Differential Roles of Smad1 and p38 Kinase in Regulation of Peroxisome Proliferator-activating Receptor γ during Bone Morphogenetic Protein 2-induced Adipogenesis

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Bone morphogenetic protein 2 (BMP2) promotes the differentiation of undifferentiated mesenchymal cells into adipocytes. To investigate the molecular mechanisms that regulate this differentiation process, we studied the relationship between BMP2 signaling and peroxisome proliferator-activating receptor γ (PPAR γ) during adipogenesis of mesenchymal cells by using pluripotent mesenchymal cell line C3H10T1/2. In C3H10T1/2 cells, BMP2 induced expression of PPAR γ along with adipogenesis. Overexpression of Smad6, a natural antagonist for Smad1, blocked PPAR γ expression and adipocytic differentiation induced by BMP2. Overexpression of dominant-negative PPAR γ also diminished adipocytic differentiation of C3H10T1/2 cells, suggesting the central role of PPAR γ in BMP2-induced adipocytic differentiation. Specific inhibitors for p38 kinase inhibited BMP2-induced adipocytic differentiation and transcriptional activation of PPAR γ , whereas overexpression of Smad6 had no effect on transcriptional activity of PPAR γ . Furthermore, activation of p38 kinase by overexpression of TAK1 and TAB1, without affecting PPAR γ expression, led the up-regulation of transcriptional activity of PPAR γ . These results suggest that both Smad and p38 kinase signaling are concomitantly activated and responsible for BMP2-induced adipocytic differentiation by inducing and up-regulating PPAR γ , respectively. Thus, BMP2 controls adipocytic differentiation by using two distinct signaling pathways that play differential roles in this process in C3H10T1/2 cells.

INTRODUCTION

Adipocytes play a major role in controlling lipid homeostasis and energy balance. Adipocytes derive from undifferentiated mesenchymal cells that have the capacity to differentiate into myoblasts, osteoblasts, and chondrocytes as well as adipocytes (Prockop, 1997; Pittenger *et al.*, 1999). A variety of hormones, growth factors, and cytokines are involved in this differentiation program of adipocytes (Spiegelman, 1998; Rosen and Spiegelman, 2000). Among these factors, bone morphogenetic protein 2 (BMP2), a member of the transforming growth factor- β (TGF- β) superfamily that is implicated in embryogenesis, organogenesis, and morphogenesis by controlling differentiation of variety types of cells (Kingsley, 1994; Hogan, 1996), promotes adipocytic differentiation of undifferentiated mesenchymal cells (Ahrens *et al.*, 1993; Ji *et al.*, 2000; Sottile and

Seuwen, 2000). However, it is unknown how BMP2 promotes differentiation of mesenchymal cells into adipocytes.

BMP2 exhibits its biological effects through the sequential activation of two types of transmembrane receptors, namely, type I (BMPRI) and type II (BMPRII), which possess intrinsic serine/threonine kinase activity (Heldin *et al.*, 1997; Massague and Wotton, 2000). After forming heterocomplex between BMPRI and BMPRII, activated BMPRI phosphorylates and activates Smad1 and Smad5, which subsequently associate with Smad4, a common partner for receptor-regulated Smads. These complexes then relocate to the nucleus and regulate the expression of target genes, leading to an elicitation of biological effects of BMP2 (Heldin *et al.*, 1997; Massague and Wotton, 2000).

In addition to Smad signaling pathway, a mitogen-activated protein (MAP) kinase, p38 kinase, which is involved in growth and differentiation of many types of cells, is also activated by BMP2 (Iwasaki *et al.*, 1999; Kimura *et al.*, 2000). In some types of cells, BMP2 activates MAP kinase kinases, including TAK1, and consequently the MAP kinase kinase elicits MKK3 or MKK6 that directly phosphorylates and activates p38 kinase (Raingeaud *et al.*, 1996;

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Shibuya *et al.*, 1996, 1998; Davis, 2000; Kimura *et al.*, 2000). The phosphorylated p38 kinase, in turn, controls gene expression in the nucleus. Although p38 kinase has been described to be implicated in regulation of adipogenesis (Engelman *et al.*, 1998, 1999), the precise role of p38 kinase in adipogenesis remains elusive.

A nuclear hormone receptor, peroxisome proliferator-activating receptor γ (PPAR γ), plays a central role in differentiation program of adipocytes by controlling the expression of specific genes for adipocytes such as lipoprotein lipase and aP2 through a specific binding element for PPAR γ in their promoter regions (Wu *et al.*, 1999; Rosen and Spiegelman, 2000). In fact, overexpression of PPAR γ in fibroblasts causes adipocytic differentiation of the cells (Tontonoz *et al.*, 1994). Furthermore, the mice deficient for PPAR γ gene showed markedly impaired adipogenesis (Kubota *et al.*, 1999; Rosen *et al.*, 1999). Because BMP2 promotes the adipocytic differentiation of mesenchymal cells, it is possible that the linkage between BMP2 and PPAR γ signaling may account for adipogenesis of mesenchymal cells.

To determine the molecular mechanisms by which BMP2 promotes adipocytic differentiation, we investigated the role of Smad signaling and p38 kinase in adipogenesis of undifferentiated mesenchymal cells by using murine pluripotent mesenchymal cell line C3H10T1/2 (Ahrens *et al.*, 1993; Asahina *et al.*, 1996). We found that Smad1 is essential for induction of PPAR γ , which is necessary for BMP2-induced adipocytic differentiation. We also shown herein that p38 kinase acts as an activator for PPAR γ in C3H10T1/2 cells. Our results indicate that two distinct signaling cascades that are concomitantly elicited by BMP2 control adipocytes differentiation of mesenchymal cells in the different manners.

MATERIALS AND METHODS

Cell, Antibodies, and Reagents

C3H10T1/2 cells were purchased from RIKEN (Saitama, Japan) and were cultured in DMEM containing 10% fetal bovine serum. Anti-PPAR γ monoclonal (E-8) and polyclonal antibody (H-100), anti-hemagglutinin polyclonal antibody, anti-Smad1 antibody, and anti- β -actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p38 kinase and anti-phospho-p38 kinase antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-Myc monoclonal, anti-Flag monoclonal, and anti-phosphoserine polyclonal antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA), Kodak Scientific Imaging Systems (New Haven, CT), and Zymed Laboratories (South San Francisco, CA), respectively, and used as described previously (Nishimura *et al.*, 1998). SB203580 and PD169316 were purchased from Calbiochem (San Diego, CA). FR167653 was kindly provided by Fujisawa Pharmaceutical (Osaka, Japan).

Constructs and Transfection

6xMyc-Smad 1 (Imamura *et al.*, 1997), Flag-Smad6 (Imamura *et al.*, 1997), dominant-negative form MKK3 (Enslen *et al.*, 1998), and TAK1 and TAB1 (Shibuya *et al.*, 1996) cDNA were kindly gifted by Drs. Kohei Miyazono, Roger Davis, and Hiroshi Shibuya. Flag-Smad1, Flag-Smad 4, PPAR γ expression vector, and PPRE-Luc reporter gene have been described previously (Hu *et al.*, 1996; Nishimura *et al.*, 1998). To generate dominant-negative mutant form of PPAR γ (Barroso *et al.*, 1999), proline 467 was point mutated into leucine by performing in vitro mutagenesis, and the sequence of the mutant PPAR γ was confirmed by DNA sequence analysis. Transfec-

tion of C3H10T1/2 cells was carried out using FuGENE6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol.

Generation of Adenovirus

The recombinant adenovirus carrying wild-type or dominant-negative form of PPAR γ , dominant-negative MKK3, or Flag-Smad6 was constructed by homologous recombination between the expression cosmid cassette and the parental virus genome in 293 cells as described previously (Miyake *et al.*, 1996) by using adenovirus construction kit (Takara, Kyoto, Japan). The viruses were confirmed to retain no proliferative activity in the cells other than 293 cells, because of lacking E1A-E1B (Miyake *et al.*, 1996). Titers of the viruses were determined by modified point assay (Miyake *et al.*, 1996).

Oil Red O Staining

C3H10T1/2 cells were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin for 20 min. After washing the cells twice with PBS and once with 60% isopropyl-alcohol, the cells were stained with Oil Red O solution (Sigma-Aldrich, St. Louis, MO). The area of the cells stained with Oil Red O was measured by ImagePro Plus analyzer (Palmerton).

Immunoprecipitation and Immunoblotting

Cells were washed three times with ice-cold buffer PBS and solubilized in lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 10% glycerol, 1% Triton X-100, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate). The lysates were centrifuged for 20 min at 4°C, 16,000 $\times$ g. The supernatants of the centrifuged cell lysates were recovered, boiled in SDS sample buffer containing 0.5 M β -mercaptoethanol, and used as total cell lysates. For immunoprecipitation, the lysates were incubated with antibodies for 4 h at 4°C, followed by immunoprecipitation with protein A-Sepharose (Zymed Laboratories) or protein G-agarose (Roche Diagnostics). Immunoprecipitates were washed five times with lysis buffer and boiled in SDS sample buffer containing 0.5 M β -mercaptoethanol, and supernatants were recovered as immunoprecipitates sample. These samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with appropriate antibodies. The samples were visualized with horseradish peroxidase coupled to protein A (KPL) or horseradish peroxidase-coupled anti-mouse IgG antibodies (Cappel Laboratories, Durham, NC), and enhanced by enhanced chemiluminescence detection kits (Pharmacia AB, Uppsala, Sweden).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from C3H10T1/2 cells by RNeasy kit (QIAGEN, Valencia, CA). After denaturation of total RNA at 70°C for 10 min, cDNA was synthesized with oligo-dT primer and reverse transcriptase (QIAGEN). PCR amplification was performed by using specific primers for PPAR γ (forward primer, 5'-TATGGAGTTCATGCTTGTTGA-3'; reverse primer, 5'-CGGGAAGGACTTTATG-TATG-3'). PCR products were loaded to agarose gel, and stained with ethidium bromide. After PCR, products were subcloned into TA-cloning vector, and DNA sequences of PCR products were determined.

Transcriptional Activity of PPAR γ

Luciferase reporter constructs containing three repeats of PPAR γ binding elements (PPRE-Luc) were cotransfected with TK-renilla luciferase construct (Promega, Madison, WI) into C3H10T1/2 cells. Two days after transfection, cells were lysed and luciferase activity was determined using specific substrates in a luminometer (Promega) according to manufacturer's protocol. Transfection efficiency was normalized by determining the activity of renilla luciferase.

Statistical Analysis

All data were analyzed by analysis of variance followed by a paired *t* test. Values shown are mean $\pm$ SD.

RESULTS

BMP2 Promotes Adipocytic Differentiation of C3H10T1/2 Cells by Inducing PPAR γ

To investigate the relationship between BMP2 signaling and PPAR γ during adipocytic differentiation, we first evaluated the effects of BMP2 and a specific ligand for PPAR γ , troglitazone (TZD) (Sarraf *et al.*, 1999), on adipocytic differentiation of C3H10T1/2 cells (Ahrens *et al.*, 1993; Asahina *et al.*, 1996). As shown in Figure 1, A and B, BMP2 induced differentiation of C3H10T1/2 cells into adipocytes, whereas TZD had little effect on adipocytic differentiation of C3H10T1/2 cells. Treatment with both BMP2 and TZD more efficiently induced adipocytes differentiation of C3H10T1/2 cells than that with BMP2 alone (Figure 1, A and B). Interestingly, we found that pretreatment with BMP2 is able to trigger the adipogenic effects of TZD on C3H10T1/2 cells (Figure 1, C and D). Collectively, these data suggest that BMP2 induced PPAR γ expression, resulting in the induction of adipocytic differentiation in C3H10T1/2 cells. We therefore assessed the role of BMP2 in the regulation of PPAR γ expression in C3H10T1/2 cells as determined by Western blotting analysis and RT-PCR experiment. Although expression of PPAR γ was not detected in untreated C3H10T1/2 cells, C3H10T1/2 cells treated with BMP2 expressed PPAR γ (Figure 1, E and F). The expression of PPAR γ was observed 3 h after BMP2 treatment and increased in a time-dependent manner (Figure 1F). The data indicate that BMP2 induced expression of PPAR γ in C3H10T1/2 cells.

We next asked the importance of PPAR γ in BMP2-induced adipocytic differentiation. To address this, we overexpressed a dominant-negative mutant of PPAR γ (Barroso *et al.*, 1999) in C3H10T1/2 cells by using adenovirus system and determined its effects on adipocytic differentiation of C3H10T1/2 cells. As shown in Figure 2A, overexpression of dominant-negative PPAR γ markedly inhibited adipocytic differentiation induced by BMP2. The data suggest that PPAR γ is critical for BMP2 to exhibit the adipogenic effect in C3H10T1/2 cells. To further verify this notion, we examined whether expression of PPAR γ is sufficient to induce adipocytic differentiation of C3H10T1/2 cells. As shown in Figure 2B, exogenous introduction of PPAR γ was able to promote the adipocytic differentiation of C3H10T1/2 cells. Interestingly, this effect of PPAR γ was profoundly enhanced by BMP2. Together, the data suggested that expression of PPAR γ is sufficient for adipocytic differentiation of C3H10T1/2 cells and that BMP2 exhibits adipogenic actions by regulating expression and function of PPAR γ .

Smad1 Is Mediator for BMP2-induced Adipocytic Differentiation in C3H10T1/2 Cells by Inducing PPAR γ Expression

Because Smad1 has been shown to play important roles in BMP2 signaling (Heldin *et al.*, 1997), we next examined whether activation of Smad1 is involved in the induction of PPAR γ and adipocytic differentiation of C3H10T1/2 cells. To address this, we overexpressed Smad6, a natural antag-

onist for Smad1 in BMP2 signaling (Imamura *et al.*, 1997), and determined the effects of Smad6 on PPAR γ expression and adipocytic differentiation. We confirmed that Smad6 was effectively overexpressed and completely blocked activation of Smad1 elicited by BMP2 in C3H10T1/2 cells (Figure 3A). Western blotting experiment indicated that overexpression of Smad6 abolished BMP2-induced expression of PPAR γ (Figure 3B). Moreover, overexpression of Smad 6 blocked adipocytic differentiation promoted by BMP2 treatment (Figure 3, C and D). These results indicated that activation of Smad1 is necessary for induction of PPAR γ and subsequent adipocytic differentiation of C3H10T1/2 cells.

BMP2 Up-Regulates Transcriptional Activity of PPAR γ Not through Smad Signaling

We observed that BMP2 enhanced the adipogenic effects of PPAR γ as shown in Figure 2B; therefore, we determined the effects of Smad signaling on the transcriptional activity of PPAR γ by using a firefly luciferase reporter construct containing PPAR γ -responsive elements, PPRE-Luc (Hu *et al.*, 1996). As shown in Figure 4A (left), BMP2 up-regulated the transcriptional activity of PPAR γ . However, overexpression of Smad6 failed to block the effects of BMP2 on transcriptional activity of PPAR γ (Figure 4A, left). Western blotting analyses indicate that treatment of BMP2 or overexpression of Smad6 had little influence on the expression of PPAR γ introduced by transfection in this experimental condition (Figure 4A, right). Thus, the data suggest that BMP2 up-regulated the function of PPAR γ not through Smad1 signaling. To confirm this, we determined whether BMP2 is able to enhance adipogenic function of PPAR γ in the condition where Smad signaling is inactivated. As shown in Figure 4B, in spite of blockade of Smad signaling by overexpression of Smad6, overexpression of PPAR γ could differentiate C3H10T1/2 cells into adipocytes. More importantly, BMP2 further promoted the adipogenic effects of PPAR γ in C3H10T1/2 cells even in the presence of Smad6. Together, these results suggested that BMP2 enhanced the adipogenic function of PPAR γ by up-regulating its transcriptional in Smad-independent mechanisms.

p38 Kinase Up-Regulates Transcriptional Activity of PPAR γ during Adipocytic Differentiation

BMP2 has been shown to activate not only Smad signaling but also elicit p38 kinase pathway in the variety types of cells (Iwasaki *et al.*, 1999; Kimura *et al.*, 2000; Hay *et al.*, 2001). In C3H10T1/2 cells, p38 kinase was activated upon treatment with BMP2, and the activation reached maximum at 10 min and thereafter declined (Figure 5A). Consistent with our results shown in Figure 4, A and B, we observed that activation of p38 kinase was only modestly inhibited by overexpression of Smad6 (Figure 3A), which abolished phosphorylation of Smad1 induced by BMP2 (Figure 3A). We, therefore, assessed whether p38 kinase is responsible for activation of PPAR γ function by using a specific inhibitor for p38 kinase, SB203580 (Enslen *et al.*, 1998; Iwasaki *et al.*, 1999). SB203580 efficiently inhibited activation of p38 kinase elicited by BMP2 without affecting the activation of Smad1 in C3H10T1/2 cells (Figure 5, B and C). As shown in Figure 5D, treatment with SB203580 inhibited the BMP2-dependent transcriptional activation of PPAR γ . In contrast, SB203580

Figure 1. BMP2 induced adipogenesis in C3H10T1/2 cells. C3H10T1/2 cells were cultured with BMP2 (300 ng/ml), troglitazone (10^{-6} M), or both for 10 d. After 10 d, the cells were stained with Oil Red O, photographed under a microscope (A), and the area stained with Oil Red O in the cells was assessed as described in the text (B). (C and D) Treatment of BMP2 initiated the adipogenic effects of troglitazone in C3H10T1/2 cells. C3H10T1/2 cells precultured with or without BMP2 for 3 d (300 ng/ml) were incubated with or without troglitazone (10^{-6} M) for 4 d. The cells were stained with Oil Red O, photographed under a microscope (C), and the areas stained with Oil Red O in the cells were assessed (D). (E) BMP2 induced expression of PPAR γ in C3H10T1/2 cells. C3H10T1/2 cells were untransfected (lanes 1 and 2) or transfected with pcDNA3 (lane 3) or PPAR γ expression vector (lane 4). Twelve hours after transfection, the cells were incubated with (lane 2) or without (lanes 1, 3, and 4) BMP2 for 3 d and lysed. The cell lysates were immunoprecipitated with anti-PPAR γ polyclonal antibody and immunoblotted with anti-PPAR γ mAb (top). The lysates were also determined by immunoblotting with anti- β -actin antibody (bottom). (F) BMP2 induced expression of PPAR γ mRNA in a time-dependent manner in C3H10T1/2 cells. Total RNA was isolated from C3H10T1/2 cells that were incubated with or without BMP2 for 3, 6, 12, or 24 h and subjected to RT-PCR experiments by using specific primers for PPAR γ (top) or β -actin (bottom).

Figure 2. (A) Dominant-negative PPAR γ blocked BMP2-induced adipogenesis of C3H10T1/2 cells. C3H10T1/2 cells infected with control or dominant-negative PPAR γ adenovirus at 100 multiplicity of infection were incubated with or without BMP2 (300 ng/ml) for 10 d. The cells were stained with Oil Red O, photographed under a microscope, and the areas stained with Oil Red O in the cells were assessed. (B) Overexpression of PPAR γ -induced adipogenesis. C3H10T1/2 cells infected with control or PPAR γ adenovirus at 100 multiplicity of infection were incubated with or without BMP2 (300 ng/ml) for 7 d. The cells were stained with Oil Red O, photographed under a microscope, and the areas stained with Oil Red O in the cells were assessed.

did not affect PPAR γ expression (Figure 5E). To further evaluate the importance of p38 kinase in the transactivation of PPAR γ , we attempted to examine the transcriptional activity of PPAR γ when p38 kinase is specifically activated in the absence of BMP2. Because p38 kinase is regulated by TAK1 (Davis, 2000), and overexpression of TAK1 together with TAB1 is able to activate the down stream signaling (Shibuya *et al.*, 1996), we examined the effects of overexpression of TAK1 and TAB1 on the transcriptional activity of PPAR γ . We observed that overexpression of TAK1 together with TAB1 efficiently leads activation of p38 kinase in C3H10T1/2 cells (Figure 6A). Overexpression of TAK1 and TAB1 in C3H10T1/2 cells dramatically enhanced the transcriptional activity of PPAR γ (Figure 6B). In addition, treatment with SB203580 inhibited this effect of TAK1 and TAB1 (Figure 6B). These results suggest that p38 kinase accounts for up-regulation of transcriptional activity of PPAR γ , and thereby promotes its adipogenic function. To assess the role of p38 kinase in BMP2-induced adipocytic differentiation, we next tested the effects of dominant-negative form of MKK3, which is a direct regulator for p38 kinase (Davis, 2000). Overexpression of dominant-negative MKK3 inhibited BMP2-induced adipocytic differentiation (Figure 6C). Consistently, we also found that treatment with SB203580 inhibited adipocyte differentiation of C3H10T1/2 cells in the presence of BMP2 (Figure 6D). To further confirm the roles

of p38 kinase in the regulation of PPAR γ function, we also examined the effects of other p38 kinase inhibitors, PD169316 (Kummer *et al.*, 1997; Assefa *et al.*, 1999) and FR167653 (Yoshinari *et al.*, 2001; Kobayashi *et al.*, 2002). Both PD169316 and FR167653 abolished BMP2-induced p38 phosphorylation (Figure 7A) and suppressed the transactivation of PPAR γ by BMP2 (Figure 7B) and BMP2-induced adipogenesis of C3H10T1/2 cells (Figure 7C). Together, these results suggested that p38 kinase promotes the BMP2-induced adipocytic differentiation by activating function of PPAR γ .

DISCUSSION

Although BMP2 is well known to induce adipogenesis of mesenchymal cells (Ahrens *et al.*, 1993; Ji *et al.*, 2000; Sottile and Seuwen, 2000), to date, the molecular mechanisms by which BMP2 promotes adipocytic differentiation are elusive. In this study, we have shown that BMP2 induces the expression of PPAR γ in association with adipocytic differentiation of C3H10T1/2 cells. We also found that dominant-negative PPAR γ markedly blocked BMP2-induced adipocytic differentiation of C3H10T1/2 cells. Furthermore, introduction of PPAR γ is enough to induce adipogenesis of C3H10T1/2 cells in the absence of BMP2. Collectively, it is most likely that BMP2 exhibits its adipogenic effects by inducing PPAR γ . This conclusion is supported by the results that pretreatment of C3H10T1/2 cells with BMP2 was able to bring out the adipogenic effects of TZD. Moreover, overexpression of Smad6, which blocked activation of Smad1 in response to BMP2 stimulation, abolished BMP2-induced adipogenesis of C3H10T1/2 cells as well as the induction of PPAR γ expression. Thus, activation of Smad family signaling is a prerequisite for the induction of PPAR γ expression, thereby inducing differentiation of C3H10T1/2 cell into adipocytes.

Recently, the data that Smad regulates the function of transcriptional factors, nuclear receptors, or coactivator through its physical association are accumulated (Massague and Wotton, 2000). We have shown herein that BMP2 increased the transcriptional activity of PPAR γ . BMP2 also enhanced adipogenesis caused by overexpression of PPAR γ . It is, therefore, possible that Smad signaling promoted the adipogenesis in cooperation with PPAR γ . However, Smad6 failed to inhibit the transcriptional activity of PPAR γ enhanced by BMP2. In addition, overexpression of Smad1 or Smad5 together with Smad4 had no effects on transcriptional activity of PPAR γ (our unpublished data). Furthermore, BMP2 enhanced PPAR γ -promoted adipogenesis of C3H10T1/2 cells in which Smad signaling is blocked by overexpression of Smad6. These data suggest that Smad signaling is implicated in the regulation of the PPAR γ expression but not in activation of its function. We found that specific inhibitors for p38 kinase, SB203580, PD169316, and FR167653, decreased the transcriptional activity of PPAR γ enhanced by BMP2 treatment to the basal level and markedly inhibited BMP2-induced adipocyte differentiation of C3H10T1/2 cells. Consistently, treatment with SB203580 or overexpression of dominant-negative MKK3 inhibited BMP2-induced adipogenesis of C3H10T1/2 cells. Furthermore, overexpression of TAK1 and TAB1, which markedly induced activation of p38 kinase, was sufficient to up-regu-

Figure 3. Overexpression of Smad6 inhibited activation of Smad1 (A), BMP2-regulated induction of PPAR γ (B), and adipogenesis (C and D). (A) C3H10T1/2 cells infected with control or Flag-Smad6 adenovirus at 100 multiplicity of infection were starved with 0.2% fetal calf serum-DMEM for 16 h and then stimulated with or without BMP2 (300 ng/ml) for 10 min and lysed. The cell lysates were immunoprecipitated with anti-Smad1

Figure 4. (A) BMP2 up-regulated the transcriptional activity of PPAR γ in C3H10T1/2 cells. Left, pcDNA3 (control) (1.6 μg) or Smad6 expression vector (1.6 μg) was transfected into C3H10T1/2 cells together with PPAR γ (0.1 μg) and RXR (0.1 μg) expression vectors and PPRE-Luc (0.1 μg) and TK-renilla (0.1 μg) reporter constructs. Twelve hours after transfection, cells were incubated with or without BMP2 (300 ng/ml) for 2 d. At the end of the culture, cells were lysed and the luciferase activity was measured and normalized by determining renilla luciferase activity as described in the text. Data were shown as mean $\pm$ SD. Right, expression of PPAR γ and Smad6 were monitored by immunoblotting with anti-PPAR γ (top) and anti-Flag (middle) antibodies in the same set of transfection experiments. The lysates were also determined by immunoblotting with anti- β -actin antibody (bottom). (B) Introduction of PPAR γ rescued the adipogenesis blocked by Smad6. C3H10T1/2 cells were infected with Flag-Smad6 (100 multiplicity of infection) and/or PPAR γ (100 multiplicity of infection) adenoviruses and incubated with or without BMP2 (300 ng/ml) for 7 d. The cells were stained with Oil Red O, photographed under a microscope, and the areas stained with Oil Red O in the cells were assessed.

antibody and immunoblotted with anti-phosphoserine antibody (top). The amount of Smad1 in the immunoprecipitates was determined by immunoblotting with anti-Smad1 (second panel). The lysates were also determined by immunoblotting with antiphosphop38 (third panel) or antip38 (fourth panel) antibodies. The expression of Flag-Smad6 was determined by immunoblotting with anti-Flag antibody (bottom). (B) C3H10T1/2 cells infected with control or Flag-Smad6 adenovirus at 100 multiplicity of infection were incubated with or without BMP2 (300 ng/ml) for 4 d and lysed. The lysates were determined by immunoblotting with anti-PPAR γ mAb followed by immunoprecipitation with anti-PPAR γ polyclonal antibody (top). The lysates were also determined by immunoblotting with anti-Flag (middle) or anti- β -actin antibodies (bottom). (C and D) 10T1/2 cells infected with control or Flag-Smad6 adenovirus at 100 multiplicity of infection were incubated with or without BMP2 (300 ng/ml) for 10 d. The cells were stained with Oil Red O, photographed under a microscope, and the areas stained with Oil Red O in the cells were assessed.

late the transcriptional activity of PPAR γ . This effect of TAK1 and TAB1 was also suppressed by treatment with SB203580. These results indicate that p38 kinase but not Smad signaling accounts for up-regulation of PPAR γ activity, which leads to further promotion of adipogenesis. The potential schematic model in which BMP2 regulates adipogenesis through activation of Smad1 and p38 kinase is delineated in Figure 8.

The mechanism for the up-regulation of PPAR γ by p38 kinase remains unknown. The direct up-regulation of PPAR γ by p38 kinase through phosphorylation is unlikely because PPAR γ possesses only one consensus phosphorylation site by MAP kinases at serine112, which is shown to be phosphorylated by extracellular signal-regulated kinase kinase, thereby inhibiting the transcriptional activity of PPAR γ (Hu *et al.*, 1996). Consistent with this report, we confirm that a specific inhibitor for mitogen-activated protein kinase kinase, PD98059, enhanced the function of PPAR γ - and BMP2-induced adipogenesis in C3H10T1/2 cells (our unpublished data). Another possibility is that PPAR γ might be up-regulated by forming the complex with a certain transcription factor. ATF-2 is known to be regulated by p38 kinase and to control transcription through cross-talk with other transcriptional factors or coactivators (Davis, 2000). We observed that BMP2 phosphorylates ATF-2 in C3H10T1/2 cells (our unpublished data). It is, therefore, possible that interaction of ATF-2 with PPAR γ might account for the up-regulation of PPAR γ by p38 kinase, although the interaction of ATF-2 and PPAR γ , and the

Figure 5. (A) Time-dependent activation of p38 kinase by BMP2 in C3H10T1/2. C3H10T1/2 cells were serum starved in 0.2% fetal calf serum-DMEM for 16 h. The cells were then stimulated with BMP2 (300 ng/ml) for 5, 10, 15, 30, or 60 min and lysed, and the cell lysates were determined by immunoblotting with anti-phospho-p38 kinase (top) and anti-p38 kinase antibodies (bottom). (B) Activation of p38 kinase in C3H10T1/2 and its inhibition by SB203580. C3H10T1/2 cells were serum starved in 0.2% fetal calf serum-DMEM for 12 h. The cells were further incubated with or without p38 kinase inhibitor SB203580 (10 μ M), for 4 h. The cells were then stimulated with BMP2 (300 ng/ml) for 10 min and lysed, and the cell lysates were determined by immunoblotting with anti-phospho-p38 kinase (top) and anti-p38 kinase antibodies (bottom). (C) SB203580 did not affect the phosphorylation of Smad1. C3H10T1/2 cells were serum starved in 0.2% fetal calf serum-DMEM for 12 h. The cells were further incubated with or without SB203580 (10 μ M) for 4 h. The cells were then stimulated with BMP2 (300 ng/ml) for 10 min and lysed. The cell lysates were immunoprecipitated with anti-Smad1 and immunoblotting with anti-phosphoserine (top) or anti-Smad1 antibodies (bottom). (D) SB203580 inhibited transcriptional activity of PPAR γ induced by BMP2. C3H10T1/2 cells were transfected into together with PPAR γ (0.1 μ g) and RXR (0.1 μ g) expression vectors, and PPRE-Luc (0.1 μ g) and TK-renilla (0.1 μ g) reporter constructs, and pcDNA3 (1.6 μ g). Twelve hours after transfection, cells were incubated with or without SB203580 (10 μ M) for 2 d. At the end of the culture, cells were lysed and the luciferase activity was measured and normalized by determining renilla luciferase activity as described in the text. Data are shown as mean $\pm$ SD. (E) SB203580 did not affect the expression of PPAR γ . C3H10T1/2 cells were incubated with BMP2 (300 ng/ml), SB203580 (10 μ M), or both for 3 d. The cells were lysed, and the expression of PPAR γ was determined by immunoblotting with anti-PPAR γ monoclonal antibody, followed by immunoprecipitation with anti-PPAR γ polyclonal antibody (top). The lysates were also determined by immunoblotting with anti- β -actin antibody (bottom).

Figure 6. (A) Overexpression of TAK1- and TAB1-induced activation of p38 kinase in C3H10T1/2 cells. C3H10T1/2 cells were transfected with pcDNA3 vector or both TAK1 and TAB1 expression constructs. Twenty hours after transfection, the cells were serum starved for 16 h and lysed. The cell lysates were determined by immunoblotting with anti-phospho-p38 kinase (top) or anti-p38 kinase (bottom) antibodies. (B) Overexpression of TAK1 and TAB1 up-regulated transcriptional activity of PPAR γ . pcDNA3 (control) (1.6 μg) or both TAK1 (0.8 μg) and TAB1 (0.8 μg) expression vectors (1.6 μg) were transfected into C3H10T1/2 cells together with PPAR γ (0.1 μg) and RXR (0.1 μg) expression vectors and PPRE-Luc

biological relevance of this interaction are needed to be addressed.

Recently, Sano *et al.* (1999) have reported the direct cooperation between Smad3 and p38 kinase signaling. Similarly, it has been shown that p38 pathway is involved in transforming growth factor- β -induced gene expression by interacting with Smad signaling (Hanafusa *et al.*, 1999; Watanabe *et al.*, 2001). In contrast, our results with SB203580 indicated that p38 kinase pathway is not involved in the regulation of PPAR γ expression, which is controlled by Smad signaling. Kimura *et al.* (2000) and Yanagisawa *et al.* (2001) reported that activation of p38 signaling was blocked by Smad6 or Smad7 in mouse hybridoma or PC12 cells. On the other hand, we showed herein that Smad6 failed to inhibit the transactivation of PPAR γ by BMP2 and that BMP2 increased adipogenic function of PPAR γ even in the presence of Smad6, suggesting that Smad6 has little effect on the up-regulation of PPAR γ by p38 kinase. Because we observed that SB203580 had no effect on phosphorylation of Smad1, and that the inhibitory effect of Smad6 on p38 activation was modest in C3H10T1/2 cells, the extent of interaction of both Smad pathway and p38 signaling might depend on types of cells or tissues. Alternatively, it is also possible that experimental models and/or levels of Smad6 expression may account for these differences. Further dissection of relationship of both pathways would solve this issue.

Because we have shown that BMP2 induced expression of PPAR γ in C3H10T1/2 cells and that Smad6 blocked the effects of BMP2, we were concerned that these effects of BMP2 or Smad6 might affect our transcriptional assay for PPAR γ . To eliminate this possibility, we exogenously introduced the appropriate amount of PPAR γ by transfection when we examined the effects of BMP2 on the transcriptional activity of PPAR γ . As we expected, the amount of PPAR γ introduced by transfection was much larger than that induced by BMP2 (Figure 1E). Moreover, when we performed the reporter assay, the amounts of PPAR γ were not affected by treatment with BMP2 or Smad6 (Figure 4A). It is, therefore, likely that our transcriptional assay for PPAR γ was independent of levels of PPAR γ induced by BMP2 treatment or effect of Smad6.

It is known that adipocytes share their origin in bone marrow with osteoblasts (Prockop, 1997; Pittenger *et al.*, 1999). We and others have demonstrated that BMP2 also differentiates pluripotent mesenchymal cells toward osteoblasts by activating Smad signaling (Yamamoto *et al.*, 1997;

(0.1 μg) and TK-renilla (0.1 μg) reporter constructs. Twelve hours after transfection, cells were incubated with or without SB203580 (10 μM) for 2 d. At the end of the culture, cells were lysed and the luciferase activity was measured and normalized by determining renilla luciferase activity as described in the text. Data were shown as mean $\pm$ SD. (C) Dominant-negative MKK3 inhibited BMP2-induced adipogenesis in C3H10T1/2 cells. C3H10T1/2 cells infected with control or dominant-negative MKK3 adenovirus at 100 multiplicity of infection were incubated with or without BMP2 (300 ng/ml). The cells were stained with Oil Red O, photographed under a microscope, and the areas stained with Oil Red O in the cells were assessed. (D) SB203580 inhibited BMP2-induced adipogenesis. C3H10T1/2 cells were incubated with BMP2 (300 ng/ml), SB203580 (10 μM), or both for 10 d. The cells were stained with Oil Red O, photographed under a microscope, and the areas stained with Oil Red O in the cells were assessed.

Figure 7. (A) Inhibition of p38 kinase activation by PD169316 and FR167653. C3H10T1/2 cells were serum starved in 0.2% fetal calf serum-DMEM for 12 h. The cells were further incubated with or without p38 kinase inhibitor SB203580 (10 μ M), PD169316 (1 μ M), or FR167653 (10 μ M) for 4 h. The cells were then stimulated with BMP2 (300 ng/ml) for 10 min and lysed, and the cell lysates were determined by immunoblotting with anti-phospho-p38 kinase (top) and anti-p38 kinase antibodies (bottom). (B) PD169316 and FR167653 inhibited transcriptional activity of PPAR γ induced by BMP2. C3H10T1/2 cells were transfected together with PPAR γ (0.1 μ g) and RXR (0.1 μ g) expression vectors and PPRE-Luc (0.1 μ g) and TK-renilla (0.1 μ g) reporter constructs, and pcDNA3 (1.6 μ g). Twelve hours after transfection, cells were incubated with or without SB203580 (10 μ M), PD169316 (1 μ M), or FR167653 (10 μ M) for 2 d. At the end of the culture, cells were lysed and the luciferase activity was measured and normalized by determining renilla luciferase activity as described in the text. Data were shown as mean $\pm$ SD. (C) PD169316 and FR167653 inhibited BMP2-induced adipogenesis. C3H10T1/2 cells were incubated with or without BMP2 (300 ng/ml), in the presence or absence of SB203580 (10 μ M), PD169316 (1 μ M), or FR167653 (10 μ M) for 10 d. The cells were stained with Oil Red O, photographed under a microscope, and the areas stained with Oil Red O in the cells were assessed.

Nishimura *et al.*, 1998). In this study, we showed that BMP2 regulated PPAR γ expression via Smad signaling. Interestingly, we also observed that overexpression of PPAR γ in bone marrow stromal cells or primary osteoblasts inhibited

Figure 8. Schematic model of BMP2-induced adipogenesis. Activation of Smad1 is essential for induction of PPAR γ expression, which is critical for adipogenic effects of BMP2. In contrast, activation of p38 kinase, presumably through MKK3 and MKK6, plays roles in the up-regulation of transcriptional activity of PPAR γ .

the osteoblastic differentiation process (Hata and Nishimura, unpublished data). Abnormally accelerated adipogenesis in bone marrow, also known as fatty marrow, is often observed in the patients with osteoporosis, which is a common metabolic bone disease characterized by the impaired function and differentiation of osteoblasts (Ducy *et al.*, 2000). Therefore, the identification of molecular mechanisms that regulate the direction between adipogenesis and osteoblastogenesis may contribute to the further understanding of pathogenesis of metabolic bone disease such as osteoporosis.

In conclusion, our data suggest that BMP2 induces the differentiation of undifferentiated mesenchymal cells into adipocytes by induction of PPAR γ expression through activation of Smad1 and up-regulation of its transcriptional activity via activation of p38 kinase. We believe that these results further our understanding of the molecular mechanism underlying the BMP2-induced adipogenesis.

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