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Oncostatin M Promotes Osteogenesis and Suppresses Adipogenic Differentiation of Human Adipose Tissue-Derived Mesenchymal Stem Cells

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Abstract Oncostatin M (OSM) is a multifunctional cytokine of the interleukin-6 family and has been implicated in embryonic development, differentiation, inflammation, and regeneration of liver and bone. In the present study, we demonstrated that treatment of human adipose mesenchymal stem cells (hADSCs) with OSM-attenuated adipogenic differentiation, as indicated by decreased accumulation of intracellular lipid droplets and down-regulated expression of adipocytic markers, such as lipoprotein lipase and PPARγ. However, OSM treatment stimulated osteogenic differentiation, as demonstrated by the increase in matrix mineralization and expression levels of osteogenic differentiation markers, including alkaline phosphatase, Runx2, and osteocalcin. OSM treatment induced activation of JAK2, JAK3, and ERK in hADSCs, and pre-treatment of hADSCs with the JAK2 inhibitor, AG490, significantly restored the OSM-induced inhibition of adipogenic differentiation. Whereas, the JAK3 inhibitor, WHI-P131, and the MEK inhibitor, U0126, had no effects on the anti-adipogenic activity of OSM. On the other hand, the pro-osteogenic activity of OSM was prevented by treatment of the cells with WHI-P131 or U0126, but not with AG490. These results indicate that distinct signaling pathways, including JAK2, JAK3, and MEK-ERK, play specific roles in the OSM-induced anti-adipogenic and pro-osteogenic differentiation of hADSCs. J. Cell. Biochem. 101: 1238–1251, 2007.

Key words: OSM; adipocytes; osteoblasts; JAK2; JAK3; mesenchymal stem cells

Mesenchymal stem cells (MSCs) are present in a variety of tissues, including bone marrow, periosteum, trabecular bone, synovium, skeletal muscle, and adipose tissues [Short et al., 2003; Barry and Murphy, 2004]. They possess self-renewal capacity, long-term viability, and differentiation potential toward diverse cell types, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages [Caplan, 1991; Beresford et al., 1992; Prockop, 1997; Pittenger et al., 1999; Short et al., 2003; Barry and Murphy, 2004]. Human adipose tissue-derived MSCs (hADSCs) can be isolated from subcutaneous adipose tissues without severe pain associated with isolation of bone marrow-derived MSCs (BMSCs). Due to their stem cell-like properties, hADSCs are thought to be an excellent tool for mesenchymal tissue regeneration in many diseases that result from impaired function of these mesenchymal cells [Caplan, 1991; Beresford et al., 1992; Prockop, 1997; Pittenger et al., 1999; Short et al., 2003; Barry and Murphy, 2004]. However, for clinical applications of hADSCs, it is imperious to elucidate

Abbreviations used: MSCs, mesenchymal stem cells; hADSCs, human adipose tissue-derived mesenchymal stem cells; BMSCs, bone marrow-derived mesenchymal stem cells; OSM, oncostatin M; LIF, leukemia inhibitory factor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; ERK, extracellular signal-regulated kinase; ALP, alkaline phosphatase; PPARγ, peroxisome proliferator-activated receptor γ; LPL, lipoprotein lipase; OC, osteocalcin.

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the extracellular factors and the molecular mechanisms involved in determination of lineage-specific differentiation of hADSCs.

Oncostatin M (OSM), which is produced by activated monocytes and T lymphocytes, is a multifunctional cytokine that influences the growth and differentiation of several cell types [Gomez-Lechon, 1999; Tanaka and Miyajima, 2003]. OSM belongs to the IL-6 family of cytokines, including IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, and cardiotrophin 1. Accumulating evidences have suggested that IL-6 family of cytokines plays a crucial role in the regulation of bone development and remodeling [Taguchi et al., 1998; Heymann and Rousselle, 2000]. The crucial roles of IL-6 family cytokines in bone are exemplified by Stüve–Wiedemann syndrome, a severe autosomal recessive genetic disease whose prominent features include bowing of long bone, cortical thickening, and altered trabecular pattern, caused by null mutations of LIF receptor [Dagoneau et al., 2004]. Furthermore, studies on a series of knockout or transgenic mice showed that IL-6 family cytokines are necessary for normal mouse bone development and bone turnover [Heymann and Rousselle, 2000]. For instance, transgenic mice overexpressing OSM develops osteopetrotic bone tissue, possibly by stimulation of bone formation and inhibition of bone resorption [Malik et al., 1995]. In vitro studies on osteoblastic models demonstrated that OSM stimulates osteogenic differentiation in BMSCs [Chipoy et al., 2004]. However, published data obtained from in vitro studies on osteoblastic models suggest that IL-6 family of cytokines positively or negatively regulates the osteogenic differentiation, depending on cell types or differentiation stages [Bellido et al., 1997; Taguchi et al., 1998; Heymann and Rousselle, 2000; Chipoy et al., 2004; Malaval et al., 2005]. In addition, IL-6 family of cytokines has been reported to inhibit adipogenic differentiation in a murine stromal cell line [Gimble et al., 1994]. In contrast, stimulatory effect of LIF on adipogenic differentiation has been demonstrated in embryonic stem cell model [Aubert et al., 1999]. Therefore, in spite of these various reports, the physiological role of OSM in the osteogenic or adipogenic differentiation of MSCs and the molecular mechanisms underlying the OSM-induced regulation of differentiation are still unclear.

OSM acts on target cells by binding to a heterodimeric membrane receptor which is composed of LIF- or OSM-specific receptor and gp130 receptor chain [Gomez-Lechon, 1999]. Binding of OSM to the cognate receptors induces activation of Janus kinases (JAKs), leading to phosphorylation of the docking sites in the receptor for SHP-2, and signal transducers and activators of transcription (STAT). SHP-2 is thought to couple-activated receptors to the extracellular signal-regulated kinase (ERK) cascade, Ras/Raf/MEK/ERK, for induction of immediate early genes, *egr-1* and c-*fos*. Phosphorylated STAT molecules, including STAT1 and STAT3, form a dimer and are translocated to the nucleus where it regulates gene expression by binding its target sequences [Gomez-Lechon, 1999; Kamimura et al., 2003]. We recently demonstrated that OSM induces proliferation and activation of JAK2, JAK3, STAT1, STAT3, and ERK in hADSCs, and that JAK3/STAT1 and MEK/ERK pathways are involved in the OSM-induced proliferation [Song et al., 2005]. However, the role of JAK/STAT and MEK/ERK pathways in the OSM-induced regulation of adipogenic or osteogenic differentiation has not yet been determined.

In the present study, we sought to determine the effects of OSM on the adipogenic or osteogenic differentiation of hADSCs, and the contributions made by OSM-induced signaling pathways to the differentiation. We show that OSM inhibits adipogenic differentiation and stimulates osteoblastic differentiation by distinct signaling pathways, involving JAK2, JAK3, and ERK.

MATERIALS AND METHODS

Materials

α-minimum essential medium, phosphate-buffered saline, trypsin, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Human recombinant OSM and IL-6 were from R&D Systems (Minneapolis, MN) and human recombinant LIF was from Chemicon (Temecula, CA). JAK3 inhibitor I (WHI-P131) and AG490 were purchased from Calbiochem (La Jolla, CA). U0126 was from BIOMOL (Plymouth Meeting, PA). LightCycler FastStart DNA Master Sybr Green I was purchased from Roche Applied Science (Mannheim, Germany). All other reagents were purchased from Sigma-Aldrich.

Cell Culture

Subcutaneous adipose tissue was obtained from elective surgeries with the patient's consent, as approved by the Institution Review Board, and hADSCs were isolated as previously reported [Kang et al., 2005]. Briefly, adipose tissues were washed at least three times with sterile phosphate-buffered saline and treated with an equal volume of collagenase type I (1 g/L in Hank's balanced salt solution with 1% bovine serum albumin) for 60 min at 37°C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction by centrifugation (300*g* for 5 min). The pellet was resuspended in α-minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and plated in tissue culture dishes at 3,500 cells/cm^2. The primary hADSCs were cultured for 4–5 days until confluence and were defined as passage "0." The passage number of hADSCs used in the experiments was 3–10. The expression profiles of the hADSCs were highly similar to those expression profiles of human BMSCs, as was observed by using flow cytometric analysis and microarray analysis [Lee et al., 2004]. The hADSCs were positive for CD29, CD44, CD90, and CD105, all of which have been reported to be marker proteins of mesenchymal stem cells. However, c-kit, CD34, and CD14, which are known as hematopoietic markers, were not expressed in hADSCs. Since hADSCs derived from different donors were shown to exhibit varying degrees of differentiation potentials either to adipocytes or osteoblasts, we used hADSCs derived from at least two different donors in the present study.

Induction of Adipogenic Differentiation

For induction of adipogenic differentiation, hADSCs were seeded onto 24-well culture plate at a density of 6×10^4 cells/well, cultured for 48 h to confluence in the growth medium, and then treated with adipogenic differentiation medium (10% fetal bovine serum, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μM insulin, and 200 μM indomethacin in α-minimum essential medium) for 1 week. The accumulation of intracellular triglyceride droplets was visualized by Oil Red-O staining as described previously [Novikoff et al., 1980] and the phase contrast images were photographed at 200× microscopic power by a digital camera equipped in an inverted microscope (Leica DM IRB). The number of cells committed to the adipogenic lineage was quantified by counting the number of cells having numerous cytoplasmic lipid-filled vacuoles in three randomly selected visual fields in the same well.

Induction of Osteogenic Differentiation

hADSCs were seeded onto 24-well culture plate at a density of 6×10^4 cells/well and cultured for 48 h to confluence in the growth medium. Osteogenic differentiation was induced by exposure of confluent hADSCs to osteogenic differentiation medium (10% fetal bovine serum, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid in α-minimum essential medium) for longer than 3 weeks, and the extracellular matrix calcification was visualized by Alizarin red S staining. Briefly, the cells were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde for 30 min. The fixed cells were incubated with 2% Alizarin red-S for 10 min with shaking. To minimize nonspecific staining, the cells were rinsed five times with deionized water and once with phosphate-buffered saline for 20 min. Osteogenic differentiation was quantified by measuring alizarin red-stained area and density using a Scion imaging software (Scion Corporation, Frederick, MD).

Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity of the cell layer was measured in triplicate cultures by rinsing twice with Hank's balanced salt solution, and then incubating the cells with 5 mM p-nitrophenyl phosphate in 50 mM glycine and 1 mM $MgCl_2$, pH 10.5, at 37°C for 20 min. The absorbance of p-nitrophenol produced by ALP activity was monitored at 405 nm by using a PowerWave$_x$ microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Cells were treated as indicated, and total cellular RNA was extracted by the Tryzol method (Invitrogen). For RT-PCR analysis, aliquots of 2 μg of RNA were subjected to cDNA synthesis with 200 U of M-MLV reverse transcriptase (Invitrogen) and 0.5 μg of oligo(dT) 15 primer (Promega, Madison, WI). The cDNA,

present in 2 μl of this reaction mixture, was amplified with 0.5 U of GoTaq DNA polymerase (Promega) and 10 pmol of each sense and antisense primers as follows: GAPDH 5′-TCCATGACAACTTTGGTATCG-3′, 5′-TGTAGCCAAATTCGTTGTCA-3′, LPL 5′-ATGGAGAGCAAAGCCCTGCTC-3′, 5′-TACAGGGCGGCCACAAGTTTT-3′, PPARγ2 5′-GCTGTTATGGGTGAAACTCTG-3′, 5′-ATAAGGTGGAGATGCAGGTTC-3′, ALP 5′-TGGAGCTTCAGAAGCTCAACACCA-3′, 5′-ATCTCGTTGTCTGAGTACCAGTCC-3′, Runx2 5′-CAGACCAGCAGCACTCCATA-3′, 5′-CAGCGTCAACACCATCATTC-3′, OC 5′-GTGCAGAGTCCAGCAAAGGT-3′, 5′-TCAGCCAACTCGTCACAGTC-3′.

The thermal cycle profile was as follows: denaturation for 30 s at 95°C, annealing for 45 s at 52–58°C depending on the primers used, and extension for 45 s at 72°C. For semi-quantitative assessment of expression levels, each PCR reaction was carried out on 30 cycles. PCR products were size fractionated on 1.2% ethidium bromide/agarose gel and quantitated under UV transillumination.

Real-Time RT-PCR

Quantitative PCR was performed and analyzed on a capillary real-time thermocycler (LightCycler, Roche Diagnostics). Amplification was done in the presence of SYBR Green I as follows: in 20 μl of final volume, cDNA was mixed with LightCycler-Fastart DNA Master SYBR Green (Roche Diagnostics), 10 pmol each of forward primer and reverse primer, and 4 mM $MgCl_2$. Glass capillaries were placed into the LightCycler rotor, and the following run protocol was used: a pre-denaturing step at 95°C for 10 min, an amplification and quantification program repeated for 50 cycles at 95°C for 10 s, annealing for 10 s at 52–58°C, depending on the primers used, and extension for 30 s at 72°C. At the end of elongation at each cycle, SYBR Green I fluorescence was measured. Data analysis was performed essentially as indicated by Roche using "Fit Point Method" in the LightCycler software 3.3 (Roche Diagnostics). Relative quantification was made against serial dilution of GAPDH cDNA used as a housekeeping gene.

Trypan Blue Exclusion Assay

For direct counting of total cell number, cells were harvested by trypsinization, suspended in phosphate-buffered saline, and incubated with an equal amount of 0.1% trypan blue. The number of trypan blue-negative cells was counted by using a hemocytometer.

Statistics

Experiments were repeated at least three times with hADSCs derived from at least two different donors. Data were expressed as mean and standard deviation, and paired parametric data were compared by Student's *t*-test.

RESULTS

OSM Inhibits Adipogenic Differentiation of hADSCs

To explore whether OSM affects differentiation of hADSCs to adipocytes, the adipogenic differentiation of hADSCs was induced by exposure of the cells to adipogenic differentiation medium in the presence of different concentrations of OSM, and accumulation of intracellular triglyceride droplets was visualized by Oil Red-O staining as described under "Materials and Methods." As shown in Figure 1A, exposure of hADSCs to adipogenic differentiation medium for 10 days, but not to growth medium, resulted in differentiation to adipocytes. However, OSM treatment dose-dependently prevented the adipogenic differentiation of hADSCs with a maximal inhibition at 10 ng/ml OSM (Fig. 1A,B), suggesting a potent anti-adipogenic activity of OSM in hADSCs.

Since OSM belongs to IL-6 family of cytokines, including IL-6 and LIF, we next compared the effects of IL-6, LIF, and OSM on the adipogenic differentiation of hADSCs. As shown in Figure 1C,D, treatment of hADSCs with either 10 ng/ml IL-6 or LIF had no effect on the differentiation, in contrast to complete prevention of adipogenesis in the presence of OSM. These results demonstrate that OSM specifically inhibits the adipogenesis of hADSCs.

Time Dependence of the OSM-Induced Inhibition of Adipogenic Differentiation

To explore time dependence of the OSM-induced inhibition of adipogenic differentiation in hADSCs, the cells were pulse-treated with 10 ng/ml OSM during the indicated time course of in vitro adipogenic differentiation for 10 days. Pulse treatment of hADSCs with OSM during day 0–3 had no significant effect on the

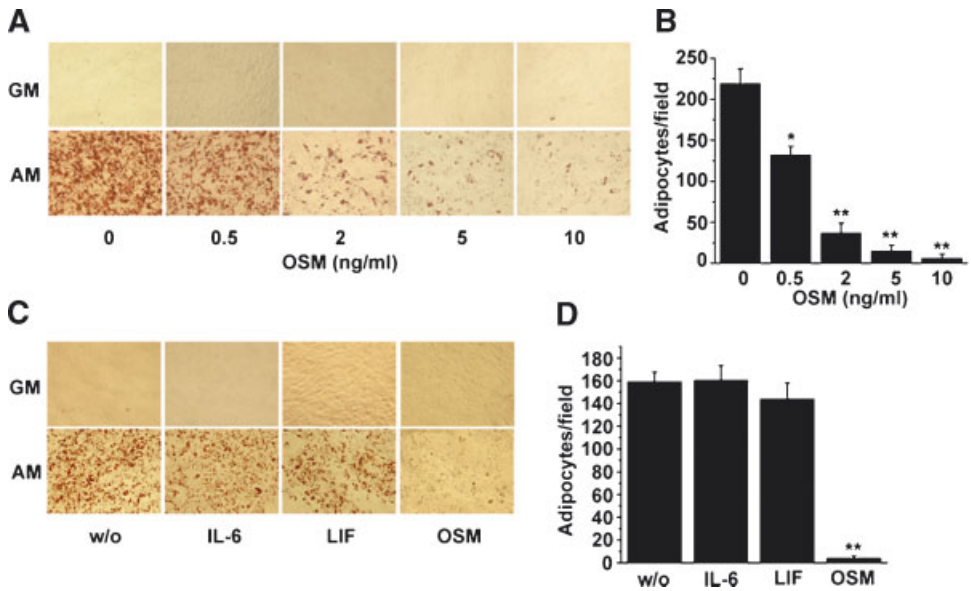

Fig. 1. Effects of IL-6 family of cytokines on adipogenic differentiation of hADSCs. **A**: hADSCs at confluence were treated with the indicated concentrations of OSM in a growth medium (GM) or adipogenic differentiation medium (AM). After 10 days, lipid droplets were visualized by Oil Red-O staining, and the phase contrast images were photographed by a digital CCD camera equipped in an inverted microscope. Data from a representative of three independent experiments are shown. **B**: The number of Oil Red O-positive cells was quantified and demonstrated as mean ± SD; n = 3 (*$P < 0.05$; **$P < 0.01$). **C**: hADSCs were treated with IL-6, LIF, or OSM (10 ng/ml each) in GM or AM for 10 days. Adipocytes were visualized by Oil Red-O staining, and the phase contrast images were photographed. Data from a representative of three independent experiments are shown. **D**: The number of Oil Red O-positive cells was quantified and demonstrated as mean ± SD; n = 3 (**$P < 0.01$ versus control (w/o)).

adipogenic differentiation of hADSCs (Fig. 2). However, exposure of hADSCs to OSM during day 0–5 significantly attenuated the accumulation of intracellular lipid droplets, and treatment of the cells with OSM during entire period (day 0–10) completely prevented the differentiation. Furthermore, pulse treatment of the cells with OSM during day 3–7 or day 5–7 also significantly inhibited the differentiation of hADSCs. These results suggest that the exposure of the cells to OSM during the periods after day 3 is critical for the OSM-induced inhibition of adipogenic differentiation.

OSM Inhibits the Expression of Adipogenic Markers in hADSCs

Adipogenesis is regulated by a transcription factor, peroxisome proliferator-activated receptor γ (PPARγ), which is essentially required for expression of adipocyte-specific genes, such as lipoprotein lipase (LPL) [Tontonoz et al., 1994]. The expression levels of the adipocyte markers during adipogenic differentiation of hADSCs were determined by RT-PCR analysis. LPL and PPARγ were highly expressed in hADSCs, when exposed for 10 days to adipogenic differentiation medium, whereas no expression of the adipogenic markers in the control hADSCs cultured with growth medium (Fig. 3A), consistent with the intracellular accumulation of lipid droplets in adipocytes differentiated from hADSCs. We next explored the effect of OSM on the expression levels of the adipogenic genes, LPL and PPARγ, by real-time RT-PCR. As shown in Figure 3B,C, expression levels of LPL and PPARγ were greatly increased, when hADSCs were exposed to adipogenic differentiation medium. However, treatment of the cells with OSM during adipogenic differentiation completely prevented the increased expression of these adipogenic markers in response to adipogenic differentiation medium. These results suggest that OSM negatively regulates the adipogenic differentiation of hADSCs by suppressing the expression of PPARγ.

Molecular Mechanism Involved in the Anti-Adipogenic Activity of OSM

We have previously reported that OSM triggered activation of JAK2, JAK3, and ERK in hADSCs, and that OSM stimulated proliferation of the cells through activation of JAK3/

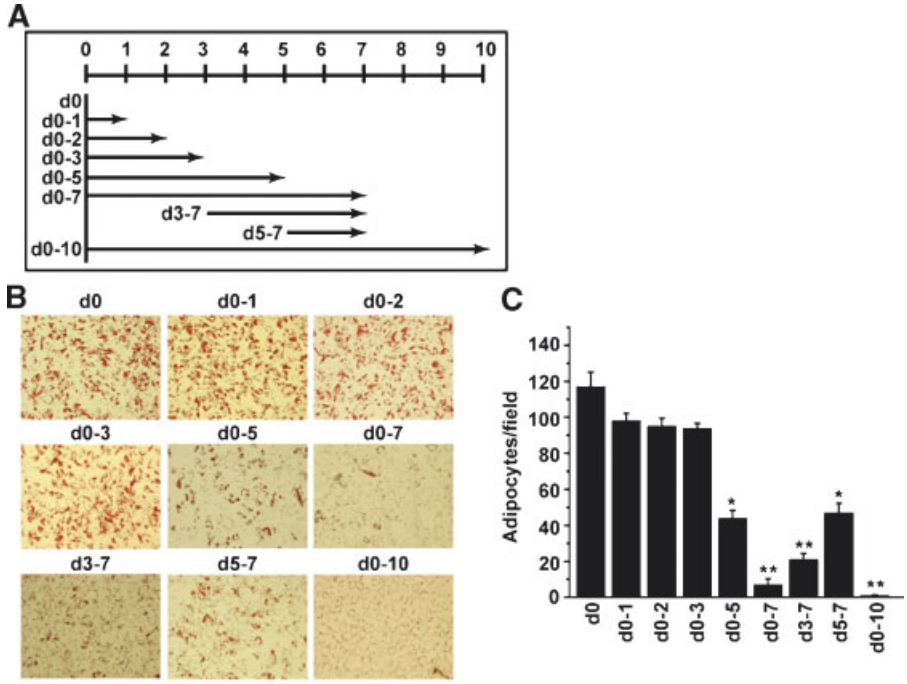

Fig. 2. Time dependence of OSM on adipogenic differentiation of hADSCs. **A**: hADSCs were exposed to AM on day 0, and pulse-treated with 10 ng/ml OSM during adipogenic differentiation. Arrows indicate the time periods for treatment of OSM. **B**: On day 10, lipid droplets were visualized by Oil Red-O staining, and the phase contrast images were photographed by a digital camera equipped on a light microscopy. Data from a representative of three independent experiments are shown. **C**: The number of Oil Red O-positive cells was quantified and demonstrated as mean ± SD; n = 3 (*$P < 0.05$; **$P < 0.01$ versus control (d0)).

STAT1 and MEK/ERK pathways [Song et al., 2005]. To gain insight into the signaling mechanism leading to the OSM-induced inhibition of adipogenesis, hADSCs were exposed to OSM along with JAK2 inhibitor AG490, JAK3-specific inhibitor WHI-P131, or MEK inhibitor U0126. As shown in Figure 4A, treatment of hADSCs with AG490 in the absence of OSM did

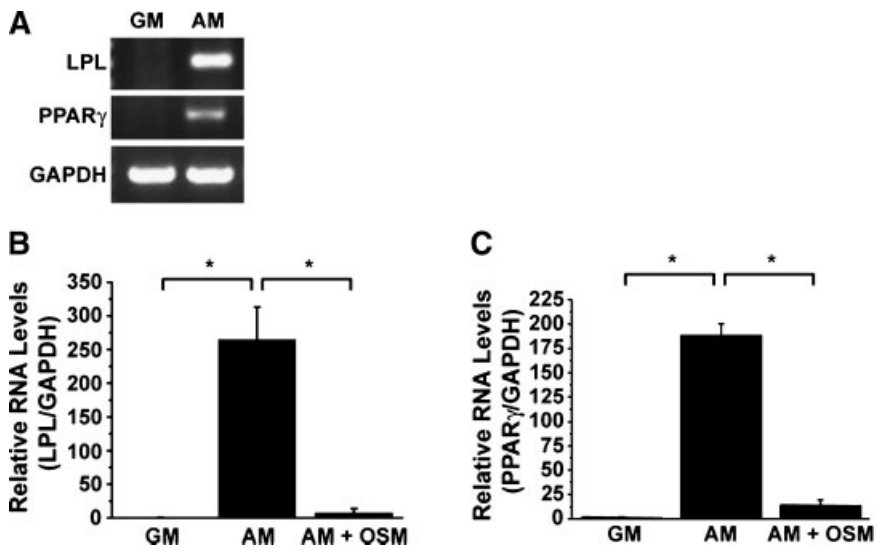

Fig. 3. Effects of OSM on the expression levels of adipogenic markers in hADSCs. **A**: hADSCs were treated with GM or AM for 10 days. The expression levels of LPL, PPARγ, and GAPDH were analyzed by RT-PCR. **B**,**C**: hADSCs were treated with GM or AM together with 10 ng/ml OSM for 10 days. Real-time PCR analysis was performed on total RNA with primers specific for LPL (B) and PPARγ (C), and the values were normalized using GAPDH as an internal control. Bars equal mean ± SD; n = 3 (*$P < 0.05$).

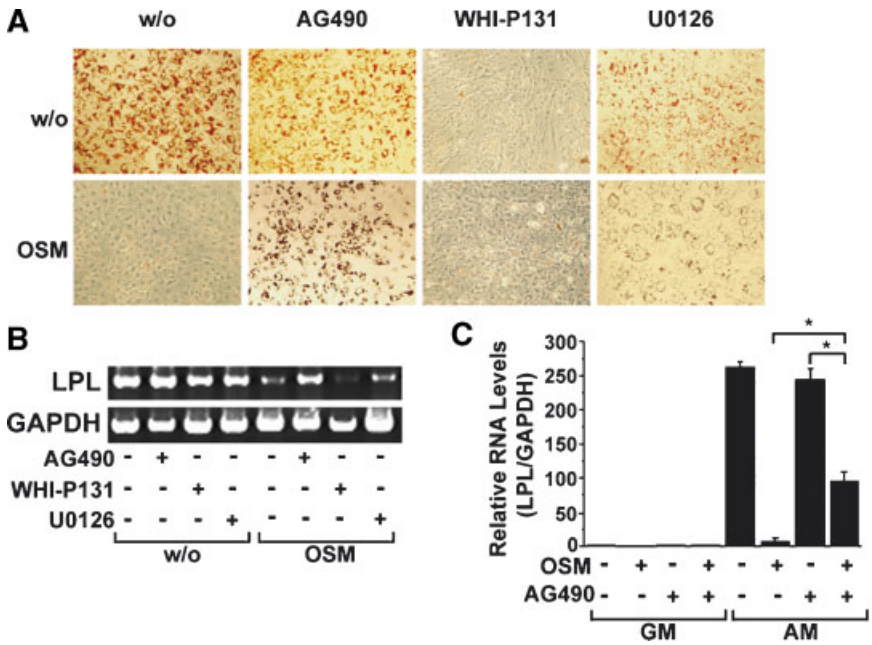

Fig. 4. Role of JAK2 in the OSM-induced inhibition of adipogenic differentiation. **A**: hADSCs were treated with AM in the absence or presence of 10 μM AG490, 30 μg/ml WHI-P131, 10 μM U0126, or 10 ng/ml OSM for 10 days. Adipocytes were visualized by Oil Red-O staining and the phase contrast images were photographed by a digital CCD camera equipped in an inverted microscope. Data from a representative of three independent experiments are shown. **B**: The expression levels of LPL and GAPDH were analyzed by RT-PCR analysis. Representative data from three independent experiments are shown. **C**: hADSCs were treated with GM or AM in the absence or presence of 10 μM AG490 or 10 ng/ml OSM for 10 days as indicated, and the mRNA levels of ALP and GAPDH were determined by real-time RT-PCR analysis. The relative levels of ALP mRNA were normalized to those of GAPDH. Bars equal mean ± SD; n = 3 (*$P < 0.05$).

not affect the adipogenic differentiation of the cells. However, exposure of the cells to 10 μM AG490 in the presence of OSM significantly recovered the OSM-induced inhibition of intracellular accumulation of lipid droplets, suggesting a specific role of JAK2 in the OSM-induced inhibition of adipogenesis. Whereas, the OSM-induced inhibition of adipogenesis was not recovered by treatment with either WHI-P131 or U0126, indicating that JAK2, but not JAK3 and ERK, is specifically involved in the OSM-induced attenuation of adipogenic differentiation.

JAK2 is Involved in the OSM-Induced Inhibition of Adipocytic Gene Expression

To confirm the involvement of JAK2 in the OSM-induced suppression of adipogenic differentiation, we next explored the effects of AG490 on the OSM-induced inhibition of expression of LPL by using semi-quantitative RT-PCR. As shown in Figure 4B, treatment of hADSCs with AG490 together with OSM significantly recovered the OSM-induced inhibition of mRNA levels of LPL. However, exposure of hADSCs to WHI-P131 or U0126 during adipogenic differentiation in the presence of OSM did not recover the OSM-induced inhibition of expression levels of LPL. To further confirm the involvement of JAK2 in the OSM-induced inhibition of LPL expression, we next determined the expression levels of LPL by real-time RT-PCR. As shown in Figure 4C, the OSM-induced decrease of LPL expression was partially, but significantly, restored by pretreatment of the cells with AG490. Although AG490 treatment could not completely recover the OSM-induced inhibition of LPL expression, these results suggest that JAK2 plays a crucial role in the OSM-induced down-regulation of adipocyte-specific gene expression.

OSM Enhances Osteogenic Differentiation of hADSCs

The effect of OSM on osteogenic differentiation of hADSCs was explored as shown in Figure 5A,B. In confluent hADSCs treated with osteogenic medium, OSM dose-dependently increased the mineral density with a maximal increase at 10 ng/ml concentration, as

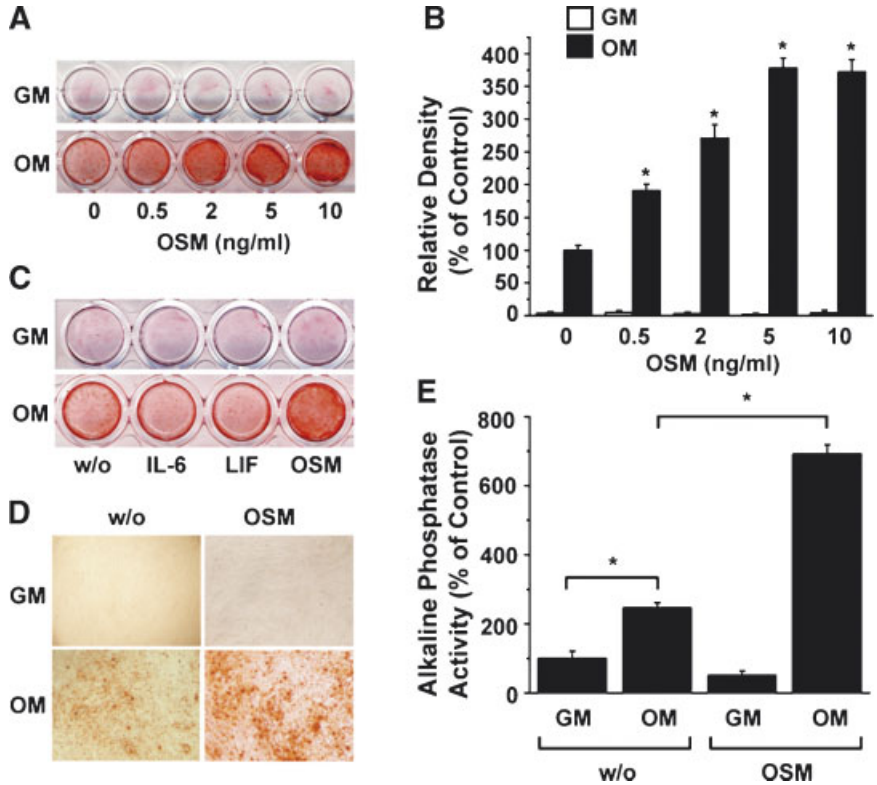

Fig. 5. Effects of IL-6 family of cytokines on osteogenic differentiation of hADSCs. **A**: hADSCs at confluence were treated with GM or osteogenic differentiation medium (OM) in the presence of indicated concentrations of OSM. After 14 days, matrix mineralization was determined by Alizarin Red-S staining and photographed by a digital camera. **B**: Relative density of each well was quantified by using Scion image program, and results from triplicate determination are shown as mean ± SD. **C**: hADSCs were exposed to GM or OM containing 10 ng/ml IL-6, LIF or OSM for 14 days as indicated. Matrix mineralization was determined by Alizarin Red-S staining and photographed by a digital camera. **D**: hADSCs were treated with GM or OM in the absence or in the presence of 10 ng/ml OSM for 14 days. Mineralized nodules were stained by Alizarin red-S and photographed by a digital CCD camera equipped in an inverted microscope (Leica DM IRB) at 200×. **E**: Alkaline phosphatase activity was determined by a colorimetric assay. Results from a representative of three experiments are shown as mean ± SD; n = 3 (*$P < 0.05$).

demonstrated by Alizarin Red-S staining. To assess whether OSM specifically stimulated the osteogenic differentiation, we next examined the effects of other members of IL-6 family cytokines, such as IL-6 and LIF, on the differentiation. In contrast to OSM, treatment of hADSCs with either IL-6 or LIF did not stimulate the mineral density (Fig. 5C). As shown in Figure 5D, the exposure of confluent hADSCs to osteogenic medium induced the mineral deposition, and treatment of the cells with OSM during osteogenic differentiation drastically increased the extracellular mineralization. Whereas, OSM treatment did not induce extracellular mineralization of hADSCs in cultures maintained in growth medium. These results specifically implicate OSM in the regulation of osteogenic differentiation.

Since ALP activity, an early marker of osteogenic differentiation, plays an important role in both the osteogenic differentiation and eventual mineralization processes, we next determined ALP activity in these cells. As shown in Figure 5E, the ALP activity in hADSCs was increased when exposed to the osteogenic medium, and treatment of the cells with OSM further stimulated the ALP activity. The OSM-induced increase of ALP activity is consistent with the increased mineralization in the OSM-treated cells during osteogenic differentiation.

Time Dependence of OSM-Stimulated Osteogenic Differentiation in hADSCs

To explore time dependence of OSM-stimulated osteogenic differentiation in hADSCs, we determined extracellular mineralization in hADSCs treated with osteogenic differentiation medium in the presence or absence of OSM.

Incubation of hADSCs in osteogenic differentiation medium time-dependently increased the mineralization and maximal differentiation occurred on day 20. However, the mineralization was accelerated by continuous treatment of the cells with 10 ng/ml OSM during osteogenic differentiation, and maximal mineralization occurred on day 10 (Fig. 6A,B). The density of deposited mineral on day 5 in the presence of OSM was almost comparable to those on day 15 in the absence of OSM (Fig. 6A,B), suggesting a potent pro-osteogenic activity of OSM on hADSCs.

To assess whether continuous treatment of hADSCs with OSM is essential for the differentiation, the cells were pulse-treated with 10 ng/ml OSM for 14 days during the indicated time course of in vitro osteogenesis. Pulse treatment of hADSCs with OSM during day 0–1 significantly increased the mineral deposition, and maximal increase occurred during day 0–2 pulse treatment (Fig. 6C,D). These results suggest that exposure of hADSCs to OSM during early stage of differentiation is essential for the OSM-stimulated osteogenic differentiation.

In a previous study, we demonstrated that OSM increased cell number of sub-confluent hADSCs [Song et al., 2005], however, OSM did not significantly stimulate proliferation of confluent hADSCs (data not shown). To elucidate whether OSM-induced increase of cell number was responsible for the OSM-stimulated mineral deposition, we examined the effect of OSM on the cell number of confluent hADSCs during osteogenesis. However, as shown in Figure 6E, OSM had no significant impact on the number of confluent hADSCs during osteogenic differentiation. Therefore, it is likely that the OSM-stimulated mineralization was not due to increased cell number during osteogenic differentiation.

Effect of OSM on Expression of Osteoblastic Markers in hADSCs

Osteoblast differentiation of MSCs occurs along with an increase in the expressions of

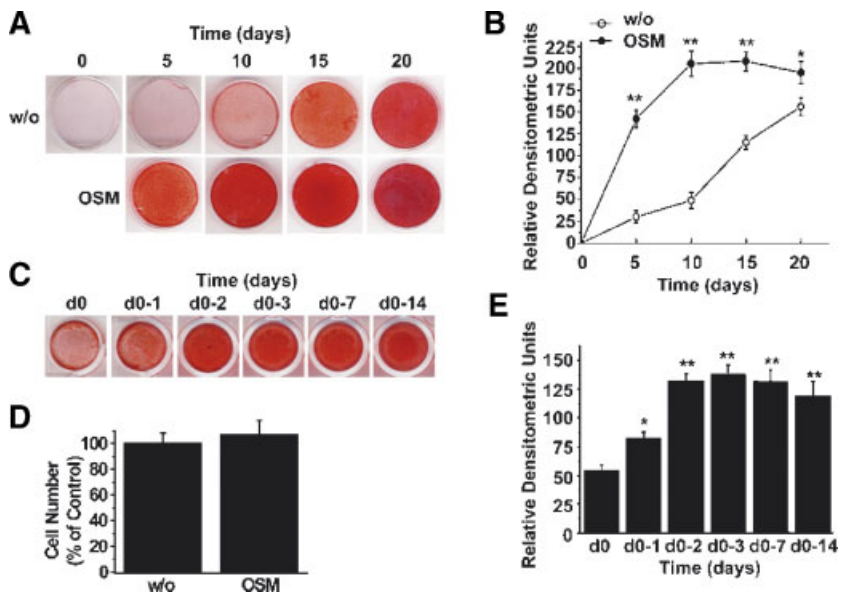

Fig. 6. Time dependence of OSM on osteogenic differentiation of hADSCs. **A**: hADSCs were treated with OM in the absence or presence of 10 ng/ml OSM for the indicated time. Matrix mineralization was determined by Alizarin Red-S staining and photographed by a digital camera. Data from a representative of three independent experiments are shown. **B**: Relative density of each well was quantified by using Scion image program and results from triplicate determination are shown as mean ± SD (*$P < 0.05$; **$P < 0.01$ vs. control (w/o)). **C**: During osteogenic differentiation, hADSCs were pulse-treated with OM containing 10 ng/ml OSM for the indicated time periods. After 14 days, matrix mineralization was assessed by Alizarin Red-S staining and photographed by a digital camera. Data from a representative of three independent experiments are shown. **D**: Relative density of each well was quantified by using Scion image program, and results from duplicate determination are shown as mean ± SD (*$P < 0.05$; **$P < 0.01$ vs. control (d0)). **E**: hADSCs were treated with OM in the absence or presence of 10 ng/ml OSM for 14 days. The number of cells was determined by trypan blue exclusion assay and values are expressed as a percentage of control (w/o) which was defined as 100%. Data are shown as mean ± SD ($n = 3$).

ALP, osteocalcin (OC), and Runx2. To confirm the pro-osteogenic effect of OSM, we next examined the expression of osteoblasts-specific genes by semi-quantitative RT-PCR analysis. The expression levels of ALP, OC, and Runx2 were slightly increased after exposure of hADSCs to osteogenic medium for 3 days (Fig. 7A). However, treatment of the cells with osteogenic medium along with OSM-exhibited robust increase in the mRNA levels of osteoblast markers, such as ALP, OC, and Runx2. In the growth media, OSM alone did not increase the expression levels of the osteogenic markers in hADSCs. To confirm these results, the mRNA levels of the osteogenic markers were determined by real-time RT-PCR. As shown in Figure 7B–D, the expression levels of ALP, OC, and Runx2 were elevated by treatment of the cells with osteogenic differentiation medium together with OSM. These results are consistent with the observations that OSM treatment stimulated the extracellular mineralization of hADSCs exposed to osteogenic medium, suggesting that OSM promotes the osteoblastic differentiation by increasing the expression levels of the osteoblastic markers.

Molecular Mechanism Involved in the Pro-Osteogenic Activity of OSM

In the above, we demonstrated that the anti-adipogenic activity of OSM was mediated by activation of JAK2, but neither JAK3 nor ERK. Therefore, to elucidate the involvement of JAK isoforms in the OSM-stimulated osteogenesis, we examined the effects of JAK inhibitors on the OSM-stimulated mineralization. As shown in Figure 8, extracellular mineralization was induced by exposure of the cells to osteogenic medium for 7 days and OSM treatment stimulated the mineralization of hADSCs. Co-treatment of the cells with AG490 did not inhibit the pro-osteogenic activity of OSM. In contrast, however, co-treatment of hADSCs with WHI-P131 completely attenuated the OSM-stimulated osteogenic differentiation. These results suggest that JAK3, but not JAK2, is specifically involved in the pro-osteogenic activity of OSM, in contrast to the involvement of JAK2 in the OSM-induced inhibition of adipogenic differentiation. To explore whether ERK activation is necessary for osteogenic differentiation, we next examined the effect of U0126 on the

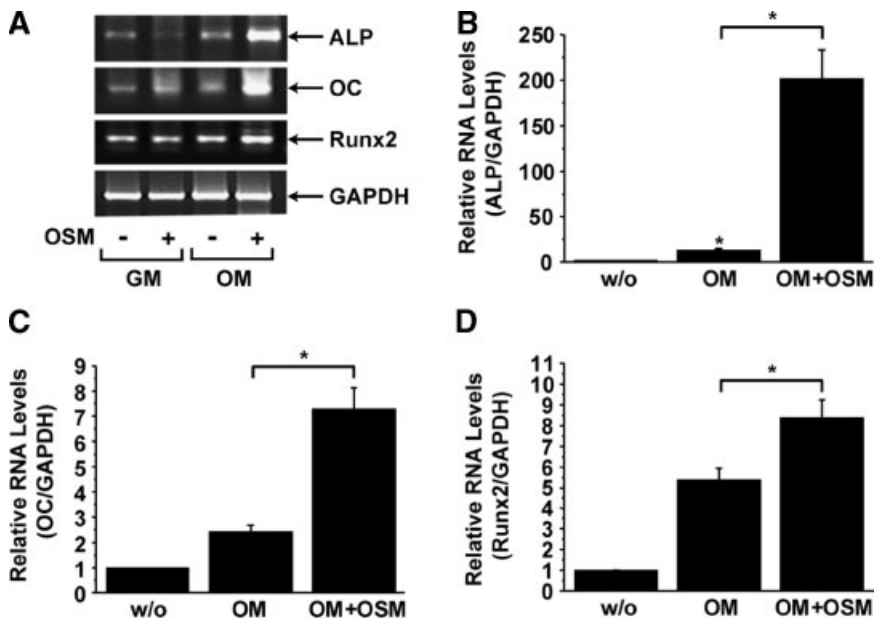

Fig. 7. Effects of OSM on the expression levels of osteoblastic markers in hADSCs. **A**: hADSCs were treated with GM or OM in the absence or presence of 10 ng/ml OSM for 3 days. The expression levels of ALP, OC, Runx2, and GAPDH were analyzed by RT-PCR. **B**,**C**: The expression levels of ALP, Runx2, and OC were quantified by real-time PCR analysis with primers specific for ALP (B), OC (C), and Runx2 (**D**). The relative mRNA levels were normalized to those of GAPDH. Bars equal mean ± SD; n = 3 (*$P < 0.05$).

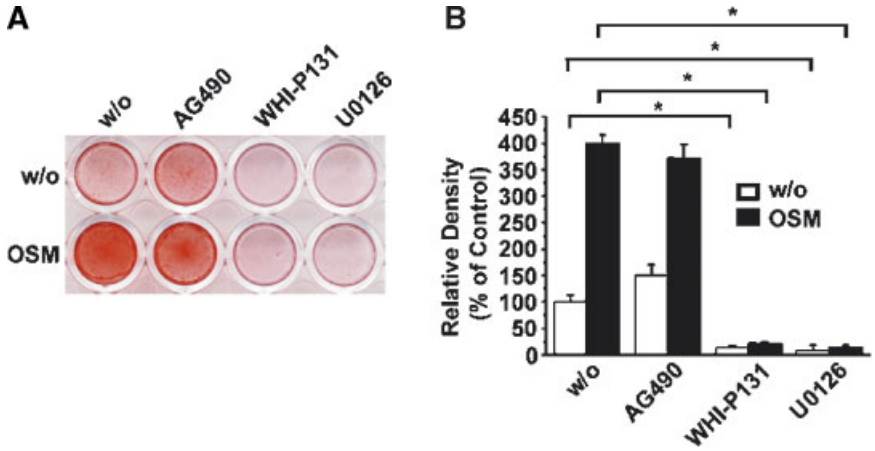

Fig. 8. Role of JAK3 and ERK in the OSM-induced stimulation of osteogenic differentiation. **A**: hADSCs were treated with OM in the absence or presence of 10 μM AG490, 30 μg/ml WHI-P131, 10 μM U0126, or 10 ng/ml OSM for 7 days. Extracellular mineralization was visualized by Alizarin Red-S staining and photographed by a digital camera. Data from a representative of three independent experiments are shown. **B**: Relative density of each well was quantified by using Scion image program and results from duplicate determination are shown as mean ± SD (*$P < 0.05$).

osteogenic differentiation in the absence or presence of OSM. Treatment of the cells with U0126 prevented the OSM-stimulated mineralization during osteogenic differentiation (Fig. 8A,B). These results suggest that both JAK3 and ERK play a key role in the OSM-stimulated osteogenic differentiation in hADSCs.

JAK3 and MEK Are Involved in the OSM-Stimulated Osteogenic Differentiation

To explore whether JAK3 and ERK play a role in the OSM-stimulated osteogenesis by enhancing the expression levels of osteogenic markers, we examined the expression levels of ALP by real-time RT-PCR. As shown in Figure 9, the expression levels of ALP was greatly increased in response to exposure of the cells to osteogenic medium containing OSM. Treatment of the cells with WHI-P131 or U0126 attenuated the mRNA levels of ALP in the OSM-treated cells. In contrast, treatment of the cells with AG490 had no significant effects on the expression levels of ALP. These results suggest that JAK3 and ERK play a crucial role in the increased expression of ALP in response to OSM during osteogenic differentiation.

DISCUSSION

In the present study, we demonstrated for the first time that OSM regulates lineage-specific differentiation of hADSCs in favor of osteoblastic and against adipogenic differentiation. Accumulating evidences have implicated IL-6 and LIF in the regulation of osteogenic differentiation of several osteoprogenitors and bone remodeling [Taguchi et al., 1998; Heymann and Rousselle, 2000]. However, neither IL-6 nor LIF duplicates the pro-osteogenic or anti-adipogenic

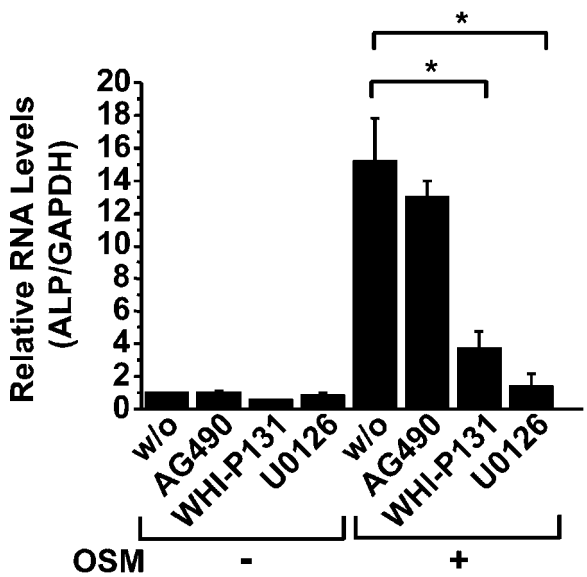

Fig. 9. Role of JAK3 and ERK in the OSM-induced expression of osteoblastic markers in hADSCs. hADSCs were treated with OM in the absence or presence of 10 μM AG490, 30 μg/ml WHI-P131, 10 μM U0126, or 10 ng/ml OSM for 3 days, and the mRNA levels of ALP and GAPDH were determined by real-time RT-PCR analysis. The relative levels of ALP mRNA were normalized to those of GAPDH. The data are shown as mean ± SD of triplicate determinations. Bars equal mean ± SD; n = 3 (*$P < 0.05$).

effects of OSM in hADSCs, suggesting a principal role of OSM in the regulation of osteogenic differentiation in hADSCs. In accordance with the present study, transgenic mice overexpressing OSM exhibit increased bone formation and enlarged hind limbs [Malik et al., 1995], and obsteoblasts derived from gp130$^{-/-}$ mice exhibit diminished ALP mRNA and protein both in vivo and in osteoblast cultures [Shin et al., 2004]. In addition, OSM has been shown to induce osteoblastic differentiation in diverse osteogenic progenitor cells, such as MG63 cells, calvaria obsteoblastic precursors and BMSCs [Bellido et al., 1998; Chipoy et al., 2004; Malaval et al., 2005]. These studies support our present finding that OSM has a potent stimulatory effect on the osteogenic differentiation of hADSCs.

We have previously reported that OSM induces activation of JAK2, JAK3, and ERK and that JAK3/STAT1- and MEK/ERK-dependent pathways are involved in the OSM-induced proliferation of hADSCs [Song et al., 2005]. Pharmacological inhibition of JAK2 activity had no effects on the OSM-stimulated proliferation of hADSCs. On the other hand, in the present study, we demonstrated for the first time that JAK2 plays a key role in the anti-adipogenic effect of OSM, since inhibition of JAK2 activity significantly restored the OSM-induced inhibition of adipogenesis without impacts on the adipogenic differentiation. However, the pharmacological inhibition of JAK2 activation could not completely restore the OSM-induced inhibition of adipogenesis, suggesting involvement of other signaling pathways in the OSM-induced suppression of adipogenesis in hADSCs. These results suggest that JAK2 may be responsible in part for the anti-adipogenic effect of OSM through STAT- and ERK-independent pathways because JAK2 activity is not involved in the OSM-stimulated activation of STAT isoforms and ERK [Song et al., 2005]. In contrast, JAK3, but not JAK2, is essential for the OSM-stimulated matrix mineralization. To the best of our knowledge, the current study provides the first evidence to indicate that JAK3 is involved in the osteogenic differentiation. To support these results, the molecular mechanism involved in the JAK3-dependent enhancement of osteogenic differentiation should further be determined. In the present study, inhibition of MEK/ERK pathway was found to attenuate OSM-stimulated osteogenic differentiation of hADSCs. ERK has been reported to play a key role in the regulation of osteoblastic differentiation. For instance, activation of ERK in human osteoblastic cells results in upregulation of expression and DNA binding activity of Cbfa1 or Runx2, the master regulator of osteogenic differentiation [Ziros et al., 2002]. Since JAK3/STAT and MEK/ERK pathways do not cross-talk with each other in hADSCs [Song et al., 2005], it is likely that these two signaling pathways independently play a role in osteogenic differentiation of hADSCs.

Pulse treatment of hADSCs with OSM for the initial 2 days was sufficient for the OSM-induced stimulation of osteogenic differentiation. In contrast, pulse exposure of the cells to OSM for the initial 3 days had no effect on the adipogenic differentiation of hADSCs. These results indicate that exposure of the cells with OSM during the early stages of the osteogenic differentiation is essential for the pro-osteogenic activity of OSM. Expression of ALP, Runx2, and OC were enhanced by the pulse treatment with OSM during the early stage of osteoblastic differentiation (Fig. 7). Consistent with the current results, pulse treatment of mOSM for 1–4 days was found to stimulate osteogenic differentiation of calvaria progenitors, indicated by increased formation of bone nodule [Malaval et al., 2005]. Similarly, treatment of BMSCs with OSM for 14 days stimulated formation of mineralized nodules, and increased expression levels of ALP and OC [Chipoy et al., 2004]. However, when the cells were exposed to OSM for longer periods (21 days), OSM was inactive or inhibitory for induction of the osteoblastic markers. These results support our current data that exposure of the cells to OSM during early stages of osteoblastic differentiation was necessary for the OSM-stimulated expression of osteoblastic markers.

Since MSCs are the progenitor cells for both osteoblasts and adipocytes [Prockop, 1997; Short et al., 2003; Barry and Murphy, 2004], differentiation of MSCs into adipocytes rather than osteoblasts may be responsible for reduction in the number of osteoblastic cells in osteoporosis and aging [Chan and Duque, 2002]. We observed that OSM stimulates osteogenic differentiation and inhibits adipogenic differentiation of hBMSCs as well as hADSCs (data not shown), suggesting that OSM can affect the adipogenic and osteogenic differentiation of

MSCs in bone tissues. It has been reported that the number of adipocytes in the bone marrow increases in parallel with a decrease in the number of osteoblasts in various types of osteoporosis [Nuttall and Gimble, 2000]. Moreover, the volume of adipose tissue in the bone increases with age in normal subjects and is substantially elevated in age-related osteoporosis [Meunier et al., 1971]. These results suggest that bone loss in age-related osteoporosis is at least in part caused by a shift from the osteoblastic to the adipocytic pathway of MSC differentiation [Nuttall and Gimble, 2000; Chan and Duque, 2002]. Therefore, it has been suggested that shift of MSC differentiation from adipocytic to osteoblastic lineage may be helpful for cell therapy of the bone diseases [Nuttall and Gimble, 2000]. These results provide a possibility that systemic or local application of hADSCs to patients together with OSM could potentially be useful for restoring bone-forming capacity in bone diseases. Further studies with animal models will allow us to better describe the anti-adipogenic and pro-osteogenic properties of OSM.

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