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Oncostatin M Inhibits Adipogenesis through the RAS/ERK and STAT5 Signaling Pathways*

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Adipocytes play a key role in energy homeostasis and several cytokines have been shown to regulate adipogenesis. While the interleukin (IL)-6 family of cytokines was previously reported to be involved in adipogenesis, roles of this family in adipogenesis and their mechanisms of action are not fully understood. Here we show that among the IL-6 family, oncostatin M (OSM) most strongly inhibits adipogenesis of 3T3-L1 cells and mouse embryonic fibroblasts (MEFs). We also demonstrate that OSM inhibits adipogenesis through the Ras/extracellular signal-regulated kinase (ERK) and signal transducer and activator of transcription (STAT) 5 signaling pathways. In addition, OSM inhibits the early phase of the differentiation without affecting cell proliferation throughout adipogenesis including mitotic clonal expansion. CCAAT/enhancer-binding protein (C/EBP) α, C/EBPβ, and peroxisome proliferator-activated receptor (PPAR) γ are known to be required for adipogenesis. Expression of C/EBPα and PPARγ was almost completely abrogated by OSM. In contrast, neither the mRNA nor protein level of C/EBPβ was affected by OSM. Forced expression of C/EBPβ induced differentiation in the presence of troglitazone, and OSM inhibited this C/EBPβ-induced differentiation. Taken together, our results indicate that OSM inhibits the onset of terminal differentiation of adipocytes through the Ras/ERK and STAT5 signaling pathways by possibly regulating C/EBPβ activity.

Adipocytes are a major energy reservoir, storing excess energy as lipids and releasing it on demand. In addition, adipocytes constitute an endocrine system by secreting soluble mediators known as adipokines, which regulate not only peripheral tissues such as muscles and adipose tissues but also the central nervous system (1). Disorders in adipose tissues are a major cause of the development of the metabolic syndrome, a common basis of type 2 diabetes and atherosclerotic vascular diseases (2). It is therefore important to understand the nature of adipocytes and the mechanism of adipocyte differentiation. The mechanism of adipocyte differentiation has been studied by using preadipocyte cell lines, primary cultured cells, and genetically manipulated mice. These studies have identified several transcription factors required for adipogenesis, *e.g.* CCAAT/enhancer-binding protein (C/EBP)[3] α, C/EBPβ, and peroxisome proliferator-activated receptor (PPAR) γ (3, 4). A number of soluble factors have also been reported to regulate adipogenesis, *e.g.* insulin-like growth factor (IGF)-I and fibroblast growth factor (FGF) 10 enhance adipogenesis (5, 6), whereas some inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, inhibit adipogenesis (4, 7).

IL-6 is an inflammatory cytokine and together with IL-11, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) constitutes the IL-6 family of cytokines, which share a common signal transducing receptor subunit, gp130. The members of this family play important roles in the development and maintenance of various biological systems such as the immune, hematopoietic, endocrine, and neuronal systems (8, 9). A number of reports have implicated the IL-6 family in adipogenesis, though their results were somewhat controversial, *e.g.* IL-6 was reported to inhibit adipocyte differentiation of 3T3-L1 cells (10, 11); LIF was purified as a lipoprotein lipase-inhibiting protein (12), but another study reported that LIF stimulated adipogenesis of 3T3-F442A and Ob1771 preadipocytes, whereas it had only a modest effect on 3T3-L1 cells (13); IL-11 was found as an inhibitor of the adipogenesis of 3T3-L1 cells (14) and bone marrow stroma cells (15); and CT-1 and CNTF did not affect adipogenesis, though they induced signals in 3T3-L1 cells (16, 17). Because these studies were conducted mostly by using cultured cells, the controversy could be due to the developmental stage of cells and tissues used by different investigators. In any case, the precise molecular mechanisms of these effects remain unclear, and it is important to evaluate the effects of these cytokines under the same conditions to clarify the mechanisms.

Although TNFα, IL-1, and IL-6 are inflammatory cytokines, their modes of action are different. Major TNFα and IL-1 signaling pathways involve the activation of nuclear factor-κB (NF-κB) by the transforming growth factor β-activated kinase (TAK)/TAK1-binding protein (TAB) 1/NF-κB inducing kinase

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[3] The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; IL, interleukin; OSM, oncostatin M; ERK, extracellular signal-regulated kinase; STAT, signal transducer and activator of transcription; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; GFP, green fluorescent protein.

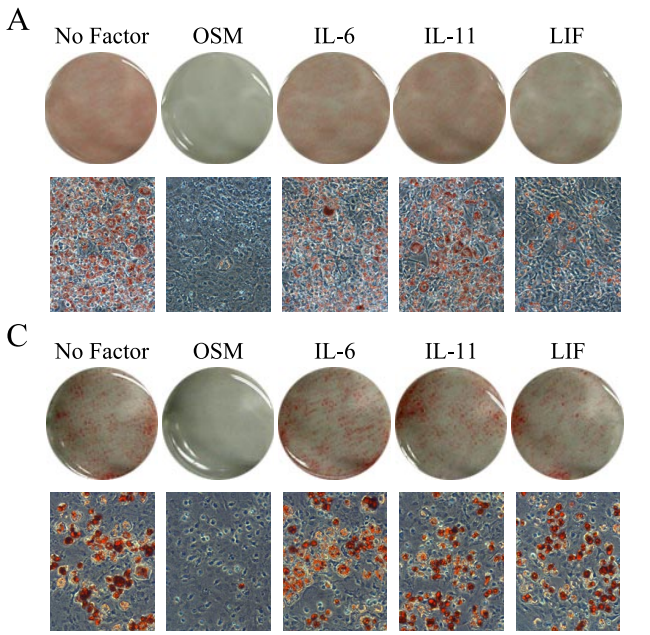

FIGURE 1. **Effects of the IL-6 family of cytokines on adipogenesis of 3T3-L1 cells and MEFs.** *A* and *C*, 3T3-L1 cells (*A*) and MEFs (*C*) were induced to differentiate into mature adipocytes in the presence of 10 ng/ml of OSM, IL-6, IL-11, or LIF, and subjected to Oil-red O staining. OSM completely inhibited the accumulation of lipids in both cell types. *B* and *D*, Northern blot analysis of aP2, C/EBPα, and PPARγ expression in 3T3-L1 cells (*B*) and MEFs (*D*) in the presence of the IL-6 family. The amount of total RNA loaded was confirmed by staining of ribosomal RNA with ethidium bromide (*EtBr*).

(NIK) cascade and the caspase cascade, whereas the IL-6 family activates mainly Ras/extracellular-signal regulated kinase (ERK) and signal transducer and activator of transcription (STAT) signaling pathways. Extensive studies on the mechanism by which TNFα and IL-1 suppress adipogenesis have shown that they suppress adipocyte differentiation from mesenchymal stem cells by inhibiting PPARγ function through NF-κB activated by the TAK/TAB1/NIK cascade (7). As TNFα and the IL-6 family of cytokines induce different signaling pathways, it is expected that the IL-6 family would affect adipogenesis through a mechanism distinct from TNFα.

In this article we attempted to clarify the roles of the IL-6 family of cytokines in adipogenesis and their mechanisms of action. We compared the effect of IL-6, IL-11, OSM, and LIF on adipocyte differentiation by using primary cultures of mouse embryonic fibroblasts (MEFs) and 3T3-L1 cells, a preadipocyte cell line. We found that among the family members tested, OSM most strongly suppressed the differentiation of both MEFs and 3T3-L1 cells. OSM exhibits a wide variety of activities being involved in immune regulation (18, 19), hematopoiesis (20), and liver regeneration (21). OSM elicits its functions through the OSM receptor consisting of gp130 and the OSM-specific subunit by activating the Ras/ERK, STAT3, and STAT5 signaling pathways. However, the roles of OSM in adipogenesis have not been well investigated. Here we demonstrate that OSM inhibits the early phase of the differentiation process through the Ras/ERK and STAT5 signaling pathways by possibly regulating C/EBPβ activity without affecting cell proliferation.

EXPERIMENTAL PROCEDURES

3T3-L1 Cell Culture and Enumeration of Cells—3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% bovine serum (BS) (Invitrogen), and 50 μg/ml gentamicin (Wako). To induce adipocyte differentiation, the medium was changed to DMEM with 10% fetal bovine serum (FBS) (Invitrogen), 50 μg/ml gentamicin, 10^{-6} M dexamethason (Dex) (Wako), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma), and 5 μg/ml human insulin (Wako) 2 days after cells became confluent and incubated for 2 days. Medium was then changed to DMEM with 10% FBS (Invitrogen), 50 μg/ml gentamicin, and 5 μg/ml human insulin. After 2 days, the medium was then changed to DMEM with 10% FBS (Invitrogen) and 50 μg/ml gentamicin, and thereafter, the medium was changed every 2 days.

For determining cell numbers, cells treated with 0.05% trypsin (Invitrogen) were resuspended in the culture medium and the number of cells was counted with a hemocytometer.

Isolation and Culture of MEFs—MEFs were isolated from mouse embryos at 14.5 days postcoitum. Briefly, embryos were chopped into pieces and incubated in 0.05% trypsin, 0.5 mM EDTA at 37 °C for 50 min with periodic agitation. Cells dispersed by pipetting were passed through a 70-μm cell strainer and washed with DMEM with 10% FBS (EQUITECH-BIO) and 50 μg/ml gentamicin. Cell culture condition and differentiation protocols were the same as for 3T3-L1 cells except that MEFs were maintained in DMEM with 10% FBS (EQUITECH-BIO) and 50 μg/ml gentamicin until confluent.

Plasmids, Reagents, and Antibodies—cDNAs encoding STAT5B1*6, STAT5B2, and ΔSTAT5A were kindly provided by Dr. T. Kitamura (Institute of Medical Science, The University of Tokyo). All the cDNAs used in this study including STAT3-C and ΔSTAT3 were inserted into the pMXs-IRES-GFP vector as previously described (22).

PD98059 and troglitazone were purchased from Cell Signaling Technology and Sigma, respectively. Cytokines used in this study were recombinant murine (rm)OSM (R&D), rmIL-6 (PeproTech), recombinant human (rh)IL-11 (Genzyme Techne), rmLIF (Genzyme Techne), and rhFGF basic (bFGF) (PeproTech).

Antibodies against ERK1/2 (9102), phosphorylated ERK1/2 (9101), STAT3 (9132), phosphorylated STAT3 (9131), and phosphorylated STAT5 (9351) were purchased from Cell Signaling Technology. Antibodies against STAT5 (sc-835), C/EBPβ (sc-150), and actin (sc-1616) were purchased from Santa Cruz Biotechnology.

Oil-red O Staining of Cells—Cells were fixed with a 20% formalin solution for 5 min and washed with water and then 60%

isopropyl alcohol. After being washed, cells were incubated in Oil-red O (Sigma) staining solution: 1.8 mg/ml in 60% isopropyl alcohol at 37 °C for 30 min. The excess staining solution was removed by washing with 60% isopropyl alcohol and water.

Retroviral Infection of 3T3-L1 Cells—The virus packaging cells PLAT-E were transfected with 6 μg of pMXs-IRES-GFP construct per 10-cm dish using FuGENE6 Transfection reagent (Roche Applied Science). Twenty-four hours after the transfection, the medium was changed, and subsequently cells were incubated for 24 h. Then, viruses were precipitated by centrifuging at 6,000 × *g* overnight, resuspended in DMEM containing 10% BS, 50 μg/ml gentamicin, and 8 μg/ml polybrene (Sigma), and used as the viral suspension. 3T3-L1 cells cultured in 6-well plates were incubated with 1 ml of viral solution for 8 h. Then, 1 ml of DMEM containing 10% BS, 50 μg/ml gentamicin, and 8 μg/ml polybrene was added and the incubation continued for 16 h. The viral solution was then washed out three times with 5 ml of phosphate-buffered saline (Invitrogen). The efficiency of infection was estimated by measuring GFP expression using flow cytometry.

Northern Blot Analysis—Total RNA was prepared with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (5 μg) resolved by electrophoresis on a 1% agarose gel was transferred to a positively charged Nylon membrane (Roche Applied Science) and RNA was cross-linked to the membrane using UV-stratalinker (Stratagene). We applied the DIG-labeling system (Roche Applied Science) following the manufacturer's instructions.

Western Blot Analysis—Total cell lysate was prepared with lysis buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (Wako), and 500 μg/ml Pefabloc (Roche Applied Science). To detect the phosphorylation of ERK1/2, STAT3, and STAT5, 30 mM sodium pyrophosphate, 50 mM NaF, and 1 mM Na_3VO_4 were added to this lysis buffer. Total cell lysate was dissolved by polyacrylamide gel electrophoresis and transferred to an Immobilon-P Membrane (Millipore). The membrane was incubated with primary antibodies in Tris-buffered saline with Tween 20 (TBSt) with 1% skim milk at 4 °C overnight. It was then incubated with secondary antibodies (anti-rabbit (Amersham Biosciences) or goat (Jackson ImmunoResearch) IgG-horseradish peroxidase) in TBSt with 1% skim milk at room temperature for 2 h. The signals were detected by Western Lightning (PerkinElmer Life Sciences) according to the manufacturer's instructions.

Bromodeoxyuridine (BrdU) Incorporation Assay—3T3-L1 cells were induced to differentiate on a cover glass in a 6-well plate. Twelve hours later, BrdU (Sigma) was added to the culture to a final concentration of 10 μM, and the cells were incubated for an additional 12 h. Then, cells were fixed with ice-cold 70% ethanol, denatured with 2 M HCl, and neutralized with 0.1 M sodium borate (pH 8.5). After blocking with ImmunoBlock (Dainipponseiyaku) at 4 °C overnight, cells were incubated with anti-BrdU antibody (Sigma) in phosphate-buffered saline containing 2% bovine serum albumin (Sigma) at room temperature for 2 h. Cells were next incubated with anti-mouse IgG-Rhodamine (Jackson ImmunoResearch) in phosphate-buffered saline containing 2% bovine serum albumin at room temperature for 1 h. The cover glass was mounted with GEL/MOUNT (biomeda) containing Hoechst33342 on a slide glass and fluorescence was observed. Rhodamine and Hoechst33342-positive cells were counted in 6 different microscopic views from 3 different cover glasses for each sample. The total number of cells in each view ranged from 120 to 182.

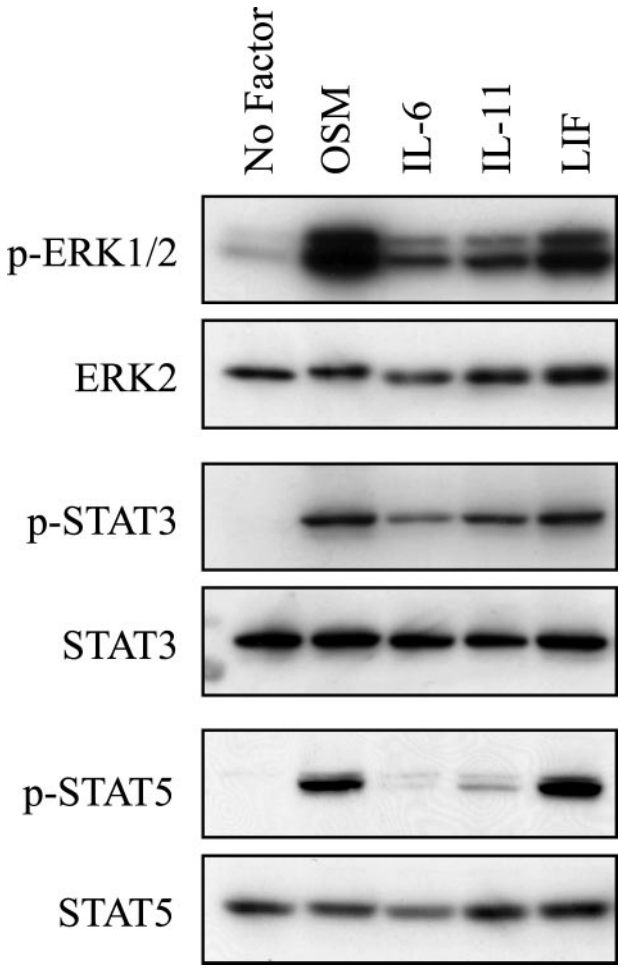

FIGURE 2. **Activation of the Ras/ERK, STAT3, and STAT5 signaling pathways by the IL-6 family in 3T3-L1 cells.** 3T3-L1 cells were harvested for Western blot analysis 10 min after the addition of 10 ng/ml of OSM, IL-6, IL-11, or LIF. *p-ERK1/2*, *p-STAT3*, and *p-STAT5* stand for the phosphorylated form of ERK1/2, STAT3, and STAT5, respectively. Note that the anti-ERK1/2 antibody used in this experiment seemed to detect predominantly ERK2 in these conditions (see manufacturer's comments) and the phosphorylation of ERK2 was also detected by the shift of a signal seen in anti-ERK2 blotting. The phosphorylation of ERK1/2 and STAT5 was selectively induced by OSM and LIF.

RESULTS

OSM Strongly Inhibits Adipogenesis of 3T3-L1 Cells and MEFs—3T3-L1 cells are induced to differentiate by Dex, IBMX, and insulin and have been extensively used as a model for adipocyte differentiation. To evaluate roles for the IL-6 family in adipogenesis, 3T3-L1 cells were induced to differentiate into mature adipocytes in the presence of 10 ng/ml of OSM, IL-6, IL-11, or LIF. The extent of the differentiation was confirmed by Oil-red O staining and also by the expression of mRNA of aP2, C/EBPα, and PPARγ, which are known to be expressed in mature adipocytes. As shown in Fig. 1, *A* and *B*, adipogenesis of 3T3-L1 cells was inhibited strongly by OSM and weakly by LIF, whereas IL-6 and IL-11 had no significant effect.

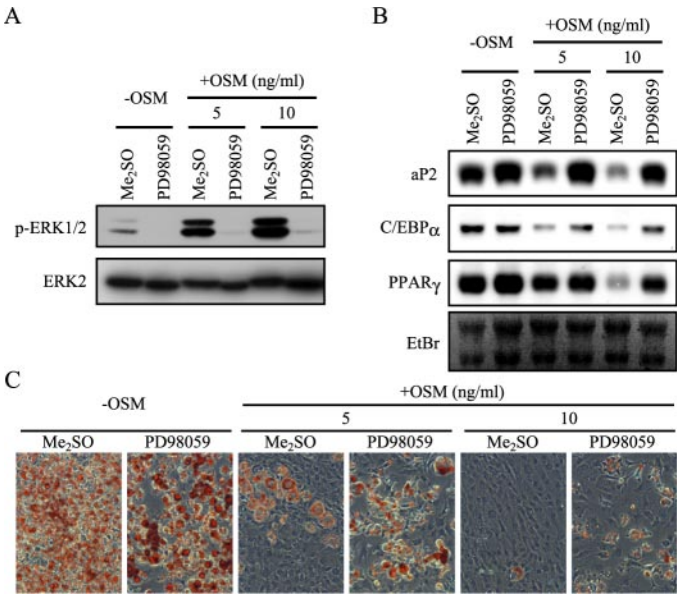

FIGURE 3. **Effect of MEK inhibition on adipogenesis in 3T3-L1 cells.** *A*, activation of ERK1/2 was analyzed by Western blotting. 3T3-L1 cells were pretreated with 50 μM of PD98059, a MEK inhibitor, or only Me_2SO for 1 h before the addition of 5 or 10 ng/ml of OSM. The cells were harvested 10 min after the challenge with OSM and proteins were extracted for Western blot analysis. PD98059 inhibited the phosphorylation of ERK1/2 efficiently even after the stimulation with OSM. *B* and *C*, effect of PD98059 on adipogenesis was analyzed by Northern blotting of aP2, C/EBPα, and PPARγ (*B*) and Oil-red O staining (*C*). 3T3-L1 cells were induced to differentiate into mature adipocytes in the presence of OSM with or without PD98059. The MEK inhibitor partially cancelled the inhibitory effect of OSM on adipogenesis in terms of both gene expression and lipid accumulation.

To investigate whether the inhibitory effect of OSM in adipogenesis is specific to 3T3-L1 cells or not, we tested another adipogenic system. Because MEFs are known to differentiate into mature adipocytes when exposed to Dex, IBMX, and insulin in a manner similar to 3T3-L1 cells, the effect of the IL-6 family was evaluated in MEFs. Oil-red O staining and northern blotting revealed that OSM also strongly inhibited adipogenesis of MEFs, whereas IL-6 and IL-11 had no effect. Unlike in 3T3-L1 cells, LIF did not significantly alter adipogenesis in MEFs (Fig. 1, *C* and *D*). Adipocyte differentiation of bone marrow stroma cells was also inhibited by OSM (data not shown). These results strongly suggest that OSM regulates adipogenesis in various tissues. Hereafter, we used 3T3-L1 cells to analyze the mechanism of the inhibitory effect of OSM on adipogenesis.

Activation of the Ras/ERK and STAT5 Signaling Pathways by OSM and LIF—Next, we investigated signaling pathways responsible for the inhibition of adipogenesis by OSM in 3T3-L1 cells. Because OSM is known to activate the Ras/ERK, STAT3, and STAT5 signaling pathways, we examined the activation of these signaling pathways by monitoring the phosphorylation of ERK1/2, STAT3, and STAT5 in 3T3-L1 cells. The phosphorylation of ERK1/2 was induced strongly by OSM and weakly by LIF, but was only marginally induced by IL-6 and IL-11. Whereas all of the IL-6 family cytokines induced phosphorylation of STAT3, only OSM and LIF phosphorylated STAT5 (Fig. 2). The fact that all four cytokines phosphorylated STAT3 indicates that receptors for these cytokines were expressed substantially. As the differentiation of 3T3-L1 cells was inhibited by OSM and LIF but not by IL-6 and IL-11 (Fig. 1, *A* and *B*), the Ras/ERK and STAT5 signaling pathways, but not STAT3, were expected to play important roles in the inhibitory effect of OSM. In fact, retroviral expression of the active (STAT3-C) or dominant-negative form (ΔSTAT3) of STAT3 (23, 24) did not affect adipogenesis of 3T3-L1 cells regardless of the presence or absence of OSM (data not shown).

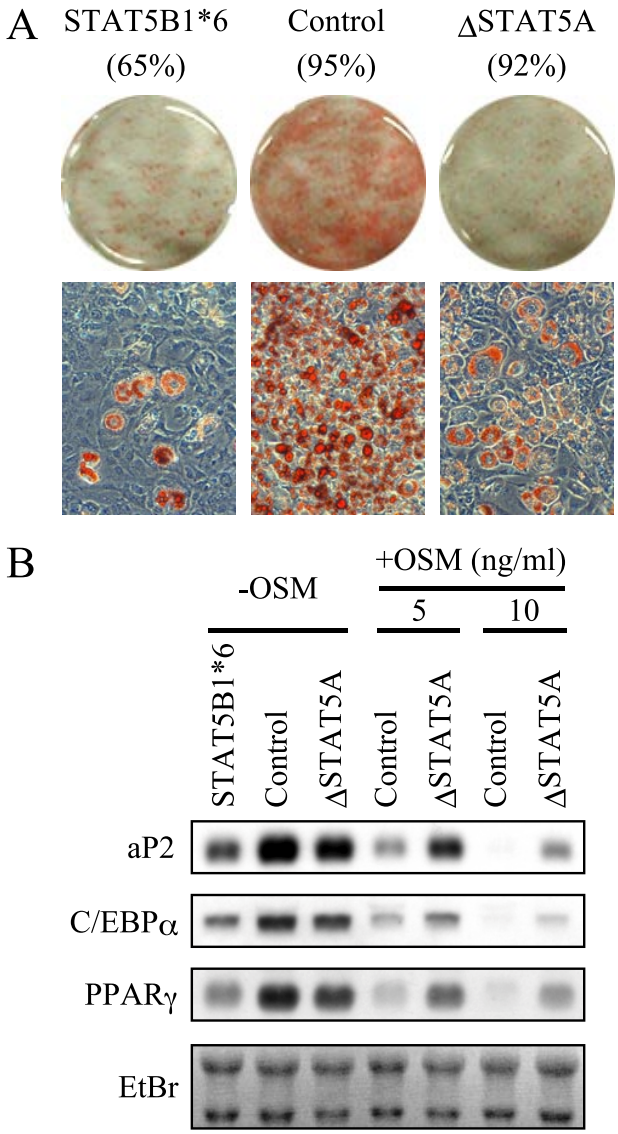

FIGURE 4. **Effect of active and dominant negative forms of STAT5 on adipogenesis in 3T3-L1 cells.** STAT5B1*6 (an active form of STAT5) and ΔSTAT5A (a dominant negative form of STAT5) were retrovirally introduced into 3T3-L1 cells. As a control, a vector without an insert was used to infect 3T3-L1 cells (*control*). These cells were induced to differentiate in the absence of OSM and subjected to Oil-red O staining (*A*) and Northern blot analysis of aP2, C/EBPα, and PPARγ, together with the cells with dominant negative STAT5 induced to differentiate in the presence of OSM (*B*). The numbers in *parentheses* are the efficiency of the viral infection.

Inhibition of the Ras/ERK Signaling Pathway Partially Recovers Adipogenesis in the Presence of OSM—To reveal whether the Ras/ERK signaling pathway is involved in the inhibitory effect of OSM, we blocked the Ras/ERK signaling with a mitogen-activated protein kinase/ERK kinase (MEK) inhibitor, PD98059, in the presence of OSM. PD98059 completely blocked the activation of ERK1/2 induced by OSM in 3T3-L1 cells as demonstrated by a lack of phosphorylated ERK1/2 (Fig. 3*A*). Then, we induced the differen-

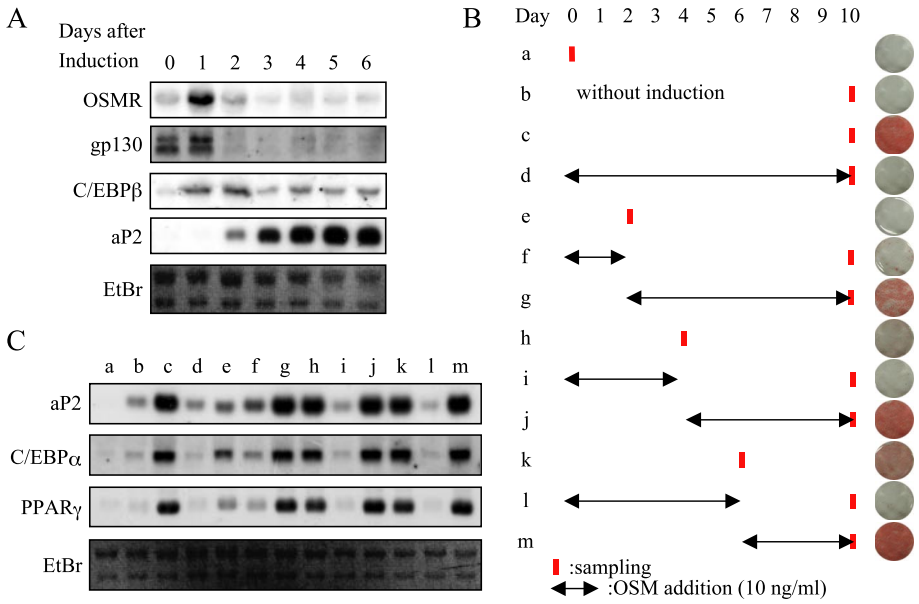

FIGURE 5. **OSM exerts its inhibitory effect on adipogenesis in an early phase of differentiation.** *A*, expression patterns of OSMR and gp130 during adipogenesis determined by Northern blot analysis. At day 0, 3T3-L1 cells were induced to differentiate. The expression patterns of C/EBPβ and aP2 indicate that the differentiation process was normal. *B*, schematic representation of the time course for the addition of OSM during adipogenesis. OSM (10 ng/ml) was added during the periods indicated as *black arrows*, and the preparation of total RNA and Oil-red O staining were done at the time points indicated as *red lines*. The *right panels* of the scheme show the results of Oil-red O staining. *C*, Northern blot analysis of aP2, C/EBPα, and PPARγ for each condition.

tiation of 3T3-L1 cells in the presence of OSM with or without PD98059. OSM inhibited the expression of aP2, C/EBPα, and PPARγ in the absence of PD98059, but the addition of PD98059 cancelled the inhibitory effect (Fig. 3*B*). However, lipid accumulation in cells was not fully recovered by PD98059 (Fig. 3*C*). These results clearly indicate that the Ras/ERK signaling pathway partially, but not entirely, contributes to the inhibition of adipogenesis by OSM.

STAT5 Mediates the Inhibitory Effect of OSM on Adipogenesis—Because of the observation that blocking of the Ras/ERK signaling pathway did not fully recover lipid accumulation in the presence of OSM (Fig. 3*C*) and different modes of signaling activated by the IL-6 family (Fig. 2), we speculated that STAT5 also plays a role in the inhibitory effect of OSM. However, it was previously shown that induction of adipocyte differentiation by Dex, IBMX, and insulin was accompanied by the activation of STAT5 and was necessary for the early phase of differentiation (25). To address the role of STAT5, we first retrovirally introduced STAT5B1*6 (an active form of STAT5) (26) and ΔSTAT5A (a dominant negative form of STAT5) (27) into 3T3-L1 cells and induced these cells to differentiate into mature adipocytes with Dex, IBMX, and insulin in the absence of OSM. The infection efficiencies of the control, STAT5B1*6, and ΔSTAT5A virus were 95, 65, and 92%, respectively. Consistent with a previous report, ΔSTAT5A inhibited the differentiation. Interestingly, STAT5B1*6 inhibited the differentiation more severely than ΔSTAT5A, though the infection efficiency of the STAT5B1*6 virus was lower than that of ΔSTAT5A (Fig. 4). Similar results were obtained with another active form of STAT5, STAT5B2 (28) (data not shown). However, when the cells were induced to differentiate in the presence of OSM, the level of expression of adipogenic genes in cells with ΔSTAT5A was significantly higher than that in control cells (Fig. 4*B*). These results suggest that STAT5 activation is necessary for the induction of differentiation, but continuous and excessive activation is inhibitory to the maturation of 3T3-L1 cells, and the STAT5 signaling pathway as well as the Ras/ERK signaling pathway contributes to the inhibitory effect of OSM.

OSM Inhibits the Onset of Terminal Differentiation without Affecting C/EBPβ Induction and Mitotic Clonal Expansion—To reveal in more detail the mechanism behind the inhibitory effect of OSM, we first investigated the expression of the OSM-specific receptor subunit, the OSM receptor (OSMR), and gp130 during the differentiation of 3T3-L1 cells into adipocytes. OSMR expression was highest 1 day after the induction of differentiation and diminished after day 3, while gp130 was expressed for the first day, and its level diminished thereafter (Fig. 5*A*). Because of the expression patterns of the receptor subunits, OSM was expected to exert its inhibitory effect in an early stage of differentiation. To test this possibility, we added 10 ng/ml of OSM for different periods during the differentiation process as indicated in Fig. 5*B*. The extent of differentiation was confirmed by Oil-red O staining and Northern blotting. The presence of OSM in the first 2 days was enough to almost completely inhibit the differentiation, while cells significantly differentiated when OSM was added after that time (Fig. 5, *B* and *C*). These observations are consistent with the expression patterns of OSMR and gp130 (Fig. 5*A*).

It is well known that in the first 2 days of the differentiation process, the expression of C/EBPβ is up-regulated and 3T3-L1 cells undergo several rounds of the cell cycle, which is known as mitotic clonal expansion. After mitotic clonal expansion, the cells undergo terminal differentiation through which they acquire the characteristics of mature adipocytes. Thus, we investigated which steps were affected by OSM. In the absence of OSM, the C/EBPβ mRNA level peaked at 1–2 days after induction of the differentiation process (Fig. 5*A*). In this period, the mRNA level of C/EBPβ was not changed by OSM (Fig. 6*A*). Three major C/EBPβ protein isoforms are known to be produced from the same C/EBPβ mRNA, namely LAP*, LAP, and LIP. LAP* and LAP are transcriptional activators, while LIP is an inhibitor. OSM affected neither of them (Fig. 6*A*). 3T3-L1 cells undergoing mitotic clonal expansion show a fibroblastic cell morphology, which indicates reentry of the cells into the cell division cycle. We observed that OSM had no effect on cell morphology in the first 2 days (Fig. 6*B*). To more directly assess the effect of OSM on cell proliferation in mitotic clonal expansion, we performed a BrdU incorpora-

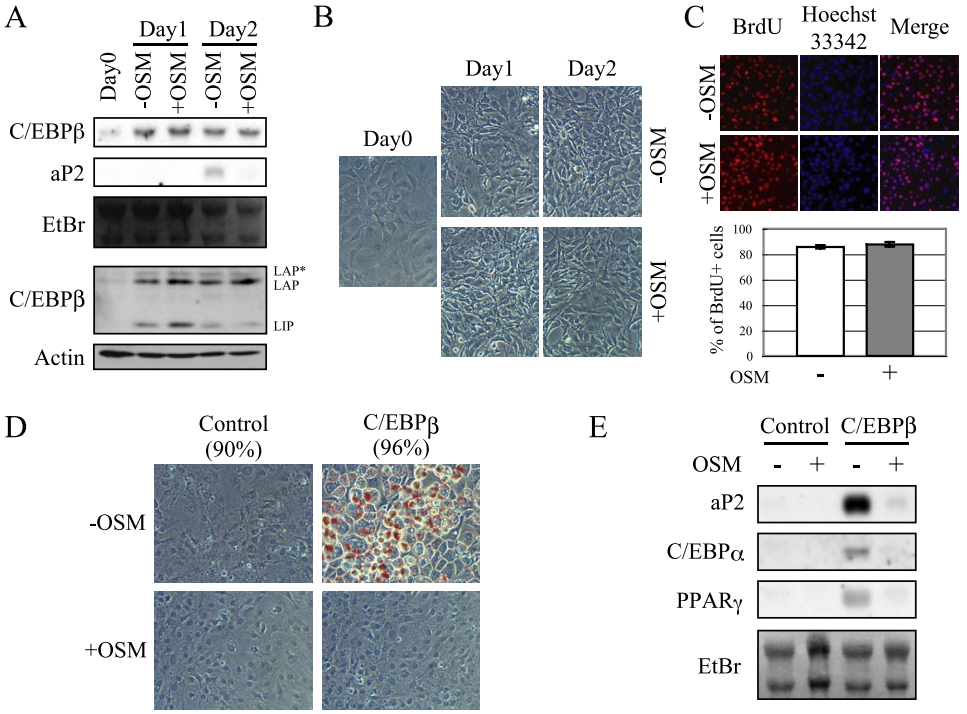

FIGURE 6. **C/EBPβ induction, mitotic clonal expansion, and C/EBPβ-induced adipogenesis in the presence of OSM.** *A*, Northern blot analysis of C/EBPβ and aP2, and Western blot analysis of C/EBPβ. Induction of C/EBPβ in the first 2 days of the differentiation process in the presence or absence of 10 ng/ml of OSM was monitored. In anti-C/EBPβ Western blotting, the bands corresponding to LAP*, LAP, and LIP are indicated. The amount of protein loaded was confirmed by anti-Actin blotting. *B*, morphology of 3T3-L1 cells in the first 2 days of the differentiation process in the presence or absence of OSM. Light microscopic photographs of the cells are shown. The elongated fibroblastic cell morphology indicates that the cells are undergoing mitotic clonal expansion. *C*, assessment of mitotic clonal expansion with the BrdU incorporation assay. The incorporation of BrdU into cells between 12 and 24 h post-induction was examined by measuring the fluorescence of rhodamine (*red*) as described under "Experimental Procedures." Hoechst33342 was used to stain nuclei (*blue*). Representative microscopic photographs are shown. Fractions of BrdU-positive cells in the total cell population are shown in a graph with the standard deviation ($n = 6$ for each sample). *D* and *E*, C/EBPβ was retrovirally introduced into 3T3-L1 cells and the cells were induced to differentiate into mature adipocytes by addition of 2 μM troglitazone instead of Dex, IBMX, and insulin. As a control, a vector without an insert was introduced into 3T3-L1 cells (*control*). The extent of differentiation was examined by Oil-red O staining (*D*) and Northern blot analysis of aP2, C/EBPα, and PPARγ (*E*). The numbers in *parentheses* give the efficiency of the viral infection. OSM almost completely abrogated both lipid accumulation and the expression of adipogenic genes.

tion assay. 3T3-L1 cells were induced to differentiate in the presence or absence of OSM, and their incorporation of BrdU from 12 to 24 h after the induction was measured. Fig. 6*C* shows representative photographs and results of a quantitative analysis of BrdU-positive cells. Consistent with the morphological observations (Fig. 6*B*), OSM did not significantly affect cell proliferation during mitotic clonal expansion. In contrast, OSM inhibited the expression of aP2 which was up-regulated 2 days post-induction in the absence of OSM (Figs. 5*A* and 6*A*). These results indicate that OSM inhibits the onset of terminal differentiation without affecting the C/EBPβ induction and mitotic clonal expansion.

OSM Attenuates C/EBPβ-induced Adipogenesis—At the onset of terminal differentiation, C/EBPβ up-regulates the transcription of C/EBPα and PPARγ and then C/EBPα and PPARγ upregulate each other's transcription, allowing the cells to acquire characteristics of mature adipocytes through terminal differentiation. It is thus possible that OSM blocks the function of C/EBPβ, leading to inhibition of the up-regulation of C/EBPα and PPARγ, and the onset of terminal differentiation, although OSM does not alter the level of C/EBPβ (Fig. 6*A*). To reveal the relation between OSM effect and C/EBPβ function, we expressed C/EBPβ in 3T3-L1 cells by using a retroviral vector and evaluated the effect of OSM on adipogenesis using these transfectants. In this case, it was assumed that endogenous C/EBPβ expression induced by Dex, IBMX, and insulin would make the interpretation of results difficult. For this reason, we utilized troglitazone, a synthetic ligand for PPARγ, to enhance the differentiation, instead of Dex, IBMX, and insulin. The efficiency of infection for the control and C/EBPβ expression vectors was 90 and 96%, respectively. We monitored the differentiation by Oil-red O staining and Northern blotting. Under the conditions, control cells did not differentiate into mature adipocytes but the introduction of C/EBPβ allowed the cells to differentiate (Fig. 6, *D* and *E*), indicating that this adipogenesis was driven by exogenously introduced C/EBPβ. Then, 10 ng/ml of OSM was added to the cells to evaluate the effect of OSM on C/EBPβ-induced adipogenesis. OSM almost completely suppressed both lipid accumulation and up-regulation of the expression of mature adipocyte-specific genes in these cells (Fig. 6, *D* and *E*). These results strongly suggest that OSM interrupts the function of C/EBPβ, and thereby inhibits the onset of terminal differentiation.

OSM Does Not Affect Cell Proliferation throughout Adipocyte Differentiation—At the end of mitotic clonal expansion, the cells cease proliferation and proceed with terminal differentiation. It is known that, however, if the cells are kept proliferative after mitotic clonal expansion, they cannot undergo terminal differentiation. To clarify whether the inhibitory function of OSM is caused by prolonged proliferation, we induced differentiation of 3T3-L1 cells in the presence of 10 ng/ml of OSM or bFGF and compared their effects. Both OSM and bFGF completely inhibited lipid accumulation and the expression of adipogenic genes, but cell density was higher in the presence of bFGF than OSM (Fig. 7, *A* and *B*). We therefore investigated whether OSM and/or bFGF affect cell proliferation during adipogenesis. We counted the cell number 2, 4, and 6 days after the induction of differentiation. OSM did not change the cell number significantly throughout adipogenesis compared with the cells without cytokines (Fig. 7*C*). These results are consistent with our observation that OSM does not affect mitotic clonal expansion (Fig. 6, *B* and *C*). In contrast, bFGF significantly increased the cell number throughout adipogenesis (Fig. 7*C*).

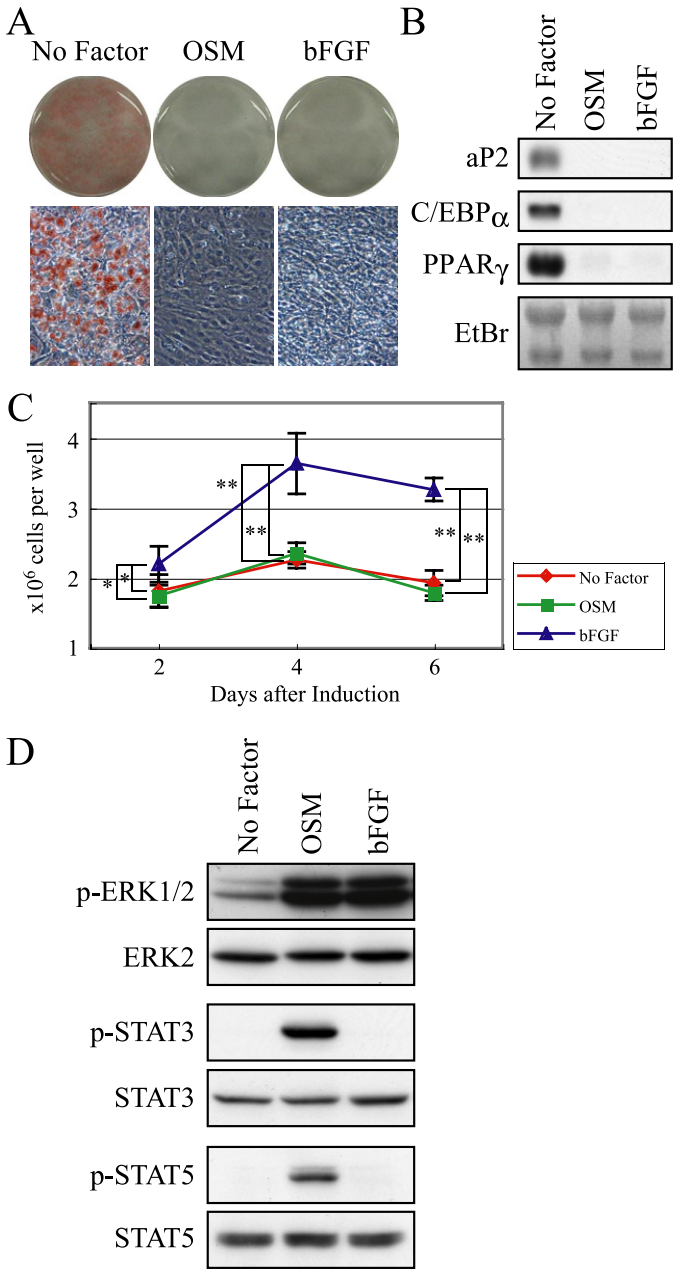

FIGURE 7. **Adipogenesis and cell proliferation in the presence of OSM or bFGF.** *A–C*, 3T3-L1 cells were induced to differentiate into mature adipocytes in the presence of 10 ng/ml of OSM or bFGF, and subjected to Oil-red O staining (*A*) and Northern blot analysis of aP2, C/EBPα, and PPARγ expression (*B*). Both cytokines completely inhibited the accumulation of lipids and expression of adipogenic genes. At 2, 4, and 6 days post-induction, the number of cells per 6 wells was counted, and the data were plotted with the standard deviation. The *p* value was calculated with Student's *t* test ($n = 5$ for each sample, *, $p < 0.05$; **, $p < 0.01$) (*C*). *D*, 3T3-L1 cells were harvested for Western blot analysis 10 min after the addition of 10 ng/ml of OSM or bFGF to monitor phosphorylation of ERK1/2, STAT3, and STAT5. *p-ERK1/2*, *p-STAT3*, and *p-STAT5* stand for the phosphorylated form of ERK1/2, STAT3, and STAT5, respectively. bFGF activated the Ras/ERK signaling pathway at a level comparable to OSM, but not the STATs signaling pathways.

These results indicate that prolonged exposure of 3T3-L1 cells to bFGF keeps the cells proliferative and inhibits adipogenesis, whereas OSM inhibits adipogenesis via a mechanism distinct from bFGF, because it does not affect cell proliferation throughout the differentiation process.

Finally, we compared the signaling pathways activated by bFGF and OSM in 3T3-L1 cells to reveal which pathways are important for the OSM-mediated inhibition of adipogenesis. bFGF activated the Ras/ERK signaling pathway at a level comparable to OSM, but bFGF failed to activate the STAT3 and STAT5 signaling pathways (Fig. 7*D*). These results suggest that OSM inhibits adipogenesis via the STATs signaling pathways as well as the Ras/ERK signaling pathway.

DISCUSSION

Almost all previous studies on roles for the IL-6 family in adipogenesis were conducted independently for each cytokine using different cell systems. Gimble *et al.* (29) reported that IL-6, IL-11, LIF, and OSM inhibit the adipogenesis of a murine stroma cell line, BMS2, and compared the effects of these cytokines. However, their results must be interpreted carefully, because they used human LIF and OSM in their study. In murine cells, human OSM activates intracellular signals through LIFR but not OSMR (30). In the present study, we have compared the effects of OSM, IL-6, IL-11, and LIF on adipocyte differentiation under the same conditions by using MEFs and 3T3-L1 cells, and demonstrated that adipogenesis was most profoundly inhibited by OSM, and to a lesser extent, by LIF. In contrast, IL-6 and IL-11 showed no significant effect (Fig. 1). Similar results were also obtained in the differentiation of bone marrow stroma cells (data not shown). OSM most strongly activated the Ras/ERK and STAT5 signaling pathways, suggesting that OSM exerts its inhibitory activity through ERK1/2 and/or STAT5 (Fig. 2). In fact, blocking the activation of ERK1/2 by PD98059 partially recovered the differentiation process in the presence of OSM, indicating that the Ras/ERK signaling pathway plays an important role in the suppression of adipogenesis (Fig. 3). Consistent with our results, the Ras/ERK signaling pathway was previously shown to inhibit adipogenesis (31, 32). However, it was also reported that the Ras/ERK signaling pathway enhanced adipogenesis (33, 34). Those apparently contradictory results may be explained by dual roles of the Ras/ERK signaling pathway in adipogenesis. The differentiation of 3T3-L1 cells into adipocytes proceeds through two steps, mitotic clonal expansion and terminal differentiation. The Ras/ERK signaling pathway is essential for the mitotic clonal expansion stage (34), whereas prolonged and excessive activation may block terminal differentiation.

It should be noted that the blocking of ERK1/2's activation by PD98059 did not completely recover adipocyte differentiation in the presence of OSM, indicating that an additional signaling pathway is involved in the inhibitory effect of OSM. Consistent with the report that STAT5 activity is required for adipogenesis (25), enforced expression of a dominant negative form of STAT5 inhibited adipogenesis in the absence of OSM. Interestingly, however, enforced expression of an active form of STAT5 also inhibited adipogenesis in the absence of OSM, and dominant negative STAT5 rescued adipogenesis from the inhibitory effect of OSM (Fig. 4). These results clearly demonstrate for the first time that like the Ras/ERK signaling pathway, deregulated activation of the STAT5 signaling pathway is inhibitory for adipogenesis. Because STAT5 is activated in the early phase of the differentiation process (25) and is a positive regulator of proliferation (35), it may be required for mitotic clonal expansion but continuous and excessive activation of STAT5 may be inhibi-

tory for terminal differentiation. This is consistent with our observation that OSM inhibits the onset of terminal differentiation without affecting mitotic clonal expansion (Fig. 6, *A–C*).

Adipogenesis requires functions of C/EBPβ, C/EBPα, and PPARγ. When growth-arrested 3T3-L1 cells are induced to differentiate, C/EBPβ is rapidly induced and after a long latency, C/EBPα and PPARγ are expressed in a mutually regulated manner. Activation of C/EBPβ by phosphorylation is required for mitotic clonal expansion and also for the expression of C/EBPα and PPARγ, which are anti-mitotic and regulate the expression of various adipogenic genes. We have demonstrated that OSM down-regulates the expression of C/EBPα and PPARγ, which is recovered by blocking the Ras/ERK or STAT5 signaling pathways (Figs. 3*B* and 4*B*). In contrast, OSM had no effect on the mRNA and protein levels of C/EBPβ (Fig. 6*A*). As reported previously, we observed up-regulation of C/EBPβ expression in the first 2 days after the induction of differentiation, when mitotic clonal expansion occurred. We also observed that the presence of OSM in this period was crucial to the inhibitory effect (Fig. 5). These results tempted us to examine the possibility that OSM interrupts the function of C/EBPβ. Enforced expression of C/EBPβ in the presence of troglitazone, a PPARγ agonist, induced adipocyte differentiation without Dex, IBMX, and insulin. In these conditions, OSM suppressed the expression of aP2, C/EBPα, and PPARγ and lipid accumulation (Fig. 6, *D* and *E*). These results strongly suggest that OSM modulates the function of C/EBPβ, which then affects the expression of C/EBPα and PPARγ. As oncogenic Ras has been reported to diminish the level of C/EBPα (36), it is tempting to speculate that OSM diminishes the expression of C/EBPα and PPARγ by modulating C/EBPβ activity.

Finally, we demonstrated that OSM did not affect cell proliferation throughout the differentiation process, whereas bFGF induced proliferation and inhibited adipogenesis (Fig. 7, *A–C*). These results indicate that OSM does not inhibit adipogenesis by stimulating mitosis. Unlike OSM, bFGF activated the Ras/ERK signaling pathway, but not the STAT3 and STAT5 signaling pathways (Fig. 7*D*). Because the excessive activation of STAT5, not STAT3, is inhibitory for adipogenesis (Fig. 4), the OSM-specific inhibitory effect should be caused by the STAT5 signaling pathway.

In conclusion, this study shows that among the IL-6 family of cytokines, OSM strongly suppresses the onset of adipocyte differentiation via the Ras/ERK and STAT5 signaling pathways by possibly modulating the function of C/EBPβ.

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