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Inhibition of IGF-1 Expression by Prolonged TGF-β1 Administration Suppresses Osteoblast Differentiation

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RUNNING TITLE
TGF-β1 regulates osteodifferentiation via the IGF/Akt signaling

Capsule
Background: TGF-β1 positively and negatively regulates osteoblast differentiation.
Results: Repeated TGF-β1 negatively regulates osteoblast differentiation caused by inhibiting IGF-1 expression and Akt phosphorylation.
Conclusion: Prolonged TGF-β1 treatment inhibits osteoblast differentiation of mesenchymal stem cells via the suppression of IGF-1 signaling pathway.
Significance: IGF-1 administration may recover the suppression of osteogenesis and promotion of bone resorption due to chronic inflammation by TGF-β1.
ABSTRACT

TGF-β1 can regulate osteoblast differentiation not only positively but also negatively. However, the mechanisms how negative regulation could undergo is not well understood. We previously established the reproducible model for studying the suppression of osteoblast differentiation by repeated or high dose treatment with TGF-β1 although single low dose TGF-β1 strongly induced osteoblast differentiation. mRNA expression and protein level of insulin-like growth factor-1 (IGF-1) were remarkably decreased by repeated TGF-β1 administration in human periodontal ligament (HPDL) cells, human mesenchymal stem cells (hMSC), and murine preosteoblasts MC3T3-E1 cells. Repeated TGF-β1 administration subsequently decreased alkaline phosphatase (ALP) activity and mRNA expression of osteoblast differentiation marker genes, such as RUNX2, ALP, and bone sialoprotein (BSP). Additionally, repeated administration significantly reduced the downstream signaling pathway of IGF-1, such as Akt phosphorylation in these cells. Surprisingly, exogenous and overexpressed IGF-1 recovered ALP activity and mRNA expression of osteoblast differentiation marker genes even with repeated TGF-β1 administration. These facts indicate that the key mechanism of inhibition of osteoblast differentiation induced by repeated TGF-β1 treatment is simply due to the downregulation of IGF-1 expression. Inhibition of IGF-1 signaling using small interfere RNA (siRNA) against insulin receptor substrate-1 (IRS-1) suppressed mRNA expression of RUNX2, ALP, BSP, and IGF-1 even with single TGF-β1 administration. The present study showed that persistence of TGF-β1 inhibited osteoblast differentiation via suppression of IGF-1 expression and subsequent downregulation of PI3K/Akt pathway. We think this fact could open the way to use IGF-1 as a treatment tool for bone regeneration in prolonged inflammatory disease.

INTRODUCTION

Insulin-like growth factor-1 (IGF-1) plays an important role in cell growth, differentiation, survival, and cell cycle progression. IGF-1 is secreted by mature osteoblasts and can be stored locally in the bone matrix until its release during bone resorption. IGFs stimulate in vitro and in vivo osteoblast proliferation and differentiation through specific membrane receptors (1-4). IGF-1 upregulation may partially mediate increased expression of bone matrix proteins and bone anabolic effects in aged, ovariectomized rats (5). Although IGF-1 does not direct undifferentiated stromal cells to differentiate into cells of an osteoblast lineage, it enhances the function of mature osteoblasts (6). IGF-1 also plays a role in the regulation of chondrocyte differentiation (7, 8) and promotes longitudinal bone growth by augmenting chondrocyte hypertrophy (8). Exogenous IGF-1 markedly improves chondrocyte matrix biosynthesis (9).

Transforming growth factor-β1 (TGF-β1) is crucial for connective tissue regeneration and bone remodeling, as demonstrated by several in vivo and in vitro studies. It affects osteoblast differentiation and bone formation (10-14) and increases mRNA levels of osteoblast differentiation markers and alkaline phosphatase (ALP) activity in murine bone marrow stromal cells (12). However, TGF-β1 also blocks osteogenesis by various mechanisms depending
on its concentration, the cell density, and differentiation stage of the cells (15-17) and blocks odontogenesis by downregulating dentin sialophosphoprotein (18). The mitogen-activated protein kinase (MAPK) pathway negatively regulates the Smad pathway and osteoblast mineralization (19, 20). Some studies have reported that TGF-β1 has biphasic and concentration-dependent effects on osteoblast differentiation (15, 21). Although the TGF-β/Smad pathway could be the major inducer of osteogenesis, the dual effect of TGF-β signaling and the mechanism by which TGF-β1 influences osteogenesis remains unexplained. TGF-β is also an anti-inflammatory cytokine. However, it induces the development of Th17 cells, which produce the proinflammatory cytokine IL-17 (22). Many cytokines, including TGF-β1, IL-1, IL-6, and TNF-α, appear to be involved in degenerative diseases such as osteoarthritis, though the extent of involvement is unknown. Like other cytokines, TGF-β1 may inhibit osteoregeneration during inflammation.

We recently established an experimental model to study the inhibitory mechanism of TGF-β1 in osteoblast differentiation and found that a single low dose TGF-β1 administration significantly promoted osteoblast differentiation, but its repeated or high dose administration inhibited osteoblast differentiation in normal human periodontal ligament (HPDL) cells (23). DNA microarray analysis revealed that repeated TGF-β1 administration markedly reduced IGF-1 expression. Therefore, we studied the effects of TGF-β1 on mRNA expression and production of IGF-1 to elucidate its action mechanism in HPDL cells and MC3T3-E1 cells. We found that repeated TGF-β1 administration caused IGF-1 downregulation and Akt phosphorylation, resulting in the inhibition of osteoblast differentiation. Moreover, the inhibition was reversed by treatment with endogenous IGF-1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Osteogenic Differentiation**—Normal HPDL cells and human mesenchymal stem cells (hMSC) were purchased from Lonza (Basel, Switzerland) and cultured in BulletKit® Stromal cell growth medium (SCGM, Lonza) and BulletKit® Mesenchymal stem cell growth medium (MSCGM, Lonza), respectively. HPDL cells and hMSC of passages 5 to 8 and 3 to 4, respectively, were seeded at a density of $1 \times 10^5$ cells/cm$^2$ for each assay. MC3T3-E1 cells were purchased from RIKEN Bio Resource Center (Ibaraki, Japan). MC3T3-E1 cells were seeded at a density of $1.6 \times 10^5$ cells/cm$^2$ for each assay. Osteoblast differentiation was induced by replacing with the osteoblast differentiation medium (OBM), comprising α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 50 μg/ml L-ascorbic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 10 mM β-glycerophosphate (Wako), with or without rhTGF-β1 (Wako), this was added on the following day. Under the single TGF-β1 administration condition, the medium was not changed until day 3 or day 4, whereas under repeated TGF-β1 administration condition, OBM containing fresh TGF-β1 was changed every 12 h. Control cells were treated identically except that they did not receive TGF-β1.

**Assay of Alkaline Phosphatase (ALP) Activity and Mineralization**—Three days after
stimulation, cells were washed two times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 5 min at room temperature, and washed three times with water. For staining, an ALP substrate solution (Roche Diagnostics, Basel, Switzerland) was added to the fixed cells for 60 min at room temperature. After staining, cells were washed three times with distilled water and images were scored. ALP activity was measured as follows, the cells were washed twice with PBS and lysed with lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, complete protease inhibitor mixture, and 1% NP-40). ALP activity was assayed using p-nitrophenylphosphate as a substrate and the protein content was measured with a DC Protein Assay Kit (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. ALP activity was then expressed as μmol p-nitrophenol (pNP)/ min/ mg of protein.

To detect calcium deposits in mineralized tissue, cells were fixed by the same method and then stained with Alizarin red S solution (pH 6.38, Wako) for 5 min at room temperature. After washing 5 times with PBS, images captured with phase-contrast microscope.

Quantitative Real Time-PCR (qRT-PCR) — qRT-PCR was used to examine the expression of osteoblast differentiation markers. Following incubation for 48 and 72 h in the differentiation medium, total RNA was extracted using QIAzol reagent (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR analysis was performed using the Premix Ex Taq™ reagent (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. Target genes included RUNX2, alkaline phosphatase (ALP), bone sialoprotein (BSP), and insulin-like growth factor 1 (IGF1) osteocalcin (OC). 18S rRNA was used as an internal control. All primers and probes are presented in Table 1 and were designed using ProbeFinder v2.45 (http://qpcr.probefinder.com/roche3.html). Relative expression of genes of interest was estimated using delta-delta-Ct method.

Protein Extraction and Immunoblotting—Cells were lysed with lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, complete protease inhibitor mixture, 1 mM sodium orthovanadate, and 1% NP-40), and the protein content was measured using a DC Protein Assay Kit (BioRad, Marnes-la-Coquette, France). Equivalent protein concentrations were resolved by electrophoresis on NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane. The membrane was probed with anti-phosphorylated Akt (1:2000; Cell Signaling Technology Inc., Danvers, MA, USA) and anti-IGF1 (1:2500; Abcam, Cambridge, UK) antibodies, followed by HRP-conjugated goat anti-rabbit IgG. Bound antibodies were visualized using a chemiluminescent substrate (ECL plus; GE Healthcare UK Ltd., Buckinghamshire) and ImageQuant LAS 4000 mini (GE healthcare).

Small Interfering RNA (siRNA) Transfection—HPDL cells were transfected with siRNA for insulin receptor substrate 1 (IRS-1) (Ambion, siRNA ID s7520) using Lipofectamine™ RNAiMAX (Invitrogen). An siRNA consisting of a scrambled sequence of similar length was
transfected as a control. siRNA was diluted in 100 µl Opti-MEM I medium without serum in each well of a new tissue culture plate. Subsequently, RNAiMAX was added to each well, followed by addition of adequate number of cells in 500 µl of growth medium without antibiotics such that they were 30%–50% confluent 24 h after plating. After 24 h, the medium was replaced with α-MEM containing 5% FBS and cells were cultured for 48 h until confluent. To determine whether IRS-1 siRNA specifically silenced IRS-1 expression, we evaluated its protein expression in transfected HPDL cells by western blotting.

**Plasmid Construction and Transfection**—A human IGF-1 expression plasmid was generated by inserting full-length IGF-1 cDNA into the expression vector pEF1/V5-His at the BamHI and EcoRI sites. MC3T3-E1 cells were plated in 12-well plates at 70%–80% confluence (1 × 10^5 cells). Cells were transfected with plasmid mixtures containing 1.6 µg/well of pEF1/IGF1 or pEF1/mock using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. After 6 h, the medium was replaced with α-MEM containing 5% FBS and cells were cultured for 72 h until confluent.

**Statistical Analysis**—All data are expressed as mean ± S.E. When ANOVA indicated differences among the groups, multiple comparisons between each experimental group were performed using the Bonferroni test. Statistical significance was defined as p < 0.05.

**RESULTS**

**Repeated TGF-β1 Administration Inhibits Osteoblast Differentiation in HPDL cells,**

**MC3T3-E1 cells and hMSC**—We found that the same dose of TGF-β1 could positively as well as negatively regulate osteoblast differentiation and reported that single treatment with 1 ng/ml TGF-β1 most induced osteoblast differentiation (23). The direction of regulation was determined by the number of administrations. In HPDL cells and hMSC, 1 ng/ml TGF-β1 was suitable to induce osteoblast differentiation and in MC3T3-E1 cells 0.1 ng/ml TGF-β1 was suitable in our preliminary experiments (data not shown). We investigated the differences between a single and repeated TGF-β1 administration by comprehensive analysis using DNA microarray and found that IGF-1 expression decreased approximately 10 fold in cells administered repeated doses of TGF-β1 compared with cells administered a single dose of TGF-β1 (data not shown). In HPDL cells and hMSC, as the number of administrations of 1.0 ng/ml TGF-β1 increased, ALP activity and mRNA expression of osteoblast differentiation-related genes (RUNX2 and ALP) was significantly decreased (Fig. 1A–C and 2A–C). The decrease in IGF-1 mRNA expression revealed by DNA microarray was confirmed by qRT-PCR (Fig. 1D and 2D). Repeated TGF-β1 administration caused the decrease of ALP activity, IGF-1 mRNA expression regardless of serum condition (supplemental Fig. S1). After 2 weeks of osteogenic culture in presence of serum, HPDL cells also showed increased mineralization by a single administration of 1.0 ng/ml TGF-β1 and showed decreased mineralization by repeated administration of 1.0 ng/ml TGF-β1 (supplemental Fig. S2). OC mRNA expression level was decreased by TGF-β1 treatment (Fig. 1E and 2E). Sowa et al. also reported that TGF-β1 treatment reduced the OC mRNA level.
in MC3T3-E1 cells (32). In MC3T3-E1 cell line, which is a well-characterized murine preosteoblast cell line for studying osteoblast differentiation, ALP activity and mRNA expression of ALP and IGF-1 were significantly decreased by repeated administration of 0.1 ng/ml TGF-β1 (Fig. 2). OC mRNA level was decreased by TGF-β1 treatment in MC3T3-E1 cells.

Repeate TGF-β1 Treatment Inhibits IGF-1 Expression and Akt Phosphorylation—We conducted Western blot analysis to examine the levels of IGF-1 protein and phosphorylated Akt (pAkt), a downstream effector of PI3K. In HPDL cells, repeated TGF-β1 administration significantly decreased IGF-1 protein at 72 and 96 h (Fig. 3). Akt phosphorylation was also remarkably reduced after repeated TGF-β1 administration at 72 and 96 h. Similarly, IGF-1 and pAkt protein level was decreased by repeated TGF-β1 treatment in both hMSC and MC3T3-E1 cells. However, both IGF-1 protein and phosphorylated Akt increased after a single TGF-β1 administration compared to the untreated control.

Inhibition of IRS-1 Inhibits Osteoblast Differentiation Initiated by a Single TGF-β1 Treatment in HPDL cells—We examined whether the inhibition of IGF-1 signaling could suppress a promotion of osteoblast differentiation by a single administration of TGF-β1 (1 ng/ml). siRNA transfection did not affect proliferation or cell density (Fig. 5A). ALP activity was suppressed by single TGF-β1 treatment in cells with knockdown of IRS-1, while single TGF-β1 treatment stimulated ALP activity in control siRNA transfected cells (Fig. 5C). Analysis by qRT-PCR showed that knockdown of ISR-1 by siRNA resulted in a marked reduction of mRNA expression of RUNX2, ALP, IGF-1, and BSP in cells administered a single dose of TGF-β1 (Fig. 5D–G).

Exogenous IGF-1 Recovers the Suppression of Osteoblast Differentiation by Repeated TGF-β1 Administration in HPDL cells—IGF-1 stimulates osteoblast differentiation and bone formation. Furthermore, IGF-1 is essential for TGF-β1–induced osteoblast differentiation, as indicated by our results. However, the effect of IGF-1 on the inhibition of osteoblast differentiation by repeated TGF-β1 administration is not well known. We therefore evaluated the effect of exogenous IGF-1 on HPDL cells. As shown in Fig. 6A, ALP activity was remarkably diminished with increased TGF-β1 administrations. Interestingly, the addition of 200 ng/ml IGF-1 fully recovered the suppression of ALP activity by repeated TGF-β1 administration. Exogenous IGF-1 also significantly increased mRNA expression of RUNX2, ALP, and BSP (Fig. 6B–D). We found that IGF-1 expression markedly increased on treatment with exogenous IGF-1 (Fig. 6E).

IGF-1 Overexpression Restores Osteoblast Differentiation in MC3T3-E1 cells—To determine whether IGF-1 was essential for osteoblast differentiation, MC3T3-E1 cells were transfected with the pEF1-IGF1 plasmid. Repeated administration of 0.1 ng/ml TGF-β1 in mock transfected cells resulted in a decrease in ALP activity and mRNA expression (Fig. 7B–D). However, ALP activity was not decreased by repeated administration of 0.1
ng/ml TGF-β1 in pEF1-IGF1 transfected cells. These pEF1-IGF1 transfected cells with repeated administration of TGF-β1 had higher ALP activity than cells that were mock transfected without treatment. These results indicate that IGF-1 enhances osteoblast differentiation only after it is induced by TGF-β1.

**DISCUSSION**

Growth factors such as TGF-β, bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) are proteins secreted by cells that act on target cells to promote bone regeneration. TGF-β1 is abundantly expressed in the periosteum, osteocytes (24), and during fracture healing *in vivo* (25, 26). TGF-β1 also exerts antiproliferative effects and functions as a tumor suppressor during early stages of tumorigenesis, and as a tumor promoter aiding in metastatic progression at later stages (27-29). Similarly, TGF-β signaling also regulates osteoblast differentiation both positively and negatively. Recombinant human TGF-β1 induces *in vivo* endochondral bone formation in extraskeletal sites of adult baboons (14, 30). TGF-β1–loaded microgranules have a high bone regenerative capacity, ALP activity, and osteocalcin content in rabbit calvarial defects (11). Smad3 null mice are osteopenic, with less cortical and cancellous bones compared with wild-type littermates (31). Overexpression of Smad3 enhances ALP activity, mineralization, and production of bone matrix proteins in MC3T3-E1 cells (32). Despite several evidences supporting its positive effect on osteoblast differentiation, TGF-β1 has been shown to inhibit osteocalcin promoter activity and osteocalcin expression in rat primary osteoblasts (19). TGF-β1 suppresses mRNA expression of osteoblast differentiation genes, such as ALP, BSP, and osteocalcin, in MC3T3-E1 cells (33). Although these inhibitory effects on osteoblast differentiation have been described, the mechanism of suppression remains unknown. We had previously established a reproducible model for studying the suppression of osteoblast differentiation by TGF-β1, showing that TGF-β1 negatively regulates osteoblast differentiation by prolonged and repeated TGF-β1 treatment (23).

In this study, we found that repeated TGF-β1 administration remarkably decreased IGF-1 expression in HPDL, hMSC and MC3T3-E1 cells. IGF-1 is an important regulator of bone homeostasis and is central to the achievement of normal longitudinal bone growth and bone mass. Therefore, the inhibition of autocrine IGF-1 expression may decrease the effects of IGF-1 on these cells and cause inhibition of osteoblast differentiation. The IGF-1 gene knockout mice revealed that the fetal mice demonstrated short-limb dwarfism delays in mineralization and increased chondrocyte apoptosis (34). Long-term recombinant human IGF-1 therapy for short children with severe IGF-1 deficiency caused an increase in height velocity from 2.8 cm/yr on average at baseline to 8.0 cm/yr during the first year of treatment (1). The combined delivery of BMP-7 and IGF-1 genes synergistically enhanced the differentiation of HPDL cells while suppressing their proliferation (4). Once activated, IGF-1 can interact with its receptor, IGF-1R, to induce signaling. Following stimulation by a ligand, the IRSs are rapidly phosphorylated on multiple tyrosine residues (35). These phosphorylated substrates bind to
proteins containing Src homology 2 domains, and later activate PI3K/Akt signaling to regulate cell differentiation, growth, and metabolism (36). We previously found that the PI3K inhibitor LY294002 completely abrogated TGF-β1–induced osteoblast differentiation of HPDL cells (data not shown). Repeated TGF-β1 administration markedly inhibited IGF-1 expression and subsequently Akt phosphorylation and then decreased osteoblast differentiation marker expression. Thus, these results suggest that the inhibition of osteoblast differentiation caused by repeated TGF-β1 administration is associated with the downregulation of IGF-1 expression and Akt inactivation.

To examine whether the IGF-1 signaling cascade plays an important role in TGF-β1–induced osteoblast differentiation, siRNA for IRS-1 were used. The inhibition of IGF-1 signaling resulted in downregulation of mRNA expression of osteoblast differentiation marker gene, although a single TGF-β1 administration induced osteoblast differentiation of HPDL cells (Fig. 5). Our results revealed that TGF-β1–induced osteoblast differentiation requires IGF-1 expression. However, repeated TGF-β1 treatment downregulates IGF-1 expression which appears to be the key mechanism for the negative effects on osteoblast differentiation caused by TGF-β1.

Interestingly, exclusive exogenous IGF-1 markedly restored the downregulated ALP staining and osteoblast differentiation marker gene expression caused by repeated TGF-β1 administration (Fig. 6). IGF-1 has been found to be very potent and is localized at healing fracture sites in rats and humans. Locally applied IGF-1 and TGF-β1 from IGF-1/TGF-β1–coated polylactide membranes distinctly increases bone area in healing fractures in a sheep model of delayed callus formation (2). According to our results, TGF-β1 is inadequate for stimulation of osteoblast differentiation. Topical IGF-1 application using gelatin hydrogels is well tolerated and may be efficacious for hearing recovery in patients with sudden sensorineural hearing loss that is resistant to systemic glucocorticoids (37). Thus, topical IGF-1 treatment may be utilized as a new therapeutic measure by inducing osteoblastic differentiation. Next, we investigated whether overexpression of IGF-1 can promote osteoblast differentiation. Osteoblast differentiation was inhibited in HPDL, MC3T3-E1 cells, and hMSC by repeated TGF-β1 administration (Fig. 1, 2, and 3). IGF-1 overexpression successfully recovered the suppression of osteoblast differentiation by repeated TGF-β1 administration. ALP expression in pEF1-IGF1-transfected cells repeatedly administered TGF-β1 was higher compared with pEF1-IGF1-transfected cells which were not administered TGF-β1 (Fig. 7B–D). We also observed that a single TGF-β1 administration is sufficient to upregulate ALP expression (data not shown). IGF-1 enhances the function of mature osteoblasts, although IGF-1 does not direct the differentiation of undifferentiated stromal cells toward the osteoblasts lineage (38). Our observations support the fact that IGF-1 acts as an enhancer only after osteoblast differentiation is initiated by TGF-β1. Therefore, IGF-1 secreted from cells could induce osteoblast differentiation through paracrine or autocrine mechanisms. IGF-1 signaling is essential for osteoblast differentiation induced by TGF-β1 administration.
TGF-β1 influences a broad range of cellular activities, including anti-inflammatory processes, growth, differentiation, and extracellular matrix synthesis. TGF-β1 also induces IL-6 release in human bronchial epithelial cells and prostate cancer cells (39, 40). Our experimental conditions involving repeated TGF-β1 administration may correspond to the suppression of osteogenesis and promotion of bone resorption due to chronic inflammation, since chronic inflammation could induce prolonged TGF-β1 expression.

In conclusion, our results reveal that TGF-β1 inhibits osteoblast differentiation via suppression of IGF-1 expression and subsequent downregulation of the PI3K/Akt pathway. Our results suggest that exclusive IGF-1 administration recovers the suppression of osteogenesis and promotion of bone resorption due to chronic inflammation, such as during periodontal disease and rheumatoid arthritis. Thus, local application of IGF-1 may be useful as a treatment tool for bone regeneration in prolonged inflammatory disease.

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FOOTNOTES
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FIGURE LEGENDS

Fig. 1.
Repeated TGF-β1 administration inhibits expression of osteoblast differentiation markers in HPDL cells. A, HPDL cells were cultured in α-MEM (1), OBM (2), OBM with a single administration of 1 ng/ml TGF-β1 (3), OBM with a double administration of 1 ng/ml TGF-β1 (4), and OBM with a triple administration of 1 ng/ml TGF-β1 (5) for 72 h. ALP activity was visualized by ALP activity staining of cells. A single TGF-β1 administration markedly induced ALP activity. However, as the number of administrations increased, ALP activity gradually decreased. B–E, Confluent cells were treated for 72 h with OBM (1), OBM with a single administration of 1 ng/ml TGF-β1 (2), and OBM with a double administration of 1 ng/ml TGF-β1 (3). Repeated TGF-β1 administration inhibited the expression of RUNX2 (B), ALP (C), IGF-1 (D), and OC (E). Expression of these genes was analyzed by qRT-PCR and their mRNA levels were normalized to that of 18S rRNA and measured in triplicate. Figures shown represent at least three independent experiments. Values represent mean ± S.E. (n = 4). Bonferroni correction for multiple comparisons was applied. *P < 0.001.

Fig. 2.
Repeated TGF-β1 administration inhibits expression of osteoblast differentiation markers in hMSC. A, The cells were cultured in OBM (1), OBM with a single administration of 1 ng/ml TGF-β1 (2), OBM with a triple administration of 1 ng/ml TGF-β1 (3) for 4 days after which ALP activity was measured. B–E, Confluent cells were treated for 96 h with OBM (1), OBM with a single administration of 1 ng/ml TGF-β1 (2), and OBM with a triple administration of 1 ng/ml TGF-β1 (3). Repeated TGF-β1 administration inhibited the expression of RUNX2 (B), ALP (C), IGF-1 (D), and OC (E). Expression of these genes was analyzed by qRT-PCR and their mRNA levels were normalized to that of 18S rRNA and measured in triplicate. Values represent mean ± S.E. (n = 4). Bonferroni correction for multiple comparisons was applied. *P < 0.001.

Fig. 3.
Repeated TGF-β1 administration inhibits expression of osteoblast differentiation markers in MC3T3-E1 cells. A, MC3T3-E1 cells were cultured in α-MEM (1), OBM (2), OBM with a single administration of 0.1 ng/ml TGF-β1 (3), and OBM with repeated administration of 0.1 ng/ml TGF-β1 (4) for 72 h. ALP activity was visualized by ALP activity staining of cells. A single TGF-β1 administration induced ALP activity. However, repeated TGF-β1 administration suppressed ALP activity. B, Confluent cells were treated for 72 h with OBM (1), OBM with a single administration of 0.1 ng/ml TGF-β1 (2), and OBM with repeated administration of 0.1 ng/ml TGF-β1 (3). Repeated TGF-β1 administration inhibited the expression of ALP (B), IGF-1 (C), and OC (D). Expression of these genes was analyzed by qRT-PCR and their mRNA levels were normalized to that of 18S rRNA, and measured in triplicate. Figures shown represent at least three independent experiments. Values
represent mean ± S.E. (n = 4). Bonferroni correction for multiple comparisons was applied. *P < 0.01, *P < 0.001.

Fig. 4.
Repeated TGF-β1 administration inhibits IGF-1/Akt signaling. Confluent cells were cultured in OBM and treated with a single or repeated TGF-β1 administration for 72 or 96 h without serum starvation culture. A, HPDL cells were cultured for 72 h or 96 h. B, hMSC were cultured for 96 h. C, MC3T3-E1 cells were cultured for 72 h. The levels of IGF-1 protein and phosphorylated Akt were determined by Western blot analysis. Blots are representative of three experiments.

Fig. 5.
Inhibition of IGF-1 signaling by a specific siRNA suppresses TGF-β1–induced expression of osteoblast differentiation marker genes. HPDL cells were transfected with IRS-1 siRNA, or control siRNA (A), and cultured in α-MEM for 3 days until confluent (B). Confluent cells were treated for 72 h in the presence or absence of 1 ng/ml TGF-β1. Transfection with IRS-1 siRNA reduced the expression of RUNX2 (D), ALP (E), IGF-1 (F), and BSP (G). Expression of these genes was analyzed by qRT-PCR and their mRNA levels were normalized to that of 18S rRNA and measured in triplicate. Figures shown represent at least three independent experiments. Values represent mean ± S.E. (n = 4). Bonferroni correction for multiple comparisons was applied. *P < 0.05, *P < 0.01.

Fig. 6.
Exogenous IGF-1 restores inhibition of osteoblast differentiation by repeated TGF-β1 administration in HPDL cells. A, HPDL cells were cultured in OBM with a single administration of 1 ng/ml TGF-β1, OBM with a double administration of 1 ng/ml TGF-β1, and OBM with a triple administration of 1 ng/ml TGF-β1 for 72 h. Exogenous IGF-1 (200 ng/ml) was added concomitantly with double or triple TGF-β1 administration. ALP activity was visualized by ALP activity staining of cells. It gradually decreased as the number of TGF-β1 administrations increased. However, exogenous IGF-1 completely restored the downregulation of ALP activity by repeated TGF-β1 administration. B, Confluent cells were treated for 72 h with OBM (1), OBM with a single administration of 1 ng/ml TGF-β1 (2), OBM with a double administration of 1 ng/ml TGF-β1 (3), and OBM with a double administration of 1 ng/ml TGF-β1 along with 200 ng/ml IGF-1 (4). Addition of IGF-1 fully recovered the expression of RUNX2 (B), ALP (C), BSP (D), and IGF-1 (E). Expression of these genes was analyzed by qRT-PCR and their mRNA levels were normalized to that of 18S rRNA, and measured in triplicate. Figures shown represent at least three independent experiments. Values represent mean ± S.E. (n = 4).

Fig. 7.
Overexpression of IGF-1 restores inhibition of osteoblast differentiation by repeated TGF-β1 administration in MC3T3-E1 cells. A, MC3T3-E1 cells were transfected with pEF1-V5-IGF1 vector
or pEF1-mock vector. Transfected cells were cultured in α-MEM for 3 days until confluent and then cells were treated with or without repeated administration of 0.1 ng/ml TGF-β1 for 72 h. Transfection with the pEF1-V5-IGF1 vector completely recovered the inhibition of ALP activity staining (B), ALP mRNA expression (C) and ALP activity (D) by repeated TGF-β1 administration. Expression of ALP was analyzed by qRT-PCR. The mRNA level of ALP was normalized to that of 18S rRNA and measured in triplicate. Figures shown represent at least three independent experiments. Values represent mean ± S.E. (n = 4). Bonferroni correction for multiple comparisons was applied. *P < 0.001.
Table 1. Primers used for quantitative real-time PCR.

| Gene symbol     | GenBank accession no. | Forward primer sequence | Reverse primer sequence |
|-----------------|-----------------------|-------------------------|-------------------------|
| human RUNX2     | NM_001024630.2        | gtccttagggcgatttca      | gcttctttactgagagtgaagg  |
| human ALP       | NM_000478.3           | caacccctggggagagac      | gcattgggttgtacgtcttg    |
| human BSP       | NM_004967.3           | caacctggtcactcactg      | tcattttgggtatgtcctct    |
| human IGF-1     | NM_000618.3           | tggagacaggggctttta      | atccaggtcctgctgtga      |
| human OC        | NM_199173.4           | tgagagccctcacactcttc    | acctgtgtggactctgcac     |
| mouse Alp       | NM_007431.2           | cggatctgacacaaaaacc     | tcagatgtccgtgtgcaat     |
| mouse Igf-1     | NM_001111274.1        | tcggctctactgtacccaat    | acgcatgatggtgatcctgtga  |
| mouse Osteocalcin| NM_007541.2           | agactccgggcctacctt     | ctcgtcacaacggggttaag    |
| 18S rRNA        | M11188.1              | cggacaggattgacagattg    | cgctccaccaactaagaagc    |
Fig. 1

A

B

C

D

E
Fig. 2

A

Relative expression (μmol/L)

B

Relative expression (F2-SS/RNA)

C

Relative expression (IFN-κBSS/RNA)

D

Relative expression (IGF1/5SS/RNA)

E

Relative expression (OC/βS/RNA)
Fig. 3

A

B

C

D

Relative expression

Relative expression

Relative expression

Relative expression
Fig. 4

**HPDL**

- **IGF-1**
- **pAkt**
- **β-actin**

**72 h**

- cont
- OBM
- TGF-β1 (single)
- TGF-β1 (repeated)

**96 h**

- cont
- OBM
- TGF-β1 (single)
- TGF-β1 (repeated)

**hMSC**

- **IGF-1**
- **pAkt**
- **β-actin**

**MC3T3-E1**

- **OBM**
- TGF-β1 (single)
- TGF-β1 (repeated)

- **OBM**
- TGF-β1 (single)
- TGF-β1 (repeated)
Fig. 6
Fig. 7

A

V5  IGF-1  β-actin
Mock  pEF1-IGF1

B

OBM
OBM
+TGF-β1
(repeated)
Mock  pEF1-IGF1

C

Relative expression
(ALP/18S rRNA)

Mock  Mock  pEF1-IGF1
TGF-β1  TGF-β1  (repeated)
(repeated)

D

ALP activity
(μmol/min/mg protein)

Mock  Mock  pEF1-IGF1  pEF1-IGF1
+  +
TGF-β1  TGF-β1  (repeated)  (repeated)

*  *

#  #