factor-β1 (TGF-β1) pathway. Furthermore, we show that reversine redirects the differentiation ability of 3T3-L1 preadipocytes toward osteogenesis under osteogenic condition. Collectively, our data suggest that reversine reciprocally regulates adipogenesis and osteogenesis in 3T3-L1 preadipocytes by upregulating the TGF-β1 pathway, thus indicating a novel function of reversine in reprogramming lineage-committed preadipocytes to osteoblasts.

2. Materials and methods

2.1. 3T3-L1 cell culture and adipogenesis

The 3T3-L1 preadipocytes were differentiated as described [14]. Briefly, preadipocytes were grown until confluence, then 2 days post-confluence (designated as Day 0), differentiation was induced with 10% fetal bovine serum (FBS)–DMEM supplemented with the standard adipogenic cocktail [167 nM insulin, 0.25 μM dexamethasone (Dex), and 0.5 mM isobutylmethylxanthine (IBMX)]. After 2 days of adipogenesis the medium was changed to 10% FBS–DMEM containing 167 nM insulin. On Day 4, the medium was changed to 10% FBS–DMEM with no additional supplements. For Oil Red O (ORO) staining, differentiated cells were fixed with 3% (v/v) formaldehyde for 1 h at room temperature and stained with ORO solution as previously described [14]. ORO-stained intracellular lipids in differentiated cells were extracted using isopropyl alcohol and then quantified using a spectrophotometric analysis at 490 nm using a microplate reader (Bio-Rad). To examine the effect of various cell signaling inhibitors on reversine-inhibited adipogenesis, 3T3-L1 preadipocytes were pretreated with 2.5 μM reversine in the absence or presence of 50 μM SB431542, SP600125, U0126, or LY204002 for 2 days (*i.e.*, Day −2 to Day 0). Adipogenesis was then initiated using the aforementioned method.

2.2. Electron microscopy

3T3-L1 preadipocytes treated with or without 2.5 μM reversine for 24 h were treated with a primary fixative 2.0% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. After the initial fixation the cells were further treated with 1% osmium tetroxide. Cells were then embedded in 1.5% agarose and then dehydrated by ethanol wash. The cells were further processed by resin infiltration, embedded in beam capsule, and polymerized for 48 h at 60 °C. Samples were imaged using a Philips CM-100 Transmission Electron Microscope (TEM) (FEI company) operated at 100 kv, 200 μm condenser aperture and 70 μm objective aperture.

2.3. Cell viability and apoptosis assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Alfa Aesar) assay was performed as previously described [15], with modifications. Proliferating preadipocytes at 80% confluence were treated with various concentrations of reversine for 48 h. Cells were then incubated with fresh 10% FCS–DMEM containing 0.5 mg/ml MTT solution for 1 h at 37 °C. The medium was then aspirated and the remaining purple formazan crystals were solubilized using dimethoxyl sulfoxide (DMSO) for spectrophotometric analysis. Apoptosis in these cells was assessed using the GloMax-Multi Detection System (Promega) according to the manufacturer's instructions. 2×10^5 3T3-L1 preadipocytes per well were seeded on a 96-well plate and treated with various concentration of reversine (0–5 μM). After the 24 h treatment, Caspase-Glo 3/7 substrate was added to the wells and incubated for 1 h at room temperature, followed by measurement of luminescence by a Multi-well Plate Reader (Promega).

2.4. Osteogenesis

3T3-L1 preadipocytes were treated with 2.5 μM reversine for 3 days. Control cells were incubated with 0.1% DMSO. To induce osteogenic transdifferentiation, reversine or DMSO-treated 3T3-L1 preadipocytes were cultured in 10% FBS–DMEM supplemented with 0.1 μM Dex, 50 μg/ml ascobate-2-phosphate, 10 mM β-glycerophosphate for 14 days. Cells were then fixed with 3% formaldehyde at room temperature for 1 h. Osteogenesis of these cells was assessed by incubation with naphthol-ASMX–phosphatate (Sigma) and Fast Red TR salt (Sigma).

2.5. Real-time PCR assay

Total RNA from cultured cells was extracted using Reliaprep™ RNA cell miniprep system (Promega). First-strand cDNA was synthesized using ImPromII reverse transcriptase system (Promega). Real-time PCR was performed using iQ™SYGn Green supermix (Bio-Rad) in the iCycler iQ real-time PCR detection system (Bio-Rad). The results of the real-time PCR for gene transcripts were analyzed by the $2^{-\Delta\Delta Ct}$ calculation. The specific primers used for real-time PCR analyses are listed in Supplementary Table 1.

2.6. Statistical analysis

The data were analyzed using the one-way ANOVA of Statistical Analysis System 9.0. Dunnett's multiple comparison was performed by a Student's *t*-test procedure to compare the treatment group to a control group. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Reversine modulates cellular morphology, viability and apoptosis of 3T3-L1 preadipocytes

Previously, reversine has been shown to alter cell cycle and apoptosis in various cell types through inhibition of multiple cellular signaling pathways [10,16–20]. Thus, we first examined the effect of reversine on viability, apoptosis and cell morphology of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured in DMEM in the presence or absence of 2.5 μM reversine for 24 h. Reversine treatment resulted in a dramatic morphological change in 3T3-L1 preadipocytes, in that many were enlarged and flattened compared with those of non-treated control cells (Fig. 1A). Electron microscopy further showed multiple nuclei in reversine treated 3T3-L1 preadipocytes (Fig. 1B). Next, the effect of reversine on cellular viability and apoptosis in 3T3-L1 preadipocytes was determined using an MTT assay and a caspase 3/7-based luminescence assay, respectively. Reversine treatment resulted in a dose-dependent inhibition of 3T3-L1 preadipocyte viability, with approximately 30% decrease in cell viability at 5 μM reversine (Fig. 1C). In addition, reversine treated cells exhibited elevated levels of caspase 3/7 activity (Fig. 1D). These results indicate that reversine alters cell morphology, and modulates cell cycle progression and viability of preadipocytes.

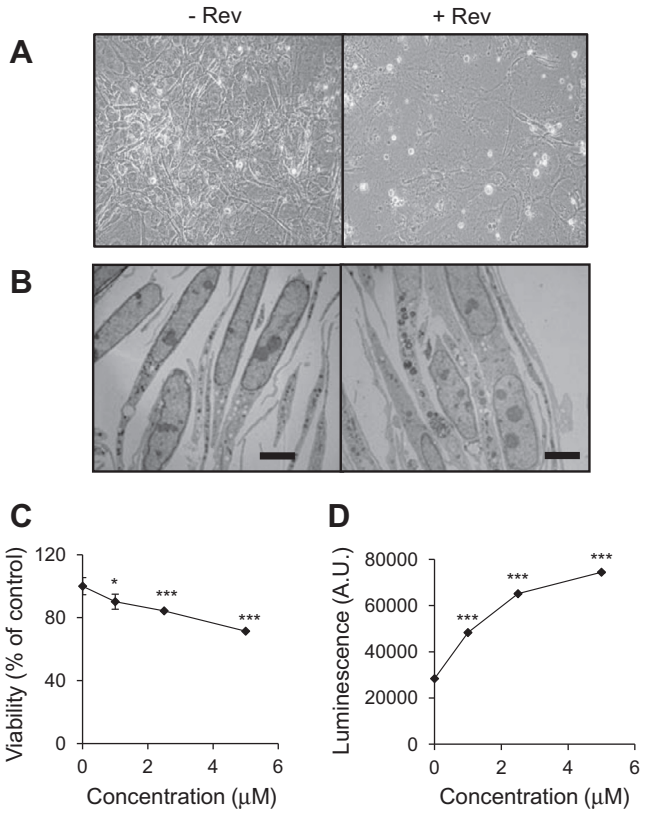

Fig. 1. Reversine alters morphology, viability and apoptosis of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were incubated with 2.5 μM reversine (Rev) (A, B), or various concentrations of reversine (C, D) for 24 h. Cells were then subjected to phase contrast microscopy (A), electron microscopy (B), MTT-based cell viability assay (C), and luminescence-based apoptosis assay (D). Scale bar, 5 μm. Data represent means ± S.E.M. ($n = 3$). $^{*}p < 0.05$; $^{***}p < 0.001$.

3.2. Reversine inhibits adipogenesis on 3T3-L1 preadipocytes

To determine the role of reversine in the differentiation fate of preadipocytes, we first examined the effect of reversine on adipogenic ability of 3T3-L1 preadipocytes by exposing differentiating cells to 2.5 μM reversine during a 6-day course of adipogenesis. As shown in Fig. 2, reversine treatment resulted in approximately 75% inhibition of lipid accumulation in differentiated adipocytes as judged by ORO staining (Fig. 2A) and quantification of ORO stained lipids (Fig. 2B). Consistently, reversine-treated adipocytes showed markedly decreased levels of adipocyte marker genes, such as *adipocyte-specific secretory factor* (*ADSF*)/*Resistin*, *CCAAT/enhancer-binding proteinα* (*C/EBPα*), *peroxisome proliferator activated receptor γ* (*PPARγ*) and *fatty acid synthase* (*FAS*), and increased, but not significant, levels of preadipocyte specific genes, such as *preadipocyte factor-1* (*Pref-1*) and *sex-determining region Y-box 9* (*Sox9*) (Fig. 2C). We further attempted to pinpoint the key adipogenesis step(s) responsible for the anti-adipogenic action of reversine. As illustrated in Fig. 2D, 3T3-L1 preadipocytes were subjected to 6-days of adipogenesis, during which 2.5 μM reversine was added at various phases of differentiation. ORO staining analysis showed that 3T3-L1 preadipocytes undergoing both a reversine pretreatment (*i.e.*, treatment 6) and a reversine treatment during the early phase (*i.e.*, treatments 3) showed an inhibition of intracellular lipid accumulation at similar level to cells from 6 days of treatment (*i.e.*, treatment 5) (Fig. 2E and F). Consistently, differentiated cells in treatments 3, 5 and 6 displayed markedly reduced levels of adipocyte marker genes, such as *PPARγ*, *C/EBPα*, and *tail-interacting protein of 47 kDa* (*TIP47*), compared with those in treatments 2 and 4 (Fig. 2G–I). These results suggest that the anti-adipogenic function of reversine is largely attributable to its inhibitory action both in cellular processes of preadipocytes and the early event of adipocyte differentiation.

3.3. Reversine inhibits adipogenesis partly through activation of TGF-β pathway in preadipocytes

Next, we sought to explore the signaling pathways responsible for mediating reversine-inhibited adipogenesis in 3T3-L1 cells. 3T3-L1 preadipocytes were first pretreated with 2.5 μM reversine for 48 h in the absence or presence of 50 μM of specific inhibitors: U0126, LY294002, SB431542 or SP600125, which inhibit extracellular signal-regulated kinases 1/2 (ERK1/2), phosphoinositide 3-kinase (PI3K), TGF-β1 receptor, and c-Jun N-terminal kinase (JNK), respectively. Adipogenesis was then initiated for 6 days in the presence of reversine and the inhibitors. While most inhibitors showed little effect on blunting the inhibitory action of reversine in adipogenesis, SB431542 pretreatment at a concentration of 50 μM partially blocked the reversine-inhibited adipogenesis (Fig. 3A). SB431542-inhibited reversine function in adipogenesis of 3T3-L1 cells was further observed in a dose-dependent manner with a maximum inhibition at 100 μM (Fig. 3B), suggesting the involvement of TGF-β1 pathway in reversine-inhibited adipogenesis of 3T3-L1 preadipocytes. Indeed, reversine treatment of 3T3-L1 preadipocytes elevated mRNA levels of genes involved in TGF-β1 pathway, such as *TGF-β1*, *TGF-β2*, *TGF-β3*, *Fibronectin*, *Smad2* and *Smad3*, in a time- and dose-dependent manner (Fig. 3C and D). These results suggest that the activation of TGF-β1 pathway is required for the anti-adipogenic function of reversine in 3T3-L1 cells.

3.4. Reversine pretreatment shifts differentiation ability of 3T3-L1 preadipocytes from adipogenesis to osteogenesis

Since reversine has been shown to reverse lineage-committed cells to MSCs [21], we next asked if reversine-treated 3T3-L1 preadipocytes could be reversed to MSCs for redifferentiation to osteoblasts. We found that different concentrations of reversine treatment increased mRNA levels of MSC marker genes, such as *CD73* and *CD90*, in preadipocytes (Fig. 4A and B). Because previous research has shown that adipocytes and osteoblasts are derived from common mesenchymal stem cells and there is a possibility of transdifferentiation between cell types, we next sought to examine whether reversine treated 3T3-L1 preadipocytes could undergo osteogenesis by culturing reversine-pretreated 3T3-L1 preadipocytes in osteogenic condition for 14 days. A 3-day of reversine pretreatment resulted in differentiation of 3T3-L1 preadipocyte to osteoblasts, as evidenced by increased alkaline phosphatase (ALP) activity, an osteogenesis marker. ALP activity was judged by ALP staining with naphthol-ASMX–phosphatate and Fast Red TR salt (Fig. 4C). Consistent with this finding, reversine-pretreated 3T3-L1 preadipocytes exposed to osteogenic condition showed a dramatic increase in *alkaline phosphatase* (*ALP*), *osteocalcin* (*OC*) and *osteopontin* (*OPN*) mRNA expression, which are specific markers of osteoblasts (Fig. 4D–F). Taken together, these results demonstrate that reversine can reverse lineage-committed 3T3-L1 preadipocytes to MSC-like cells which are capable of being differentiated into osteoblasts *in vitro*.

4. Discussion

Adipocytes and osteoblasts are derived from a common MSC origin, there is evidence of transdifferentiation between the two cell types. Various factors, such as androgens [22], osteopontin [23], and oncostatin M [24], Wnt/β-catenin [25], and the

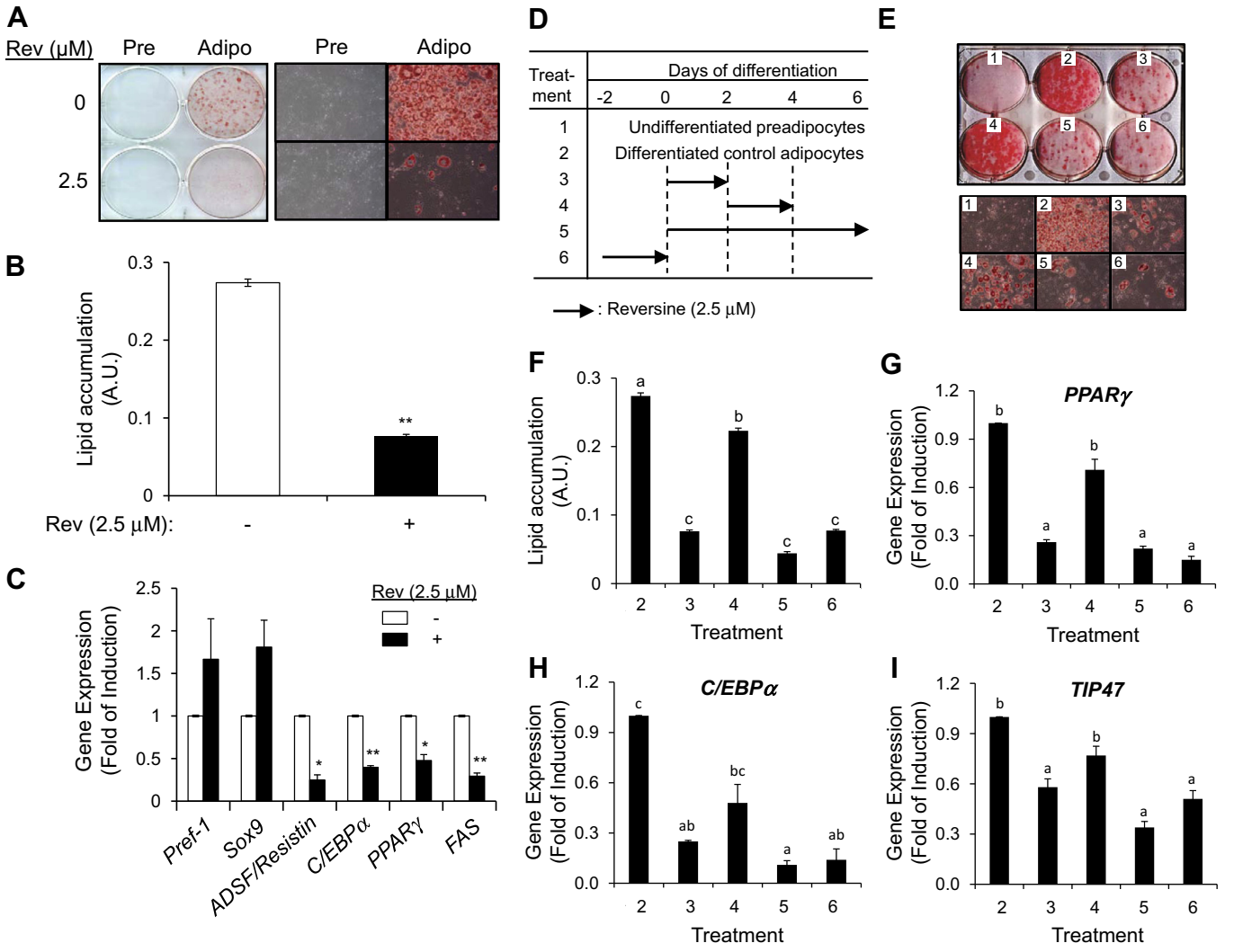

Fig. 2. Effect of reversine on adipogenesis of 3T3-L1 preadipocytes. Two-day postconfluent 3T3-L1 preadipocytes (Pre) were subjected to a 6-day of adipocyte (Adipo) differentiation in the presence of 0.1% DMSO (v/v) or 2.5 μM reversine (Rev) dissolved in DMSO. After differentiation, cells were subjected to ORO staining for photographic and microscopic analyses of lipid accumulation (A), followed by a quantitative analysis of ORO-stained intracellular lipids (B). (C) Expression levels of *Pref-1* and *Sox9*, preadipocyte marker genes, and *ADSF/Resistin*, *C/EBPα*, *PPARγ* and *FAS*, adipocyte-specific genes, in these cells were analyzed by real-time PCR analysis. The signals were normalized to *β-actin* internal control, and the results were expressed as relative fold of induction. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation for 6 days with 2.5 μM reversine at various adipogenesis time points as illustrated in (D). After adipogenesis, cells were subjected to ORO staining (E), quantitative analysis of ORO-stained intracellular lipids (F), and real-time PCR analysis to examine mRNA levels of *C/EBPα*, *PPARγ* and *TIP47* by (G–I). Data represent means ± S.E.M. ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.005$. Different letters represent significant differences at $P < 0.05$.

microRNAs, such as miR-17-5p and miR-106a [26], have been shown to modulate the differentiation of MSCs to adipocytes and osteoblasts. Regulation of the balance between adipogenesis and osteogenesis in MSCs is suggested to modulate progression of various human diseases, such as obesity and osteoporosis. Despite the proposed role of reversine in regenerative medicine, the role of reversine in modulating the plasticity of preadipocytes toward other mesenchymal lineage has remained elusive.

In the present study, we demonstrated an anti-adipogenic property of reversine *in vitro*. Exposure of preadipocytes to reversine during the growth or the early phase of differentiation resulted in inhibition of adipogenesis of 3T3-L1 preadipocytes through a concomitant induction of the TGF-β1 pathway, an anti-adipogenic cellular signaling pathway [27,28]. Moreover, we found that reversine pretreatment reversed lineage-committed 3T3-L1 preadipocytes to MSC-like cells which are capable of differentiating into osteoblasts. Contrary to our findings, Kim et al. reported that reversine promoted adipogenesis, despite its inhibitory effect on the insulin signaling pathway [29]. Unlike our study, this report showed that a 6-day reversine treatment at a relatively high concentration (*i.e.*, 10 μM) had little effect on cell morphology during the course of adipogenesis [29]. Moreover, 3T3-L1 preadipocytes employed to Kim et al.'s study as control seemingly showed poor basal adipogenic ability, as shown by sporadic lipid accumulation after 6-day of adipogenesis under standard adipogenic condition [29]. In contrast, most of 3T3-L1 preadipocytes used in our study were differentiated to adipocytes with significant accumulation of lipid droplets. This difference in lipid accumulation could be attributed to the different response to reversine. Nevertheless, our study clearly showed that a pre-exposure of preadipocytes to a low dose of reversine, as well as the addition of reversine during the early phase of differentiation, directly contributed to the reprogramming of preadipocytes to osteoblasts with impaired adipogenesis *in vitro*. Supporting to this notion, we demonstrated that reversine treatment resulted in the expression of MSC marker genes, such as *CD73* and *CD90* [30,31], in lineage-committed

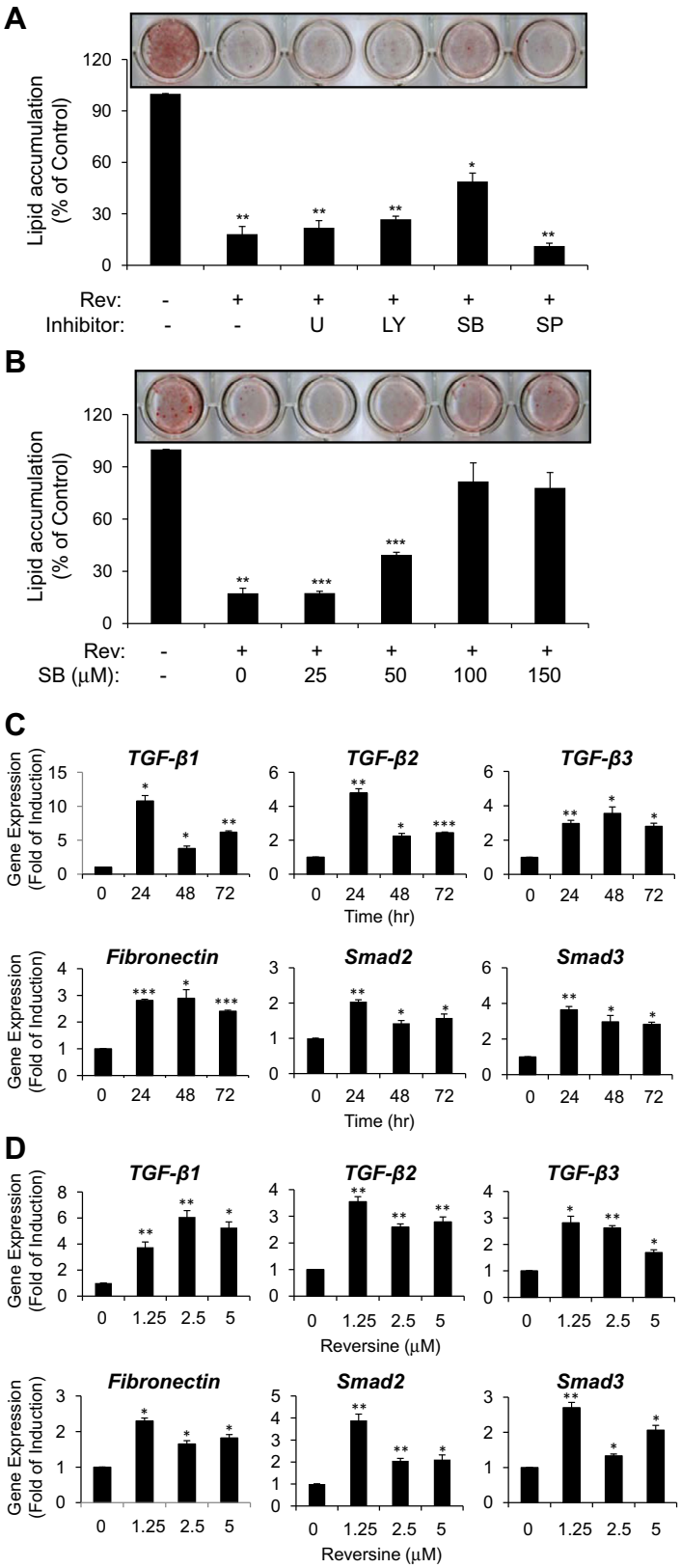

Fig. 3. TGF-β pathway partly mediates reversine-inhibited adipogenesis of 3T3-L1 preadipocytes. (A) 3T3-L1 preadipocytes pretreated with 2.5 μM reversine (Rev) in the absence or presence of 50 μM U0126 (U), LY294002 (LY), SB431542 (SB) or SP600125 (SP) for 48 h were subjected to standard adipogenesis for 6 days. (B) 3T3-L1 preadipocytes pretreated with 2.5 μM reversine in the absence or presence of various concentrations of SB431542 (SB) for 48 h were subjected to standard adipogenesis for 6 days. Differentiated cells were then subjected to ORO staining of intracellular lipid droplets, followed by a quantitative analysis of ORO-stained intracellular lipids. 3T3-L1 preadipocytes treated with 2.5 μM reversine for indicated time (0–72 h) (C) or various concentrations of reversine (0–5 μM) for 48 h (D). Cells were then subjected to real-time PCR analysis to determine the mRNA levels of genes involved in TGF-β pathway. The signals were normalized to *β-actin* internal control, and the results were expressed as relative fold of induction. Data represent means ± S.E.M. ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$.

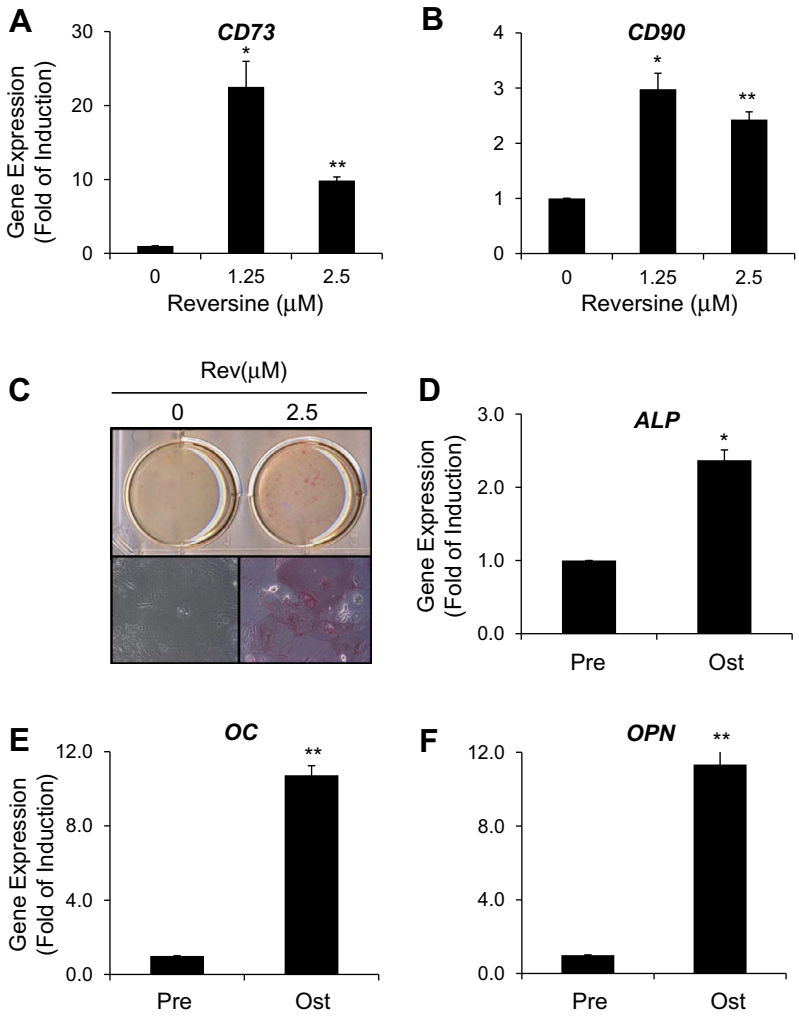

Fig. 4. Reversine pretreatment promotes differentiation of 3T3-L1 preadipocytes to osteoblasts. 3T3-L1 preadipocytes (Pre) treated with various concentrations of reversine (Rev) (0–2.5 μM) for 72 h were subjected to real-time PCR analysis to determine the mRNA levels of *CD73* (A) and *CD90* (B). (C) These cells were subjected to osteogenesis for 14 days. Alkaline phosphatase activity in the differentiated osteoblasts (Ost) was then measured by staining with naphthol-ASMX–phosphatate and Fast Red TR salt for photographic (top) microscopic (bottom) analyses. These cells were also subjected to real-time PCR analysis to determine the mRNA levels of *ALP* (D), *OC* (E) and *OPN* (F). The signals were normalized to *β-actin* internal control, and the results were expressed as relative fold of induction. Data represent means ± S.E.M. ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.005$.

preadipocytes. Moreover, reversine treatment in 3T3-L1 preadipocytes activated the TGF-β pathway. Given the critical role of the TGF-β pathway in MSC lineage decisions, adipogenic ability of committed preadipocytes [32], and osteogenesis [33], our finding of reversine-induced reprogramming of preadipocytes to osteoblasts is likely resulted from the concurrent induction of the TGF-β pathway and the transcriptional activation of MSCs by reversine in preadipocytes. However, the precise mechanisms underlying reversine-induced activation of the TGF-β pathway in preadipocytes still remain to be fully clarified.

Taken together, our findings demonstrate that reversine increases the plasticity of lineage-committed preadipocytes toward osteogenesis by inhibition of adipogenesis through induction of TGF-β pathway *in vitro*. These findings could contribute to the development of new approaches for adipose tissue-based regeneration of osteoblasts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.036.

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