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ER stress-inducible ATF3 suppresses BMP2-induced ALP expression and activation in MC3T3-E1 cells



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ABSTRACT

Endoplasmic reticulum (ER) stress suppresses osteoblast differentiation. Activating transcription factor (ATF) 3, a member of the ATF/cAMP response element-binding protein family of transcription factors, is induced by various stimuli including cytokines, hormones, DNA damage, and ER stress. However, the role of ATF3 in osteoblast differentiation has not been elucidated. Treatment with tunicamycin (TM), an ER stress inducer, increased ATF3 expression in the preosteoblast cell line, MC3T3-E1. Overexpression of ATF3 inhibited bone morphogenetic protein 2-stimulated expression and activation of alkaline phosphatase (ALP), an osteogenic marker. In addition, suppression of ALP expression by TM treatment was rescued by silencing of ATF3 using shRNA. Taken together, these data indicate that ATF3 is a novel negative regulator of osteoblast differentiation by specifically suppressing ALP gene expression in preosteoblasts.

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1. Introduction

The endoplasmic reticulum (ER) has various functions including the synthesis, localization, folding, and secretion of proteins [1]. ER stress, which is included by ultraviolet light, viral infection and several micro-environmental stimuli [2], plays a key role in signal transduction that is important for metabolism, differentiation, and apoptosis of mammalian cells [3]. Tunicamycin (TM) induces ER

stress, the unfolded protein response, apoptosis, and cellular dysfunction [4,5]. A recent study demonstrated that TM blocks differentiation of osteoblasts by stimulating ER stress [6].

Osteoblasts are a type of mesenchymal cell and constitute bone along with chondrocytes, osteoclasts, and bone marrow stromal cells [7]. These cells express various markers, such as osteocalcin (OC), alkaline phosphatase (ALP), bone sialoprotein, osteonectin, collagen type 1, osteopontin and osteonectin [8,9]. Osteoblasts differentiate from bone precursor cells in response to various hormones, cytokines and transcription factors, to form mineralized bone [10,11]. Bone morphogenetic protein-2 (BMP2) stimulates bone formation by activating the Smad1/5/8 pathway and Runt-related transcription factor 2 (Runx2), which controls expression of osteogenic markers such as OC, bone sialoprotein, collagen type 1, and osteopontin [12–14]. BMP2 is a member of the transforming growth factor superfamily and plays important roles in various cellular functions, including signal transduction, development, cell growth, and repair of bone fractures [15–17]. A recent study reported that differentiation of osteoblasts is regulated by a type of ER stress that occurs via BMP2 induction [18].

Activating transcription factor (ATF) 3 is a member of the ATF/cAMP response element-binding protein (CREB) family of transcription factors and is induced by various signals including

Abbreviations: β -GP, β -glycerophosphate; AA, ascorbic acid 2-phosphate; AD-shATF3, anti-ATF3 shRNA; ALP, alkaline phosphatase; ATF, activating transcription factor; BMP2, bone morphogenetic protein-2; CREB, cAMP response element-binding; ER, endoplasmic reticulum; MOI, multiplicity of infection; OC, osteocalcin; TM, tunicamycin.

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growth factors, cytokines, hormones, hypoxia, DNA damage, and a variety of ER stresses [19,20]. Several recent studies suggested that ATF3 functions in general adaptive responses, such as environmental, emotional and nutritional alterations [21–23]. ATF3 is expressed in various cells and its dysfunction is closely related to various inflammatory diseases and cancer [20]. In addition, ATF3 is stimulated by the ER stress inducer, TM [24]. ATF3 plays key roles in atherosclerosis, hyperlipidemia [25,26], adipocyte differentiation [27], and chondrocyte differentiation [28]. However, it is not known whether ATF3 influences the differentiation of osteoblasts. Glucose metabolism is closely correlated with bone metabolism, and TM regulates differentiation of osteoblast.

This study investigated whether ATF3 regulates osteoblast differentiation. BMP2 treatment reduced ATF3 expression at the mRNA and protein levels, and overexpression of ATF3 negatively regulated BMP2-induced ALP gene expression. Furthermore, silencing of ATF3 rescued TM-induced suppression of osteoblast differentiation.

2. Materials and methods

2.1. Reagents

Recombinant human BMP2 protein was obtained from Cowell-medi Co. (Pusan, Korea). TRIzol reagent and Lipofectamine LTX were purchased from Invitrogen (Carlsbad, CA). Anti-ATF3 and anti- β -actin antibodies were purchased from Santa Cruz Biotechnology (CA). Ascorbic acid 2-phosphate (AA), β -glycerophosphate (β -GP), and TM were purchased from Sigma Aldrich Co. (St. Louis, MO).

2.2. Plasmids and adenovirus

The ALP-Luc (–1783 bp to +135 bp) reporter construct was kindly provided by Dr. Franceschi (University of Michigan). pcDNA3.1-ATF3 and –1850-ATF3-Luc [25] reporter construct were kindly provided by Dr. Kitajima (Gunma University). ATF3 shRNA was designed by iGENE, Ltd (Tsukuba, Japan).

2.3. Cell culture and transient transfection

MC3T3-E1 cells were cultured in α -minimal essential medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) in humidified air containing 5% CO₂ at 37 °C. Cells were transiently transfected with the indicated plasmid DNA using Lipofectamine LTX. The dual-luciferase system (Promega) was used to measure promoter activity. The firefly luciferase signal was normalized to the Renilla luciferase signal. Luminescence was detected using a Wallac 1420 VICTOR2 microplate reader (Perkin Elmer, USA). For viral infection, cells were treated with AD-shATF3 at the designated multiplicity of infection (MOI) in 2% serum. After 4 h, an equivalent volume of medium containing 10% fetal bovine serum was added, and cells were incubated for an additional 24–48 h.

2.4. RT-PCR analysis

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions. Reverse transcription was performed using 2 μ g total RNA. PCR conditions were as follows: initial denaturation of 95 °C for 5 min; 22–36 cycles of 94 °C for 30 s, the optimum annealing temperature for 30 s, and 72 °C for 30 s; followed by a final extension of 72 °C for 5 min. The primer sequences were as follows: ATF3 forward, 5'-TTCGCCATCCAGAATAAACAC-3' and reverse, 5'-TTTCTGCAGGCACTGTCTT-3'; ALP forward, 5'-CTGCCT

ACTTGTGTGGCGTGA-3' and reverse, 5'-CCACCCATGATCACGTG-CATA-3'; OC forward, 5'-CTCCTGAGTCTGACAAAGCCTT-3' and reverse, 5'-GCTGTGACATCCATTACTTGC-3'; GAPDH forward, 5'-AC CACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCTGTGCT GTA-3'. Quantitative real-time PCR was performed using the TOP-real qPCR SYBR kit and a Rotor-Gene Q thermal cycler (Qiagen, USA). The thermal cycling conditions were as follows: initial denaturation of 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s.

2.5. ALP staining

Cells were fixed in 70% ethanol for 30 min, and then rinsed three times with deionized water. Fixed cells were treated with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP®/NBT) solution (Sigma Aldrich Co.) for 30 min.

2.6. Statistical analysis

All experiments were repeated at least three times, and statistical analysis was performed using a Student's *t*-test or analysis of variance followed by Duncan's multiple comparison tests. A *P*-value < 0.05 was considered significant. Results are expressed as mean \pm SD of triplicate independent samples.

3. Results

3.1. ATF3 expression decreases in MC3T3-E1 cells cultured in osteogenic conditions

BMP2 increases the expression levels of ER stress markers such as Bip, C/EBP homologous protein, ATF4, and ATF6 [17,18]. ATF3 is involved in various cellular functions and is induced by ER stress [20]. To identify the role of ATF3 in osteoblast differentiation, we first examined ATF3 expression in MC3T3-E1 cells treated with BMP2. RT-PCR analysis revealed that BMP2 reduced the mRNA level of ATF3, but increased the mRNA levels of ALP and OC, which are osteoblast differentiation markers (Fig. 1A). Furthermore, quantitative real-time PCR revealed that these changes in mRNA levels occurred in a time dependent manner (Fig. 1B and C). BMP2 treatment also reduced the protein level of ATF3 (Fig. 1D). As an alternative way of inducing osteoblast differentiation, cells were treated with AA and β -GP following which the mRNA level of ATF3 was reduced and the mRNA levels of ALP and OC were increased (Fig. 1E). Real-time PCR and western blot revealed that these changes occurred (Fig. 1F–H). To confirm that BMP2 and AA/ β -GP suppress ATF3 transcription, a transient transfection assay was performed using a luciferase reporter containing the promoter of the ATF3 gene. Consistently, both BMP2 and AA/ β -GP decreased TM-induced promoter activity of the ATF3 (Fig. 1I and J). Taken together, ATF3 is significantly reduced by treatment with osteogenesis inducing factors such as BMP2 and AA/ β -GP in MC3T3-E1 cells.

3.2. TM-induced ER stress inhibits BMP2-induced osteoblast differentiation and increases ATF3 expression

Our previous study showed that ER-stressor TM suppressed BMP2-induced osteoblast differentiation, and activates ATF6 [6,17]. Therefore, we examined whether ATF3 expression is regulated by ER stress in MC3T3-E1 cells. TM, which potentially induces ER stress, significantly inhibited BMP2-induced ALP gene expression in a dose-dependent manner (Fig. 2A). TM treatment also reduced ALP staining, an indicator of ALP activity, in MC3T3-E1 cells in a dose-dependent manner (Fig. 2B). However, TM treatment

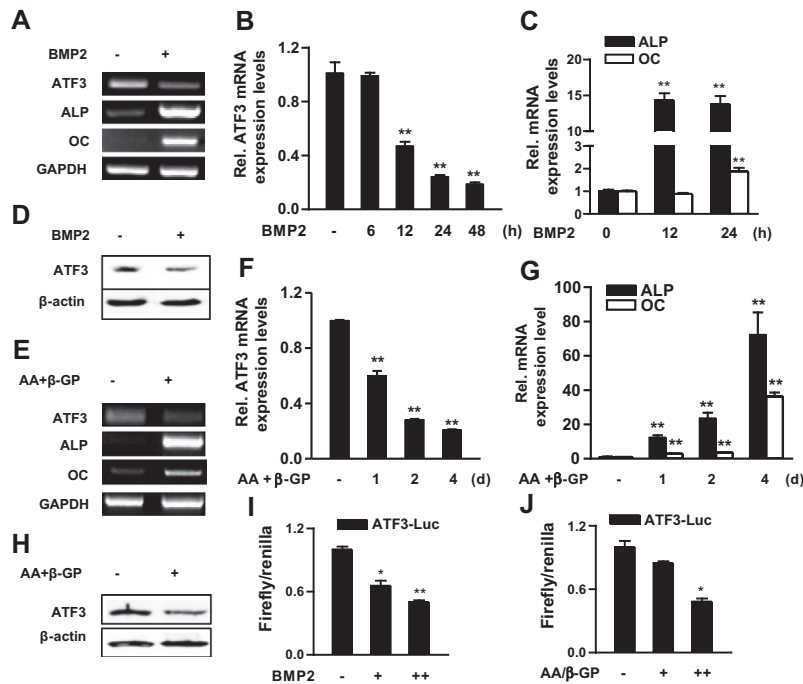


Fig. 1. ATF3 expression in MC3T3-E1 cells is reduced by BMP2- and AA/β-GP-induced osteogenic differentiation. (A) RT-PCR analysis of ATF3, ALP, and OC mRNA levels in MC3T3-E1 cells cultured with BMP2 (200 ng/ml) for 2 days. (B, C) Real-time PCR analysis of ATF3 (B), ALP and OC mRNA levels in MC3T3-E1 cells cultured with BMP2 (200 ng/ml) for various amounts of time. (D) Western blot analysis of the ATF3 protein level in MC3T3-E1 cells cultured with BMP2 (200 ng/ml) for 2 days. (E) RT-PCR analysis of ATF3, ALP, and OC mRNA levels in MC3T3-E1 cells cultured with AA (50 μg/ml) and β-GP (10 mM) for 2 days. (F, G) Real-time PCR analysis of ATF3 (F), ALP and OC (G) mRNA levels in MC3T3-E1 cells cultured with AA (50 μg/ml) and β-GP (10 mM) for up to 4 days. (H) Western blot analysis of the ATF3 protein level in MC3T3-E1 cells cultured with AA (50 μg/ml) and β-GP (10 mM) for 2 days. (I, J) Luciferase activity of the ATF3 gene promoter in MC3T3-E1 cells treated with BMP2 (+, 200 ng/ml; ++, 400 ng/ml) (I) or AA/β-GP (+, AA: 50 μg/ml β-GP: 10 mM; ++, AA: 100 μg/ml β-GP: 20 mM) (J). Graphs represent mean values plus S.D. of $n = 2$ experiments $^{**}P < 0.01$.

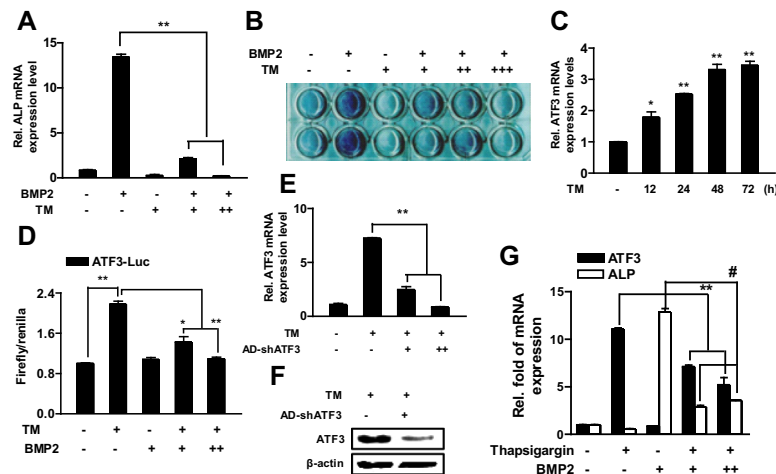


Fig. 2. ATF3 is involved in the inhibition of BMP2-induced ALP expression by TM. (A) Real-time PCR analysis of the ALP mRNA level in MC3T3-E1 cells cultured with BMP2 (200 ng/ml) and TM (+, 0.2 μg/ml; ++, 0.4 μg/ml) for 2 days. (B) ALP staining in MC3T3-E1 cells treated with BMP2 (200 ng/ml) and TM (+, 0.2 μg/ml; ++, 0.4 μg/ml; +++, 0.8 μg/ml) for 2 days. (C) Real-time PCR analysis of the ATF3 mRNA level in MC3T3-E1 cells treated with TM (0.2 μg/ml) for up to 72 h. (D) Luciferase activity of the ATF3 gene promoter in MC3T3-E1 cells treated with TM (0.2 μg/ml) and BMP2 (+, 200 ng/ml; ++, 400 ng/ml) for 48 h. (E) Real-time PCR analysis of the ATF3 mRNA level in MC3T3-E1 cells treated with TM (0.2 μg/ml) in the presence or absence of AD-shATF3 (+, 50 MOI; ++, 100 MOI) for 2 days. (F) Western blot analysis of the ATF3 protein level in MC3T3-E1 cells treated with TM (0.2 μg/ml) and AD-shATF3 (50 MOI) for 2 days. (G) Real-time PCR analysis of the ATF3 and ALP mRNA level in MC3T3-E1 cells treated with thapsigargin (10 nM) and BMP2 (+, 200 ng/ml; ++, 400 ng/ml) for 24 h. Graphs represent mean values plus SD of $n = 2$ experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{#}P < 0.01$.

significantly increased the mRNA level of ATF3 in a time-dependent manner (Fig. 2C). To confirm that TM stimulates ATF3 transcription, a transient transfection assay was performed using a luciferase reporter containing the promoter of the ATF3 gene. Consistently, TM significantly increased the promoter activity of the ATF3 gene, and BMP2 decreased TM-induced ATF3 promoter activ-

ity (Fig. 2D). To determine whether TM directly affects ATF3 expression, we examined the mRNA level of ATF3 following TM and AD-shATF3 treatment. AD-shATF3 significantly reduced the TM-induced mRNA level of ATF3 (Fig. 2E). This result was confirmed at the protein level (Fig. 2F). In addition, we examined whether thapsigargin, other ER-stress inducer, regulated ATF3

expression and osteoblast differentiation. Real-time PCR showed that thapsigargin dramatically increased ATF3 mRNA but decreased BMP2-induced ALP expression (Fig. 2G). Taken together, these results suggest that the ER-stress inducer TM and thapsigargin increases ATF3 expression and prevents BMP2-induced ALP gene expression

3.3. ATF3 inhibits BMP2-induced ALP expression and activation

To understand the role of ATF3 in osteoblast differentiation, we overexpressed ATF3 in MC3T3-E1 cells and then examined the expression of osteoblast differentiation markers. RT-PCR analysis revealed that ATF3 overexpression reduced BMP2-induced ALP expression (Fig. 3A). Real-time PCR revealed that ATF3 overexpression significantly reduced the BMP2-induced ALP mRNA level (Fig. 3B). However, ATF3 overexpression did not affect BMP2-induced OC expression, suggesting that OC is not a target of ATF3. To examine whether ATF3 affects transcription of ALP, a transient transfection assay was performed using a luciferase reporter construct containing the promoter of the ALP gene. ATF3 significantly reduced the BMP2-induced promoter activity of the ALP gene in a dose-dependent manner (Fig. 3C). ALP staining revealed that ATF3 overexpression reduced BMP2-induced ALP activity in a dose-dependent manner (Fig. 3D). These results demonstrate that ATF3 suppresses BMP2-induced ALP expression and activation.

3.4. Silencing of ATF3 expression rescues TM-induced suppression of osteoblast differentiation

To investigate whether silencing of ATF3 expression rescues the TM-induced reduction in ALP expression, MC3T3-E1 cells were infected with AD-shATF3 in the presence or absence of TM, together with BMP2. AD-shATF3 significantly rescued the TM-induced reduction in the mRNA level of ALP (Fig. 4A). Furthermore,

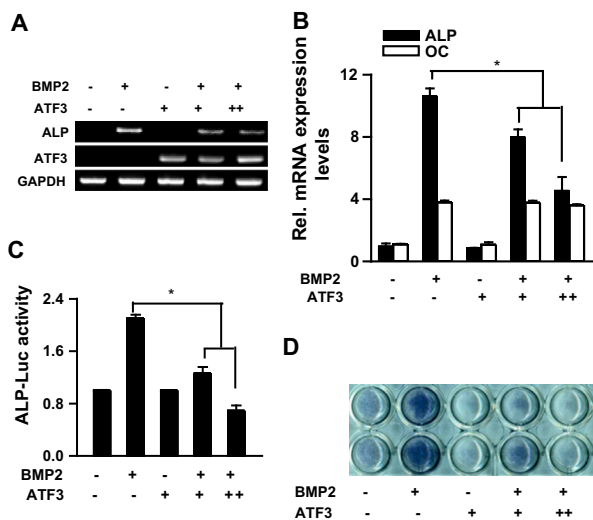


Fig. 3. ATF3 inhibits BMP2-induced ALP expression, but not OC expression. (A, B) MC3T3-E1 cells were transfected with pcDNA3.1-ATF3 (+, 200 ng/well; ++, 400 ng/well), and were then incubated in the absence or presence of BMP2 (200 ng/ml) for 2 days. (A) RT-PCR analysis of ALP and OC mRNA levels. (B) Real-time PCR analysis of ALP and OC mRNA levels. (C) MC3T3-E1 cells were transfected with an ALP-Luc reporter construct with or without pcDNA3.1-ATF3 (+, 200 ng/well; ++, 400 ng/well), and were then incubated in the absence or presence of BMP2 (200 ng/ml) for 2 days. ALP gene promoter activity was measured using a dual-luciferase assay. (D) ALP staining of MC3T3-E1 cells transfected with pcDNA3.1-ATF3 (+, 200 ng/well; ++, 400 ng/well) and then incubated in the absence or presence of BMP2 (200 ng/ml) for 2 days. Graphs represent mean values plus SD of $n = 2$ experiments. * $P < 0.05$.

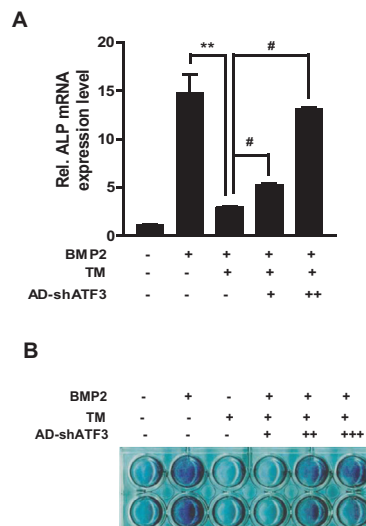


Fig. 4. Silencing of ATF3 expression rescues TM-induced suppression of osteoblast differentiation. (A) Real-time PCR analysis of the ALP mRNA level in MC3T3-E1 cells treated with BMP2 (200 ng/ml), TM (0.2 µg/ml), and AD-shATF3 (+, 50 MOI; ++, 100 MOI) for 2 days. (B) ALP staining of MC3T3-E1 cells treated with AD-shATF3 (+, 50 MOI; ++, 100 MOI; +++, 150 MOI), BMP2 (200 ng/ml), and TM (0.2 µg/ml) for 2 days. Graph represents mean values plus SD of $n = 2$ experiments. ** $P < 0.01$, # $P < 0.01$.

AD-shATF3 rescued the TM-induced reduction in ALP staining (Fig. 4B). These results indicate that silencing of ATF3 rescues TM-induced reduction in ALP expression and activation.

4. Discussion

In this study, ATF3 expression was increased by TM but reduced by BMP2 in MC3T3-E1 cells. Treatment with AA and β -GP, which also induces osteogenesis, reduced the level of endogenous ATF3. When MC3T3-E1 cells were cultured in osteogenic conditions, overexpression of ATF3 suppressed the expression and activation of ALP. In addition, TM-induced suppression of ALP expression and activation was rescued by silencing of ATF3. These data indicate that ATF3 plays a critical role in osteogenic differentiation by regulating the expression and activation of ALP.

BMP2 strongly induces bone formation and osteoblast differentiation [14,29]. BMP2 dramatically increases expression of osteogenic marker genes, such as those encoding ALP, osteonin, bone sialoprotein and OC [17,29–31]. BMP2 induces mild ER stress and stimulates osteoblast differentiation by regulating expression of osteogenic genes. In this study, mRNA and protein levels of ATF3 were suppressed during BMP2-induced osteoblast differentiation.

ATF3 is a CREB protein and is induced by several cellular stresses, including ER stress [4,5,19,26]. ATF3 regulates various cellular functions including chondrogenic differentiation [28], expression of cyclin D1 in chondrocytes [32], androgen receptor function in prostate cancer [33], and adipocyte differentiation [27]. The induction of ATF3 expression by ER stress is relevant to these effects. We previously reported that the ER stress inducer, TM, inhibits differentiation of osteoblasts. In addition, chondrocytes and osteoblasts are highly coordinated during bone formation. Therefore, we hypothesized that induction of ATF3 expression by TM can regulate osteoblast differentiation. As expected, TM-induced ATF3 dramatically reduced BMP2-induced expression and activation of ALP in MC3T3-E1 cells. Moreover, silencing of ATF3 rescued the TM-induced reduction in ALP expression. These data suggest that ATF3 can regulate osteoblast differentiation by suppressing ALP expression and activation. Interestingly, ATF3 did not regulate BMP2-induced OC expression. Previously, our study reported that, BMP2 stimulates osteoblast differentiation through Runx2-dependent

ATF6 expression, which directly regulated OC expression. On the contrary, this study showed that ATF3 expression was repressed by BMP2 and negatively regulates osteoblast differentiation via suppressing BMP2-induced ALP expression. These results suggest that even though both ATF3 and ATF6 were activated by ER stress, but their roles were diametrically opposed in osteoblast differentiation. In MC3T3-E1 cells, ATF3 slightly reduces FGF2/FSK-induced OC promoter activity, but does not affect FSK-induced OC promoter activity [34]. Another study suggested that ATF3 indirectly affects Runt-related transcription factor 2 activity, which is a master regulator of OC, via suppressing cyclin D1 in chondrocytes [28]; however, this study did not examine that ATF3 regulates OC expression. Therefore, we suggest that ATF3 specifically regulates expression and activation of ALP in preosteoblasts.

ALP catalyses the hydrolysis of phosphomonoesters and is expressed in various cells [35]. ALP is highly expressed and has critical roles in bone and hard tissue formation [36]. A mutation in the ALP gene gives rise to the genetic disease hypophosphatasia, which is characterized by the undermineralisation of bone [37]. High levels of ALP leads to the mineralisation of neotissue, and ALP is detected during the early stage of osteoblast differentiation [38,39]. Therefore, we suggest that ATF3 can suppress osteoblast differentiation and bone formation via regulating ALP expression and activation. In promoter analysis of ALP gene, our study showed that ATF3 inhibited ALP transcription. Therefore, we investigated whether the ALP promoter contained ATF3 binding sequence motifs 5'-TGACGT(A, C)(A, G)-3' [40]. As a result, ALP promoter had two candidate binding motifs (TGAGGTCA) at -1196 to -1189 and (TGACGGA) at -422 to -416. However, we needed to further confirm the molecular mechanism of inhibiting ALP transcription by ATF3.

In conclusion, ATF3 expression was increased by TM treatment and reduced during BMP2- and AA/ β -GP-induced osteogenic differentiation. ATF3 specifically suppressed ALP expression and activation in preosteoblast MC3T3-E1 cells.

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Conflict of interest

None of the authors have a conflict of interest.

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