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Original Article

The period circadian clock 2 gene responds to glucocorticoids and regulates osteogenic capacity

Takahiro Abe [a, *], Tomoya Sato [b], Tetsuya Yoda [c], Kazuto Hoshi [a]

[a] Oral and Maxillofacial Surgery, Department of Sensory and Motor System Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[b] Department of Plastic and Reconstructive Surgery, Saitama Medical University, Saitama, Japan
[c] Department of Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

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ABSTRACT

Introduction: The central regulatory system that generates biological rhythms is regulated by circadian clock genes expressed by cells in the suprachiasmatic nucleus. Signals from this system are converted to adrenocortical hormones through the sympathetic nervous system and transmitted to peripheral organs. Another system releases glucocorticoids (GCs) in response to stress through the HPA-axis. Here we investigated the second messenger GC, which is shared by these systems and influences the expression of circadian clock genes of cells of the musculoskeletal system and in viable bone tissue.

Methods: We used mouse-derived cell lines, which differentiate into osteoblasts (MC3T3-E1, C2C12, and 10T1/2) as well as primary cultures of mouse osteoblasts to determine the expression levels of circadian clock genes that respond to GC. Mice (mPer2$^{m/m}$) with an inactivating mutation in the period circadian clock 2 gene (*Per2*) exhibit marked dysrhythmia. Here we compared the bone morphologies of mPer2$^{m/m}$ mice with those of wild-type (WT) mice.

Results: The expression of major circadian clock genes was detected in each cell line, and their responsiveness to GC was confirmed. We focused on *Per2*, a negative regulator of the circadian clock and found that a *Per2*-loss-of-function mutation increased the proliferative capacity of osteoblasts. Treatment of mutant mice with slow-release GC and bisphosphonate affected the maturation of bone tissue, which reflects a tendency to retard calcification.

Conclusion: Our investigations of the mechanisms that regulate circadian rhythm function in tissues of the musculoskeletal system that respond to the stress hormone GC, reveal that *Per2* is required for the maturation of bone tissue. Thus, the influences of the systems that control circadian rhythms and the responses to stress by regenerating tissue used for regenerative medicine must be considered and studied in greater detail.

1. Introduction

The 2017 Nobel Prize in Physiology or Medicine was awarded to researchers who identified genes responsible for controlling the circadian rhythm and their mechanisms of action (https://www.nobelprize.org/prizes/medicine/2017/summary/). The central control system generating circadian rhythm resides in the suprachiasmatic nucleus (SCN) and is regulated by factors expressed by circadian clock genes through negative feedback mechanisms [1,2]. The master regulators of the circadian clock system CLOCK and ARNTL/BMAL1 form a heterodimer and bind to an enhancer sequence called the E box in circadian clock genes such as *Per* and *Cry* to promote their transcription. Proteins encoded by *Per* (PER1, 2,

Abbreviations: CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; HPA-axis, hypothalamic-pituitary-adrenal-axis; ASPS, advanced sleep phase syndrome; BMSCs, bone marrow stem cells; BV/TV, bone volume/tissue volume; OV/TV, Osteoid volume/Tissue volume; OV/BV, Osteoid volume/ Bone volume; OS/BS, Osteoid surface/ Bone surface; Tb.Th, Trabecular thickness; ES/BS, Eroded surface/ Bone surface; MS/OS, Mineralizing surface/Osteoid surface; G.P.Th, growth plate thickness.

* Corresponding author. The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan. Fax: +81 3 5800 6832.
E-mail address: abetakahiro.dream@gmail.com (T. Abe).

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and 3) and *Cry* (CRY1 and 2) form a complex that suppresses transcriptional activation by the CLOCK–ARNTL complex to generate the core loop of a circadian rhythm [1,2].

Circadian clock genes are intimately associated with sleep. For example, a mutation in *Per2* causes a sleep disorder, advanced sleep phase syndrome (ASPS), which is inherited and caused by a missense mutation (S662G) in *PER2*, which alters the circadian period [3]. Further, *Per2* function is associated with other pathologies. For example, *Per2* is involved in suppressing tumors through other genes that affect cell proliferation and death such as cyclin-dependent kinase 1, cyclin A1, transformed mouse 3T3 cell double minute 2, and growth arrest and DNA-damage-inducible 45 alpha [4]. Therefore, disruption of the circadian clock machinery may increase the risk of developing cancer [4,5]. Further, *Per2* regulates time-of-day-dependent variation in the passive cutaneous anaphylactic reaction of mice by controlling the rhythmic secretion of glucocorticoids (GCs) from the adrenal glands, gating the responses to GCs of mast cells at certain times of day, or both [6]. These findings suggest that the circadian clock system is intimately involved in allergic diseases such as asthma, allergic rhinitis, and chronic urticaria through its effects on GC levels.

Adrenocortical hormones are secreted in a rhythmic manner. The amplitudes of their levels undergo circadian variations during intervals of approximately 24 h [7]. The phase of the rhythm in humans peaks upon waking, while the phase in mice is nocturnal [8]. The circadian signal emitted by the SCN is regulated through preganglionic sympathetic neurons in the spinal cord [9]. GCs subsequently produced by the adrenal cortex serve as messengers to transmit the clock signal to peripheral cells. Thus, the signaling pathway through which the activity of the SCN-sympathetic nerves, which function as the central control system of rhythm, is altered to produce GCs, and the rhythmic input signal of GCs is transmitted to cells distributed throughout the body [5]. In contrast, GCs produced by the adrenal cortex play a central role in host defenses in response to stress, and together with mineral corticoids and androgens, mediate the regulation of electrolyte levels, blood pressure, and sugar metabolism [10]. The secretion of GCs under stress is controlled by the so-called hypothalamic pituitary adrenal (HPA) axis, whose components are located in the hypothalamus, which releases corticotropin-releasing hormone (CRH), which in turn binds to a receptor that mediates the release of adrenocorticotrophic hormone (ACTH) from adenohypophysial cells [11]. ACTH facilitates the secretion of GCs (cortisol in humans and corticosteroids in rodents) from the adrenal cortex, independent of SCN-sympathetic nerves [11].

Evidence indicates that GCs act directly and indirectly in bone tissue to cause osteoporosis [reviewed in 12]. However, we are aware of only a few studies [13–15,18] on the interactions of bone with circadian clock genes. Moreover, the expression of clock genes in osteoblasts is regulated by the sympathetic nervous system and the hormone leptin, which regulates bone remodeling, a homeostatic function that maintains a constant bone mass [13]. In homozygous *Arntl/Bmal1*-deficient ($Arntl^{-/-}$) mice, osteoclast differentiation is increased by downregulating the expression of osteoprotegerin [14]. Further, osteoclast-specific *Arntl/Bmal1*-knockout mice have increased bone mass because of diminished osteoclast differentiation [15], and ARNTL/BMAL1-deficient osteoblasts have a higher ability compared with wild-type (WT) mice to support osteoclastogenesis by inducing the synthesis of RANKL, which is an endogenous osteoclast-activating factor secreted mainly by osteoblasts and activated T cells [16,17]. These results suggest that bone resorption and mass are control by a complex mechanism employed by the osteoblastic Clock system [18]. These studies of the association of ARNTL/BMAL1 with bone metabolism include insufficient data about the influence of PER2 on bone tissues. Here we used *Per2*-mutant mice ($mPer2^{m/m}$) to determine how GCs, the second messengers that regulate rhythmic and stress responses, influence the expression of circadian clock genes of cells that regulate bone homeostasis, and assessed the effects of PER2 on bone tissue.

2. Methods

2.1. Cell culture

The mouse preosteoblastic cell line MC3T3-E1, the premyoblastic cell line C2C12, and the fibroblast-like cell line 10T1/2 were cultured in α-modified Minimum Essential Medium (α-MEM) (WAKO, Osaka, Japan) with 10% fetal bovine serum (FBS) (BioWest, Nuaillé, France); DMEM with 10% FBS, Basal medium Eagle (BME) with 10% FBS; RPMI 1640 with 10% FBS; and α-MEM with 10% FBS and 50 μg/ml M-CSF, respectively. The growth- and conditioned media were changed every three days. All cultures were maintained at 37 °C in humidified air in an atmosphere containing 5% CO_2 and were passaged every 7 days. Dr. T. Ogasawara provide the MC3T3-E1 and 10T1/2 cell lines were obtained from the RIKEN BRC-Cell bank (Tsukuba, Japan).

2.2. Mice

Animal experiments were conducted according to the ARRIVE Guidelines for Reporting Animal Research [19] as previously described [20]. Adult female (15 weeks old) *Per2* mutant mice ($mPer2^{m/m}$) [21] with an ICR background and the same strain of wild-type mice were housed in a temperature-controlled (24 ± 1 °C) room under a 12-h light:12-h dark cycle. The $mPer2^{m/m}$ mice carry an in-frame deletion in the sequence encoding the PER-ARNT-SIM–B (PAS) domain and are deficient in mPer2-mediated transcriptional regulation [21]. These mice were provided by S. Shibata. Primary murine osteoblasts (pOBs) were isolated from the skulls of newborn mice. Neonatal mouse skulls were dissected free of adherent soft tissue, washed in PBS, and sequentially digested with 0.2% dispase and 0.1% collagenase. The experiments were conducted according to the institutional ethical guidelines for research involving animals. The Institutional Review Board of Saitama Medical University approved this study.

2.3. RT-PCR analysis of clock gene expression

Confluent cultures were incubated in the presence of 0–10^3 nM dexamethasone (Dex) for 0–8 days. Total RNA (2 μg) was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan) and subjected to RT-PCR using a SuperScript One-Step RT-PCR System with a Platinum Taq Kit (Invitrogen, California, USA) according to the manufacturer's instructions. Gene-specific primer pairs are shown in Table 1. For RT-PCR analysis, cDNA synthesis was performed for 30 min at 45 °C, and the products were denatured for 2 min at

Table 1
RT-PCR primers.

Genes	Upstream (5′-3′)	Downstream (5′-3′)
Per1	ccatggacatgtctact	agaggaccaggggacat
Per2	ctacctggtcaaggtgcaagag	ggtttgaatcttgccactgg
Per3	tcctgatggtaagacattccag	gcgtgaacaatcacactcactt
Cry1	cgtctgtttgtgattcgggg	attcacgccacaggagttgc
Cry2	agaaggtgaagaggaacagcac	tagatgtatcgagaggggaagc
Arntl/Bmal1	tcaagaatgcaagggaggcc	aacaggtagaggcgaagtcc
Clock	ctatgcttcctggtaacgcg	gcctattattggtggtgccc
Gapdh	tgaaggtcggtgtgaacggatttggc	tgaaggtcggtgtgaacggatttggc

94 °C. PCR amplification was performed as follows: 26 cycles of denaturation for 60 s at 94 °C with primer annealing for 90 s at 48 °C (*Arntl/Bmal1*), 50 °C (*Per1*, *Per3*, *Cry1*, and *Cry2*) and 55 °C (*Per2*, *Clock*); and extension for 180 s at 72 °C. *Gapdh* mRNA was used as a loading control. Expression of mRNA was detected using a semiquantitative RT-PCR assay. The PCR conditions were determined so that the band intensity was a linear function of the number of cycles. Bands were quantified using a LAS-3000mini luminescent image analyzer (Fuji Film, Japan), and the intensities were normalized to those of *Gapdh*.

2.4. Cell proliferation

Cells (4×10^3 cells per well) were added to the wells of 96-well plates. To assess the effects of Dex on pOBs derived from WT and mPer2$^{m/m}$ mice, the cells were incubated in growth medium with 100 nM Dex in the presence or absence of 100 μM zoledronic acid (ZA). Concentrations were determined the approximate value required to express clock genes by the dilution method using concentrations between 1 and 1000 (DEX: nM, ZA: μM). The sample cells were quantified using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA), according to the manufacturer's instructions. Briefly, 20 μl of MTS was added to 100 μl of culture medium. After incubation for 2 h at 37 °C, the absorbance was measured at 490 nm using a Model 680 XR plate reader (BIO RAD, USA). The measurements represent the mean of at least three independent experiments (six replicates per data point).

2.5. Experiments using mice

Slow-release pellets (Innovative Research of America, Sarasota, FL) containing prednisolone (PSL) (15 mg/21 day) were implanted into the lateral side of the neck according to the manufacturer's protocol. Bisphosphonate (BP), in the form of ZA, was provided by Novartis Pharma AG (Basel, Switzerland). We treated WT and mPer2$^{m/m}$ mice with once-weekly subcutaneous injections of 100 μg/kg ZA. PSL was released from pellets implanted into the mice for 21 days, and the weekly injections of ZA continued for 21-days. ZA inhibits bone resorption by inducing osteoclasts to undergo apoptosis and was therefore used to assess osteogenic capacity.

2.6. Microfocus X-ray computed tomography (μ-CT)

Microfocus X-ray computed tomography (μCT) (ELE SCAN; Nittetsu Elex Co. Ltd., Tokyo, Japan), performed according to the manufacturer's protocol, was used to obtain cross-sectional views of the secondary spongiosa in the distal tibial metaphysis, approximately 0.28 mm distal to the growth plate. μCT settings were as follows: energy, 67.0 kV; current, 100 μA; and slice thickness, 13.09 μm (n = 4 mice per group). Three-dimensional (3D) digital images were reconstructed using above software.

2.7. Bone histomorphometry

Bone specimens were fixed, left undecalcified, and embedded in a volume of ethanol (70–80%), which was 25-times the volume of the sample, as previously described [20]. Calcein (16 mg/kg) (Dojindo, Kumamoto, Japan) was injected intraperitoneally 4 days and 1 day before the mice were killed. The dose for subcutaneous administration was 0.1 mL/10 g body weight. A computer and digitizer tablet (Histometry RT Camera; System Supply Co., Ltd., Nagano, Japan) were used for histomorphometric analysis, which was conducted by the Niigata Bone Science Institute (Niigata, Japan). The nomenclature, symbols, and units follow the recommendations of the Nomenclature Committee of the American Society for Bone and Mineral Research [22]. The perimeter, trabecular thickness (Tb.Th, mcm) is structural indices of cancellous bone tissue. We calculated the bone formation parameters as follows: bone volume

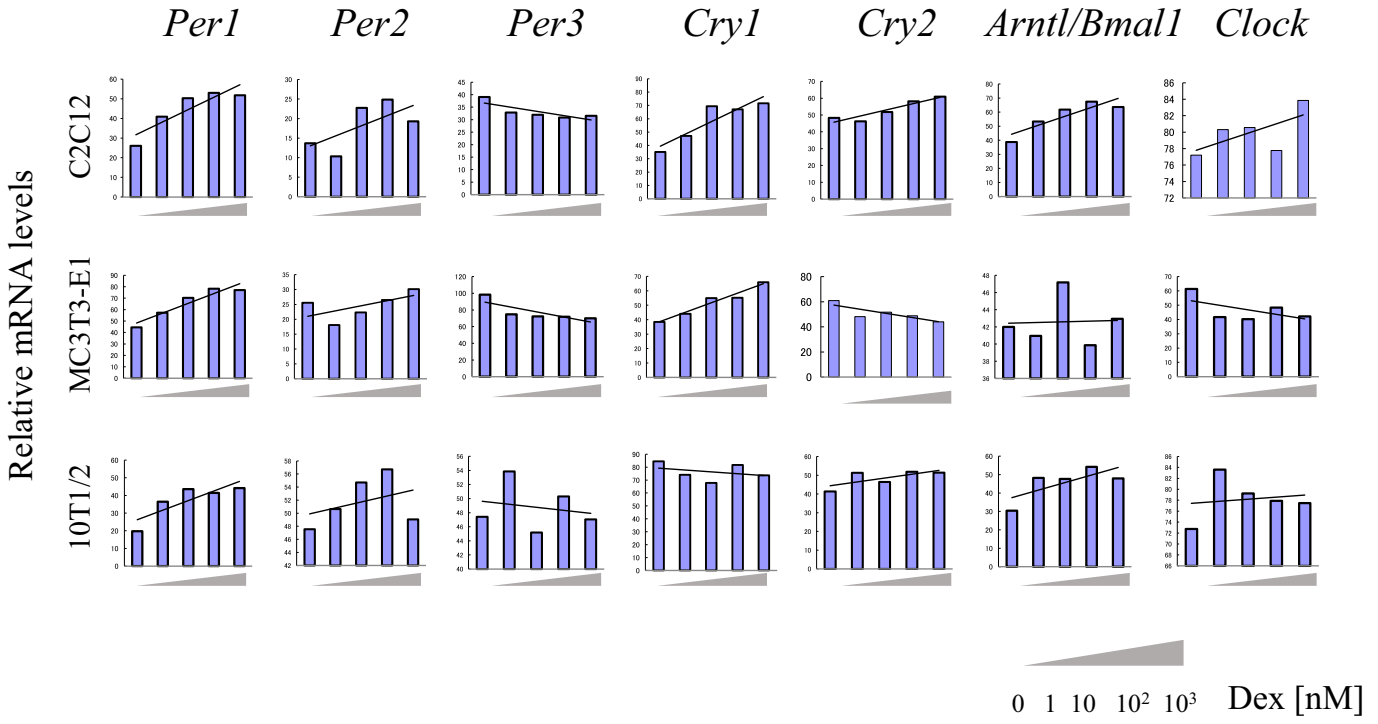

Fig. 1. Analysis of the expression of circadian clock genes in mouse cell lines treated with dexamethasone (Dex). A. RT-PCR analysis of mRNAs encoding Clock proteins expressed by the C2C12, MC3T3-E1, and 10T1/2 cell lines. Cultures were treated with 0–10^3 nM Dex.

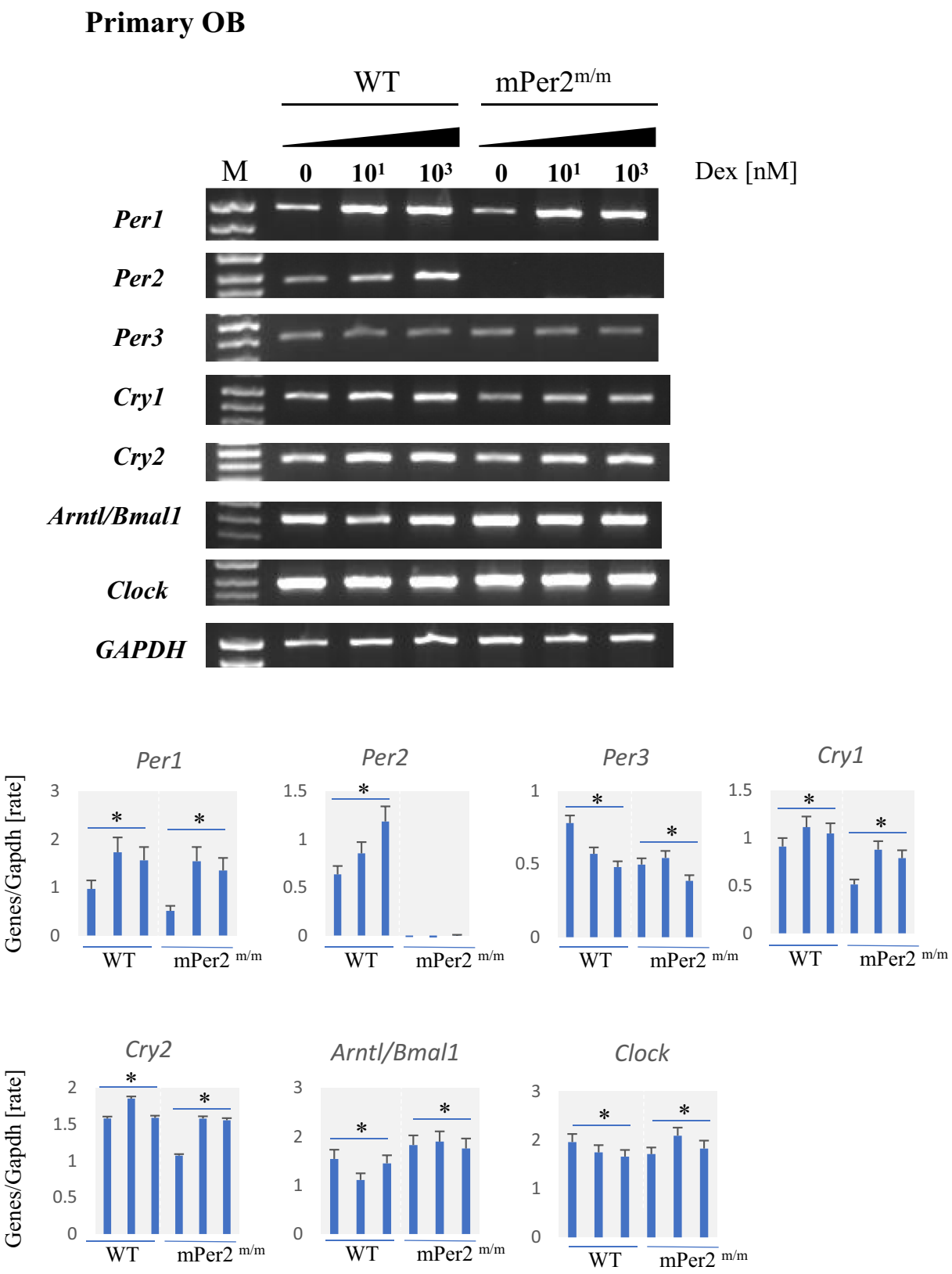

Fig. 2. Analysis of the expression of circadian clock genes in primary cultures of osteoblasts of wild-type (WT) and mPer2$^{m/m}$ mice treated with Dex. Top panel. Agarose gel electrophoresis of Clock gene expression in primary osteoblast (pOB) cultures treated with different concentrations of Dex. Bottom panel. Quantitation of mRNA levels normalized to those of *Gapdh* mRNA (*$p < 0.05$).

(BV/TV, %), osteoid volume (OV/TV, %; OV/BV, %), and osteoid surface (OS/BS, %) were calculated. The bone resorption parameters were as follows: the amount of eroded surface (ES/BS, %) calculated as a percentage of the total bone surface. Mineralizing surface (MS/OS, %) is kinetic indices of cancellous bone tissue. We calculated the dynamic histomorphometric parameters of the growing long bone metaphysis, the growth plate thickness (G.P.Th, μm).

2.8. Statistical analysis

Results are expressed as the mean ± SEM of three replicates, comparisons were performed using one-way analysis of variance or the Student *t* test, and $p < 0.05$ indicates a significant difference.

3. Results

3.1. Effect of Dex on the expression of circadian clock genes in vitro

The relevant phenotypes of the cell lines analyzed for their transcriptional responses to Dex are as follows: The osteoblast-like cell line MC3T3-E1 differentiates into osteoblasts [23]. The C2C12 cell line exhibits a myoblast-like phenotype [24]. The 10T1/2 cell line exhibits fibroblast-like characteristics [25]. Generally, the levels of *Per1*, *Per2*, and *Cry1* mRNAs increased as a function increasing Dex concentrations in MC3T3-E1 and C2C12 cells, whereas the levels of *Per3* mRNA decreased. The levels of *Cry2*, *Arnt/Bmal1*, and *Clock* mRNAs did not change in a manner that demonstrated a convincing effect of Dex concentrations. In

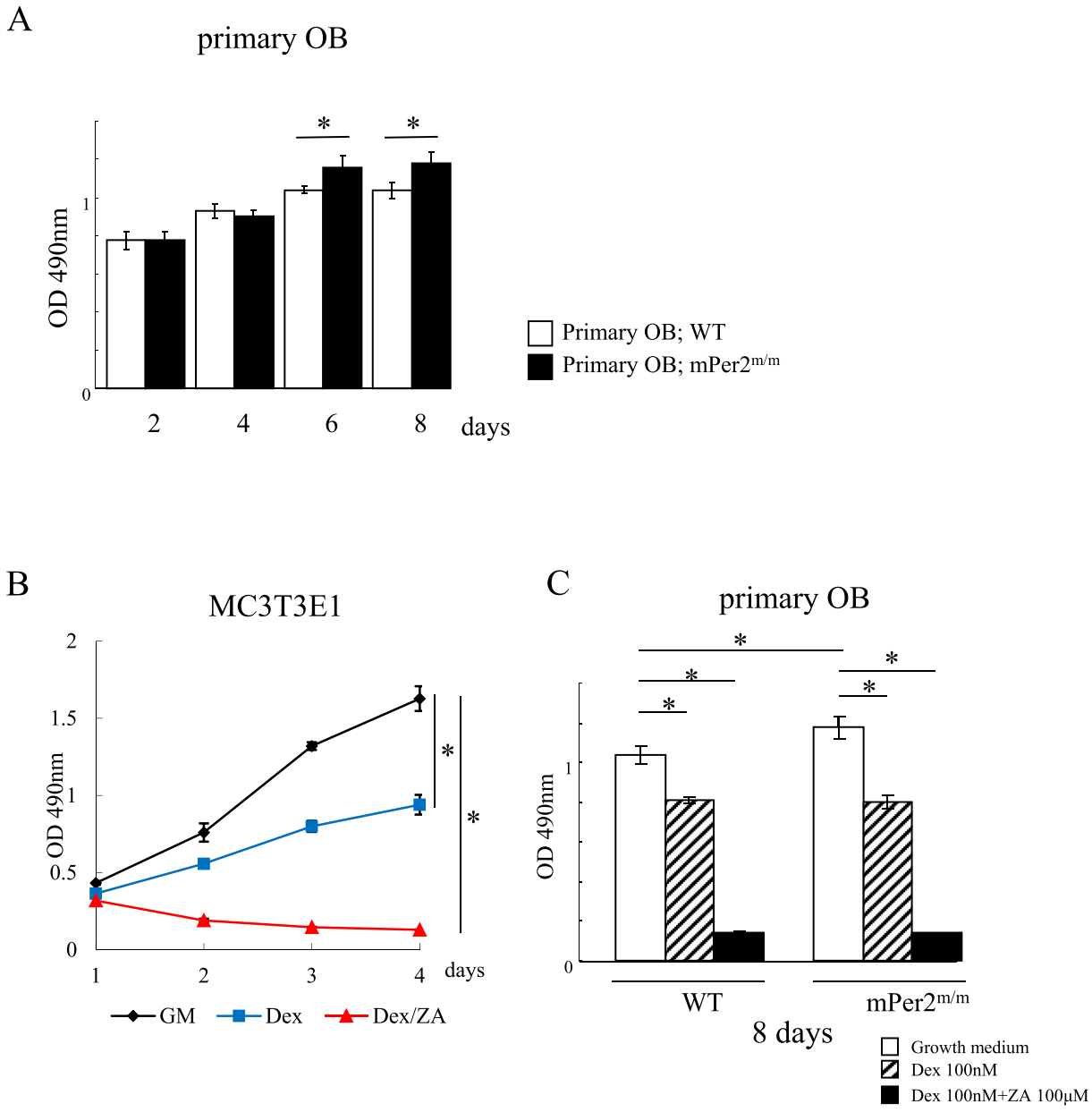

Fig. 3. Analysis of the proliferation of osteoblast. A. Proliferation of pOBs of wild-type (WT) and mPer2$^{m/m}$ mice (*$p < 0.05$). B. Proliferation of MC3T3-E1 cells in the presence and absence of 100 nM each of Dex and zoledronic acid (ZA). Growth medium without supplements (black line), with Dex (blue line), and Dex plus ZA (red line). C. Proliferation of pOBs of WT and mPer2$^{m/m}$ mice after 8 days in culture in the absence or presence (100 nM each) of Dex, ZA, and Dex plus ZA (*$p < 0.05$).

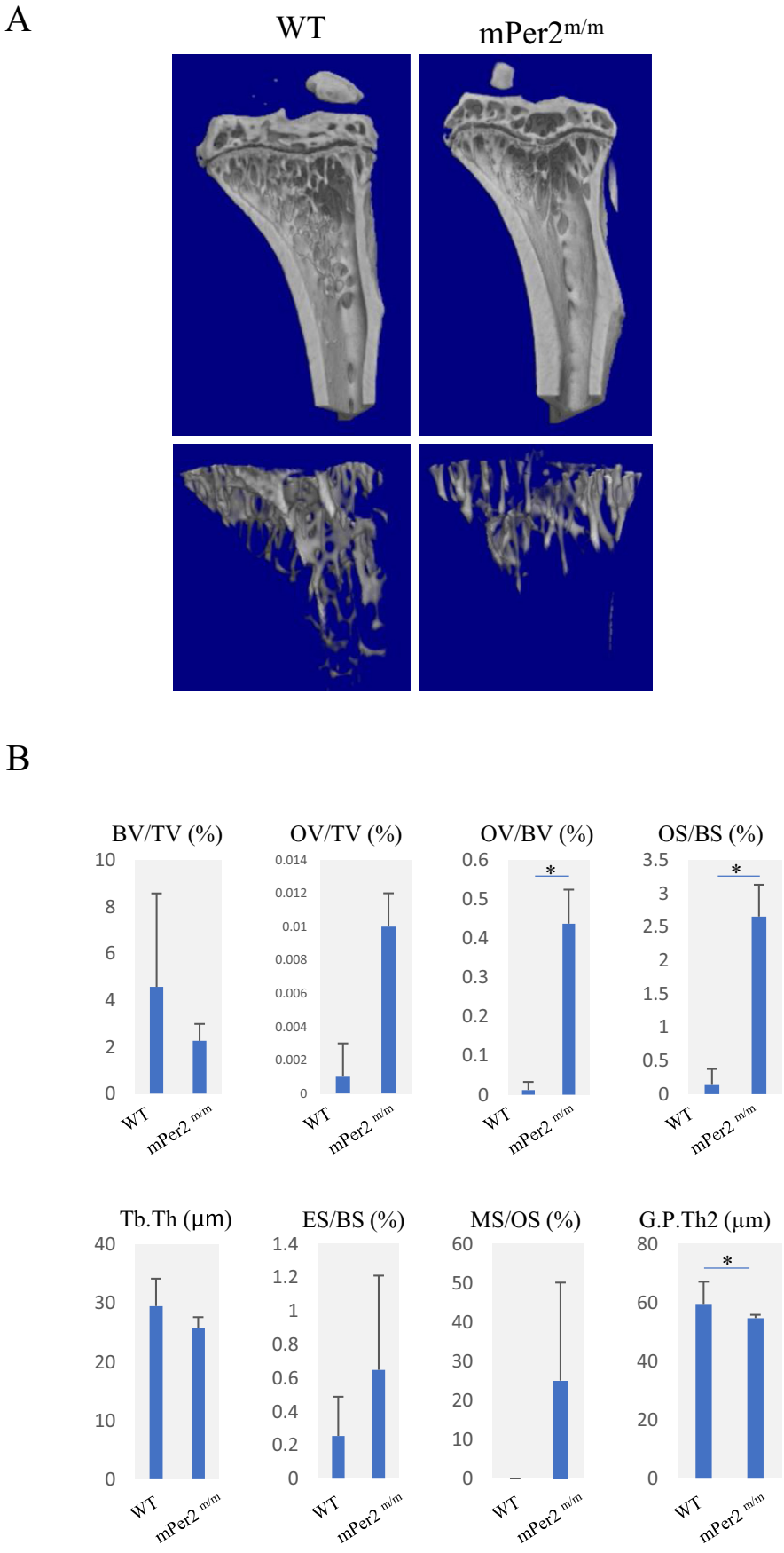

Fig. 4. Morphometric analysis of the fibulae of WT and mPer2$^{m/m}$ treated with ZA. ZA was administered daily for 21 days to WT and mPer2$^{m/m}$ mice injected with slow-release pellets containing prednisolone (PSL). A. μ-CT 3D images of fibulae. *Left*: sagittal view (upper panel) and axial view (lower panel) of WT mice demonstrating the usual cortical bone and trabeculae. *Right*: sagittal view (upper panel) and axial view (lower panel) of mPer2$^{m/m}$ mice revealing more thicker cortical bone and smaller trabeculae than those of WT. B. Morphometric analysis of the fibulae of WT and mPer2$^{m/m}$ mice treated with ZA (*$p < 0.05$).

undifferentiated 10T1/2 cells, the levels of mRNAs encoding circadian clock genes varied, except those of *Per1* and *Per2* (Fig. 1).

3.2. Expression of circadian clock genes in pOBs of WT and *mPer2*$^{m/m}$ mice

In the primary culture of osteoblasts from WT and mPer2m/m mice, the levels of *Per1, Per2(only in those of WT)* and *Cry1* mRNAs generally increased, according to the increase in concentrations of Dex, whereas the levels of *Per3* mRNA decreased. These data are consistent with those of the cell lines (Section 2.2). The quantification using the luminescent image analyzer also showed that the expression patterns of circadian clock genes in mPer2$^{m/m}$ mice were almost similar to those of WT mice (Fig. 2).

3.3. Osteoblast proliferation and inhibition by Dex and ZA

The proliferation of pOBs isolated from WT and mPer2$^{m/m}$ mice differed, starting on day 6 (Fig. 3A). The proliferative capacity of pOBs derived from mPer2$^{m/m}$ mice was significantly higher. Fig. 3B shows that the proliferation of MC3T3-E1 cells was significantly inhibited (approximately 2-fold) by the administration of 100 nM Dex. Further addition of 100 μM ZA completely inhibited proliferation. The proliferative capacity of pOBs from WT and mPer2$^{m/m}$ mice was also inhibited by approximately 25% by Dex and completely inhibited when mice were administered Dex combined with ZA, consistent with the data for MC3T3-E1 cells (Fig. 3C).

3.4. μ-CT morphometric analysis and bone histomorphometry

To evaluate osteogenic capacity, we treated the mice with PSL and ZA, which inhibits osteoblast proliferation (Fig. 3C). Representative 3-D images of bone structures in the tibiae of WT and mPer2$^{m/m}$ mice treated with PSL and ZA are shown in Fig. 4A. Compared with the trabeculae of WT mice treated with both ZA and PSL, those in PSL/ZA-treated mPer2$^{m/m}$ mice were smaller (Fig. 4A, lower panels). Bone morphometry analysis (Fig. 4B) showed that although PSL/ZA-treated mPer2$^{m/m}$ mice had tended to decrease BV/TV than compared with PSL/ZA-treated WT, the OV/BV and OS/BS values of PSL/ZA-treated mPer2$^{m/m}$ mice seemed higher compared with those of WT, suggesting that PSL/ZA-treated mPer2$^{m/m}$ mice showed a tendency to retard calcification.

4. Discussion

Increases in the levels of stress hormones, particularly in people whose cortisol response to the administration of CRH is increased, suffer from sleep abnormalities, which are worsened in association with the exacerbation of stress symptoms [10]. These findings suggest that the normal response to stress, which is controlled by the HPA-axis, is required for normal sleep. The presence of abnormal activity rhythms in *mPer2*-deficient mice demonstrate that the GC rhythm is absent. Further, diurnal feeding rhythm is undetectable in *mPer2*-deficient mice [26]. Inadequate sleep leads to increased appetite, obesity [24], and increased mortality [25], demonstrating the deep relationship between sleep disorders and stress, which significantly affects the body, although stress is not addressed by these studies. However, the responses to restraint stress, hypoglycemia, and ACTH injection are intact in mPer2 deficient mice [26]. Therefore, *Per2* may not significantly influence the regulation of the activity of the HPA-axis, at least in rodents. In contrast, the *Per1* promoter region harbors a GC response element (GRE), and the transcription of *Per1* is controlled by GC in hepatic cells (HepG2) [27]. Further, GC signaling resets the circadian cycle in peripheral tissues as described in above [11]. In our present study of osteogenic cells, we show that *mPer2* responses to GC were similar to those of mPer1. The GRE-like region may therefore exist in *Per2*.

The regulation of cartilage differentiation is mediated by direct stimulation of *mPer1* expression by PTH through a protein kinase A-CREB-dependent mechanism in chondrocytes, which regulates the subsequent *Arntl*/*Clock*-dependent synthesis of the extracellular matrix [28,29]. Further, circadian clock genes are expressed by chondrocytes to regulate the synthesis of the extracellular matrix in cartilage [28], and the promoter activity of the Indian hedgehog gene (*Ihh*) is regulated by PER1 and ARNTL/BMAL1, which are coordinately expressed in the growth plate [29]. A deficit in ARNTL/BMAL1 expression leads to the disruption of the rhythmic expression profiles of *Per1* and *Ihh* in the growth plate [29]. IHH controls the differentiation and proliferation of growth plate chondrocytes [30]. These findings indicate that endochondral ossification may be regulated by the Clock gene product PER1 expressed in chondrocytes during postnatal skeletogenesis through a mechanism that regulates the rhythmic expression of *Ihh* [29]. We found that the concomitant use of BP and GC changes the concentration of RANKL in the bloodstream and that the epiphyseal growth plates widen in the femurs of mice administered BP because of the expansion of hypertrophic chondrocyte layers [20,31]. Here we show that in the absence of *Per2* expression, the characteristic widening of growth plates was suppressed and the trabeculae decreased further (Fig. 4), suggesting their involvement in bone growth related to endochondral ossification.

The loss of *Per2* expression causes abnormalities of erythrocytes and impairs oxygen transport, which significantly shortens the life span of erythrocytes [32]. Therefore, PER2 function in the bone marrow may influence the regulation of life span of circulating erythrocytes. Recently, Zhou et al. (2018) reported that osteogenic differentiation of bone marrow stem cells (BMSCs) *in vitro* is enhanced when RNA interference techniques are used to inhibit the expression of *Arntl/Bmal1* or *Per2*, indicating that ARNTL/BMAL1 and PER2 negatively regulate the osteogenic potential of BMSCs and may have a synergistic effect on the osteogenic differentiation of BMSCs [33]. We show here that the proliferation of osteoblast derived from mPer2$^{m/m}$ mice was enhanced compared with those of WT mice, suggesting that an absence of PER2 shortens the cell cycle and the biological clock. Increasing calcification in untreated mPer2$^{m/m}$ mice was repressed by treatment with PSL, thus inhibiting the generation of viable, mature bone that frames the trabecula in a cancellous bone. Therefore, these findings suggest that *Per2* regulates bone homeostasis to contribute to the stabilization of osseous tissue. Future molecular genetic studies should focus on revealing the complex mechanisms that interact to regulate the expression of circadian clock genes *in vivo*.

5. Conclusions

The current study demonstrates that bone metabolism is influenced by *Per2*. To advance regenerative medicine, it is important to deepen our understanding of the biology of sleep, circadian rhythms, and tissue repair [34].

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