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RESEARCH ARTICLE

β-adrenergic receptor signaling regulates *Ptgs2* by driving circadian gene expression in osteoblasts

Takao Hirai, Kenjiro Tanaka and Akifumi Togari*

ABSTRACT

The sympathetic nervous system modulates bone remodeling and mediates the expression of core clock genes in part through the β-adrenergic receptor (β-AR) in osteoblasts. In this study, we show that in MC3T3-E1 osteoblastic cells that isoproterenol (Iso), a non-selective β-AR agonist, upregulated the transcriptional factor Nfil3, and induced rhythmic mRNA expression of prostaglandin-endoperoxide synthase 2 (*Ptgs2,* also known as Cox2). The rhythmic effects of Iso on *Ptgs2* expression were mediated by interplay between the *Per2* and *Bmal1* clock genes in osteoblasts. In addition, *Ptgs2* was significantly decreased in bone after continuous Iso treatment. Overexpression of Nfil3 decreased *Ptgs2* expression in MC3T3-E1 cells. Knockdown of Nfil3 upregulated the expression of *Ptgs2* in MC3TC-E1 cells, indicating that Nfil3 negatively regulated *Ptgs2* in osteoblasts. Furthermore, Iso acutely induced the expression Nfil3 and increased the binding of Nfil3 to the *Ptgs2* promoter in MC3T3-E1 cells. These results suggest that Iso-mediated induction of Nfil3 in osteoblasts regulates the expression of *Ptgs2* by driving the expression of circadian clock genes. These findings provide new evidence for a physiological role of circadian clockwork in bone metabolism.

KEY WORDS: Clock gene, Osteoblast, Ptgs2, Cox2, Nfil3, β-adrenergic receptor

INTRODUCTION

Bone is a metabolically active organ that maintains continuous remodeling throughout life. Osteoblasts, osteocytes and osteoclasts are involved in bone remodeling processes in mammals, and these are regulated by endocrine, autocrine and paracrine signals (Raisz, 2005). Several studies have demonstrated that the sympathetic nervous system also regulates bone remodeling (Takeda et al., 2002; Elefteriou et al., 2005; Togari and Arai, 2008; Kondo and Togari, 2011) and mediates the expression of core clock genes, in part through the β-adrenergic receptor (β-AR), in osteoblasts (Fu et al., 2005).

The molecular machinery underlying circadian clocks in the suprachiasmatic nucleus (SCN; also known as the central pacemaker of the circadian clock) and peripheral tissues is composed of an autoregulatory transcriptional–translational feedback loop involving multiple clock genes, such as circadian locomoter output cycles protein kaput (*Clock*), brain and muscle Arnt-like protein-1 (*Bmal1*, also known as *Arntl*), period 1–3 (*Per1–Per3*) and cryptochrome 1 and 2 (*Crys1* and *Crys2*), and their proteins, which are required for the generation of endogenous circadian oscillations (Ko and Takahashi, 2006). Circadian rhythms are ubiquitous in mammals, and are not only present in the SCN but also in various peripheral tissues (regarded as peripheral circadian oscillators) (Dibner et al., 2010). Indeed, an increasing number of investigations are focusing on circadian oscillators in bone. Sympathetic signaling and dexamethasone (Dex) have been shown to entrain bone circadian oscillators and the rhythmic expression of core clock genes in human osteoblasts (Komoto et al., 2012). This finding indicated that cells in bone share a similar molecular clock mechanism to that of the SCN. However, in spite of an increasing number of studies on circadian oscillators in osteoblasts, most have focused on the existence of circadian clock genes and their upstream stimulating factors. How the circadian oscillator in osteoblasts coordinates and regulates bone physiology in response to the sympathetic nervous system has yet to be elucidated.

In the present study, we determined that isoproterenol (Iso), a non-selective β-AR agonist, induced rhythmic mRNA expression of prostaglandin-endoperoxide synthase 2 [*Ptgs2*, also known as cyclooxygenase 2 (Cox2)] in MC3T3-E1 osteoblastic cells. We also demonstrated that nuclear factor interleukin-3 [Nfil3; also known as E4-binding protein 4 (E4BP4)], a negative regulator of circadian oscillation, played an important role in regulating *Ptgs2*, a most important regulator of bone metabolism.

RESULTS

β-AR signaling stimulated transcriptional rhythmicity in osteoblasts

To determine whether β-AR signaling in murine osteoblasts orchestrated rhythmic expression of the canonical clock gene *Per2*, MC3T3-E1 osteoblastic cells were treated with Iso at 1 μM for 2 h and *Per2* expression was determined using quantitative real-time PCR (qRT-PCR) analysis at the indicated time points between 1 h and 56 h. Exposure to Iso acutely increased *Per2* expression in a concentration-dependent manner (Fig. 1A) and induced rhythmic expression of *Per2* mRNA and protein in MC3T3-E1 osteoblastic cells (the period of oscillation was 24.7 h) (Fig. 1B,C). Bmal1 is known to be indispensable in sustaining the transcriptional–translational feedback loop of Per2 (Kumaki et al., 2008). Therefore, we examined whether Bmal1 mediated *Per2* expression in murine osteoblasts. Previous studies with continuous administration of Iso have revealed that chronic stimulation of β-AR by Iso reduced bone mass by increasing bone resorption and suppressing bone formation (Kondo and Togari, 2011). Therefore, we used a model of continuous stimulation of β-AR by administering Iso daily for 2 weeks with an osmotic pump, as described in the Materials and Methods. The results showed that *Bmal1* and *Per2* expression displayed a circadian

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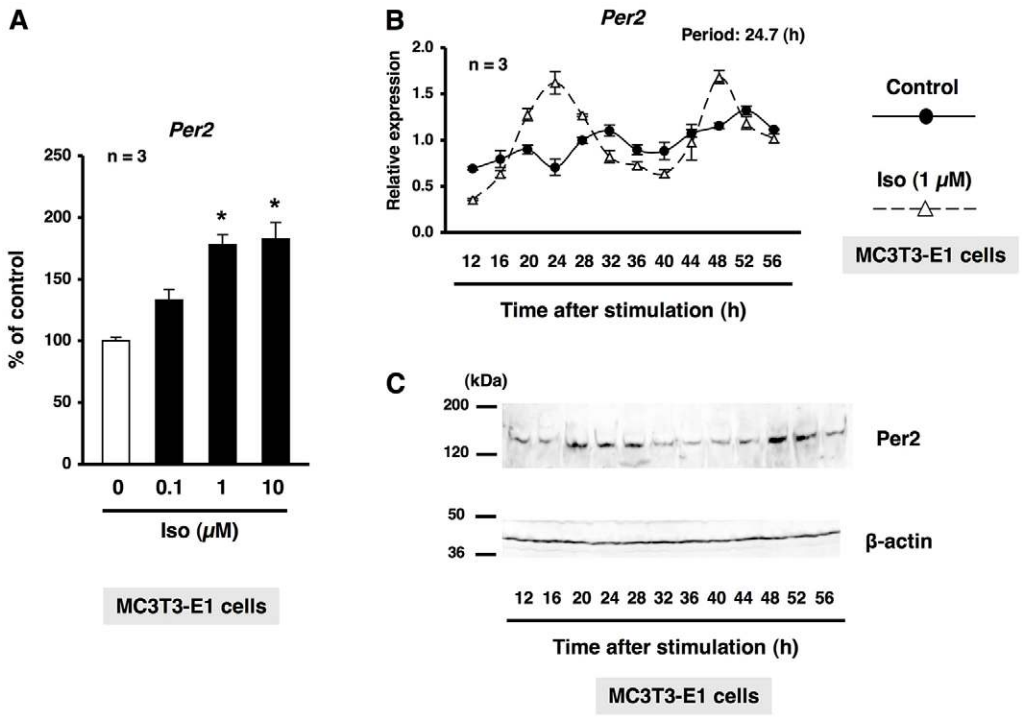

Fig. 1. β-AR signaling mediated circadian rhythmicity in MC3T3-E1 osteoblastic cells. (A) *Per2* mRNA was upregulated by Iso in a concentration-dependent manner in MC3T3-E1 cells. Cells were treated with Iso at 0.1 to 10 μM for 1 h, then harvested and processed for real-time qRT-PCR. *Gapdh* was used as an internal control. A representative result of three individual experiments is shown. Each value represents the mean±s.e.m. of three separate experiments. *$P<0.05$ compared with control value obtained in MC3T3-E1 cells cultured in the absence of Iso. (B) Expression profiles of the *Per2* transcript generated by Iso in MC3T3-E1 osteoblastic cells. Total RNA samples were collected at the indicated times after the Iso 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.m. of three separate experiments. The Cosinor analysis method was used to determine the rhythmic expression of *Per2*; the dashed lines show the fitting curves. (C) Expression profiles of the Per2 protein at the indicated times after Iso treatment in MC3T3-E1 osteoblastic cells. Western blot analyses were performed using the indicated specific antibody. β-actin was used as an internal control. A representative result of three individual experiments is shown.

pattern with opposing phases in bone (Fig. 2A). Furthermore, continuous treatment with Iso at 100 μg/g of body weight/day significantly increased *Per2* expression at zeitgeber time (ZT)0, 12, 16 and 20 and decreased *Bmal1* expression at ZT0 and ZT4 in bone (Fig. 2A). As shown in Fig. 2B, forced overexpression of the Bmal1–Clock complex significantly increased the expression of *Per2* in MC3T3-E1 cells. We then attempted to elucidate the mechanisms regulating *Per2* gene expression in MC3T3-E1 cells transfected with small interfering RNA (siRNA) for the knockdown of Bmal1 expression. Cells were transfected with siRNA for Bmal1, and the levels of *Bmal1* were determined by real-time qRT-PCR. The results demonstrated that *Bmal1* levels were significantly decreased in MC3T3-E1 cells transfected with *Bmal1* siRNA 24 h after transfection (Fig. 2C). In addition, a significant decrease in *Per2* expression was observed in MC3T3-E1 cells under these conditions (Fig. 2C). These finding indicate that circadian oscillators were impaired in osteoblasts treated with *Bmal1* siRNA, which provides a prerequisite for the further detection of circadian-regulated genes in osteoblasts.

Ptgs2 is a circadian-regulated gene in osteoblasts

To understand the physiological function of the circadian clockwork on bone metabolism and to identify circadian-regulated genes affected by β-AR signaling in osteoblasts, we evaluated the expression of canonical core clock genes and *Ptgs2* in bone after continuous Iso treatments by osmotic minipumps. As shown in Fig. 3A, we observed rhythmic expression of *Ptgs2* over 24 h. In addition, continuous treatment of Iso decreased *Ptgs2* expression at ZT0 and ZT12 and delayed the oscillation of *Ptgs2* from ZT12 to ZT16 (Fig. 3A). Furthermore, *Ptgs2* was significantly decreased at ZT0 by the continuous treatment with Iso at 25 and 100 μg/g of body weight/day in a dose-dependent manner (Fig. 3B), which indicated that *Ptgs2* is one of circadian genes regulated by the sympathetic nervous system in bone. In order to determine whether β-AR signaling in osteoblasts could mediate the rhythmic expression of *Ptgs2*, cells were treated with Iso at 1 μM for 2 h and *Ptgs2* expression were determined using real-time qRT-PCR analysis. Exposure to Iso entrained *Ptgs2* with rhythmic expression in MC3T3-E1 osteoblastic cells (the period of oscillation was 28.5 h) (Fig. 4A). To further establish whether Bmal1-mediated transcription activation was necessary for circadian regulation of *Ptgs2* mRNA in osteoblasts, we attempted to elucidate the regulation of *Ptgs2* expression in MC3T3-E1 cells transfected with siRNA either against *Bmal1* or *Per2*. Cells were transfected separately with siRNA against *Bmal1* or *Per2*, followed by the determination of *Ptgs2* levels using real-time qRT-PCR. On the one hand, a significant decrease was observed in *Ptgs2* expression in Bmal1-knockdown MC3T3-E1 cells (Fig. 4B). On the other hand, a significant increase was observed in *Ptgs2* expression in *Per2*-knockdown MC3T3-E1 cells (Fig. 4C). Taken together, these results suggest that *Ptgs2* is one of the circadian genes regulated in response to β-AR signaling in osteoblasts.

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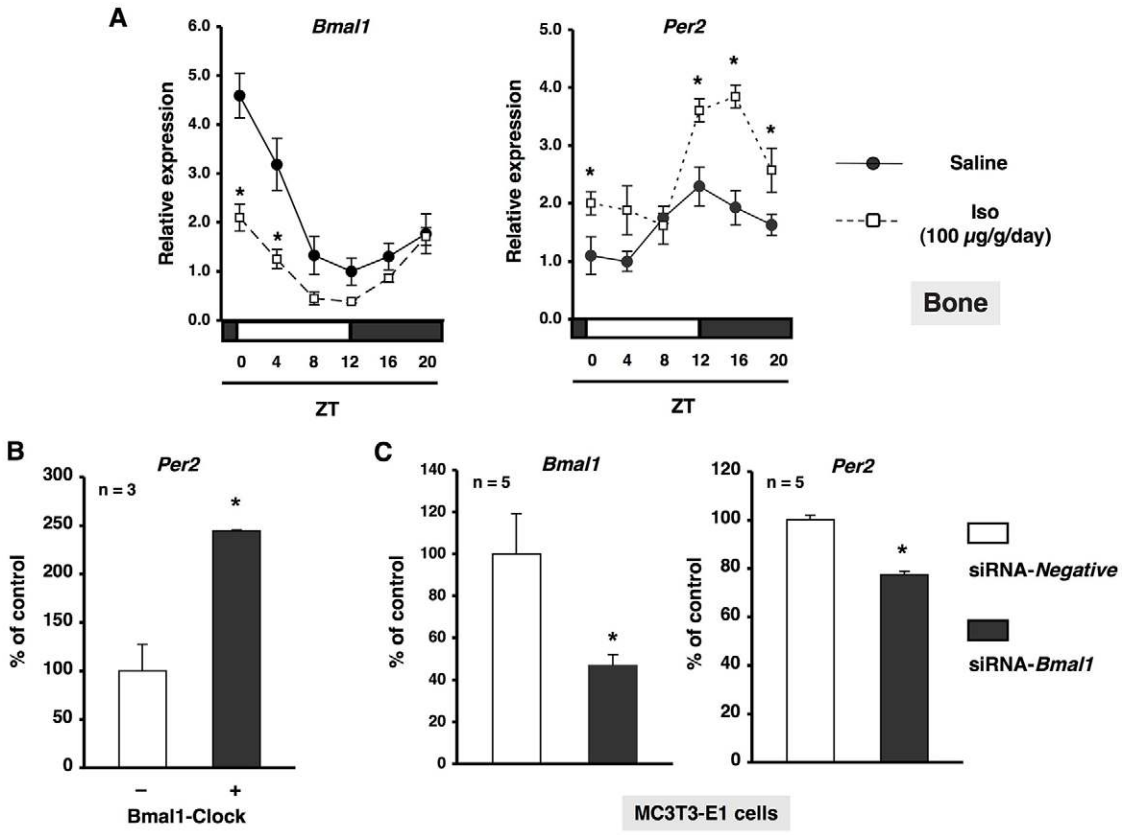

Fig. 2. β-AR signaling mediated circadian clock genes in bone. (A) A representation of the expression of circadian clock genes in bone from C57BL/6J mice treated with saline or Iso at 100 μg/g per body weight/day under light–dark cycle conditions. Bone was obtained from C57BL/6J mice every 4 h. Total RNA was isolated, and the level of mRNA was determined by real-time qRT-PCR using specific primers for *Bmal1* (left panel) and *Per2* (right panel). Relative mRNA levels were normalized to the *Gapdh* level. Data represent the mean±s.e.m. of four animals. White bars, light period; black bars, dark period. (B) Effects of the overexpression of Bmal1 and Clock in MC3T3-E1 cells. MC3T3-E1 cells were transiently transfected with expression vectors for Bmal1 and Clock, followed by further cultivation for 48 h and subsequent determination of the *Per2* levels by real-time qRT-PCR. The relative mRNA expression was normalized to *Gapdh* expression. Each value represents the mean±s.e.m. of three separate experiments. **P*<0.05 compared with control. (C) Bmal1-knockdown by siRNA in MC3T3-E1 cells. MC3T3-E1 cells were treated with *Bmal1* siRNA (siRNA-*Bmal1*) or non-silencing RNA (siRNA-*Negative*), followed by further cultivation for 48 h and subsequent determination of the *Bmal1* (left panel) and *Per2* (right panel) mRNA levels by real-time qRT-PCR. Each value represents the mean±s.e.m. of five separate experiments. **P*<0.05 compared with control.

Nfil3 negatively regulated Ptgs2 expression in MC3T3-E1 cells

Using a bioinformatics approach (http://www.cbrc.jp/research/db/TFSEARCH.html), we identified a putative consensus Nfil3/E4BP4 binding site in the *Ptgs2* promoter from −723 to −712 bp upstream of the transcription start site. Nfil3 binds 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 β-AR signaling that regulated the circadian expression of *Ptgs2* in osteoblasts. To investigate whether Nfil3 regulated *Ptgs2* gene expression, we transduced MC3T3-E1 cells with either empty pcDNA3 (pcDNA3-Negative) or pcDNA3-Nfil3. Expression of Nfil3 was confirmed by western blotting (supplementary material Fig. S1). Our results showed that forced overexpression of Bmal1–Clock upregulated *Ptgs2* gene expression in MC3T3-E1 cells, whereas forced overexpression of Nfil3 repressed it. Furthermore, overexpression of Nfil3 suppressed Bmal1–Clock-mediated upregulation of *Ptgs2* expression in MC3T3-E1 cells (Fig. 5A). These results suggest that Nfil3 is involved in rhythmic expression of *Ptgs2* in MC3T3-E1 osteoblastic cells. We then attempted to elucidate the relationship between Nfil3 and Ptgs2 in MC3T3-E1 cells. Based on our results (Hirai et al., 2014), transfecting MC3T3-E1 cells with siRNA against Nfil3, resulted in a significant decrease in Nfil3 by 24 h after transfection. Under these conditions, we observed a significant increase in *Ptgs2* expression in MC3T3-E1 cells (Fig. 5B). These results indicate that Nfil3 negatively regulates *Ptgs2* expression in MC3T3-E1 osteoblastic cells.

β-AR signaling regulated *Nfil3* in MC3T3-E1 osteoblastic cells

We next characterized the expression of *Nfil3* mRNA by β-AR signaling in bone. The systemic administration of Iso at 10 and 50 μg/g significantly increased the expression of *Nfil3* mRNA in bone (Fig. 6A). Furthermore, in total RNA extracted from MC3T3-E1 osteoblastic cells following exposure to Iso for 1, 2, and 4 h, the expression of *Nfil3* mRNA was significantly increased within 1 and 2 h after exposure to Iso. However, no significant alterations were observed in *Nfil3* expression at 4 h following the exposure to Iso (Fig. 6B). As shown in Fig. 6C, exposure to Iso induced Nfil3 protein expression in MC3T3-E1 osteoblastic cells. In addition, pretreatment with the β-AR antagonist propranolol completely prevented Iso-induced *Nfil3* expression in MC3T3-E1 osteoblastic cells, suggesting that upregulation of *Nfil3* expression by Iso is mediated by β-AR signaling (Fig. 6D).

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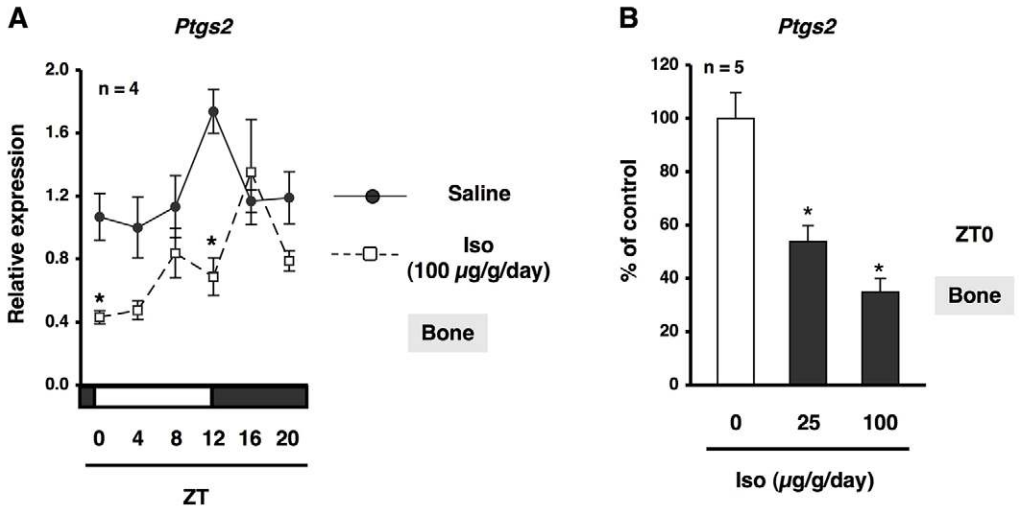

Fig. 3. Expression of *Ptgs2* after continuous treatment with Iso. Expression profile of *Ptgs2* mRNA in bone from C57BL/6J mice treated with saline or Iso at 100 μg/gram of body weight/day under light–dark cycle conditions. Bone was obtained from C57BL/6J mice every 4 h. Total RNA was isolated, and the level of *Ptgs2* mRNA was determined by real time qRT-PCR using specific primers. Relative mRNA levels were normalized to the *Gapdh* level. Data represent the mean±s.e.m. of 4 animals. White bars, light period; black bars, dark period. (A) Effect of the continuous administration of Iso at 25 and 100 μg/g of body weight/day on *Ptgs2* expression in bone. Total RNA was isolated from bone, and the level of *Ptgs2* mRNA was determined by real-time qRT-PCR using specific primers. Relative mRNA levels were normalized to the *Gapdh* level. Data represent the mean±s.e.m. of five animals. **P*<0.05 compared with control.

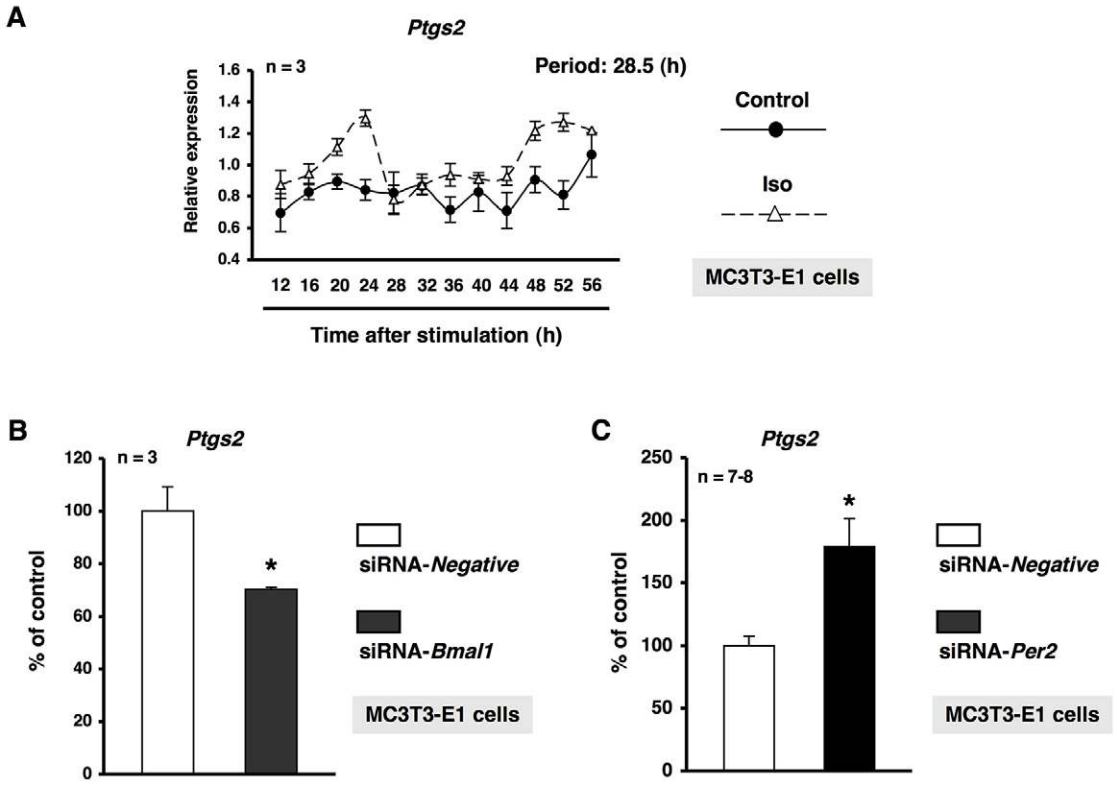

Fig. 4. Bmal1 regulated the circadian gene *Ptgs2* in MC3T3-E1 osteoblastic cells. (A) Expression profiles of the *Ptgs2* transcript generated by Iso in MC3T3-E1 osteoblastic cells. Total RNA samples were collected at the indicated times after the Iso treatment. qRT-PCR analyses of transcript levels were performed using their specific primers. *Gapdh* was used as an internal control. Each value represents the mean±s.e.m. of three separate experiments. The Cosinor analysis method was used to determine the rhythmic expression of the examined genes; the lines show the fitting curves. (B) Effect of *Bmal1* siRNA treatment on *Ptgs2* expression in MC3T3-E1 cells. MC3T3-E1 cells were treated with *Bmal1* siRNA (siRNA-*Bmal1*) or non-silencing RNA (siRNA-*Negative*), followed by further cultivation for 48 h and subsequent determination of *Ptgs2* mRNA levels by real-time qRT-PCR. Relative mRNA expression was normalized to *Gapdh*. Each value represents the means±s.e.m. of three independent determinations. **P*<0.05 compared with control. (C) Effect of *Per2* siRNA treatment on *Ptgs2* expression in MC3T3-E1 cells. MC3T3-E1 cells were treated with *Per2* siRNA (siRNA-*Per2*) or non-silencing RNA (siRNA-*Negative*), followed by further cultivation for 48 h and subsequent determination of *Ptgs2* mRNA levels by real-time qRT-PCR. Relative mRNA expression was normalized to *Gapdh*. Each value is the mean±s.e.m., *n*=7–8 independent experiments. **P*<0.05 compared with control.

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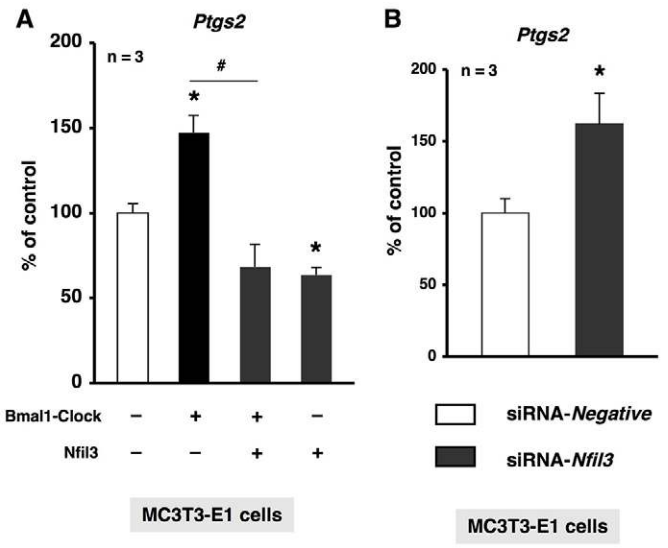

Fig. 5. Nfil3 regulated *Ptgs2* in MC3T3-E1 osteoblastic cells. (A) Effects of the overexpression of Bmal1, Clock and Nfil3 in MC3T3-E1 cells. MC3T3-E1 cells were transiently transfected with expression vectors for Bmal1, Clock and Nfil3, followed by further cultivation for 48 h and subsequent determination of *Ptgs2* levels by real-time qRT-PCR. Each value represents the mean±s.e.m. of three separate experiments. $^{*}P<0.05$ compared with the value obtained in cells transfected with the control vector. $^{\#}P<0.05$ compared with the value obtained in cells transfected with the Bmal1 and Clock expression vector. (B) Effects of Nfil3-knockdown by siRNA on the *Ptgs2* transcript in MC3T3-E1 cells. MC3T3-E1 cells were treated with *Nfil3* siRNA (siRNA-*Nfil3*) or non-silencing RNA (siRNA-*Negative*), followed by further cultivation for 48 h and subsequent determination of *Ptgs2* levels by real-time qRT-PCR. Relative mRNA expression was normalized to *Gapdh*. Each value represents the means±s.e.m. of three independent determinations. $^{*}P<0.05$ compared with control.

To determine whether Nfil3 directly regulated endogenous expression of the *Ptgs2* gene in osteoblasts, we used the chromatin immunoprecipitation (ChIP) assay to confirm that Nfil3 was bound to the *Ptgs2* promoter in MC3T3-E1 cells. Database analysis revealed a potential Nfil3 binding sites at −723 to −712 in the promoter, and at +2079 to +2090 (intron4), and +3344 to +3355 (intron5) and at +4147 to +4158 (intron7) in the *Ptgs2* gene. ChIP experiments on MC3T3-E1 cells were conducted using rabbit polyclonal anti-sera, followed by agarose gel electrophoresis. These assays revealed basal occupancy of Nfil3 to the element located at −723 to −712 in the *Ptgs2* promoter (Fig. 7A,B). Moreover, Nfil3 binding to the D-box-containing *Ptgs2* promoter region was increased in MC3T3-E1 cells after the Iso treatments (Fig. 7C). Taken together, these results indicate that the expression of Nfil3 induced by β-AR signaling directly bound to the *Ptgs2* promoter and inhibited *Ptgs2* expression in osteoblasts.

DISCUSSION

In the present study, we attempted to unravel the physiological implications of circadian oscillators in response to sympathetic nervous system activation in osteoblasts. Our results suggest that β-AR signaling increases the expression of Nfil3 and, consequently, its binding to the *Ptgs2* promoter, which in turn inhibits *Ptgs2* expression in osteoblasts (supplementary material Fig. S2).

Recent studies conducted in our laboratory have shown that treatments with Iso or Dex induce circadian expression of the clock genes *Per1*, *Per2*, *Per3* and *Bmal1* in human osteoblasts. On the one hand, Iso induced oscillations in the osteoblast-related gene *Col1a1*, but not in *ALP* or osteocalcin (Komoto et al., 2012). On the other hand, we did not observe any significant effects of Iso in osteoclasts, which indicates that Dex rather than Iso signaling governs the transcriptional rhythmicity of the molecular clock in osteoclasts (Fujihara et al., 2014). These findings suggest that the Iso-mediated effects on *Per2* expression in bone were reflected in osteoblasts. The results of the present study supply further evidence that Iso induces rhythmic mRNA expression of the clock gene *Per2* in MC3T3-E1 osteoblastic cells (Fig. 1). Additionally, *Per2* was significantly downregulated by the *Bmal1* siRNA, which implies that the molecular machines underlying circadian clocks in osteoblasts, as well as in other cell types, are composed of transcriptional–translational feedback loops involving clock genes that are mediated by E-box elements located in their promoter regions (Sato et al., 2006). However, we cannot deny the possibility that they might be under the regulation of clock-regulated genes, rather than core clock genes.

Ptgs2 is an important rate-limiting enzyme that determines the production of prostaglandin E2 (PGE_2), which mediates inflammatory responses, in addition to being one of the most important regulators of bone metabolism (Blackwell et al., 2010). Osteoblasts and osteocytes increase the expression of Ptgs2 and release PGE_2 in response to fluid flow stress (Wadhwa et al., 2002; Cherian et al., 2003). In addition, Ptgs2 regulates mesenchymal cell differentiation into the osteoblast lineage (Zhang et al., 2002) and osteoclast maturation (Blackwell et al., 2010). Rhythmic expression of *Ptgs2* mRNA levels was confirmed by the Cosinor method (Fig. 4A), indicating that circadian oscillators in osteoblasts might modulate diverse physiological processes by regulating *Ptgs2*, which in turn might regulate PGE_2 secretion and bone remodeling. The bioinformatics approach used in our studies revealed that the *Ptgs2* gene contains E-box and D-box sequences within the promoter, suggesting that *Ptgs2* is a circadian gene that is regulated by circadian oscillators in osteoblasts. Furthermore, downregulation of Bmal1 significantly decreased the expression of *Ptgs2* mRNA (Fig. 4B), which illustrated that rhythmic expression of *Ptgs2* is generated by its own transcriptional–translational feedback loop. This feedback loop might involve a set of clock genes that are regulated by the Bmal1–Clock heterodimer in MC3T3-E1 osteoblastic cells. Previous reports have shown that *Per2* genes have a cyclic AMP response element as well as D- and E-box-binding elements in their promoter (Yoo et al., 2005; Yagita and Okamura, 2000; Ohno et al., 2007). In the present study, acute Iso treatment increased *Per2* expression in MC3T3-E1 cells (Fig. 1A), whereas chronic Iso infusions increased *Per2* expression and delayed the oscillation of *Ptgs2* in bone (Fig. 2A; Fig. 3A). In addition, knockdown of *Per2*, which repressed *Bmal1* transcription, significantly increased *Ptgs2* expression (Fig. 4C). Therefore, Iso-mediated upregulation of *Per2* might play a role in the mechanism of Iso-mediated regulation of *Ptgs2* in osteobasts.

The activity of the sympathetic nervous system displays circadian rhythmicity (Herzog, 2007). In addition, β-AR signaling is involved in mediating circadian rhythms in bone metabolism (Fu, et al., 2005), in negatively regulate bone mass by inhibiting osteoblast proliferation (Takeda et al., 2002; Elefteriou et al., 2005) and in activating osteoclast activity (Togari and Arai, 2008; Kondo and Togari, 2011). Consistent with previous studies (Komoto et al., 2012), β-AR signaling mediated clock genes such

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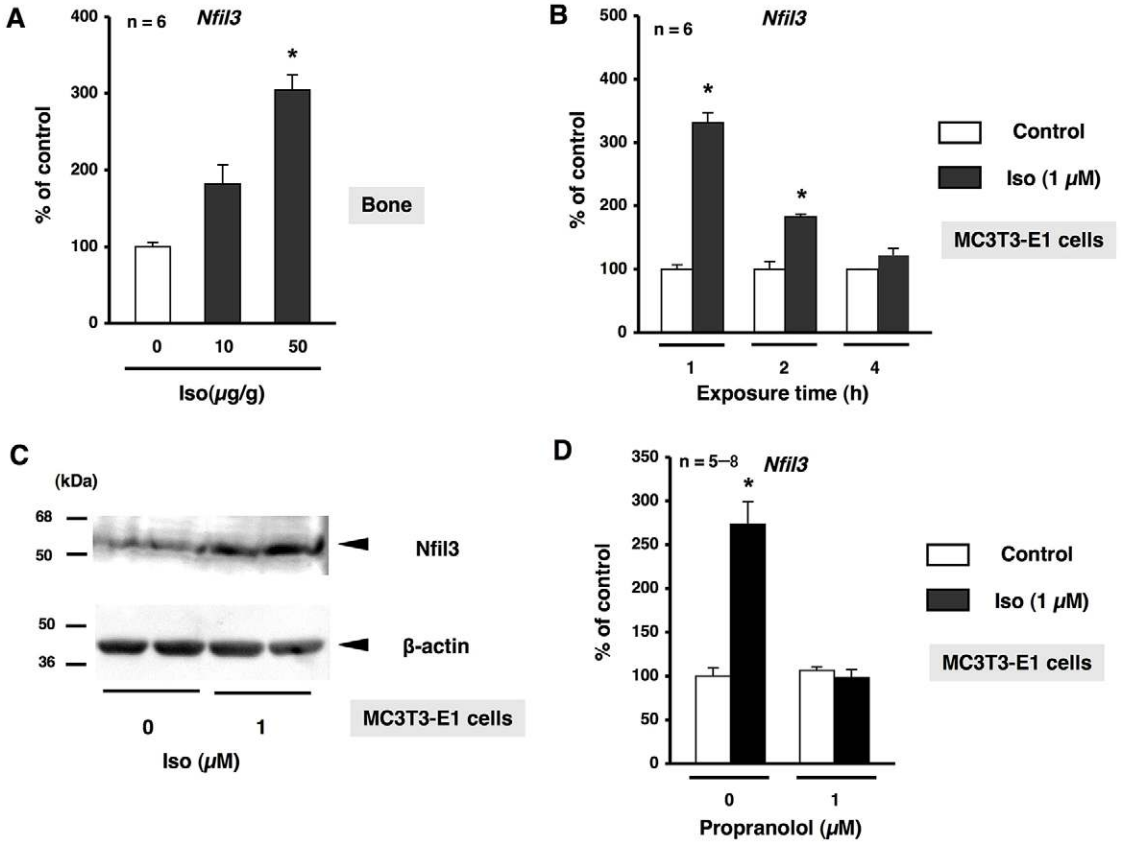

Fig. 6. β-AR signaling upregulated the clock gene *Nfil3*. (A) Effect of the intraperitoneal administration of Iso at 10 and 50 μg/gram of body weight on *Nfil3* mRNA expression in bone. Total RNA was isolated from C57BL/6J mice 4 h after the administration of Iso, and the level of Nfil3 mRNA was determined by real-time qRT-PCR using specific primers. Each value represents the mean±s.e.m. of six animals. **P*<0.05 compared with control. (B) *Nfil3* mRNA expression in MC3T3-E1 cells. Cells were treated with 1 μM Iso for 1, 2 and 4 hours, then harvested and processed for real-time qRT-PCR. A representative result of three individual experiments is shown. Each value represents the mean±s.e.m. of six separate experiments. **P*<0.05 compared with control value obtained in MC3T3-E1 cells cultured in the absence of Iso. (C) Nfil3 was increased by Iso in MC3T3-E1 cells. Cells were treated with Iso at 1 μM for 4 h, harvested and then processed for western blotting. β-actin was used as an internal control. Each lane indicates a sample from one well in the panel. A representative result of three individual experiments is shown. (D) β-AR signaling mediated *Nfil3* mRNA expression after the Iso stimulation in MC3T3-E1 cells. Cells were incubated for 1 h in the presence of Iso with propranolol at a concentration of 1 μM, followed by the determination of Nfil3 levels by real time qRT-PCR. Each value is the mean±s.e.m., *n*=5–8 independent experiments. **P*<0.05 compared with control.

as *Per2* and *Bmal1* in osteoblasts (Fig. 2). The bZIP transcription factor Nfil3/E4BP4 is a mammalian homolog of *vrille*, which functions as a key negative component of the *Drosophila* circadian clock (Blau and Young, 1999; Cyran et al., 2003, Glossop et al., 2003) and is believed to be involved in the mammalian circadian clock (Mitsui et al., 2001; Ueda et al., 2005; Akashi et al., 2006). Additionally, Nfil3 plays an essential role in natural killer (NK) cell development and IgE class switching (Gascoyne et al., 2009; Kamizono et al., 2009; Kashiwada et al., 2010). In the present study, we showed that Nfil3 was upregulated by β-AR signaling in osteoblasts. The detailed mechanisms by which Nfil3 is upregulated in osteoblasts remain to be established. Previous reports have shown that the expression of *Nfil3* is upregulated by elevated intracellular Ca^{2+} concentration and by activation of cAMP-response-element-binding protein (MacGillavry et al., 2009; Nishimura and Tanaka, 2001). Considering that Nfil3 was upregulated in MC3T3-E1 cells exposed to Iso, it is possible that cAMP/PKA pathways might trigger upregulation of Nfil3 in MC3T3-E1 cells. However, given that the expression of *Nfil3* mRNA is not sustained beyond 4 h, it would suggest that additional posttranscriptional mechanisms such as mRNA destabilization play a later role. Physiologically, it appeared that Nfil3 exerted a negative regulatory effect on *Ptgs2* expression in osteoblasts because *Ptgs2* transcripts were downregulated in Nfil3-overexpressed MC3T3-E1 cells and upregulated in Nfil3-silenced MC3T3-E1 cells (Fig. 5).

Our results reveal that the effects of Iso on *Ptgs2* expression were exerted by a complex counter regulatory mechanism. On the one hand, the rhythmic effects of Iso on *Ptgs2* expression that were described in Fig. 4 were mediated by an interplay between the Per2 and Bmal1 clock proteins at the E-box of the *Ptgs2* promoter (supplementary material Fig. S2). On the other hand, Iso-mediated chronic activation β-AR signaling increased the expression of Nfil3 (RT-PCR) and its binding to D-box elements in the *Ptgs2* promoter (ChIP assay), which in turn inhibited the expression of *Ptgs2* (supplementary material Fig. S2). These results provide a molecular mechanism for the control of bone metabolism by circadian rhythms.

In conclusion, our results suggest that β-AR signaling in osteoblasts regulate *Ptgs2* through upregulation of Nfil3, a negative regulator of circadian oscillations, and thus provide new evidence for the physiological importance of the circadian clockwork in bone metabolism.

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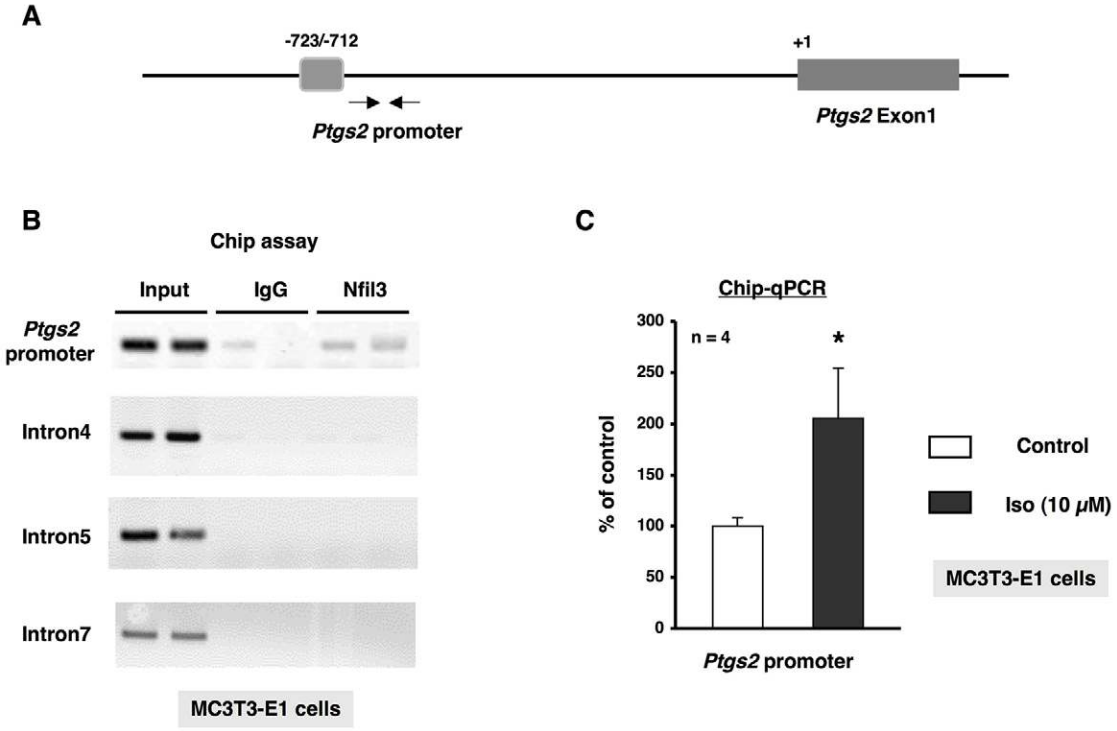

Fig. 7. β-AR-signaling-mediated promoter element binding of Nfil3 to the *Ptgs2* gene in MC3T3-E1 cells. (A) Schematic diagram showing one potential binding site of the proximal D-box in the putative promoter element of the *Ptgs2* gene. Closed boxes indicate the sites that match the consensus D-box element or E-box sequences in promoter element in the mouse *Ptgs2* gene. (B) ChIP assays with antibodies against Nfil3 from MC3T3-E1 cells. PCR was performed using sequences found in the 5′ flanking region (−723 to −712), the intron 4 region (+2079 to +2090), the intron 5 region (+3344 to +3355) and the intron 7 region (+4147 to +4158) of the mouse *Ptgs2* gene as primers. A representative picture of ChIP agarose gel electrophoresis. Anti-IgG antibodies were used as a control for specificity. Nfil3 occupancy to this promoter region of the *Ptgs2* gene was increased in MC3T3-E1 cells. PCR-amplified 136-bp *Ptgs2* promoter bands in chromatin precipitated by Nfil3 antibodies. IgG, nonimmune rabbit IgG; Nfil3, rabbit Nfil3 antibodies; Input, DNA input. (C) Iso increased the promoter element binding of Nfil3 to the *Ptgs2* gene. ChIP analysis revealed the increased binding of Nfil3 to the binding site of the *Ptgs2* promoter element in MC3T3-E1 cells following the Iso treatments. Each value represents the means±s.e.m. of four independent determinations. **P*<0.05 compared with the value obtained in MC3T3-E1 cells cultured in the absence of Iso.

MATERIALS AND METHODS

Mice

Male eight-week-old C57BL/6J mice (Japan SLC, 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℃) and humidity (50±10%) under a 12-hour-light–12-hour-dark cycle or under constant dark conditions. Zeitgeber time (ZT) 0 under the light–dark cycle was designated as lights on and ZT12 as lights off.

Drug treatments

Mice were administered isoproterenol (Sigma-Aldrich) at 100 µg/g body weight daily for 2 weeks or saline as a vehicle control by osmotic minipumps (Alzet model 2002; Durect, Cupertino, CA) implanted into the subcutaneous tissue in the back over a period of 14 days, according to the manufacturer's instructions. On the last day of the experiment, all mice were killed by cervical dislocation under ether anesthesia, and the femora and tibiae were collected (Fujihara et al., 2014). Bone tissue samples were dissected and kept at −80℃ until assayed for total RNA.

MC3T3-E1 cell cultures

MC3T3-E1 cells were purchased from the RIKEN Cell Bank. MC3T3-E1 cells were cultured in α-MEM (Life Technologies) containing 10% FBS and 1% penicillin-streptomycin at 37℃ under 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.

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 small interfering RNA (siRNA) targeting genes or non-silencing RNA diluted in Opti-MEM using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocol. Silencer Select siRNAs were used (Ambion/Applied Biosystems). *Bmal1*, *Per2*, *Nfil3* and non-silencing siRNAs 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.

Construction of expression plasmids and transfection

The entire coding sequence of mouse Nfil3 cDNA was amplified by KOD-plus Neo DNA polymerase (Toyobo, Japan) and inserted into the mammalian expression vector pcDNA3. The primers used were as follows: forward 5′-CGCGGATCCTGGGGAAGTGATTTGTCTCC-3′ and reverse 5′-CCGCTCGAGTCCTCCTCCATGCATAGCTC-3′. Amplified DNA was cloned into the pcDNA3 vector at the *Bam*HI and *Xho*I restriction enzyme sites (underlined). The correct sequences of the subcloned cDNA fragment were confirmed by complete nucleotide sequencing. The other plasmids used in this study were obtained from Addgene. mClock and mBmal1 cDNA were cloned into pcDNA4 to produce pCKPC4, which expresses hexahistidine- and Flag-tagged mouse CLOCK (pcDNA-Clock), and also into pcDNA3 to produce pBMPC3, which expresses hexahistidine-tagged mouse BMAL1 (pcDNA-Bmal1). MC3T3-E1 cells were plated at a density of 1.0×10^5 cells/cm^2. Cells were transfected after 24 h with pcDNA-Nfil3, pcDNA-Bmal1, pcDNA-Clock

or the empty vector using FugeneHD reagent (Promega) according to the manufacturer's instructions.

RNA extraction and quantitative RT-PCR

As an *in vivo* experiment, total RNA was extracted from the femur (cancellous bone). Total RNA was isolated with an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. A total of 1 µg 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℃ for 2 min, 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ 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 qRT-PCR assay. The relative quantity for each sample was normalized to the average level of the constitutively expressed housekeeping gene *Gapdh* (Komoto et al., 2012). The following primers were used: *Gapdh*, forward 5′-TGGAGAAACCTGCCAAGTATG-3′, reverse 5′-GGAGACAACCTGGTCCTCAG-3′; *Nfil3*, forward 5′-CAGTGCAGGTGACGAACATT-3′, reverse 5′-TTCCACCACACCTGTTTTGA-3′; *Ptgs2*, forward 5′-TGCCTGGTCTGATGATGTATG-3′, reverse 5′-GGGGTGCCAGTGATAGAGTG-3′; and *Per2*, forward 5′-ATGCTCGCCATCCACAAGA-3′, reverse 5′-GCGGAATCGAATGGGAGAAT-3′.

Western blotting 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 previously described inhibitors of phosphatases and proteases (Hirai et al., 2011). Cell homogenates were mixed at a volume ratio of 4:1 with 10 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% Bromophenol Blue and 5% mercaptoethanol, followed by boiling at 100℃ for 5 min, as previously described (Hirai et al., 2006). Each aliquot 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 onto a polyvinylidene fluoride membrane. The membranes were subjected to blocking with 5% low-fat 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 and Per2 (Santa Cruz Biotechnology) adequately diluted in 1% low-fat milk in TBST. Finally, proteins were reacted with the anti-rabbit-IgG antibody conjugated to horseradish peroxidase and detected with the aid of ECL detection reagents.

Chromatin immunoprecipitation assay

The ChIP assay was performed using ChIP-IT express (Active Motif) according to the manufacturer's instructions. Cell extracts were immunoprecipitated with polyclonal rabbit anti-Nfil3 (Santa Cruz). Antibody specificity was assayed with normal rabbit IgG (Santa Cruz Biotechnology). Purified DNA fragments were subjected to PCR, assessing for the *Ptgs2* promoter region −665 to −530, using the following primers: 5′-CGATGAAGTGGAGCTCAGCA-3′ and 5′-TCCTCTGTCAGGGTCCACAT-3′. PCR products were also analyzed on 2.5% agarose gels.

Data analysis

All data are expressed as the mean±s.e.m. Differences between groups were tested for statistical significance using the unpaired two-tailed Student's *t*-test. $P<0.05$ was considered to indicate significance. 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 by using the chronobiometric ellipse test (Komoto et al., 2012).

Competing interests

The authors declare no competing interests.

Author contributions

Study design was undertaken by T. H. Study conduct was undertaken by T. H. and T. K. Data interpretation was undertaken by T. H. and T. K. Manuscript preparation was undertaken by T. H. Drafting of the manuscript was undertaken by T. H. Manuscript revision was undertaken by T. H. Approving final version of manuscript was undertaken by T. H., T. K. and A. T.; T. H. and A. T. take responsibility for integrity of the data analysis.

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Supplementary material

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