BMP2 Protein Regulates Osteocalcin Expression via Runx2-mediated Atf6 Gene Transcription*

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Background: BMP2 activates UPR transducers during osteoblast differentiation. Results: BMP2 signaling increased ATF6 expression and cleavage, and activated ATF6 increased OC expression. Conclusion: BMP2 induced osteoblast differentiation through Runx2-dependent ATF6 expression, which directly regulates OC transcription. Significance: BMP2-induced mild ER stress positively regulates osteoblast differentiation via activation of UPR transducers, including ATF6.

Bone morphogenetic protein 2 (BMP2) activates unfolded protein response (UPR) transducers, such as PERK and OASIS, in osteoblast cells. ATF6, a bZIP transcription factor, is also a UPR transducer. However, the involvement of ATF6 in BMP2-induced osteoblast differentiation has not yet been elucidated. In the present study, BMP2 treatment was shown to markedly induce the expression and activation of ATF6 with an increase in alkaline phosphatase (ALP) and OC expression in MC3T3E1 cells. In contrast, ATF6 activation by BMP2 was not observed in the Runx2−/− primary calvarial osteoblasts, and Runx2 overexpression recovered BMP2 action. BMP2 stimulated ATF6 transcription by enhancing the direct binding of Runx2 to the osteoblast-specific cis-acting element 2 (OSE2, ACCACA, −205 to −200 bp) motif of the Atf6 promoter region. In addition, the overexpression of ATF6 increased the Oc promoter activity by enhancing the direct binding to a putative ATF6 binding motif (TGACGT, −1126 to −1121 bp). The inhibition of ATF6 function with the dominant negative form of ATF6 (DN-ATF6) blocked BMP2- or Runx2-induced OC expression. Interestingly, OASIS, which is structurally similar to ATF6, did not induce Oc expression. ALP and Alizarin red staining results confirmed that BMP2-induced matrix mineralization was also dependent on ATF6 in vitro. Overall, these results suggest that BMP2 induces osteoblast differentiation through Runx2-dependent ATF6 expression, which directly regulates Oc transcription.

Osteoblast differentiation is tightly regulated by a range of hormones, cytokines, and multiple transcription factors (1, 2). Bone morphogenetic protein 2 (BMP2) is one of the most important cytokines in this regard and plays several important roles in a variety of cellular functions ranging from embryogenesis, cell growth, and differentiation to bone development and the repair of bone fractures (3, 4). BMP2 exhibits this osteogenic action by activating Smad1/5/8 signaling and regulating the transcription of osteogenic genes, including distal-less homeobox 5 (Dlx5), which is a key mediator of BMP2-induced expression of Runx-related transcription factor 2 (Runx2) (5, 6). Runx2 regulates the expression of several osteoblastic genes, including collagen type 1, osteopontin, osteocalcin (OC), and bone sialoprotein (7, 8), by binding to the osteoblast-specific cis-acting element 2 (OSE2; ACCACA) (9). Recently, another BMP2 signaling pathway in osteoblasts, mediated by the unfolded protein response (UPR) of endoplasmic reticulum (ER) stress, was introduced by Murakami et al. (10). For example, the expression levels of the ER stress markers, IgH chain-binding protein (BIP), C/EBP homologous protein (CHOP),

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activating transcription factor 4 (ATF4), and ER degradation-enhancing α-mannosidase-like protein (EDEM), were up-regulated by BMP2 stimulation (10).

PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6) have been studied as the major transducers of UPR (11–15). PERK leads to phosphorylation of the α-subunit of the eukaryotic inactivation factor 2 (eIF2α), enhancing ATF4 translation and inhibiting global protein synthesis. ATF4-deficient mice exhibited a marked decrease or delay in bone mineralization, including frontal and parietal bones, clavicles, and long bones (16). The old osteocyte specifically induced substance (OASIS), another UPR transducer, is an ER membrane-bound bZIP (basic leucine zipper) transcription factor (17, 18). OASIS−/− mice exhibited severe osteopenia, involving a decrease in type I collagen in the bone matrix (10).

ATF6 is also an ER membrane-bound bZIP transcription factor, of which the structure and mode of action is similar to OASIS. ATF6 is also cleaved by regulated intramembrane proteolysis in response to ER stress, and its N-terminal fragment, including bZIP and transcriptional activation domains, moves to the nucleus to activate target gene expression via a consensus DNA binding site, TGACGTG (19–21). However, the role of ATF6 in osteoblast differentiation has not yet been elucidated. This study demonstrates for the first time that BMP2-induced osteoblast differentiation mediates mild ER stress-activated ATF6 and directly regulates OC expression.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human BMP2 peptide was obtained from R&D Systems (Minneapolis, MN). The antibody specific to ATF6 was supplied by ABCam (Cambridge, UK). The antibodies against Runx2 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Plasmids and Adenoviruses—The reporter construct containing the mouse osteocalcin promoter (OG2-Luc) was kindly provided by Dr. Franceschi (University of Michigan School of Dentistry, Ann Arbor, MI). The full-length and nuclear forms of the ATF6 plasmid were kindly provided by Dr. Ron Prywes (Department of Biological Science, Columbia University, New York). The DN-ATF6 was constructed by PCR amplification of the bZIP domain of ATF6 (21). Adenovirus (Ad) encoding the nuclear form of ATF6 (Ad-ATF6) and Ad-DN-ATF6 were constructed using methods described previously (22).

The mouse Atf6 promoter was PCR-amplified from mouse genomic DNA and inserted into the pGL3 basic vector using the SacI and Xhol restriction enzyme sites. For the translocation of ATF6 into the nucleus, the full-length and nuclear forms of Atf6 were subcloned in the pcDNA3/Gal4 vector using EcoRV and XbaI restriction sites. For DNA binding analysis, the point mutant form of the Atf6-Luc and OG2-Luc reporters were constructed using the site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) and the following primers: Atf6, 5′-CACAACAGACACACAAAAACCCACC-3′ (forward) and 5′-GGCTGATGTCTGACGGTTC-3′ (reverse); OG2, 5′-TCGATGTCAGAAGTGGTAC-3′ (forward) and 5′-GAGTACGTTGACACACTT-3′ (reverse). The primer sequences used for the 5′ deletion construct from OG2-Luc to Del-OG2-Luc were as follows: 5′-AGGTTATGAGCCACACACACACACACG-3′ (forward) and 5′-TCAATGATCTGAGTGAAGGGAATTACTAC-3′ (reverse). All constructs were confirmed by DNA sequence analysis.

Cell Culture, Transient Transfection Assays, and Viral Infection—MC3T3E1 (mouse calvarial preosteoblast) cells and Runx2-deficient primary calvarial osteoblast cells were cultured in α-minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics in a humidified atmosphere containing 5% CO2 at 37 °C. Transient transfections were performed as described previously (6). For viral infection, the cells were treated with the indicated viruses at the designated multiplicity of infection (MOI) under serum-free conditions. After 4 h, an equivalent volume of medium containing 10% FBS was added, and the cells were incubated for an additional 24–48 h.

Silencing of ATF6—The siRNAs for Atf6 (siATF6-I and -II) were synthesized chemically (ST Pharm, Siheung, Korea), deprotected, annealed, and transfected according to the manufacturer’s instructions. The MC3T3E1 cells were transfected with the siATF6-I and siATF6-II using Lipofectamine 2000 (Invitrogen). The sequences of siRNA were as follows: siATF6-I sense, 5′-GAGCCAUCUUCUUUAUAGGdtdTdT3′; siATF6-II sense, 5′-GAAGAAGAAGAAGAAGUGdtddTdT3′; scrambled sense, 5′-GUUACCCCGUCGCCTGAGGdtdTdT3′.

RT-PCR Analysis—Total RNA was isolated from the cultures using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RT-PCR was performed using 0.8 μg of the total RNA. Each reaction consisted of initial denaturation at 94 °C for 1 min followed by three-step cycling: denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, and extension at 72 °C for 30 s. After the required number of cycles (25–30 cycles), the reactions underwent a final extension at 72 °C for 5 min. The primer sequences were as follows: Atf6, 5′-GGATTGTAGCCTTGGAGATCA-GAC-3′ (forward) and 5′-ATTTTTTCTTTGAGTCCGATCT-CTAC-3′ (reverse); Oc, 5′-CTCTGAGAGTCGCAAGAGCCTT-3′ (forward) and 5′-GCTGTGACATCCATTACTTGC-3′ (reverse); Alp, 5′-GATCATTTCCACGTTCAC-3′ (forward) and 5′-TGCGGGCTTGTGGGACCTGC-3′ (reverse); Runx2, 5′-GAGGCCAAATGGTCTAC-3′ (forward) and 5′-CGCTCCGCGCCACCACATCTC-3′ (reverse); Colla1, 5′-CCCACACCCTGGAACAGAC-3′ (forward) and 5′-GGTCACAGTGGTGGTAC-3′ (reverse); β-actin, 5′-TTCTTGTCCAGCTCCTTGCGG-3′ (forward) and 5′-TGGATGGTACATGGTCCTG-3′ (reverse).

Isolation of Nuclear and Cytoplasmic Protein—MC3T3E1 cells were cultured in the presence of BMP2 for 4 days. The nuclear and cytoplasmic protein fractions were prepared using a Thermo Scientific NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer’s instructions.

Alkaline Phosphatase (ALP) Staining, Alizarin Red Staining, and OC Production Assay—For mineralization analysis, MC3T3E1 and Runx2−/− primary osteoblasts were cultured with ascorbic acid (50 μg/ml), β-glycerophosphate (2 mM), BMP2 (200 ng/ml), and the indicated viruses for 4 days. Alizarin
red and ALP staining was performed using the standard protocols. Briefly, the cultured cells were fixed with 70% ethanol, rinsed three times with deionized water, and treated with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (Sigma-Aldrich) or 40 mM Alizarin red stain solution (pH 4.2) for 15 min, respectively. The stained cultures were then photographed. The level of OC secreted into the culture medium was measured using a mouse osteocalcin ELISA kit (Biomedical Technologies Inc., Stoughton, MA) according to the manufacturer’s instructions.

Chromatin Immunoprecipitation (ChIP) Assay—The MC3T3E1 cells were treated with BMP2 for the designated times. ChIP assays were performed as described previously (23). The DNA samples were quantified by PCR using two pairs of primers. The primer sequences for the Runx2 binding region of the Atf6 promoter were as follows: 5′- CGGTAAAATCTTGGCTGCTCT-3′ (forward) and 5′-TTCCCCATCGAGATTCTTCAAGCA-3′ (reverse). The primer control sequences for the Atf6 gene were as follows: 5′-TTCCAGTGGAATCCGTGA-3′ (forward) and 5′-TCCCCAGTCTTAACTTTCC-3′ (reverse). The primer sequences for the Atf6 binding region of the Oc promoter were as follows: 5′-GATGCCCTTCTTGTGTTGGTCTT-3′ (forward) and 5′-TCCCCAGTCTTAACTTTCC-3′ (reverse). The control primer sequences for the Oc gene were as follows: 5′-GGATGTCTCTCAACACAAAGAGCA-3′ (forward) and 5′-GAAGAGCCCTAGGCAATTGTG-3′ (reverse).

Statistical Analysis—The results were expressed as the mean ± S.E. of three independent measurements. All experiments were repeated at least three times, and statistical analysis was performed using Student’s t test or analysis of variance analyses followed by Duncan’s multiple-comparison test. p values of <0.05 were considered significant.

RESULTS

BMP2 Induces ATf6 Gene Expression and Protein Cleavage—This study examined whether ATf6, one of the ER-bound transcriptional factors in response to ER stress, is also involved in BMP2-induced osteoblast differentiation in MC3T3E1 cells. RT-PCR analysis showed that BMP2 significantly increased Atf6 mRNA expression with Alp and Oc genes, which are markers of the matured osteoblast phenotype, in a time-dependent manner (Fig. 1A). The expression levels of Atf6 and Oasis were examined by real-time PCR. BMP2 induced more Atf6 expression than Oasis (Fig. 1B). Western blot analysis also showed that BMP2 increased the level of ATf6 protein expression in a time-dependent manner. Interestingly, the cleaved form (nuclear form) of ATf6 was also increased by the BMP2 treatment, as well as by a treatment with tunicamycin, a potent ER stress-inducing agent (Fig. 1C). To further confirm whether BMP2 stimulates Atf6 transcription, a transient transfection assay was performed using a luciferase reporter containing the Atf6 gene promoter. Consistently, BMP2 also increased the promoter activity of Atf6 in a dose-dependent manner (Fig. 1D). Overall, these results suggest that ATf6 plays a role in BMP2-induced osteoblast differentiation.

Runx2 Directly Regulates Atf6 Gene Transcription—To determine how BMP2 induces Atf6 expression, this study examined the effects of Runx2, downstream of the BMP2 signal, on Atf6 expression. Runx2 overexpression increased Atf6 expression significantly in a dose-dependent manner (Fig. 2A), which is similar to the BMP2 treatment experiments shown in Fig. 1A. In addition, the full-length ATf6 protein expression was increased by Runx2 overexpression. On the other hand, the level of the activated (cleaved) form of the ATf6 protein was not changed significantly by Runx2 (Fig. 2B). To confirm whether ATf6 expression was regulated under differentiation conditions instead of BMP2, primary calvarial cells were treated with ascorbic acid (AA) and β-glycerophosphate (β-GP). The AA and β-GP increased Atf6 expression with osteogenic gene expression in a time-dependent manner (Fig. 2C). Interestingly, full-length and nuclear forms ATF6 protein levels were elevated with increasing Runx2 expression by AA and β-GP treatment (Fig. 2D).

According to previous reports, Runx2 binds directly to OSE2 (ACCACA) and regulates the transcription of the target gene (9, 24). The promoter of the Atf6 gene also contains an OSE2 region that is located at −205 to −200 bp. A promoter study was performed using an OSE2-mutated reporter construct (M-Atf6-Luc) and a ChIP assay to determine if BMP2 regulates Atf6 expression via the activation of Runx2. Both BMP2 and Runx2 increased the luciferase activity of the wild type Atf6-Luc, but they did not stimulate the mutant type Atf6-Luc (Fig. 2E). The ChIP assay also showed that BMP2 enhanced the DNA binding of endogenous Runx2 to the OSE2 motif of the Atf6 promoter (Fig. 2F). These results suggest that Runx2
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FIGURE 2. Runx2 increases *Atf6* expression by interacting directly with the *Atf6* promoter region. A, changes of *Atf6* expression with overexpression of Runx2. MC3T3E1 cells were infected with Ad-Runx2 (+, 50 MOI; ++, 100 MOI) or Ad-GFP (50 MOI) for 24 h, and the cells were harvested for total RNA isolation. RT-PCR (top) or real-time PCR (bottom) were carried out using *Atf6* and β-actin primers. *p* < 0.05 compared with the untreated control. B, protein levels of *Atf6* with Ad-Runx2 treatment. The MC3T3E1 cells were infected with Ad-Runx2 (100 MOI) or Ad-GFP (100 MOI) for 24 h. The protein extracts were used for Western blot analysis with the indicated antibodies. C and D, expression of *Atf6* during AA- and β-GP-induced osteoblast differentiation. The primary calvarial cells were cultured with AA (50 μg/ml) and β-GP (10 mM) or BMP2 (200 ng/ml) for 8 days. At the designated day, the cells were harvested for total RNA isolation, and RT-PCR was carried out using *Atf6*, *Alp*, *Oc*, and β-actin primers (C). The total proteins were extracted for Western blot analysis using anti-*Atf6*, Runx2, and β-actin antibody (FL, full-length form; N, nuclear form) (D). E, luciferase activities of *Atf6*-Luc and M-*Atf6*-Luc with BMP2 or Runx2 treatment. Top, a schematic diagram of the Runx2 binding motif (OSE2; ACCACCA) on the *Atf6* promoter and point-mutated OSE2 region (ACCACA → ACCACCA). MC3T3E1 cells were transfected with 200 ng of *Atf6*-Luc or M-*Atf6*-Luc reporter plasmid and 100 ng of PMCV-β-galactosidase as an internal control with BMP2 (+, 100 ng/ml; ++, 200 ng/ml) or Runx2 (+, 100 ng/well; ++, 200 ng/well). Luciferase activity was normalized to β-galactosidase, and the relative activity is shown in the lower panel. *p* < 0.05; **, *p* < 0.01, compared with the untreated control, respectively. F, ChIP assay for binding of endogenous Runx2 to OSE2 motif on the *Atf6* promoter. MC3T3E1 cells were cultured with BMP2 (200 ng/ml) for 24 h. Soluble chromatin was prepared and immunoprecipitated with the antibody against Runx2 or IgG only as indicated. 10% of the soluble chromatin was used as the input. PCR was performed to determine and quantify the complex of the *Atf6* promoter gene with endogenous Runx2. Two pairs of primers were used for PCR, including the Runx2 binding region (−278 to −154bp) and distal region (−734 to −602 bp), which was used as a control. Error bars, S.E.

may mediate the BMP2 or AA/β-GP induction of *Atf6* expression but not mediate the BMP2-induced cleavage of *Atf6*. To further determine if Runx2 is required for BMP2-induced *Atf6* expression, the effects of BMP2 on *Atf6* expression were examined using Runx2-deficient (Runx2−/−) primary calvarial cells. RT-PCR and Western blot analysis showed that BMP2 did not affect the expression and cleavage of *Atf6* in Runx2−/− cells when compared with wild type primary calvarial cells (Fig. 3, A and B). On the other hand, overexpression of Runx2 using Ad-Runx2 in Runx2−/− cells recovered the BMP2 action on the expression and cleavage of *Atf6* (Fig. 3, C and D). In particular, Runx2 overexpression itself induced the expression of the ER-bound form of *Atf6* but did not affect the induction of the cleaved form of *Atf6*, which is consistent with the results shown in Fig. 2B (Fig. 3D). Overall, these results suggest that Runx2 is essential to the BMP2 induction of *Atf6* expression but is not needed to determine the subcellular localization of *Atf6*.

**BMP2 Increases Translocation of *Atf6* to Nucleus—ER-bound ATP6 acts as a transcriptional factor after being cleaved into its N-terminal fragment, including bZIP and transcriptional activation domains, and moving to the nucleus (19, 20). This study examined whether BMP2 can induce the translocation of *Atf6* into the nucleus using the Gal4-based transcription system. The expression vectors encoding the DNA-binding domain of the yeast transcription factor Gal4 fused to the full-length *Atf6* (Gal4-FL-ATF6) or nuclear form of *Atf6* (Gal4-N-ATF6) were co-transfected with a luciferase reporter construct containing a Gal4-binding site (Gal4-Luc; Fig. 4A, top). Gal4-FL-ATF6 increased the luciferase activities of Gal4-Luc by BMP2 treatment, whereas Gal4-N-ATF6 enhanced Gal4-Luc activity without BMP2 (Fig. 4A, bottom). Tunicamycin also increased the activity. In addition, Western blot analysis of the subcellular protein fraction showed that BMP2 increased the level of both the cytosolic and nuclear *Atf6* in a dose-dependent manner (Fig. 4B). These results suggest that BMP2 elicits**
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ATF6 Regulates Osteocalcin Gene Expression through Direct Binding to Oc Gene Promoter—The osteocalcin gene has been the most thoroughly studied of all bone-specific genes and serves as a model for regulation by 1,25-(OH)2-vitamin D3, hormones, and the transcription factors Runx2/Cbfa1 and osterix (25–27). This study further investigated whether ATF6, which is affected by BMP2 or Runx2, also functions as a transcriptional factor of osteogenic Oc gene expression. PCR analyses showed that overexpression of ATF6 by Ad-ATF6 increased the osterix, Alp, and Oc mRNA expression in a dose-dependent manner, which was similar to the effects of the BMP2 treatment. However, Runx2 and Col1a1 mRNA expression was not affected by Ad-ATF6 (Fig. 5A). The level of Oc protein secreted into the culture medium was also elevated by Ad-ATF6 (Fig. 5B). A transient transfection assay using two separate reporters containing the Oc gene promoter (OG2-Luc, −1321 to +16 bp) or 5′-deleted Oc promoter (Del-OG2-Luc, −611 to +16 bp) showed that the BMP2 treatment or overexpression of ATF6 increased the OG2-Luc activity and that BMP2 and ATF6 did not activate the Del-OG2-Luc. The addition of the dominant negative form of ATF6 (DN-ATF6) attenuated the BMP2-induced OG2-Luc activity (Fig. 5C).

ATF6 recognizes the TGACGT sequences as a consensus DNA binding site (21). This study found that the consensus DNA binding site for ATF6 was located −1126 to −1121 bp from the transcriptional initiation site of the Oc gene. A point-mutated reporter construct (TGACGT → ACACGT) (M-OG2-Luc) was produced to determine if this region was critical to the ATF6 function in regard to Oc transcription (Fig. 5D, top). The overexpression of ATF6 did not affect the activity of M-OG2-Luc unlike that of wild type OG2-Luc (Fig. 5D, bottom). Interestingly, OASIS did not even affect the OG2-Luc activities. In addition, the ChIP assay demonstrated that the BMP2 treatment enhanced the binding of endogenous ATF6 to the Oc promoter, which contained the ATF6-binding consensus sequences (Fig. 5E). According to a previous report, Runx2 and ATF4 regulate OC expression through directly binding to the OSE2 and OSE1 region on Oc promoter, respectively (28). To confirm whether these proteins work in a cooperative manner with ATF6 in controlling Oc transcription, transient transfection assays were performed. As shown in Fig. 5F, the promoter activities were increased with a combination of Runx2, ATF4, and ATF6 compared with individual treatment.

To confirm the regulatory role of ATF6, the effect of the down-regulation of ATF6 on Oc gene expression was examined using Ad-DN-ATF6 or siATF6. Three types of oligo-siATF6 were tested for silencing ATF6 in MC3T3E1 cells (data not shown). Two types reduced Atf6 mRNA expression. For knockdown of Atf6 expression, a mixture of two oligo-siATF6s was used. RT-PCR and real-time PCR consistently showed that BMP2-induced Oc expression was suppressed by Ad-DN-ATF6 in a dose-dependent manner (Fig. 6, A and D). In addition, BMP2 downstream transcription factor Runx2-induced Oc expression was also suppressed by Ad-DN-ATF6 or siATF6 treatment (Fig. 6, B, C, and E). BMP2-induced OC protein production in the medium was decreased by Ad-DN-ATF6 (Fig. 6F). Overall, these results suggest that ATF6 may stimulate Oc gene expression through direct binding to the Oc promoter and elevate OC protein level in the medium.

ATF6 Mediates BMP2-induced Extracellular Matrix Mineralization—Given that ATF6 regulates Oc gene expression directly, the effects of ATF6 on matrix mineralization in MC3T3E1 cells were examined through ALP and Alizarin red

the translocation of ATF6 from the ER to the nucleus in a similar manner as the ER stress inducer tunicamycin.

BMP2 changes the subcellular localization of ATF6. A luciferase activities of Gal4-Luc by BMP2 treatment. A schematic diagram of Gal4-Luc reporter and Gal4-ATF6 construct is shown in the upper panel. The MC3T3E1 cells were co-transfected with 200 ng of Gal4-Luc and Gal4-DBD (100 ng) or 100 ng of Gal4-ATF6 (Gal4-FL-ATF6 or Gal4-N-ATF6). 12 h after transfection, the cells were treated with BMP2 (+, 100 ng/ml; +++, 200 ng/ml) for 48 h. The luciferase assay was performed, and the results were expressed as fold activity relative to the control (bottom). *** p < 0.01 compared with the Gal4-FL-ATF6 only group. UAS, upstream activating sequences; DBD, DNA binding domain; MP, minimal promoter; TM, transmembrane domain. B, level of ATF6 protein in subcellular fraction. The cells were treated for 4 days with BMP2 (+, 100 ng/ml; +++, 200 ng/ml), and the nuclear and cytoplasmic fractions of the proteins were isolated using the NE-PER nuclear and cytoplasmic extraction kit. The protein extracts were used for Western blot analysis with the indicated antibodies. Error bars, S.E.
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FIGURE 5. ATF6 increases Oc transcription via directly binding to Oc promoter. A, expression of Oc by ATF6 overexpression. MC3T3E1 cells were infected with Ad-ATF6 (+, 50 MOI; +++, 100 MOI) or Ad-GFP (50 MOI) for 24 h or treated with BMP2 (200 ng/ml) for 4 days as a control. The cells were harvested for total RNA isolation, and RT-PCR was performed using Atf6, osterix, Alp, Oc, Col1a1, Runx2, and β-actin primers. B, changes of Oc protein production by ATF6. MC3T3E1 cells were treated with or without BMP2 (200 ng/ml) for 4 days and infected with Ad-ATF6 (+, 50 MOI; +++, 100 MOI) or Ad-GFP (50 MOI) for an additional 24 h. The level of OC in the culture medium was measured using an osteocalcin-specific ELISA kit. *, p < 0.05; **, p < 0.01 compared with the untreated control, respectively. C, luciferase activities of OG2-Luc and Del-OG2-Luc by BMP2, ATF6, or DN-ATF6. The MC3T3E1 cells were transfected with 200 ng of OG2-Luc or Del-OG2-Luc reporter plasmid and 100 ng of pCMV-β-galactosidase as an internal control with Atf6 (+, 100 ng/well; +++, 200 ng/well) or DN-ATF6 (+, 100 ng/well; +++, 200 ng/well). 12 h after transfection, the cells were treated with or without BMP2 (200 ng/ml) for 48 h. A luciferase assay was performed, and the results were expressed as fold activity relative to the control. *, p < 0.05; **, p < 0.01 compared with the untreated control. #, p < 0.05 compared with indicated group. D, luciferase activities of OG2-Luc and M-OG2-Luc by ATF6 or OASIS. A schematic diagram of the ATF6 binding motif (ACTGCA) and the point mutation in this region (TGACGT → ACACGT) on the Oc promoter (top). MC3T3E1 cells were transfected with 200 ng of OG2-Luc or M-OG2-Luc reporter plasmid and 100 ng of pCMV-β-galactosidase as an internal control with ATF6 (+, 100 ng/well; +++, 200 ng/well) or OASIS (+, 100 ng/well; +++, 200 ng/well) plasmid. *, p < 0.05; **, p < 0.01 compared with the untreated control, respectively (bottom). E, ChIP assay. The MC3T3E1 cells were cultured with BMP2 (200 ng/ml) for 4 days. Soluble chromatin was prepared and immunoprecipitated with an antibody against ATF6 or IgG only as indicated. 10% of the soluble chromatin was used as the input. PCR was performed to determine and quantify the complex formation between Oc promoter and endogenous ATF6. Two pairs of primers were used for PCR, including the ATF6 binding region (~1156 to ~1035 bp) and nonspecific region (~785 to ~635 bp) as a control. F, luciferase activities of OG2-Luc by Runx2, ATF4, and/or ATF6. The MC3T3E1 cells were transfected with 200 ng of OG2-Luc and 100 ng of pCMV-β-galactosidase as an internal control with 100 ng of Runx2, ATF4 and/or ATF6. A luciferase assay was performed, and the results were expressed as the fold activity relative to the control. **, p < 0.01, and ***, p < 0.001 compared to the untreated control. #, p < 0.05, and ##, p < 0.01 compared to the indicated group. Error bars, S.E.
As shown in Fig. 7, A and B, the ALP staining level was increased by Ad-ATF6 and/or BMP2 treatment. In contrast, Ad-DN-ATF6 blocked the BMP2-stimulated ALP staining levels in a dose-dependent manner. Alizarin red staining of Runx2/H11002/H11002 cells revealed that overexpression of the nuclear form of ATF6 or Runx2 increased matrix mineralization (Fig. 7C). In contrast, BMP2 did not induce matrix mineralization in the Runx2-deficient condition in vitro (Fig. 7C, lane 2). Interestingly, the BMP2 treatment with Runx2 overexpression enhanced matrix mineralization synergistically (Fig. 7C, lane 6). These results suggest that the BMP2-Runx2-ATF6 signal pathway positively regulates osteoblast differentiation and extracellular matrix mineralization.

DISCUSSION

This study demonstrated for the first time that ER stress-activated transcriptional factor ATF6 mediates BMP2-induced osteoblast differentiation, and osteocalcin is a target gene of ATF6. In addition, BMP2 induces Atf6 expression via Runx2 and can elicit the translocation of ATF6 from the ER membrane to the nucleus, as has been observed during ER stress responses.

Under normal conditions, ATF6 is retained in the ER membrane through an interaction with the ER protein chaperone BiP/GRP78 (15). Upon the accumulation of unfolded or misfolded proteins in the ER lumen, ATF6 is released from BiP and transits to the Golgi compartment, where it is cleaved by site 1 protease and site 2 protease in a manner similar to the sterol regulatory element-binding proteins (29). The cleaved ATF6 cytosolic domain traffics to the nucleus to activate the transcription of the target genes. In this study, BMP2 treatment induced Atf6 expression, which resulted in the increased expression of osteoblast differentiation marker genes. Western blot analysis showed that BMP2 increased the cleaved

staining. As shown in Fig. 7, A and B, the ALP staining level was increased by Ad-ATF6 and/or BMP2 treatment. In contrast, Ad-DN-ATF6 blocked the BMP2-stimulated ALP staining levels in a dose-dependent manner. Alizarin red staining of Runx2/H11002/H11002 cells revealed that overexpression of the nuclear form of ATF6 or Runx2 increased matrix mineralization (Fig. 7C). In contrast, BMP2 did not induce matrix mineralization in the Runx2-deficient condition in vitro (Fig. 7C, lane 2). Interestingly, the BMP2 treatment with Runx2 overexpression enhanced matrix mineralization synergistically (Fig. 7C, lane 6). These results suggest that the BMP2-Runx2-ATF6 signal pathway positively regulates osteoblast differentiation and extracellular matrix mineralization.

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Under normal conditions, ATF6 is retained in the ER membrane through an interaction with the ER protein chaperone BiP/GRP78 (15). Upon the accumulation of unfolded or misfolded proteins in the ER lumen, ATF6 is released from BiP and transits to the Golgi compartment, where it is cleaved by site 1 protease and site 2 protease in a manner similar to the sterol regulatory element-binding proteins (29). The cleaved ATF6 cytosolic domain traffics to the nucleus to activate the transcription of the target genes. In this study, BMP2 treatment induced Atf6 expression, which resulted in the increased expression of osteoblast differentiation marker genes. Western blot analysis showed that BMP2 increased the cleaved

staining. As shown in Fig. 7, A and B, the ALP staining level was increased by Ad-ATF6 and/or BMP2 treatment. In contrast, Ad-DN-ATF6 blocked the BMP2-stimulated ALP staining levels in a dose-dependent manner. Alizarin red staining of Runx2/H11002/H11002 cells revealed that overexpression of the nuclear form of ATF6 or Runx2 increased matrix mineralization (Fig. 7C). In contrast, BMP2 did not induce matrix mineralization in the Runx2-deficient condition in vitro (Fig. 7C, lane 2). Interestingly, the BMP2 treatment with Runx2 overexpression enhanced matrix mineralization synergistically (Fig. 7C, lane 6). These results suggest that the BMP2-Runx2-ATF6 signal pathway positively regulates osteoblast differentiation and extracellular matrix mineralization.

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FIGURE 7. ATF6 stimulates extracellular matrix mineralization. A and B, ALP staining. MC3T3E1 cells were infected with Ad-ATF6 (+, 50 MOI; ++, 100 MOI), Ad-DN-ATF6 (+, 50 MOI; + +, 100 MOI), or Ad-GFP (50 MOI) for 24 h and treated with or without BMP2 (200 ng/ml) for 4 days. The cells were stained with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution for ALP staining. C, Alizarin red staining. Runx2−/− cells were infected with Ad-ATF6 (+, 50 MOI; + +, 100 MOI) and/or Ad-Runx2 (+, 50 MOI; + +, 100 MOI) or Ad-GFP (50 MOI) for 24 h and treated with or without BMP2 (200 ng/ml) for 12 days. The cells were stained with an Alizarin red staining (AR-S) solution to evaluate extracellular matrix mineralization. Graphs represent relative intensity of stained levels. *, p < 0.05; **, p < 0.01, compared with the Ad-GFP-treated group. Error bars, SE.

form as well as full-length (ER membrane-bound form) ATF6 expression. Interestingly, when AA and β-GP treatment was used for osteoblast differentiation instead of BMP2, ATF6 expression and cleavage also increased. In this study, we focused on examining how BMP2 regulates ATF6 expression and activation as a transcriptional factor. In the bone-forming process, the action of BMP2 is mediated by the enhanced Runx2 transcriptional activity, which induces the expression of osteoblast differentiation-related genes (30–32). The Atf6 gene promoter has a Runx2-binding motif (OSE2; ACCACA) at −205 to −200 bp. Therefore, it was hypothesized that Runx2 might be involved in BMP2-induced Atf6 expression. Indeed, a series of gain- or loss-of-function studies showed that Runx2 can regulate Atf6 expression directly by binding to the OSE2 region. These results, however, revealed that Runx2 does not appear to participate in the cleavage process of the ATF6 from the ER membrane. Overexpression of Runx2 did not increase the level of the cleaved form of ATF6. Based on previous reports, the activation (cleavage) of ATF6 by BMP2 might result from the ER stress responses (10, 33). The molecular size of ATF6 cleaved by BMP2 was the same as that by tunicamycin, a potent ER stress inducer. In the designed experiment using Gal4-Luc reporter and GAL4-FL-ATF6, BMP2 increased the Gal4-Luc activity, which demonstrated that BMP2 stimulates the cleavage and translocation of ATF6 into the nucleus in a manner similar to the tunicamycin treatment. These findings suggest that BMP2, which is a potent inducer of bone formation, might also induce ER stress responses. Further studies will be needed to determine if BMP2 induces some ER proteolytic enzyme to cleave the ER-bound ATF6.

Recently, the roles of UPR transducers during in vivo osteogenesis and differentiation marker gene regulation have been well defined using knock-out mice and extensive in vitro null cell cultures. The PERK−/− mice showed severe osteopenia, which involved decreases in cortical and trabecular bone thickness (34). Osteopenia was caused by a deficiency in the number of mature osteoblasts (35). The in vivo bone phenotype of ATF4−/− mice was similar to that of PERK−/− mice. Extensive in vitro studies have shown that the PERK-eIF2α-ATF4 pathway might be involved in bone formation or osteoblast differ-

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entiation mediated by ER stress or BMP2 (10, 33). More recently, the IRE1α-XBP1 pathway of UPR was demonstrated to mediate osteoblast differentiation by promoting osterix transcription (36). The OASIS−/− mice exhibited severe osteopenia, involving a decrease in type I collagen in the bone matrix and a decrease in the activity of osteoblasts (10). The phenotype of ATF6−/− mice and the transcriptional targeting of ATF6 for osteoblast differentiation have not yet been reported. Therefore, the roles of ATF6 have never been mentioned. On the other hand, ATF6 is expected to play a similar role as OASIS because ATF6 is structurally similar to OASIS and also acts as an ER membrane-bound transcriptional factor in the response to ER stress. The gain- or loss-of-function studies showed that ATF6 plays a positive role in Oc gene expression and matrix mineralization. In addition, ATF6 regulates several osteogenic genes, including Oc, osterix, and Alp. These results suggest that ATF6 has a more critical role during osteogenic differentiation through the regulation of osterix, ALP, and OC than OASIS function.

Osteocalcin is the most abundant non-collagenous protein expressed in bone, with its expression limited specifically to cells of an osteoblast lineage, including mature osteoblasts, osteocytes, and hypertrophic chondrocytes (37–39). Transcription factors, such as Runx2 and ATF4, which are also activated by BMP2, regulate Oc transcription by directly binding to the OSE region of the Oc gene (9, 33). In contrast, ATF6 recognizes TGACGT as a core consensus DNA binding sequence and regulates the expression of target genes, such as the ER stress-related GRP78 gene (21). Endogenous ATF6 appears to mediate the core consensus DNA binding sequences because the dominant negative forms of ATF6 block the induction of the GRP78 gene (21). A database search revealed that the Oc gene also has a putative ATF6 binding motif at −1126 to −1121 bp. In the present study, ATF6 overexpression expectedly increased the level of Oc mRNA and protein expression and OG2-Luc activity in a manner similar to BMP2 but did not produce the action in the mutated ATF6 binding motif of the Oc genes (Del-OG2-Luc and M-OG2-Luc). In addition, DN-ATF6 suppressed BMP2- or Runx2-induced Oc expression and OG2-Luc activity. The ChIP results showed that ATF6 bound directly to the putative motif. Overall, these results indicate that the Oc gene is a direct target of ATF6 and a novel stimulatory factor for osteoblast differentiation. In addition, this study provides evidence that ATF6 can stimulate extracellular matrix mineralization even without the influence of Runx2.

Although ATF6 is similar to OASIS in terms of its structure and mode of cleavage, there seem to be some functional differences. For example, ATF6 targeted the Oc gene, but OASIS did not stimulate the OG2 reporter. Another study also showed that OASIS overexpression did not alter many osteoblast differentiation-related genes, including OC in MC3T3-E1 cells (10). They confirmed that OASIS increased Coll1a1 transcription via direct binding to a UPR element-like sequence (CGACGTGG) on the Coll1a1 promoter. However, our study showed that Coll1a1 was not regulated by ATF6. Therefore, ATF6 and OASIS positively regulate osteoblast differentiation by increasing the different target gene expression.

From our results, we suggest that BMP2 regulates osteoblast differentiation via Runx2-dependent Atf6 expression and activation of ATF6 via ER stress-induced intramembrane proteolysis process. In addition, ATF6 increases Oc expression by directly binding to the TGACGT sequences on the Oc promoter gene (Fig. 8). Overall, the ER stress transducer ATF6 might be a novel positive mediator of BMP2-induced osteoblast differentiation and extracellular matrix mineralization.

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