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Revised version

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1 **Peroxisome proliferator-activated receptor activity is involved in the**  
2 **osteoblastic differentiation regulated by bone morphogenetic proteins**  
3 **and tumor necrosis factor- $\alpha$ .**

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13 *Running title:* BMP and PPAR actions in osteoblast differentiation

14 *Key words:* bone morphogenetic protein (BMP), peroxisome proliferator-activated  
15 receptor (PPAR), osteoblast, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )

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17 *Disclosure statement:* All authors have nothing to disclose.

18

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24

25 *Abbreviations:*

26 ALK, activin receptor-like kinase

27 ActRII, activin type-II receptor

28 BMP, bone morphogenetic protein; BMPRII, BMP type-II receptor

29 ERK, extracellular signal-regulated kinase

30 MAPK, mitogen-activated protein kinase

31 NF $\kappa$ B, nuclear factor- $\kappa$ B

32 I $\kappa$ B, inhibitory- $\kappa$ B

33 PPAR, peroxisome proliferator-activated receptor

34 SAPK/JNK, stress-activated protein kinase / c-Jun NH2-terminal kinase

35 TGF- $\beta$ , transforming growth factor- $\beta$

36 TNF- $\alpha$ , tumor necrosis factor- $\alpha$

37 TNFR, tumor necrosis factor receptor

38



1 phosphorylation of MAPKs, NFκB, IκB and Stat pathways was inhibited in the  
2 presence of PPARα and PPARγ agonists with reducing TNF-α receptor expression. In  
3 view of the finding that inhibition of SAPK/JNK, Stat and NFκB pathways reversed the  
4 TNF-α suppression of osteoblast differentiation, we conclude that these cascades are  
5 functionally involved in the actions of PPARs that antagonize TNF-α-induced  
6 suppression of osteoblast differentiation. It was further discovered that the PPARα  
7 agonist enhanced BMP-4-induced Smad1/5/8 signaling through downregulation of  
8 inhibitory Smad6/7 expression, whereas the PPARγ agonist impaired this activity by  
9 suppressing BMPRII expression. On the other hand, BMPs increased the expression  
10 levels of PPARα and PPARγ in the process of osteoblast differentiation. Thus, PPARα  
11 actions promote BMP-induced osteoblast differentiation, while both activities of  
12 PPARα and PPARγ suppress TNF-α actions. Collectively, our present data establishes  
13 that PPAR activities are functionally involved in modulating the interaction between the  
14 BMP system and TNF-α receptor signaling that is crucial for bone metabolism.

15

## INTRODUCTION

Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)- $\beta$  superfamily, play pivotal regulatory roles in mesoderm induction and dorso-ventral patterning of developing limb buds and are known to promote differentiation of mesenchymal stem cells into chondrocytes and osteoblasts as well as differentiation of osteoprogenitor cells into osteoblasts ([Lieberman et al., 2002](#)).

BMPs are also known to have critical roles in governing various aspects of embryological development, including development of the brain, heart, kidney and eyes

([Reddi, 1997](#)). In addition to the developmental actions of BMPs, various physiological actions of BMPs in endocrine and vascular tissues have recently been elucidated ([Shimasaki et al., 2004; Otsuka, 2010; Otsuka et al., 2011](#)). The biological functions of BMPs are mediated through the Smad signal transduction pathway via specific combinations of the proper BMP receptors ([Shimasaki et al., 2004](#)).

Osteoblast differentiation is a complex process regulated by various endocrine, paracrine and autocrine factors. Osteoblasts, which arise from mesenchymal stem cell

1 precursors, undergo differentiation in response to a number of factors, including BMPs,  
2 TGFs, insulin-like growth factor-I (IGF-I), vascular endothelial growth factor (VEGF),  
3 and steroids ([McCarthy et al., 1989](#); [Celeste et al., 1990](#); [Midy and Plouet, 1994](#);  
4 [Hughes et al., 1995](#); [Spelsberg et al., 1999](#)). Once matrix synthesis begins in cultured  
5 osteoblast cells, the cells differentiate and osteoblastic markers, including alkaline  
6 phosphatase (ALP), type-I collagen and osteocalcin, are subsequently activated.  
7 Osteoblasts then embed in the extracellular matrix consisting of collagen fibrils, and the  
8 matrix is mineralized and extended in collagen fibrils. Deposition and maintenance of  
9 mineralized skeletal elements are further regulated by various growth factors including  
10 BMPs and cytokines such as interleukins and tumor necrosis factors (TNFs).

11           Among the various cytokines, TNF- $\alpha$  receptor signaling plays a predominant  
12 role in bone loss in arthritis. TNF- $\alpha$  is also involved in controlling osteoblast survival  
13 and function in addition to the induction of osteoclast differentiation leading to bone  
14 resorption ([Kudo et al., 2002](#)). The effectiveness of blocking TNF- $\alpha$  actions in  
15 treatment of active rheumatoid arthritis established the clinical significance of TNF- $\alpha$  in  
16 the pathogenesis of inflammatory bone diseases ([Feldmann and Maini, 2001](#); [Scott and](#)

1 [Kingsley, 2006](#)). However, the underlying mechanism of TNF- $\alpha$  in the regulation of  
2 differentiation of osteoblasts has not been fully elucidated.

3 Peroxisome proliferator-activated receptors (PPARs) [including](#) PPAR $\alpha$ ,  
4 PPAR $\beta/\delta$  and PPAR $\gamma$  [are categorized](#) to the family of nuclear hormone receptors  
5 [\(Desvergne and Wahli, 1999\)](#). PPAR $\gamma$  is activated by natural ligands such as  
6 polyunsaturated fatty acids [and](#) metabolites [of prostaglandins](#) and synthetic ligands,  
7 thiazolidinediones, such as rosiglitazone, pioglitazone and troglitazone [\(Willson et al.,](#)  
8 [2000\)](#). Recent studies have provided evidence that PPAR $\gamma$  [activity may](#) directly  
9 inhibit bone formation by diverting mesenchymal stem cells from the osteogenic  
10 [process to the](#) adipocytic lineage [\(Grey, 2008\)](#). Clinical studies have also revealed that  
11 thiazolidinediones decrease markers of bone formation with reduction in bone mass and  
12 increase in fracture [incidence](#) in women [\(Grey et al., 2007; Grey, 2008\)](#). However, the  
13 underlying mechanism by which PPARs affect osteoblastic differentiation has yet to be  
14 clarified.

15 The pluripotent mesenchymal precursor cell line, C2C12, a subclone of a  
16 mouse myoblastic cell line, has been widely used as a model to investigate the early

1 stages of osteoblast differentiation during bone formation in muscular tissues.  
2 Treatment of C2C12 cells with various TGF- $\beta$  superfamily ligands has distinct effects  
3 on differentiation, and BMPs inhibit myoblast differentiation of C2C12 cells and  
4 promote osteoblastic cell differentiation ([Katagiri et al., 1994](#); [Ebisawa et al., 1999](#)).  
5 In the present study, we investigated the cellular mechanisms by which PPAR agonists  
6 interact in the process of osteoblastic differentiation regulated by the activation of BMP  
7 and TNF- $\alpha$  with a focus on the interaction between BMP-Smad and PPAR signaling.

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1

2 *Cell culture and morphological examination*

3 The mouse myoblast cell line C2C12 was obtained from American Type Culture  
4 Collection (Manassas, VA). C2C12 cells were cultured in DMEM supplemented with  
5 10% fetal calf serum (FCS) and penicillin-streptomycin solution at 37°C under a humid  
6 atmosphere of 95% air/5% CO<sub>2</sub>. Changes in cell morphology were monitored using an  
7 inverted microscope.

8

9 *RNA extraction, RT-PCR, and quantitative real-time PCR analysis*

10 To prepare total cellular RNA, C2C12 cells were cultured in a 12-well plate ( $1 \times 10^5$   
11 viable cells/well) and treated with the indicated concentrations of TNF- $\alpha$  and BMPs in  
12 combination with a PPAR $\alpha$  agonist (fenofibric acid), a PPAR $\gamma$  agonist (pioglitazone)  
13 and various inhibitors including GW6471, U0126, SB203580, SP600125, AG490,  
14 NF $\kappa$ B inhibitor and SH-5 in serum-free DMEM. After 48-h culture, the medium was  
15 removed, and total cellular RNA was extracted using TRIzol® (Invitrogen Corp.,  
16 Carlsbad, CA), quantified by measuring absorbance at 260 nm. The extracted RNA

1 (1.0 µg) was subjected to an RT reaction using the First-Strand cDNA synthesis  
2 system® (Invitrogen Corp.) with random hexamer (50 ng/µl), reverse transcriptase (200  
3 U), and deoxynucleotide triphosphate (2.5 mM) at 42°C for 55 min and at 70°C for 10  
4 min. Subsequently, hot-start PCR was performed using MgCl<sub>2</sub> (50 mM),  
5 deoxynucleotide triphosphate (2.5 mM), and 1.5 U of *Taq* DNA polymerase (Invitrogen  
6 Corp.) under the conditions we previously reported ([Mukai et al., 2007](#); [Matsumoto et](#)  
7 [al., 2010](#)). PCR primer pairs, custom-ordered from Invitrogen Corp., were selected  
8 from different exons of the corresponding genes as follows: PPAR $\alpha$ , 1769-1789 and  
9 1950-1969 (from NM\_011144); PPAR $\gamma$ , 567-588 and 838-858 (from NM\_011146); Id-1,  
10 225-247 and 364-384 (from NM\_010495); runt-related transcription factor 2 (Runx2),  
11 1041-1062 and 1379-1400 (from NM\_009820); osteocalcin, 125-144 and 312-331  
12 (NM\_007541); type-1 collagen (collagen-1), 3872-3891 and 3922-3941 (NM\_007742);  
13 ALP, 1365-1385 and 1549-1568 (NM\_007431); TNFR1, 931-951 and 1211-1231  
14 (BC052675); TNFR2, 142-162 and 1142-1162 (Y14622); and a house-keeping gene,  
15 ribosomal protein L19 (RPL19), 373-393 and 547-567 (from NM\_009078). Primer  
16 pairs for mouse BMP type-1 and type-2 receptors and Smads were selected as we

1 reported previously ([Otani et al., 2007](#); [Takeda et al., 2007](#)). The expression of  
2 PPAR $\alpha$ , PPAR $\gamma$  and RPL19 mRNAs was detected by RT-PCR analysis. Aliquots of  
3 PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium  
4 bromide staining. For the quantification of mRNA levels of PPARs, Runx2,  
5 osteocalcin, collagen-1, ALP, TNFRs, BMP receptors, Smads and Id-1, real-time PCR  
6 was performed using the StepOnePlus<sup>®</sup> real-time PCR system (Applied Biosystems,  
7 Foster City, CA) under optimized annealing conditions, following the manufacturer's  
8 protocol with the following profile: 40 cycles each at 95°C for 3 sec and 60-62°C for 30  
9 sec. The threshold cycle (Ct) values were calculated using the StepOnePlus<sup>™</sup> system  
10 software (Applied Biosystems). The relative expression of each mRNA was calculated  
11 by the  $\Delta$ Ct method, in which  $\Delta$ Ct is the value obtained by subtracting the Ct value of  
12 RPL19 mRNA from the Ct value of the target mRNA, and the amount of target mRNA  
13 relative to RLP19 mRNA was expressed as  $2^{-(\Delta Ct)}$ . The data are expressed as the ratio  
14 of target mRNA to RPL19 mRNA.

15

16 *Western immunoblot analysis*

1 Cells ( $1 \times 10^5$  viable cells/well) were precultured in 12-well plates in DMEM  
2 containing 10% FCS for 24 h. After preculture, the medium was replaced with  
3 serum-free fresh medium, and then cells were treated with the indicated concentrations  
4 of the PPAR $\alpha$  agonist (fenofibric acid) and PPAR $\gamma$  agonist (pioglitazone) for 24 h  
5 before addition of TNF- $\alpha$  and BMP-4. After stimulation with growth factors for 15  
6 and 60 min, cells were solubilized in 100  $\mu$ l RIPA lysis buffer (Upstate Biotechnology,  
7 Inc., Lake Placid, NY) containing 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM sodium fluoride, 2% sodium  
8 dodecyl sulfate, and 4%  $\beta$ -mercaptoethanol. For detecting protein expression of  
9 PPAR $\alpha$  and PPAR $\gamma$ , cell lysates were collected from cells treated with BMP-4 for 48 h  
10 and 72 h. For detecting protein expression of TNFRs, cell lysates were collected from  
11 cells treated with PPAR $\alpha$  and PPAR $\gamma$  agonists for 48 h. The cell lysates were then  
12 subjected to SDS-PAGE/immunoblotting analysis as we previously reported using  
13 anti-phospho-Smad1/5/8 antibody (Cell Signaling Technology, Inc., Beverly, MA),  
14 anti-phospho- and anti-total-extracellular signal-regulated kinase (ERK) 1/2 MAPK  
15 antibodies (Cell Signaling Technology, Inc.), anti-phospho- and anti-total-p38 MAPK  
16 antibodies (Cell Signaling Technology, Inc.), anti-phospho- and

1 anti-total-stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK)  
2 MAPK antibodies (Cell Signaling Technology, Inc.), anti-phospho- and anti-total  
3 NFκB-p65 antibodies, and anti-phospho- and anti-total IκB-α antibodies (Cell  
4 Signaling Technology, Inc.), anti-phospho- and anti-total-Stat3 and Stat5 antibodies  
5 (Cell Signaling Technology, Inc.), anti-phospho- and anti-total-Akt antibodies (Cell  
6 Signaling Technology, Inc.), anti-PPARα (H-98) and anti-PPARγ (H-100) antibodies  
7 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-TNFR1 (H-271) and  
8 anti-TNFR2 (H-202) antibodies (Santa Cruz Biotechnology, Inc.), and anti-actin  
9 antibody (Sigma-Aldrich Co. Ltd.). The relative integrated density of each protein  
10 band was digitized by NIH image J 1.34s.

11

12 *Transient transfection and luciferase assay*

13 C2C12 cells ( $5 \times 10^4$  viable cells) were precultured in 12-well plates in DMEM with  
14 10% FCS. The cells were then transiently transfected with 500 ng of BRE-Luc or  
15 Id-1-Luc reporter plasmids and 50 ng of cytomegalovirus-β-galactosidase plasmid  
16 (pCMV-β-gal) using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) for

1 12 h. The cells were then treated with the indicated concentrations of PPAR $\alpha$  and  
2 PPAR $\gamma$  agonists in combination with BMP-4 in serum-free fresh medium for 24 h.  
3 The cells were washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo,  
4 Osaka, Japan). Luciferase activity and  $\beta$ -galactosidase ( $\beta$ -gal) activity of the cell  
5 lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan). The data are  
6 shown as the ratio of luciferase to  $\beta$ -gal activity.

7

#### 8 *Statistical analysis*

9 All results are shown as means  $\pm$  SEM of data from at least three separate experiments,  
10 each performed with triplicate samples. Differences between groups were analyzed  
11 for statistical significance using ANOVA with Fisher's protected least significant  
12 difference (PLSD) test or unpaired *t*-test, when appropriate, to determine differences  
13 (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). *P* values < 0.05 were  
14 accepted as statistically significant.

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1 concentration dependently.

2 To elucidate the mechanism by which PPAR agonists modulate the expression  
3 levels of osteoblastic markers in C2C12 cells, effects of PPAR agonists on BMP  
4 receptor signaling were subsequently examined. It was found that the PPAR $\alpha$  agonist  
5 (3  $\mu$ M) significantly enhanced promoter activity of the BMP-responsive element  
6 represented by BRE-Luc activity induced by BMP-4 (100 ng/ml) (**Fig. 1C**). In  
7 contrast, the PPAR $\gamma$  agonist (3  $\mu$ M) reduced BRE-Luc activity induced by BMP-4 (100  
8 ng/ml) (**Fig. 1C**). Similar results were obtained by a promoter assay using the BMP  
9 target gene Id-1-Luc (data not shown). Furthermore, the PPAR $\alpha$  agonist (3  $\mu$ M)  
10 significantly reduced mRNA levels of inhibitory Smad6 and Smad7, while treatment  
11 with the PPAR $\gamma$  agonist (3  $\mu$ M) decreased the expression level of BMPRII mRNA in  
12 C2C12 cells (**Fig. 1D**). With regard to the BMP receptor system, several preferential

13 combinations of BMP ligands and receptors have been recognized to date ([Shimasaki et](#)  
14 [al., 2004](#)). BMP-2 and -4 most readily bind to ALK-3 and/or ALK-6 in combination  
15 with the type-2 receptor BMPRII. Since ALK-6 is not expressed in C2C12 cells  
16 ([Mukai et al., 2007](#)), the major functional complex for the osteoblastic differentiation



1 induced by BMP-4 is likely ALK-3/BMPRII. Thus, PPAR $\gamma$  activity was found to  
2 suppress BMP-Smad signaling by inhibiting BMPRII expression, whereas PPAR $\alpha$   
3 enhanced BMP receptor signaling by suppressing inhibitory Smad6/7. On the other  
4 hand, BMP-2, -4, -6 and -7 (100 ng/ml) induced increases in mRNA levels of PPAR $\alpha$   
5 and PPAR $\gamma$  in C2C12 cells cultured for 48 h (**Fig. 1E**). It was also found that BMP-4  
6 (100 ng/ml) stimulated the expression of PPAR $\alpha$  and PPAR $\gamma$  protein levels in 48 h to 72  
7 h cultures (**Fig. 1F**).

8 We next studied the effects of PPAR activities on the interaction between  
9 BMP and TNF- $\alpha$  in C2C12 cells. Osteoblastic differentiation induced by BMP-2, -4,  
10 -6 and -7 (100 ng/ml) was suppressed by co-treatment with TNF- $\alpha$  (10 ng/ml), with the  
11 most pronounced effects on BMP-2- and BMP-4-induced differentiation (**Fig. 2A**).  
12 Importantly, the inhibitory effects of TNF- $\alpha$  (10 ng/ml) on BMP-4 (100 ng/ml)-induced  
13 mRNA expression of osteoblastic markers including Runx2, osteocalcin and collagen-1  
14 were reversed by co-treatment with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M), although the  
15 impact of PPAR $\gamma$  agonist on TNF- $\alpha$  inhibition of BMP-4-induced ALP expression was  
16 not significant (**Fig. 2B**). Smad1/5/8 phosphorylation induced by BMP-4 (100 ng/ml)

1 was suppressed by treatment with TNF- $\alpha$  (100 ng/ml) (**Fig. 2C**). Of note, the  
2 inhibitory actions of TNF- $\alpha$  (10 ng/ml) on BMP-induced Smad1/5/8 phosphorylation  
3 were reversed in the presence of either the PPAR $\alpha$  or PPAR $\gamma$  agonist (3  $\mu$ M) (**Fig. 2C**).  
4 In accordance with the results for Smad phosphorylation, suppression by TNF- $\alpha$  (10  
5 ng/ml) of BMP target gene Id-1 transcription induced by BMP-4 was also partially  
6 reversed by co-treatment with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M) (**Fig. 2D**). The  
7 effects of TNF- $\alpha$  are mediated through two distinct receptors: type 1, also called  
8 p60/p55 receptor (TNFR1), and type 2, also called p80/p75 receptor (TNFR2) ([Grell et](#)  
9 [al., 1994](#)). PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M) decreased the expression levels of  
10 TNFR1 and TNFR2 mRNAs (**Fig. 2E**). In addition, the changes in TNFR expression  
11 by PPAR $\alpha$  and PPAR $\gamma$  agonist (3  $\mu$ M) were evaluated by immunoblot analysis using  
12 anti-TNFR1 and TNFR2 antibodies, showing that the protein level of TNFR2 was also  
13 decreased by treatments with PPAR $\alpha$  and PPAR $\gamma$  agonists (**Fig. 2F**). The mechanism  
14 by which PPAR $\alpha$ / $\gamma$  activities reduced TNF- $\alpha$  actions may be contributed to the  
15 downregulation of TNFR signaling in C2C12 cells. Thus, both actions of PPAR $\alpha$  and  
16 PPAR $\gamma$  agonists antagonize suppression by TNF- $\alpha$  of osteoblastic differentiation

1 induced by BMP-4 with restoration of TNF- $\alpha$ -induced suppression of Smad1/5/8  
2 phosphorylation and Id-1 transcription.

3 Subsequently, the effects of PPAR $\alpha$  and PPAR $\gamma$  agonists on TNF- $\alpha$ -induced  
4 cellular signaling were investigated. TNF- $\alpha$  (100 ng/ml) readily stimulated MAPK  
5 phosphorylation including ERK1/ERK2, p38-MAPK, SAPK/JNK pathways in C2C12  
6 cells (**Fig. 3A**). The TNF- $\alpha$  actions were not significantly altered by treatment with  
7 BMP-4 (100 ng/ml). Notably, TNF- $\alpha$  (100 ng/ml)-induced phosphorylation of  
8 MAPKs including p38-MAPK and SAPK/JNK pathways (**Fig. 3A**) was inhibited in the  
9 presence of either the PPAR $\alpha$  or PPAR $\gamma$  agonist (3  $\mu$ M). NF $\kappa$ B, I $\kappa$ B and Stat3  
10 pathways were also stimulated by TNF- $\alpha$  (100 ng/ml), and the stimulation of these  
11 pathways was not affected by co-treatment with BMP-4 (100 ng/ml) (**Fig. 3B**).  
12 TNF- $\alpha$ -induced activation of NF $\kappa$ B signaling was significantly suppressed by the  
13 PPAR $\gamma$  agonist (3  $\mu$ M), while I $\kappa$ B and Stat3 phosphorylation induced by TNF- $\alpha$  was  
14 inhibited by either the PPAR $\alpha$  or PPAR $\gamma$  agonist (3  $\mu$ M). The Akt pathway was also  
15 stimulated by TNF- $\alpha$  (100 ng/ml); however, PPAR $\alpha$  or PPAR $\gamma$  agonist (3  $\mu$ M) failed to  
16 suppress Akt phosphorylation (**Fig. 3B**).

1           To further explore the major pathways for TNF- $\alpha$  receptor signaling in  
2 BMP-4-induced osteoblastic differentiation, cells were treated with specific inhibitors  
3 for ERK1/ERK2, p38-MAPK SAPK/JNK, Stat and NF $\kappa$ B. Inhibition of SAPK/JNK,  
4 Stat and NF $\kappa$ B pathways with SP600125, AG490 and NF $\kappa$ B inhibitor, respectively,  
5 reversed the suppression by TNF- $\alpha$  (10 ng/ml) of Runx2 (**Fig. 4A**) and osteocalcin (**Fig.**  
6 **4B**) mRNA expression induced by BMP-4 (100 ng/ml). On the other hand,  
7 ERK1/ERK2 and p38 inhibition by U0126 and SB203580, respectively, failed to restore  
8 the suppression by TNF- $\alpha$  (10 ng/ml) of Runx2 (**Fig. 4A**) and osteocalcin (**Fig. 4B**)  
9 mRNA levels amplified by BMP-4 (100 ng/ml). We thus conclude that SAPK/JNK,  
10 Stat and NF $\kappa$ B signaling plays an important role in PPAR $\alpha$  and PPAR $\gamma$  antagonizing the  
11 suppression by TNF- $\alpha$  of osteoblastic differentiation.

12



1 Although no bone abnormalities were identified in PPAR $\alpha$  knockout mice  
2 (Wu et al., 2000), there is substantial evidence that PPAR $\alpha$  has activities in bone  
3 development and in bone metabolism (Lecka-Czernik (Curr Osteoporos Rep 8: 84-90,  
4 2010) provides a comprehensive review of relevant literature (Lecka-Czernik, 2010)).  
5 In particular, the collective data suggests that PPAR $\alpha$  may regulate bone metabolism  
6 and bone marrow conditions by providing energy through fatty acid oxidation and by  
7 controlling cell commitment within hematopoietic lineages rather than affecting the  
8 differentiation of bone cells (Lecka-Czernik, 2010). Based on our present data,  
9 PPAR $\alpha$  has beneficial effects, at least in part, in the early process of osteoblastic  
10 differentiation preferentially in combination with the activity of BMP-4, and both  
11 PPAR $\alpha$  and PPAR $\gamma$  elicit anti-TNF $\alpha$  actions in the process of osteoblast differentiation.

12 PPAR $\gamma$  is a critical transcription factor for the induction of adipocyte  
13 differentiation based on the experimental and clinical studies using PPAR $\gamma$  agonists,  
14 thiazolidinediones (Grey, 2008). PPAR $\gamma$  transcripts are expressed in osteoblasts  
15 (Johnson et al., 1999; Jackson and Demer, 2000; Jeon et al., 2003) and osteoclast  
16 precursors (Mbalaviele et al., 2000; Chan et al., 2007). PPAR $\gamma$  agonists promote

1 adipogenesis instead of osteoblastogenesis in vitro (Gimble et al., 1996). PPAR $\gamma$   
2 heterozygous-deficient mice demonstrate increased bone mass by stimulating  
3 osteoblastogenesis (Akune et al., 2004). Taken together, it appears that PPAR $\gamma$  activity  
4 preferentially promotes adipogenetic cascade instead of the process of  
5 osteoblastogenesis. In the present study, PPAR $\gamma$  activity was found to decrease  
6 Smad1/5/8 and its downstream signaling induced by BMP-4 by suppressing BMPRII  
7 expression in C2C12 cells, suggesting an inhibitory role of PPAR $\gamma$  activity in the early  
8 process of BMP-induced osteoblastic differentiation. However, the activity of PPAR $\gamma$   
9 did not seem likely enough to attain the biological inhibition of BMP-induced osteoblast  
10 differentiation evaluated by the levels of Runx2, osteocalcin and collagen-1 expression.  
11 The interaction between PPAR and BMPs may be involved in the dual actions of  
12 adipogenesis and osteogenesis by BMPs. According to an analysis of BMPs on the  
13 mesenchymal stem cell differentiation, BMP-2, -4, -6, -7 and -9 activated adipogenic  
14 and osteogenic differentiation of mesenchymal stem cells (Kang et al., 2009).  
15 Interestingly, overexpression of PPAR $\gamma$ 2 facilitated both osteogenic and adipogenic  
16 differentiation and PPAR $\gamma$ 2 knockdown inhibited not only adipogenic differentiation but

1 also BMP-induced ossification ([Kang et al., 2009](#)), suggesting that PPAR $\gamma$  activity is  
2 also, at least in part, involved in promoting osteogenic differentiation.

3           Imbalanced functions of osteoclasts and osteoblasts lead to bone damage seen  
4 in patients with inflammatory bone diseases such as rheumatoid arthritis. Since bone  
5 loss in arthritis is related to activation of the TNF- $\alpha$  system, it can be hypothesized that  
6 TNF- $\alpha$  directly controls osteoblast survival and/or function in addition to its induction  
7 of osteoclast differentiation leading to excess bone resorption ([Kudo et al., 2002](#)). In  
8 this regard, we previously reported that TNF- $\alpha$  suppresses BMP-2-induced expression  
9 of osteoblast markers such as Runx2, osteocalcin and ALP ([Mukai et al., 2007](#)), in  
10 which MAPK and NF $\kappa$ B are involved in the suppression by TNF- $\alpha$  of BMP-2 activity  
11 in C2C12 cells ([Mukai et al., 2007](#); [Yamashita et al., 2008](#); [Matsumoto et al., 2010](#)).

12 The present results further demonstrated that, among BMP ligands, BMP-4 most  
13 effectively augments PPAR $\alpha$  activity leading to promotion of osteoblastic  
14 differentiation. BMP-4 also increased the sensitivity of PPAR agonists by  
15 upregulating the expression of PPAR $\alpha$  and PPAR $\gamma$  in the process of osteoblastic  
16 differentiation. Moreover, the activities of PPAR $\alpha$  and PPAR $\gamma$  are involved in



1 antagonizing the TNF- $\alpha$  signaling that is a negative factor for osteoblastic  
2 differentiation induced by BMP-4.

3           In our earlier study, the expression of other nuclear receptors such as estrogen  
4 receptors (ER $\alpha$  and ER $\beta$ ) and glucocorticoid receptor (GCR) in C2C12 cells was  
5 significantly increased by BMP-2 stimulation ([Matsumoto et al., 2010](#)). BMP-2  
6 increased the sensitivities of ERs and GCR, whereas estrogen and glucocorticoid  
7 differentially regulated BMP-Smad signaling, and these steroids antagonized TNF- $\alpha$   
8 signaling in a different manner ([Matsumoto et al., 2010](#)). In the present study, in  
9 addition to ER and GCR actions, PPARs were also found to antagonize TNF- $\alpha$  activities  
10 in osteoblastic differentiation. Further studies are needed to utilize the efficacious  
11 actions of PPAR $\alpha$  but modulate PPAR $\gamma$  activity in osteoblasts in relation to the activities  
12 of other nuclear receptor family molecules.

13           Collectively, PPARs are functionally involved in the process of osteoblast  
14 differentiation directed by BMP-4 and TNF- $\alpha$ . BMP-4 increases the sensitivities of  
15 PPARs, PPAR $\alpha$  in turn upregulates and PPAR $\gamma$  represses BMP-Smad signaling, and  
16 PPARs antagonize TNF- $\alpha$  signaling in a different manner (**Fig. 5**). Understanding the

- 1 integrated mechanisms behind BMP- and TNF- $\alpha$ -regulated osteoblastic differentiation
- 2 may lead to the development of novel therapeutic strategies for osteoporosis and/or
- 3 inflammatory bone disorders.

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16  
17  
18  
19

1 **FIGURE LEGENDS**

2

3 **Fig. 1. Effects of PPAR $\alpha$  and PPAR $\gamma$  agonists on BMP-induced osteoblast**

4 **differentiation.** A) After preculture, C2C12 cells were treated with BMP-2, -4, -6 and

5 -7 (100 ng/ml) in combination with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M) for 48 h.

6 Total cellular RNA was extracted and then subjected to PCR reaction. Real-time PCR

7 analysis was performed for quantification of Runx2, osteocalcin and collagen-1 mRNA

8 levels. The expression levels of target genes were standardized by RPL19 level in

9 each sample. B) Cells were treated with BMP-4 (100 ng/ml) in combination with

10 PPAR $\alpha$  agonist (3  $\mu$ M) and the PPAR $\alpha$  antagonist GW6471 (1 to 10  $\mu$ M) for 48 h.

11 Total cellular RNA was extracted and then subjected to real-time PCR analysis for

12 quantification of Runx2 and collagen-1 mRNA levels. The expression levels of target

13 genes were standardized by RPL19 level in each sample. C) Cells were transiently

14 transfected with BRE-Luc reporter plasmid (500 ng) and pCMV- $\beta$ -gal. The cells were

15 then treated with BMP-4 (100 ng/ml) and with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M) for

16 24 h. The cells were lysed and the luciferase activity and  $\beta$ -galactosidase ( $\beta$ -gal)



1 activity were measured by a luminometer. The data were expressed as the ratio of  
2 luciferase to  $\beta$ -gal activity. D) Cells were treated with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  
3  $\mu$ M) for 48 h, and total cellular RNA was extracted. Real-time PCR analysis was  
4 performed for quantification of BMPRII, ActRII, ALK-2, ALK-3, Smad6 and Smad7  
5 mRNA levels. The expression levels of target genes were standardized by RPL19  
6 level in each sample. E) Cells were treated with BMP-2, -4, -6 and -7 (100 ng/ml) for  
7 48 h, and total cellular RNA was extracted. Real-time PCR analysis was performed  
8 for quantification of PPAR $\alpha$  and PPAR $\gamma$  mRNA levels. The expression levels of target  
9 genes were standardized by RPL19 level in each sample. F) For protein analysis, cells  
10 were treated with BMP-4 (100 ng/ml) for 48 h and 72 h. The cells were then lysed and  
11 subjected to SDS-PAGE/immunoblot (IB) analysis using antibodies that detect PPAR $\alpha$   
12 and PPAR $\gamma$ , and actin as an internal control. Results (A-E) are shown as means  $\pm$   
13 SEM of data from at least three separate experiments, each performed with triplicate  
14 samples. The results (F) shown are representative of those obtained from three  
15 independent experiments. The results were analyzed by ANOVA with Fisher's post  
16 hoc test (A-E). For each result within a panel, \*,  $P < 0.05$  vs. control in each set of

1 comparisons or between the indicated groups; and the values with different superscript  
2 letters are significantly different at  $P < 0.05$ .

3

4 **Fig. 2. Effects of TNF- $\alpha$  and PPAR $\alpha$  and PPAR $\gamma$  agonists on BMP-induced**  
5 **osteoblast differentiation and TNF receptor (TNFR) expression in C2C12 cells. A,**

6 B) After preculture, the cells were treated with BMP-2, -4, -6 and -7 (100 ng/ml),  
7 TNF- $\alpha$  (10 ng/ml), and PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M) for 48 h. Total cellular  
8 RNA was extracted and subjected to PCR reaction. Real-time PCR analysis was  
9 performed for quantification of Runx2, osteocalcin, collagen-1 and ALP mRNA levels.

10 The expression levels of target genes were standardized by RPL19 level in each sample.

11 C) After preculture, the cells were pretreated with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M)

12 for 24 h prior to addition of BMP-2 (100 ng/ml) and TNF- $\alpha$  (100 ng/ml). After

13 60-min culture, the cells were lysed and subjected to SDS-PAGE/immunoblot (IB)

14 analysis using antibodies that detect phosphorylated Smad1/5/8 (pSmad1/5/8) and actin

15 as an internal control. The results shown are representative of those obtained from

16 three independent experiments. The relative integrated density of each protein band

1 was digitized by NIH image J 1.34s, pSmad1/5/8 levels were normalized by actin levels  
2 in each sample, and then pSmad1/5/8 levels after 60-min stimulation were expressed as  
3 fold changes. D) Cells were treated with BMP-4 (100 ng/ml) and TNF- $\alpha$  (10 ng/ml) in  
4 combination with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M) for 48 h and total RNA was  
5 extracted. Real-time PCR analysis was performed for the quantification of Id-1  
6 mRNA levels. The expression levels of target genes were standardized by RPL19  
7 level in each sample. E) Cells were treated with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M)  
8 for 48 h and total RNA was extracted. Real-time PCR analysis was performed for  
9 quantification of TNFR1 and TNFR2 mRNA levels. The expression levels of target  
10 genes were standardized by RPL19 level in each sample. F) For protein analysis, cells  
11 were treated with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M) for 48 h. The cells were then  
12 lysed and subjected to SDS-PAGE/immunoblot (IB) analysis using antibodies that  
13 detect TNFR1 and TNFR2, and actin as an internal control. The results shown are  
14 representative of those obtained from three independent experiments. Results (A-E)  
15 are shown as means  $\pm$  SEM of data from at least three separate experiments, each  
16 performed with triplicate samples. The results were analyzed by the unpaired *t*-test

1 (A) or ANOVA with Fisher's post hoc test (B-E). For each result within a panel, \*,  $P$   
2  $< 0.05$  vs. control in each set of comparisons; and the values with different superscript  
3 letters are significantly different at  $P < 0.05$ .

4

5 **Fig. 3. Effects of BMP-4 and PPAR $\alpha$  and PPAR $\gamma$  agonists on TNF- $\alpha$ -induced**  
6 **MAPK, NF $\kappa$ B, I $\kappa$ B, Stat and Akt activation in C2C12 cells.** A, B) After preculture,  
7 cells were treated with PPAR $\alpha$  and PPAR $\gamma$  agonists (3  $\mu$ M) for 24 h prior to addition of  
8 BMP-4 (100 ng/ml) and TNF- $\alpha$  (100 ng/ml). After 15- and 60-min culture, cells were  
9 lysed and subjected to SDS-PAGE/immunoblot (IB) analysis using anti-phospho- and  
10 anti-total-ERK1/ERK2 (pERK and tERK) antibodies, anti-phospho- and anti-total-p38  
11 (pP38 and tP38) antibodies, anti-phospho- and anti-total-SAPK/JNK (pJNK and tJNK)  
12 antibodies, anti-phospho- and anti-total-NF $\kappa$ B-p65 (pNF $\kappa$ B and tNF $\kappa$ B) antibodies,  
13 anti-phospho- and anti-total-I $\kappa$ B (pI $\kappa$ B and tI $\kappa$ B) antibodies, anti-phospho- and  
14 anti-total-Stat3 (pStat3 and tStat3) antibodies, and anti-phospho- and anti-total-Akt  
15 (pAkt and tAkt) antibodies. The results (A, B) shown are representative of those  
16 obtained from three independent experiments. The relative integrated density of each

1 protein band was digitized by NIH image J 1.34s and shown as phospho-/total-protein  
2 levels in each panel. Results (A, B) are shown as means  $\pm$  SEM of data from at least  
3 three separate experiments, each performed with triplicate samples. The results were  
4 analyzed by ANOVA with Fisher's post hoc test (A, B). For each result within a panel,  
5 the values with different superscript letters are significantly different at  $P < 0.05$ .

6

7 **Fig. 4. Inhibitory effects of MAPK, NF $\kappa$ B, Stat and Akt pathways on Runx2 and**  
8 **osteocalcin expression regulated by BMP-2 and TNF- $\alpha$ .** A, B) After preculture,  
9 cells were treated with BMP-4 (100 ng/ml) and TNF- $\alpha$  (10 ng/ml) in the presence or  
10 absence of indicated concentrations of the SAPK/JNK inhibitor SP600125, p38  
11 inhibitor SB203580, ERK inhibitor U0126, JNK/STAT inhibitor AG490, NF $\kappa$ B  
12 activation inhibitor IV and Akt inhibitor SH-5 for 48 h and total cellular RNA was  
13 extracted. Real-time PCR was performed for quantification of Runx2 and osteocalcin  
14 mRNA levels. The expression levels of target genes were standardized by RPL19  
15 level in each sample. Results (A, B) are shown as means  $\pm$  SEM of data from at least  
16 three separate experiments, each performed with triplicate samples. The results were

1 analyzed by ANOVA with Fisher's post hoc test (A, B). For each result within a panel,  
2 the values with different superscript letters are significantly different at  $P < 0.05$ .

3

4 **Fig. 5. Possible interaction of BMP-4, TNF- $\alpha$  and PPARs in the regulation of**

5 **osteoblast differentiation.** BMP-4 upregulates the expression of PPAR $\alpha$  and PPAR $\gamma$

6 in C2C12 cells. A PPAR $\alpha$  agonist, but not a PPAR $\gamma$  agonist, upregulates Runx2,

7 osteocalcin and collagen-1 expression induced by BMP-4 through downregulating

8 inhibitory Smads (Smad6/7) expression. On the other hand, the PPAR $\gamma$  agonist

9 suppressed BMP type-2 receptor (BMPRII) expression, leading to impairment of

10 BMP-Smad1/5/8 signaling. TNF- $\alpha$ -induced activation of MAPK, NF $\kappa$ B and Stat

11 pathways suppresses the BMP-4-induced osteoblast differentiation. PPAR $\alpha$  and

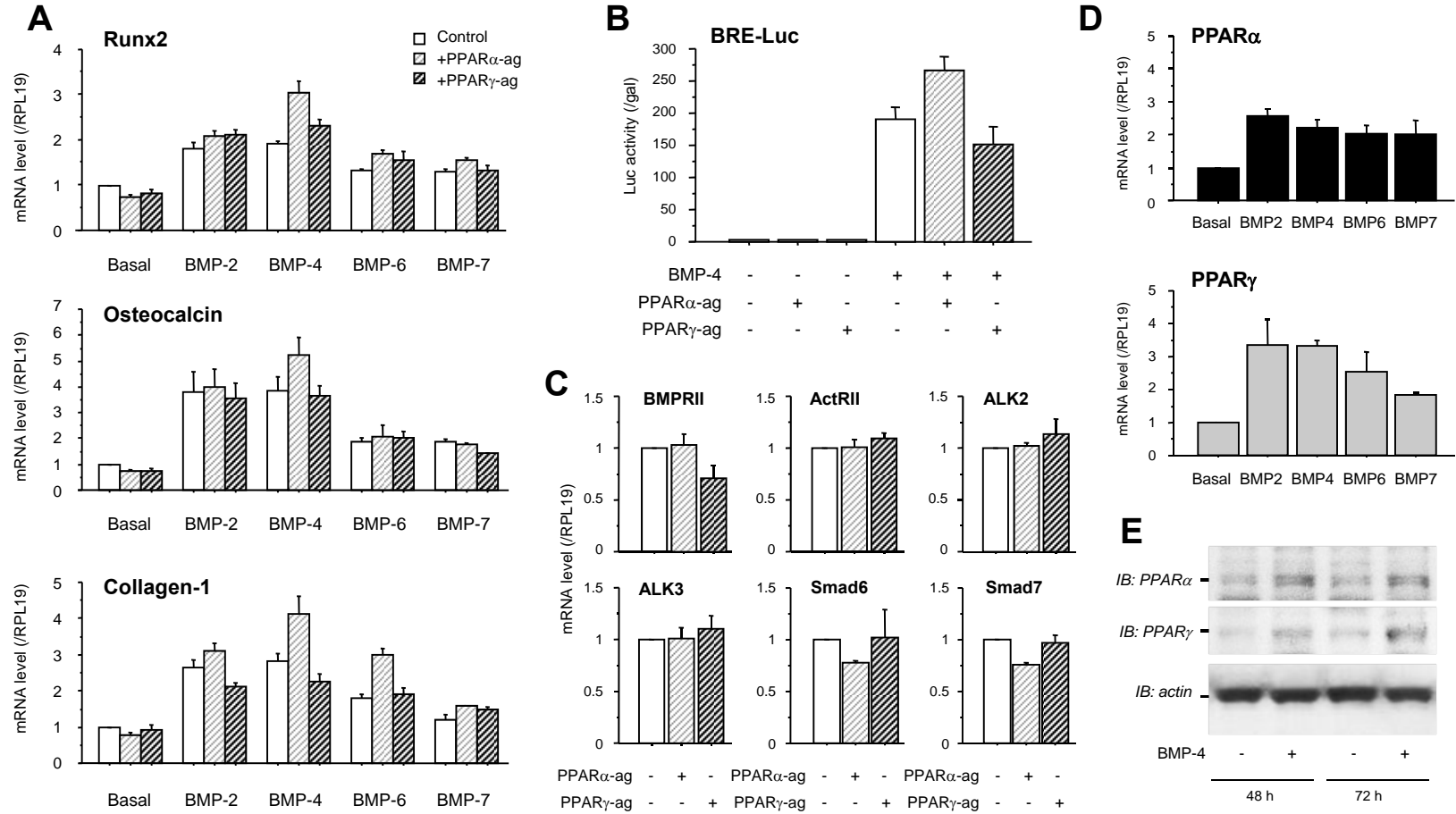
12 PPAR $\gamma$  agonists reversed suppression by TNF- $\alpha$  of BMP-4-induced osteoblast

13 differentiation through suppressing SAPK/JNK, NF $\kappa$ B and Stat signaling with reduction

14 of TNF receptor expression.

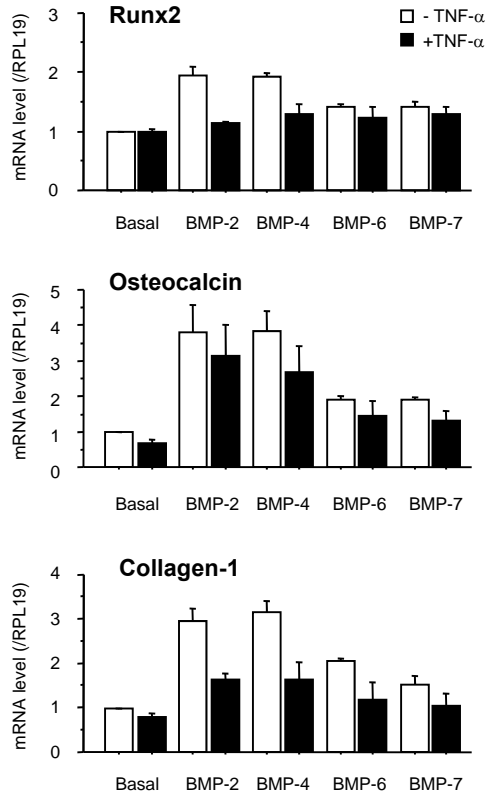
15

# Fig. 1

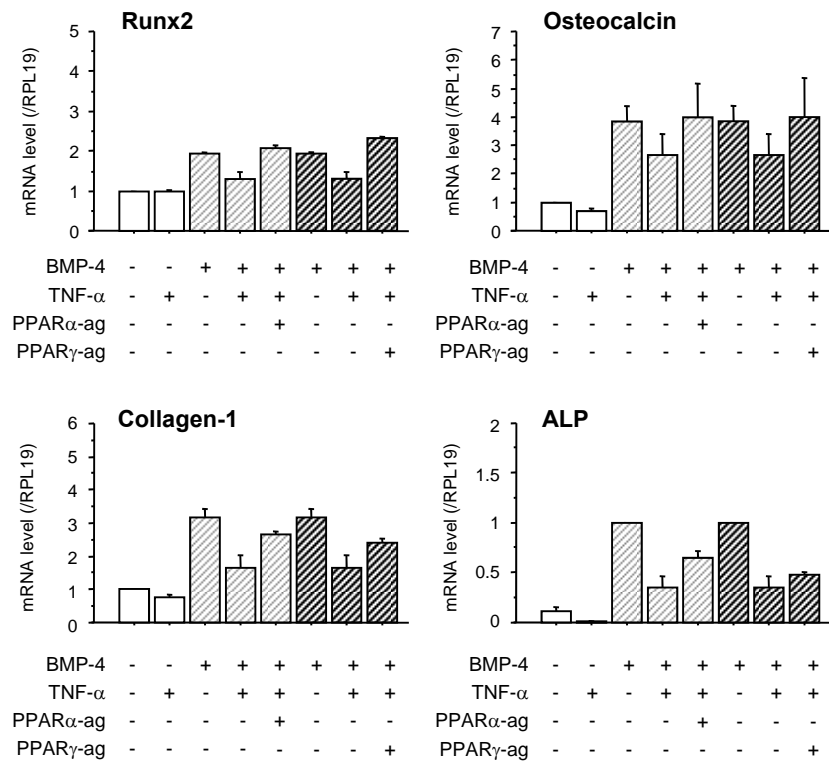


# Fig. 2

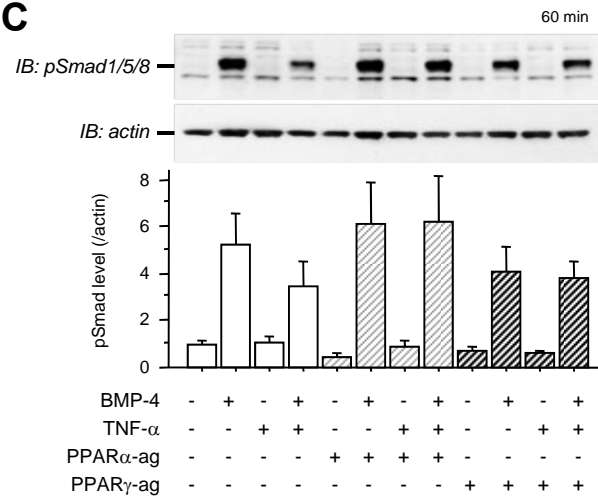
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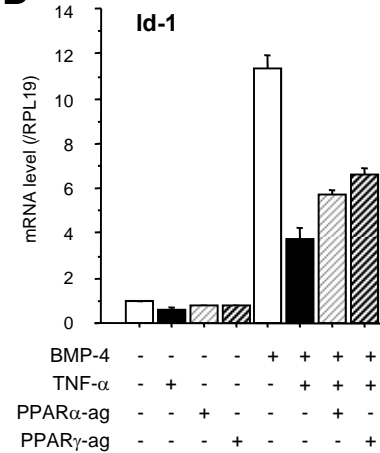
**B**



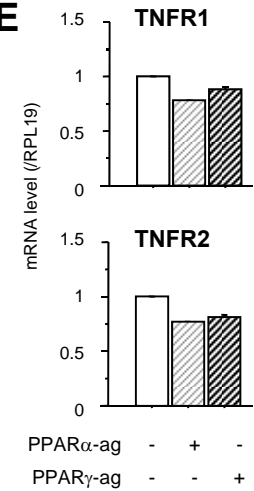
**C**



**D**

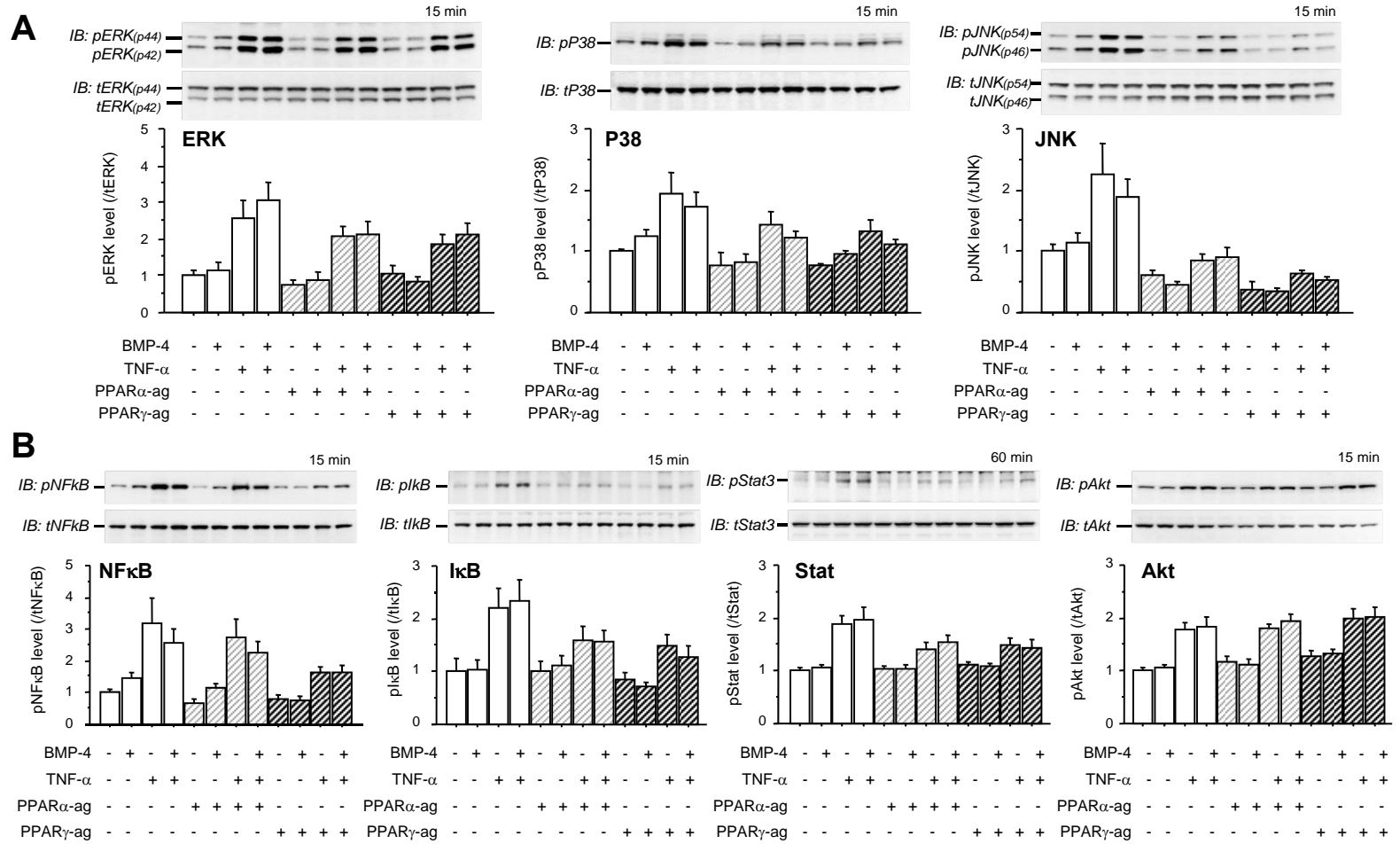


**E**

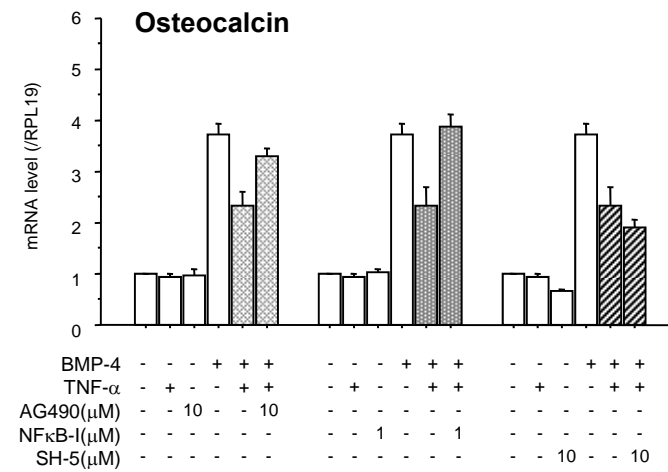
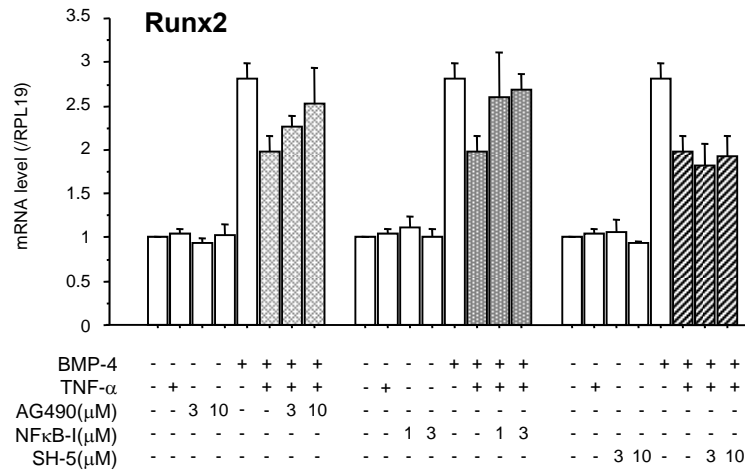
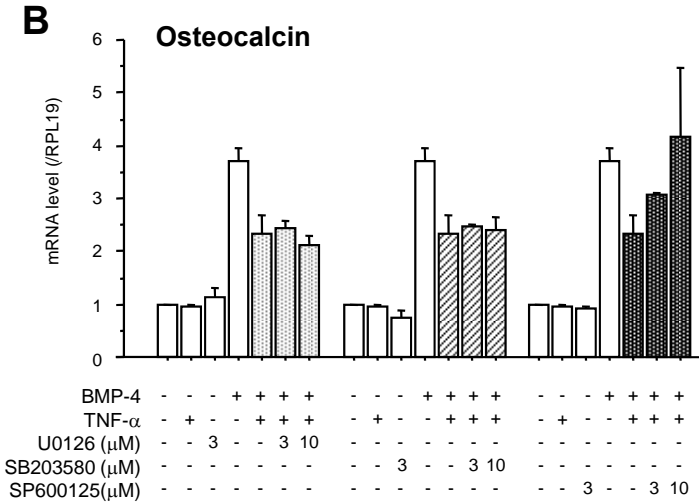
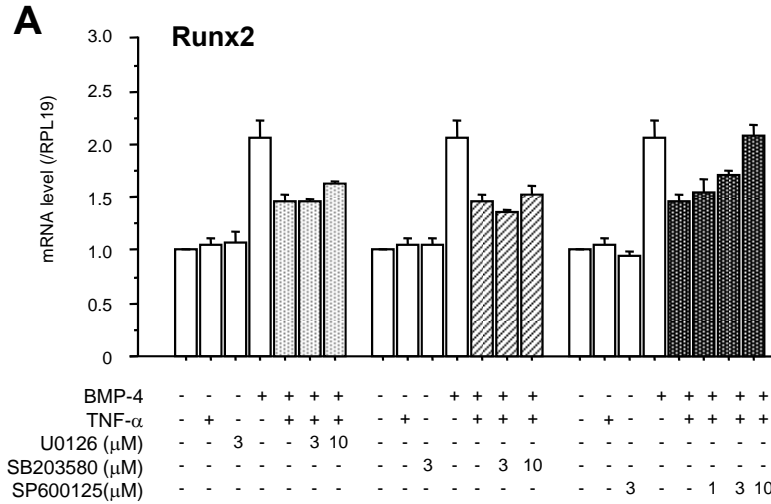




# Fig. 3



# Fig. 4



**Fig. 5**

