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α1-Adrenergic Receptor Signaling in Osteoblasts Regulates Clock Genes and Bone Morphogenetic Protein 4 Expression through Up-regulation of the Transcriptional Factor Nuclear Factor IL-3 (Nfil3)/E4 Promoter-binding Protein 4 (E4BP4)*

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Background: The physiological role of α1-adrenergic receptor (AR) signaling in bone is largely unknown.
Results: α1-AR signaling negatively regulates *Bmp4* expression by up-regulating the transcriptional factor Nfil3/E4BP4 in osteoblasts.
Conclusion: α1-AR signaling regulates clock genes and *Bmp4* expression in osteoblasts.
Significance: The regulation of clock genes by α1-AR signaling in osteoblasts may contribute to the physiological significance of the circadian clockwork in bone metabolism.

Several studies have demonstrated that the α1-adrenergic receptor (AR) plays an important role in regulating cell growth and function in osteoblasts. However, the physiological role of α1-AR signaling in bone metabolism is largely unknown. In this study, the stimulation of phenylephrine (PHE), a nonspecific α1-AR agonist, increased the transcriptional factor Nfil3/E4BP4 and led to the rhythmic expression of bone morphogenetic protein 4 (Bmp4) in MC3T3-E1 osteoblastic cells. We also showed that *Bmp4* mRNA expression peaked in bone near zeitgeber time 8 in a 24-h rhythm. Furthermore, the expression of *Nfil3* and *Bmp4* displayed a circadian pattern with opposing phases, which suggested that *Nfil3* repressed the expression of the *Bmp4* gene during a circadian cycle. On a molecular level, both loss-of-function and gain-of-function experiments demonstrated that Nfil3/E4BP4 negatively regulated *Bmp4* expression in osteoblasts. Furthermore, the systemic administration of PHE increased the expression of *Nfil3* mRNA in bone, whereas it decreased that of *Bmp4* mRNA. The expression of *Bmp4* mRNA was decreased significantly by exposure to PHE, and this was concomitant with the increase in Nfil3 binding to the D-box-containing *Bmp4* promoter region in MC3T3-E1 cells, which indicates that the expression of Nfil3 by α1-AR signaling can bind directly to the *Bmp4* promoter and inhibit *Bmp4* expression in osteoblasts. Our results suggest that α1-AR signaling regulates clock genes and *Bmp4* expression in osteoblasts. Moreover, α1-AR signaling negatively regulated *Bmp4* expression by up-regulating the transcriptional factor Nfil3/E4BP4 in osteoblasts.

The biological effects of norepinephrine and epinephrine are mediated by α- and β-adrenergic receptor (AR)[2] subtypes, which show distinct tissue distribution patterns and transmit signals through distinct biochemical pathways (1). α1-ARs have been shown to be crucially involved in controlling vascular contractility, glucose metabolism, and behavioral responses (2). α1-ARs are seven-transmembrane domain receptors that are coupled to the heterotrimeric G proteins of the $G_{q/11}$ and G_{12}/G_{13} families (3). $G_{q/11}$ family G proteins couple numerous G protein-coupled receptors to the activation of phospholipase C-β (PLC-β), PKC, and intracellular calcium release and, thereby, regulate the basic functions of these diverse cell types.

Bone is a metabolically active organ that maintains continuous remodeling throughout life. Several studies, including ours, have demonstrated that the sympathetic nervous system regulates bone remodeling in part through the β-AR (4–6). On the other hand, it has been suggested that α1-AR signaling also plays an important role in the regulation of cell growth and function through the sympathetic nervous system in osteoblasts (7–10). Norepinephrine has been shown to increase cell proliferation by suppressing K^+ channels via $G_{i/o}$-coupled α1B-ARs in human osteoblasts (11). However, their physiological role in bone remains unclear.

The mammalian circadian timing system, in which daily light-dark cycles phase-entrain the master clock in the suprachiasmatic nucleus which, in turn, synchronizes subsidiary oscillators in most peripheral cells and drives the daily rhythms of physiology and behavior (12). Circadian clocks are found in most or all tissues in mammals (13) and play important roles in local and systemic physiology (14–16). Circadian rhythms are driven by networks of transcriptional-translational autoregulatory loops and have a cycle that lasts ~24 h. The basic helix-loop-helix transcription factor brain and muscle ARNT-like 1 (Bmal1) and circadian locomotor output cycle kaput (Clock)

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[2] The abbreviations used are: AR, adrenergic receptor; PHE, phenylephrine; ZT, zeitgeber time; α-MEM, α-minimal essential medium; Luc, luciferase; qRT-PCR, quantitative RT-PCR; Bmp, bone morphogenetic protein.

heterodimerize and drive the transcription of the core clock genes *Period* (*Per1*, *Per2*, and *Per3*) and the *Cryptochrome* (*Cry1* and *Cry2*) genes by binding E-box elements within gene sequences (17). In addition to core clock factors, genes involved in basic cellular events are also regulated by transcriptional-translational autoregulatory loops (17). The circadian system coordinates rhythmic physiological processes at both cellular and tissue levels by regulating the rhythmic expression of these clock-controlled genes. A growing number of studies have recently focused on circadian oscillators in bone and growth plate cartilage (18–20). Sympathetic signaling and dexamethasone have been shown to entrain circadian oscillators and the rhythmic expression of core clock genes such as *Bmal1* and *Period* (*Per1*, *Per2*, and *Per3*) in human osteoblasts (18). Furthermore, dexamethasone mediated circadian timing to osteoclasts (21). Therefore, suprachiasmatic nucleus-controlled circadian hormone rhythms and sympathetic tone appear to play a central role in this process. However, how the circadian oscillator coordinates and regulates bone physiology in response to the sympathetic nervous system and α1-AR signaling in osteoblasts remains elusive.

In this study, we examined the possible involvement of α1-AR signaling in osteoblasts to the circadian rhythms caused by the sympathetic nervous system in bone metabolism. We showed that phenylephrine (PHE), a nonspecific α1-AR agonist, induced the rhythmic mRNA expression of bone morphogenetic protein-4 (Bmp4), which is one of the most important regulators of bone metabolism in MC3T3-E1 osteoblastic cells. Moreover, α1-AR signaling negatively regulated *Bmp4* expression by up-regulating the transcriptional factor Nfil3/E4BP4 in osteoblasts.

EXPERIMENTAL PROCEDURES

Mice—Male, 8-week-old C57BL/6J mice (Japan SLC Inc., Hamamatsu, Japan) were randomized by weight, assigned to groups, and acclimated to their cages for 1 week prior to the experiment. They were treated in accordance with the Guidelines for Animal Experiments at the School of Dentistry, Aichi-Gakuin University. Food and water were available *ad libitum*. Animals were housed together in automatically controlled conditions of temperature (23 ± 1 °C) and humidity (50 ± 10%) under a 12-hour light, 12-hour dark cycle or constant dark conditions. Zeitgeber time (ZT) 0 under the light/dark cycle was designated as lights on and ZT12 as lights off.

Drugs and Treatment—α1-Adrenergic receptor pathways were stimulated using the nonspecific α1-AR agonist PHE (Sigma-Aldrich). Mice were killed by CO_2 asphyxiation after the drug treatment. Bone tissue samples were dissected and kept at −80 °C for total RNA until assayed.

MC3T3-E1 Cell Cultures—MC3T3-E1 cells were purchased from the RIKEN Cell Bank. MC3T3-E1 cells were cultured in α-MEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. To induce differentiation, the culture medium was replaced with α-MEM containing 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate. The culture medium was changed every 2–3 days.

Generation of MC3T3-E1 Cell Lines with the Stable Expression of Nfil3—MC3T3-E1 cells were maintained in α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. 2 μg of pcDNA3-negative and full-length-Nfil3 were stably transfected into MC3T3-E1 cells by FuGENE HD, as specified by the method of the manufacturer (Promega), followed by drug selection with 800 μg/ml of the neomycin analog G418. Resistant colonies were selected and expanded. Twenty stable lines (Nfil3 series) were established. All further experiments were conducted with three of these lines, Nfil3–1, Nfil3–2, and Nfil3–3, which expressed high levels of Nfil3, respectively.

siRNA Nucleofection—MC3T3-E1 cells were grown in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin to ~70% confluency, followed by transient transfection with either siRNA targeting Nfil3 or non-silencing RNA diluted in Opti-MEM using Lipofectamine RNAiMAX (Invitrogen) according to the protocol of the manufacturer. Silencer Select siRNAs were used (Ambion/Applied Biosystems). Both the Nfil3 siRNAs and non-silencing RNA were used at final concentrations of 10 nM. The medium was then replaced with fresh medium. Cells were harvested for total RNA extraction at the indicated time points.

RNA Extraction and Quantitative RT-PCR—Total RNA was isolated with an RNeasy mini kit (Qiagen) according to the protocol of the manufacturer. One microgram of RNA was reverse-transcribed into cDNA using the QuantiTect reverse transcription kit according to the protocol of the manufacturer (Qiagen). Gene expression was analyzed with the Step-One-Plus real-time PCR system with Step One software v2.0 (Applied Biosystems). Reactions were performed in 20-μl volumes using a QuantiTect SYBR Green PCR kit (Qiagen). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative quantification of each mRNA was performed using the comparative quantity (copies) method, creating standard curves. Relative standard curves were created with serially diluted cDNA samples that were reverse-transcribed from RNA samples. This concentration range of standard curve samples was determined to be well within the detection level and sensitivity of the quantitative PCR assay. The relative quantity for each sample was normalized to the average level of the constitutively expressed housekeeping gene *Gapdh*. The following primers were used: Gapdh, 5′-TGGAGAAACCTGCCAAGTATG-3′ (forward) and 5′-GGAGACAACCTGGTCCTCAG-3′ (reverse); Nfil3, 5′-CAGTGCAGGTGACGAACATT-3′ (forward) and 5′-TTCCACCACACCTGTTTTGA-3 (reverse); Per2, 5′-ATGCTCGCCATCCACAAGA-3′ and 5′-GCGGAATCGAATGGGAGAAT-3′ (reverse); Bmp4, 5′-GTGACACGGTGGGAAACTTTCGAT-3′ (forward) and 5′-CACCTCAATGGCCAGCCCATAAT-3′ (reverse).

Western Blot Analysis—Cells were washed with PBS and collected in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, and the aforementioned inhibitors of phosphatases and proteases. Cell homogenates were mixed at a volume ratio of 4:1 with 10 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 0.01% bromphenol blue, and 5% mercaptoethanol, followed by boiling at 100 °C for 10 min, as

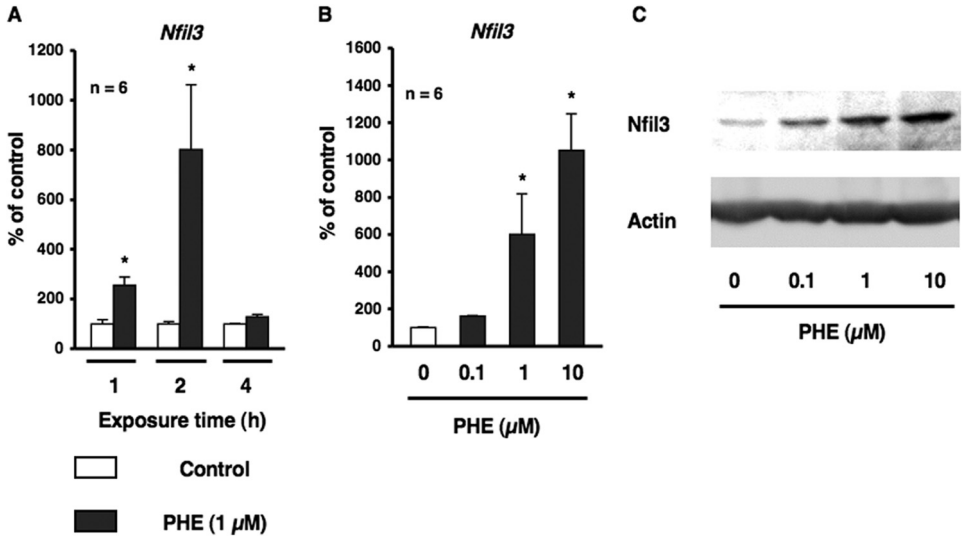

FIGURE 1. **α1-AR signaling up-regulated the transcriptional factor Nfil3 in MC3T3-E1 cells.** *A*, *Nfil3* mRNA expression in MC3T3-E1 cells. Cells were treated with 1 μM PHE for 1, 2, and 4 h, harvested, and then processed for real-time qRT-PCR. Each value represents the mean ± S.E. of six separate experiments. *, $p < 0.05$, significantly different from each control value obtained in MC3T3-E1 cells cultured in the absence of PHE. *B*, *Nfil3* mRNA was up-regulated by PHE in a concentration-dependent manner in MC3T3-E1 cells. Cells were treated with PHE at 0.1–10 μM for 2 h, harvested, and processed for real-time qRT-PCR. Each value represents the mean ± S.E. of six separate experiments. *, $p < 0.05$, significantly different from each control value obtained in MC3T3-E1 cells cultured in the absence of PHE. *C*, the transcriptional factor Nfil3 was increased by PHE in a dose-dependent manner in MC3T3-E1 cells. Cells were treated with PHE at 0.1–10 μM for 4 h, harvested, and then processed for Western blotting. A representative result of three individual experiments is shown.

described previously (22). Each aliquot of 20 μg of protein was subjected to electrophoresis on a polyacrylamide gel containing 0.1% SDS at a constant current of 10 mA/plate at room temperature and was subsequently transferred to a polyvinylidene fluoride membrane. The membranes were subjected to blocking with 5% skim milk dissolved in TBST (20 mM Tris-HCl (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20), followed by a reaction with antibodies against Nfil3 adequately diluted in 1% skim milk/TBST. Finally, proteins were reacted with an anti-rabbit IgG antibody conjugated with peroxidase and detected with the aid of ECL detection reagents.

Construction of Plasmids and Luciferase Reporter Gene Assays—MC3T3-E1 cells were seeded into 12-well tissue culture plates and cultured for 24 h in α-MEM containing 10% FBS. Cells were transiently cotransfected with 200 ng of firefly luciferase reporter plasmids and 400 ng of pcDNA3 plasmids by using FuGENE HD (Promega). Firefly luciferase (Luc) and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system and GloMax 20/20n luminometer 48 h after transfection according to the instructions of the manufacturer (Promega). The *Renilla* luciferase plasmid pRL-TK (Promega) was used as an internal control for transfection efficiency. For knockdown experiments, cells were cotransfected with 20 nmol Stealth Select RNAi siRNA (Ambion/Applied Biosystems), 200 ng of luciferase reporter plasmids, and 10 ng of pRL-TK plasmid by using Lipofectamine 2000 (Invitrogen). A 712-bp fragment (−2245 to −1534) of the *Bmp4* promoter was amplified by KOD-plus Neo DNA polymerase (Toyobo) with mouse genomic DNA and the forward primer containing a KpnI restriction site (5′-cggggtaccCAGCTGTCCCCAGAGATAACA-3′) and the reverse primer containing a KpnI restriction site (5′-cccaagcttGGTCTCCATGAGCCCTTTCT-3′). Following digestion with restriction enzymes (underlined), the purified PCR product was cloned into the KpnI and HindIII sites of the pGL3 luciferase vector (Promega). The deletion mutant of the *Bmp4* promoter plasmid (ΔDboxBmp4Luc, deletion of the putative Nfil3/E4BP4 binding site (−1815 to −1785)) was derived from the Bmp4-luc plasmid by PCR amplification using Prime STAR mutagenesis (Takara) and the following primers: 5′-CCCCAGGGAGTTTTAATTTTGTGTT-3′ and 5′-TAAAACTCCCTGGGGCATCAGGCTCT-3′. The correct sequences of the subcloned fragments were confirmed by complete nucleotide sequencing.

ChIP Assay—The ChIP assay was performed using ChIP-IT Express (Active Motif) according to the instructions of the manufacturer. Cell extracts were immunoprecipitated with polyclonal rabbit anti-Nfil3 antibody (Santa Cruz Biotechnology) and protein G magnetic beads for 4 h at 4 °C. Purified DNA fragments were subjected to PCR. PCR was addressed for the *Bmp4* promoter region (−1906 to −1758) and the exon 1 region (+243 to +461) and performed using the following primers: Bmp4 promoter region, 5′-GCACCCCTAAGGACAACGAA-3′ and 5′-CAGGCTCTGTGCTCCCATAG-3′; exon 1 region, 5′-TCTAGAGGTCCCCAGAAGCA-3′ and 5′-AGGTGCCTTTTAGCCTCCAT-3′. PCR products were also analyzed on 2.5% agarose gels.

Data Analysis—All data are expressed as the mean ± S.E. Two-tailed Student's *t* test combined with Bonferroni's correction following a one-way analysis of variance was used for multiple comparisons. Differences with $p < 0.05$ were considered significant. The circadian rhythmicity in gene expression was determined by the single Cosinor method using Timing Series Analysis Single Cosinor 6.3 software (Expert Soft Tech). The period was determined using a chronobiometric ellipse test (18).

RESULTS

α1-AR Signaling Up-regulated the Transcriptional Factor Nfil3 in Osteoblasts—On the basis of previous findings in which sympathetic signaling, particularly β-AR in osteoblasts, has been shown to entrain circadian oscillators and also induced the transcription of *nuclear factor IL-3* (*Nfil3*), which encodes E4-binding protein 4 (E4BP4) (18, data not shown), we investigated whether α1-AR signaling contributed to the regulation of core clock genes in osteoblasts. To address this hypothesis, we initially characterized *Nfil3* mRNA expression by α1-AR signaling in MC3T3-E1 osteoblastic cells. Total RNA was extracted from MC3T3-E1 osteoblastic cells following exposure to PHE for 1, 2, and 4 h and was subsequently analyzed by real-time qRT-PCR. As shown in Fig. 1*A*, the expression of *Nfil3* mRNA was significantly increased ~3- and 8-fold after 1 and 2 h following exposure to PHE. The exposure to PHE induced *Nfil3* mRNA and protein expression in a concentration-dependent manner (Fig. 1, *B* and *C*). In addition, pretreatment with the α1-AR antagonist prazosin completely inhibited PHE-induced *Nfil3* expression, as determined by real-time qRT-PCR analysis in MC3T3-E1 osteoblastic cells, which suggests that PHE-induced *Nfil3* expression in osteoblasts may be mediated by α1-AR signaling (Fig. 2).

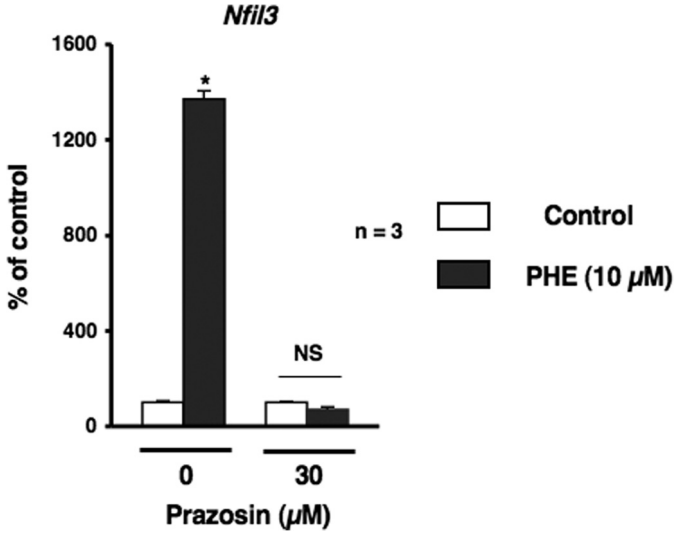

FIGURE 2. **α1-AR signaling mediated *Nfil3* mRNA expression after the PHE stimulation in MC3T3-E1 cells.** Cells were incubated for 2 h in the presence of PHE with prazosin at a concentration of 10 μM, followed by the determination of *Nfil3* levels by real-time qRT-PCR. Each value represents the mean ± S.E. of three separate experiments. *, $p < 0.05$, significantly different from each control value. *NS*, not significant.

Bmp4 Is a Circadian-regulated Gene in Osteoblasts—BMPs are members of the TGF-β superfamily, which regulates cell growth, differentiation, and function, and plays an important role in regulating normal physiological functions (23). To understand the physiological function of the circadian clockwork in bone metabolism and identify circadian-regulated genes affected by α1-AR signaling in osteoblasts, we next evaluated the expression of *Nfil3* and *Bmp4* in bone. We found that *Nfil3* mRNA expression peaked near ZT20 in a 24-h rhythm in mouse bone samples harvested during a circadian cycle. In contrast, bone *Bmp4* mRNA expression, which peaked at ZT8, displayed a circadian pattern with opposing phases, which suggested that *Nfil3* repressed *Bmp4* gene expression during a circadian cycle (Fig. 3). To determine whether α1-AR signaling in osteoblasts could mediate the rhythmic expression of a subset of genes, including canonical core clock genes and *Bmp4*, cells were treated with PHE at 10 μM, and mRNA expression was determined by real-time qRT-PCR analysis at the indicated time points between 12 and 52 h. The exposure to PHE entrained *Per2*, *Bmal1*, *Per2*, *Nfil3*, and *Bmp4* with rhythmic expression in MC3T3-E1 osteoblastic cells (period of oscillation: Bmal1, 22.7 h; Per2, 24.5 h; Nfil3, 23.7 h; Bmp4, 24.8 h), which indicates that *Bmp4* is one of the circadian-regulated genes that are regulated in response to α1-AR signaling in osteoblasts (Fig. 4).

Nfil3 Negatively Regulated Bmp4 Expression in MC3T3-E1 Cells—To investigate the physiological function of α1-AR signaling in bone metabolism and identify the target molecules of Nfil3 in osteoblasts, we stably transduced MC3T3-E1 cells with

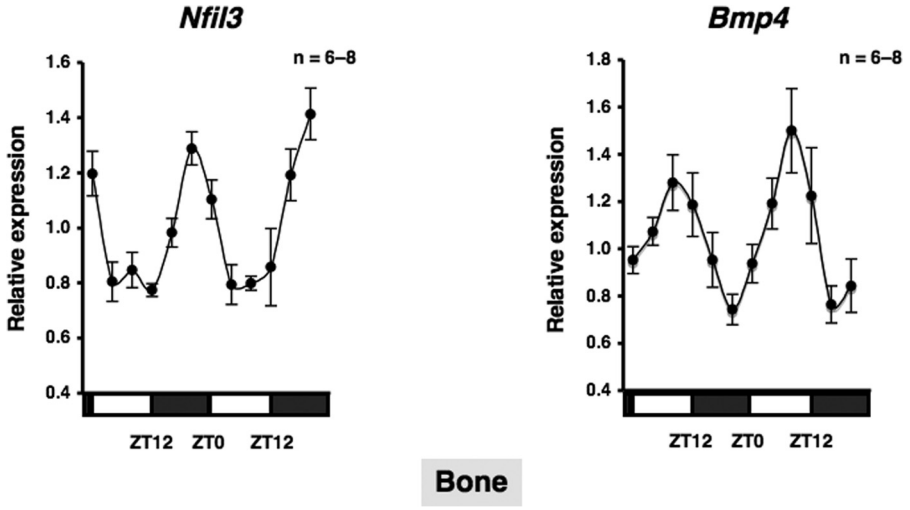

FIGURE 3. ***Nfil3* and *Bmp4* in bone.** A representation of the expression of *Nfil3* and *Bmp4* in bone from C57BL/6J mice under light/dark cycle conditions. Bone was obtained from C57BL/6J mice every 4 h. Total RNA was isolated, and the level of mRNA was determined by real-time qRT-PCR using specific primers for *Nfil3* and *Bmp4*. Relative mRNA levels were normalized to the *Gapdh* level. Data represent the mean ± S.E., $n = 6–8$ animals. *White boxes*, light period; *black boxes*, dark period.

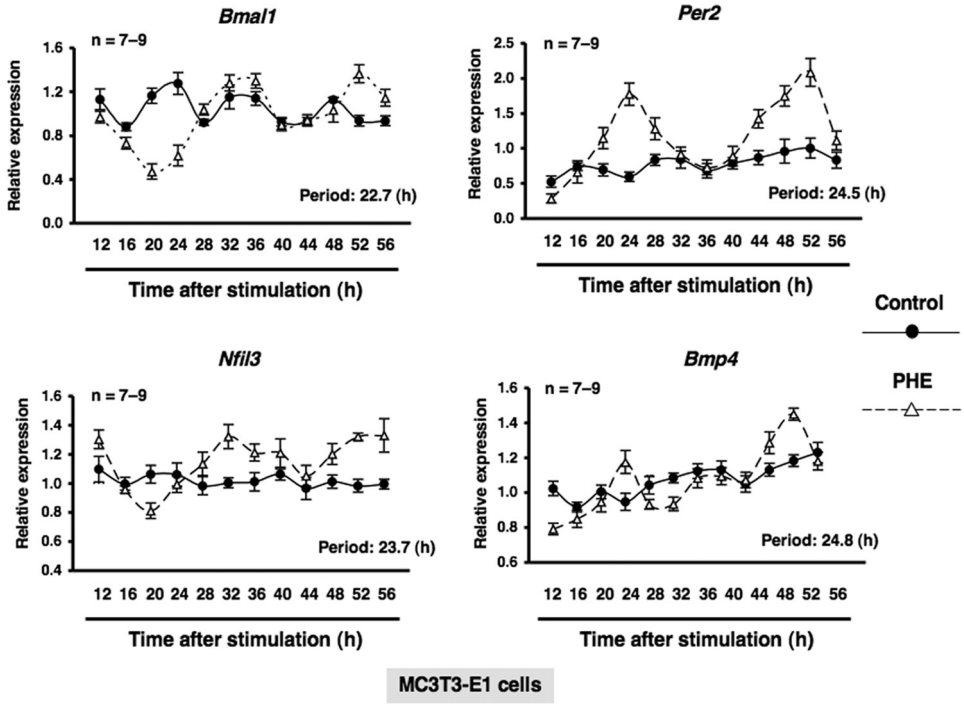

FIGURE 4. **α1-AR signaling mediated circadian rhythmicity in MC3T3-E1 cells.** Expression profiles of the *Bmal1* (*upper left*), *Per2* (*upper right*), *Nfil3* (*lower left*), and *Bmp4* (*lower right*) transcript generated by PHE in MC3T3-E1 osteoblastic cells. Total RNA samples were collected at the indicated times after PHE treatment. qRT-PCR analyses of transcript levels were performed using their specific primers. *Gapdh* was used as an internal control. Each value represents the mean ± S.E. ($n = 7–9$ independent experiments). *, $p < 0.05$, significantly different from each control value. The Cosinor analysis method was used to determine the rhythmic expression. The *lines* show the fitting curves.

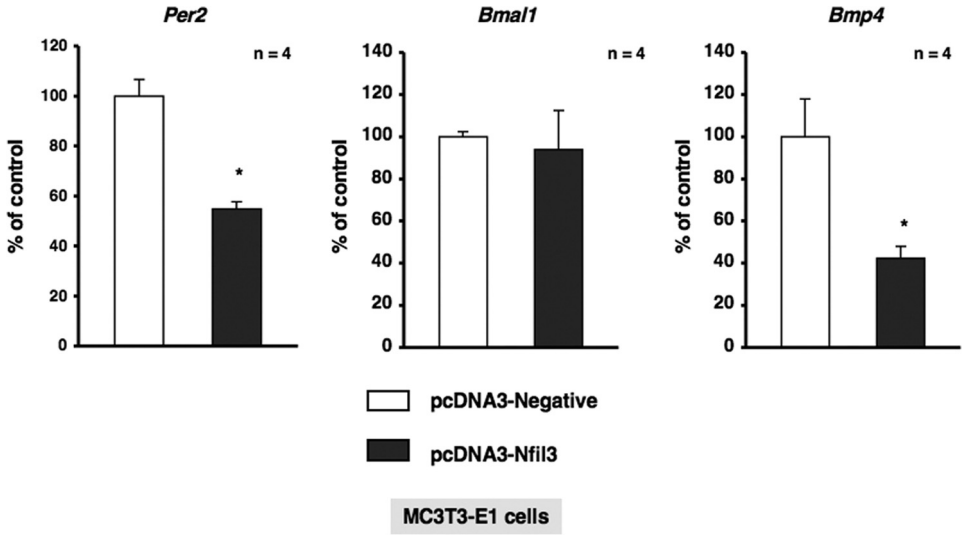

FIGURE 5. **Nfil3 negatively regulated *Per2* and *Bmp4* in MC3T3-E1 cells.** Effects of the overexpression of Nfil3 in MC3T3-E1 cells. MC3T3-E1 cells were stably transfected with expression vectors for Nfil3 (*pcDNA-Nfil3*) or control (*pcDNA-Negative*), followed by further cultivation for 48 h and the subsequent determination of *Per2*, *Bmal1*, and *Bmp4* levels by real-time qRT-PCR. Each value represents the mean ± S.E. of four separate experiments. *, $p < 0.05$, significantly different from the value obtained in cells transfected with the control vector.

either empty pcDNA3 or pcDNA3-Nfil3 and evaluated the expression of a subset of genes, including canonical core clock genes and Bmp4. As shown in Fig. 5, the forced overexpression of Nfil3 repressed *Per2* mRNA expression without altering *Bmal1* expression in MC3T3-E1 cells. Furthermore, the overexpression of Nfil3 suppressed *Bmp4* expression in MC3T3-E1 cells, which suggested that Nfil3 mediated the expression of *Bmp4* under the regulation of circadian-related molecules in MC3T3-E1 cells. We then attempted to elucidate the regulation of *Bmp4* gene expression in MC3T3-E1 cells transfected with siRNA for the knockdown of Nfil3. Cells were transfected with siRNA for Nfil3, followed by the determination of *Nfil3* levels using real-time qRT-PCR. *Nfil3* levels were decreased significantly in MC3T3-E1 cells transfected with Nfil3 siRNA when measured 24 h after transfection. A marked reduction in *Nfil3* mRNA was found after 24 h in cells transfected Nfil3 siRNA (Fig. 6*A*). Further, significant increases were observed in *Bmp4* expression and *Bmp4* promoter activity in MC3T3-E1

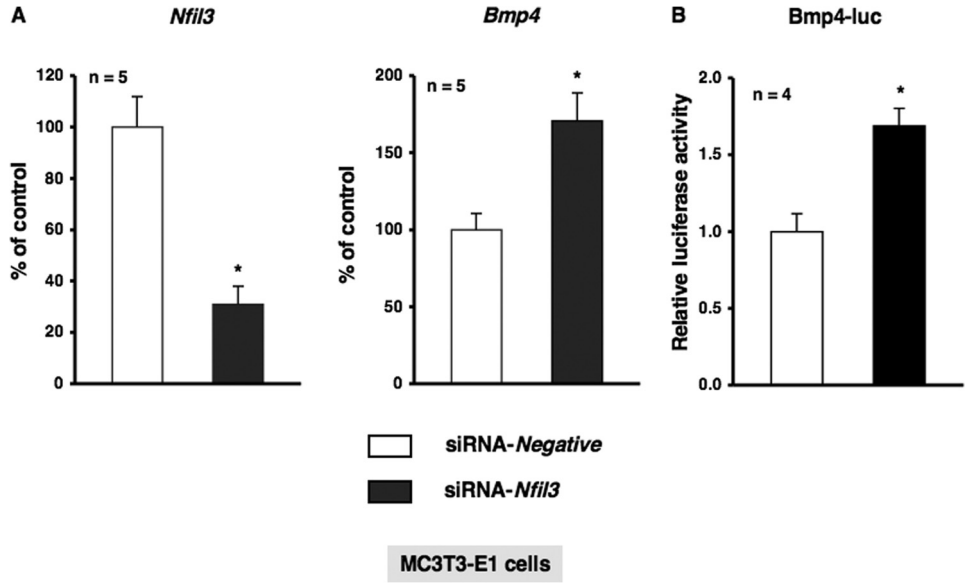

FIGURE 6. **Nfil3 knockdown by siRNA on the *Bmp4* transcript in MC3T3-E1 cells.** *A*, MC3T3-E1 cells were treated with Nfil3 siRNA (*siRNA-Nfil3*) or non-silencing RNA (*siRNA-Negative*) according to the indicated protocols. Real-time qRT-PCR analyses of transcript levels were performed using their specific primers for Nfil3 and Bmp4. Relative mRNA expression was normalized to *Gapdh*. Each value represents the mean ± S.E. of five independent determinations. *, $p < 0.05$, significantly different from each control value. *B*, MC3T3-E1 cells were transiently transfected with Bmp4-Luc in the presence of siRNA-*Nfil3* or siRNA-*Negative* according to the indicated protocols. The luciferase activity was determined 48 h after transfection. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Each value is the mean ± S.E. of four independent experiments. *, $p < 0.05$, significantly different from each control value.

cells under these conditions (Fig. 6, *A* and *B*). These results indicate that Nfil3 negatively regulates *Bmp4* expression in MC3T3-E1 cells. Using a bioinformatics approach, we next identified three putative consensus Nfil3/E4BP4 binding sites in the *Bmp4* promoter (−1815/−1804, 5′-AGTAATGTA-ATA-3′; −1802/−1791, 5′-AGTAATGTAATG-3′; and −1797/−1786, 5′-TGTAATGTAATG-3′). Nfil3/E4BP4 has been shown to bind to a D-box element of the promoter region of target genes to repress transcription. Therefore, we expected Nfil3 to be one of the target molecules of α1-AR signaling that regulates the circadian cycle in osteoblasts. To examine the functional significance of the putative Nfil3/E4BP4 binding site involved in *Bmp4* gene expression, we cloned upstream of the *Bmp4* transcript and generated luciferase reporter constructs (Bmp4-Luc) covering the potential elements that encompass the putative Nfil3/E4BP4 binding sites and deletion mutant of Bmp4-Luc (ΔDboxBmp4-Luc) that should render the Nfil3/E4BP4 site in the *Bmp4* promoter inactive. As shown in Fig. 7*A*, the overexpression of Nfil3 significantly decreased luciferase activity in cells transfected with Bmp4-Luc, which includes all three putative Nfil3/E4BP4 binding sites. In contrast, deletion of the three D-box sites elements abolished the Nfil3-mediated suppression of the *Bmp4* transcription (Fig. 7*B*). This indicates that the Nfil3/E4BP4 binding sites of the 5′ flanking region of *Bmp4* was responsible for the suppression of *Bmp4* transcription by Nfil3/E4BP4.

α1-AR Signaling Negatively Regulated Bmp4 Expression in MC3T3-E1 Cells—We next characterized the expression of *Bmp4* mRNA by α1-AR signaling in MC3T3-E1 osteoblastic cells. Total RNA was extracted from MC3T3-E1 osteoblastic cells following exposure to PHE and was subsequently analyzed by real-time qRT-PCR. As shown in Fig. 8, the expression of *Bmp4* mRNA was decreased significantly by the exposure to

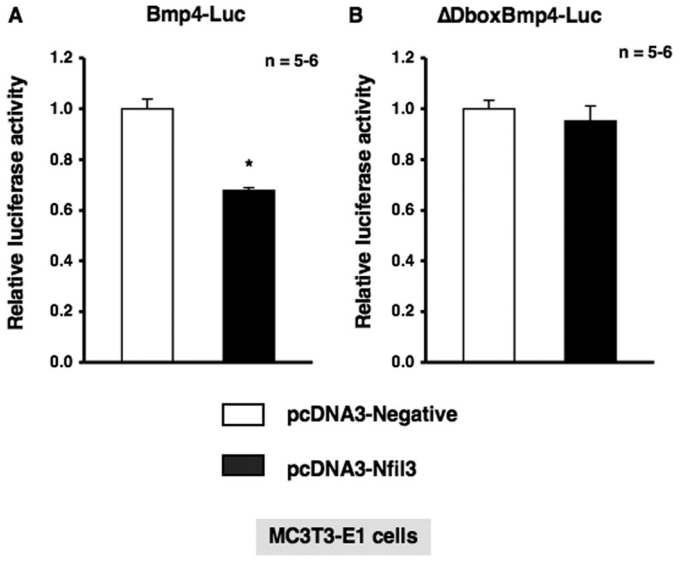

FIGURE 7. **Deletion of putative D-box elements abolished Nfil3-mediated suppression of *Bmp4* luciferase activity.** *A*, MC3T3-E1 cells were transiently transfected with Bmp4-Luc in either the presence or absence of the Nfil3 expression plasmid. Luciferase activity was determined 48 h after transfection. The total amount of DNA transfected was standardized with an empty vector. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Each value is the mean ± S.E. ($n = 5–6$ independent experiments). *, $p < 0.05$, significantly different from each control value. *B*, MC3T3-E1 cells were transfected with ΔDboxBmp4-Luc in either the presence or absence of the Nfil3 expression plasmid. The luciferase activity was determined 48 h after transfection. The total amount of DNA transfected was standardized with an empty vector. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Each value is the mean ± S.E. ($n = 5–6$ independent experiments).

PHE for 4, 8, and 12 h in MC3T3-E1 osteoblastic cells. In addition, the rhythmic expressions of *Bmal1*, *Per2*, and *Nfil3* in bone were altered by systemic administration of 10 μg/g PHE at ZT0. The expression of *Nfil3* mRNA was increased significantly at ZT4, whereas *Bmp4* was decreased significantly at ZT4 and

ZT8 after systemic administration of PHE (Fig. 9). Taken together, these results indicate that α1-AR signaling negatively regulates *Bmp4* expression in osteoblasts. The transcriptional inhibition of *Bmp4* mRNA by α1-AR signaling suggests the involvement of transcriptional regulation by Nfil3 in osteoblasts. To test this possibility, we performed a ChIP analysis to identify the binding of Nfil3 to the putative binding sites in the promoter elements of *Bmp4* genes. Database analysis revealed three potential Nfil3 binding sites in the region of the *Bmp4* promoter (Fig. 10*A*). Specific primers were designed and synthesized to cover potential D-box binding sites and exon 1 for the *Bmp4* gene. ChIP experiments on MC3T3-E1 cells revealed the basal occupancy of Nfil3 on this binding element by agarose gel electrophoresis with rabbit polyclonal antisera to Nfil3 in MC3T3-E1 cells (Fig. 10*B*). ChIP assays with primers flanking exon 1 of the coding region showed no response to PHE (data not shown). Moreover, Nfil3 binding to the D-box-containing *Bmp4* promoter region was increased in MC3T3-E1 cells after PHE treatments (Fig. 10*C*), which indicated that the expression of Nfil3 induced by α1-AR signaling can bind directly to the *Bmp4* promoter and inhibit *Bmp4* expression in MC3T3-E1 cells. Taken together, the above data indicate that Nfil3/E4BP4 binding to the promoter element of the *Bmp4* mediates Nfil3-mediated *Bmp4* suppression in response to PHE stimulation in osteoblasts.

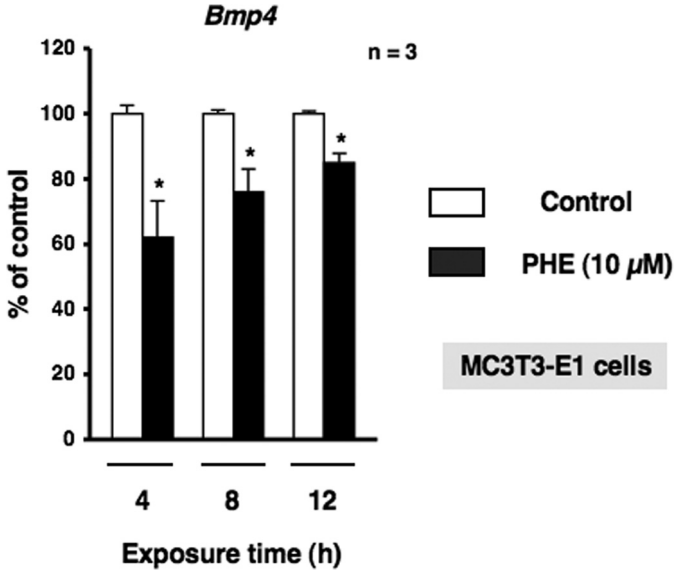

FIGURE 8. **α1-AR signaling down-regulated *Bmp4* mRNA in MC3T3-E1 cells.** MC3T3-E1 cells were treated with 10 μM PHE for 4, 8, and 12 h, harvested, and then processed for real-time qRT-PCR. Each value represents the mean ± S.E. of three separate experiments. *, $p < 0.05$, significantly different from each control value obtained in MC3T3-E1 cells cultured in the absence of PHE.

DISCUSSION

The results of this study demonstrate that α1-AR signaling in osteoblasts regulates clock genes and the putative interaction between Bmp4 and the circadian clock network in osteoblasts. In addition, the transcriptional factor Nfil3, in response to α1-AR signaling, negatively regulated *Bmp4* expression in

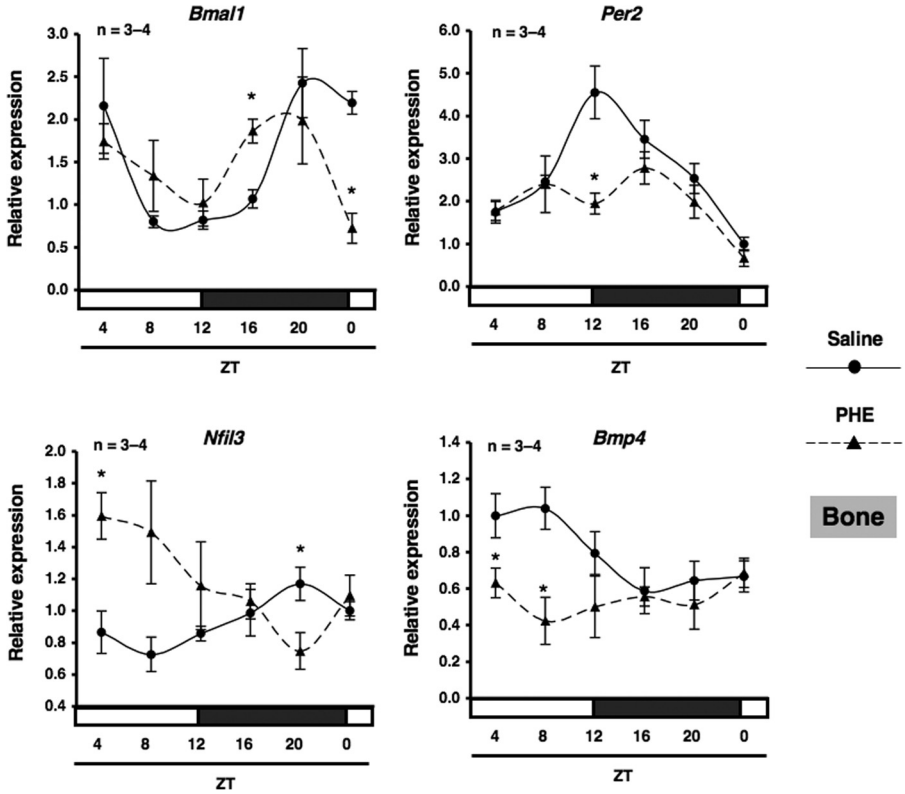

FIGURE 9. **α1-AR signaling regulated clock genes and *Bmp4* in bone.** The effect of the intraperitoneal administration of PHE at 10 μg/g on rhythmic expression of *Bmal1*, *Per2*, *Nfil3*, and *Bmp4* mRNA in bone is shown. C57BL/6J mice were maintained under a 12:12-hour light/dark cycle for 2 weeks, and then PHE was administered intraperitoneally at ZT0. Total RNA was isolated from the femurs (cancellous bone) of saline-treated and PHE-treated C57BL/6J mice. The mRNA levels of *Bmal1*, *Per2*, *Nfil3*, and *Bmp4* were determined by real-time qRT-PCR using specific primers. Each value is the mean ± S.E. ($n = 3$ or 4 in each group). *, $p < 0.05$, significantly different from each control value.

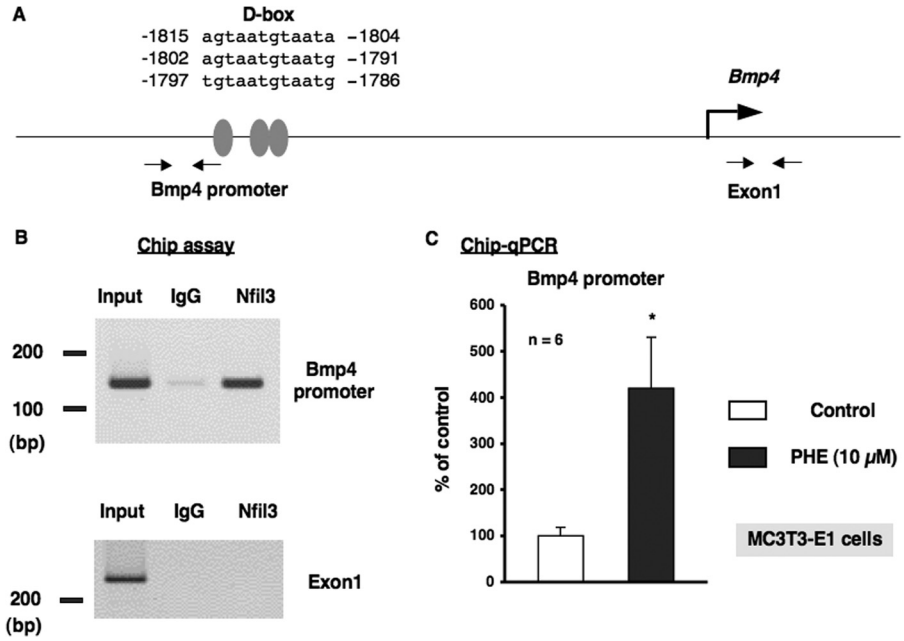

FIGURE 10. **α1-AR signaling regulated the promoter element binding of Nfil3 to the *Bmp4* gene in MC3T3-E1 cells.** *A*, schematic showing the three potential binding sites of the proximal D-box in the putative promoter element of the *Bmp4* gene. *Grey ovals* indicate the sites that match consensus D-box element sequences in the mouse *Bmp4* gene. PCR was performed using sequences found in the 5′ flanking region (−1906 to −1887 and −1777 to −1758) and the exon 1 region (+243 to +262 and +442 to +461) of the mouse *Bmp4* gene as primers. *B*, ChIP assays with antibodies against Nfil3 from MC3T3-E1 cells. A representative image of ChIP agarose gel electrophoresis. Nfil3/E4BP4 occupancy of this promoter region of the *Bmp4* gene was enriched in MC3T3-E1 cells. Chromatin was prepared from MC3T3-E1 cells and immunoprecipitated with anti-Nfil3, followed by PCR using primers from the *Bmp4* promoter or exon 1 of the *Bmp4* gene as a control. Ten percent of the input was loaded as a control. Anti-IgG antibodies were used as a control for specificity. PCR-amplified bands of the *Bmp4* promoter (*top panel*) and exon 1 of the *Bmp4* genes (*bottom panel*) in chromatin were precipitated by Nfil3 antibodies. *IgG*, nonimmune rabbit IgG; *Nfil3*, rabbit Nfil3 antibodies; *Input*, DNA input. *C*, PHE increased the promoter element binding of Nfil3/E4BP4 to the *Bmp4* gene. ChIP analysis revealed the increased binding of Nfil3 to the binding site of the *Bmp4* promoter element in MC3T3-E1 cells following the PHE treatments. Each value represents the mean ± S.E. of six separate experiments. *, $p < 0.05$, significantly different from the value obtained in MC3T3-E1 cells cultured in the absence of PHE.

osteoblasts. To the best of our knowledge, this study is the first to demonstrate the regulation of clock genes by α1-AR signaling in osteoblasts and the physiological significance of the circadian clockwork in bone metabolism.

An increasing number of studies are focusing on circadian oscillators in bone. It has been reported that treatments with isoprenaline, a nonspecific β-AR agonist, or dexamethasone induced the circadian expression of clock genes in human osteoblasts. Moreover, isoprenaline entrained oscillations in the osteoblast-related gene *Col1a1* (18). Recent studies in our laboratory reported that osteoclast-related genes such as *cathepsin K* and *nuclear factor of activated T-cells, cytoplasmic 1* showed the circadian rhythmicity in bone as well as the canonical core clock genes such as *Per1*, *Per2*, and *Bmal1* (21). The results of this study indicate that α1-AR signaling induces the rhythmic expression of the clock gene *Per2* and *Bmp4* mRNA in MC3T3-E1 osteoblastic cells. BMPs are members of the TGF-β superfamily that regulate cell growth, differentiation, and function (23) and play an important role in the regulation of normal physiological functions (24). BMP signaling is known to be mediated by the activation of type I and type II serine/threonine kinase receptors (25). Bmpr1a is a type I receptor that has a high affinity for BMP2 (26) and BMP4 (27), which are expressed in bone. BMPs have been shown to exhibit potent osteogenic activity *in vitro* (28), and the constitutive activation of BMPs or exogenous application of BMPs can induce ectopic bone formation *in vivo* (29). Therefore, *Bmp4* mRNA showed a rhythmic expression pattern as well as the circadian clock gene *Per2* in osteoblasts, which indicated that circadian oscillators regulate Bmp4 expression and may modulate diverse physiological processes in bone metabolism.

Previous studies, including ours, showed that the mRNAs of the α1-AR and β-AR subtypes were expressed in mouse and human osteoblasts (30, 31). β-AR signaling in osteoblasts has been shown to stimulate bone resorption by up-regulating *receptor activator of nuclear factor κB ligands* (Rankl). In addition, the physiological action of epinephrine has been demonstrated to be mediated by α1-AR signaling as well as β-AR signaling in osteoblasts. Epinephrine enhanced replication and alkaline phosphatase activities through α1-AR coupled to the G_i protein, which suggests that the pertussis toxin-sensitive G protein may be a potent mediator of cellular proliferation and alkaline phosphatase activities in MC3T3-E1 cells (9, 10). On the other hand, the activity of the sympathetic nervous system is known to have circadian rhythms (32). β-AR signaling in osteoblasts was shown to be involved in circadian rhythms in bone metabolism (33). Recent studies conducted in our laboratory showed that β-AR signaling entrained the circadian oscillation of clock genes (18). In this study, we showed that the transcriptional factor Nfil3/E4BP4, which functions as a key negative component of the *Drosophila* circadian clock (34–36), was up-regulated by α1-AR signaling, leading to the suppression of *Bmp4* gene expression in bone. We are particularly interested in the repression of *Bmp4* by Nfil3/E4BP4 in the context of circadian regulation because such transcription repressors of *Bmp4* have not yet been reported. The bioinfor-

matic approaches used in our studies have shown that the *Bmp4* gene contains three D-box elements within its promoters, and this finding indicates that *Bmp4* is a circadian-controlled gene that is regulated by circadian oscillators in osteoblasts. Because *Bmp4* transcripts were down-regulated in Nfil3-overexpressed MC3T3-E1 cells and up-regulated in Nfil3-silenced MC3T3-E1 cells, Nfil3 promoted the negative regulation of *Bmp4* expression in osteoblasts. Taken together, these findings suggest that Nfil3 represses *Bmp4* directly, possibly by binding DNA in their regulatory regions. Our ChIP-quantitative PCR results confirmed Nfil3 binding enrichment in the regulatory regions of *Bmp4* in MC3T3-E1 cells, which suggests that *Bmp4* is directly under the regulation of Nfil3 in osteoblasts. Therefore, these findings suggest that α1-AR signaling mediates osteoblast function through a mechanism relevant to the regulation of *Bmp4* expression in bone. However, BMP signaling is complex in bone. BMPs are potent inducers of ectopic bone formation (37–39), and mice with the postnatal inactivation of Bmpr1a, which is activated by the major BMP ligands BMP2 and BMP4, have been shown to have a greater bone mass (40), which is associated with the decreased expression of the Wnt antagonists sclerostin and dickkopf 1 (41). Therefore, the physiological significance of α1-AR signaling in bone remodeling has yet to be elucidated. The possibility that the regulation of *Bmp4* by α1-AR signaling may play a role in the regulation of bone metabolism will be investigated further in future studies.

In conclusion, our results revealed that sympathetic signaling by α1-AR regulated clock genes and *Bmp4* expression in osteoblasts. Moreover, the regulation of *Bmp4* through the up-regulation of Nfil3 by α1-AR signaling in osteoblasts may participate in bone metabolism controlled by circadian rhythms.

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