

Circadian production of melatonin in cartilage modifies rhythmic gene expression

Shanqi Fu¹, Miho Kuwahara¹, Yoko Uchida², Sei Kondo¹, Daichi Hayashi¹, Yuji Shimomura¹,
Asami Takagaki¹, Takashi Nishida¹, Yusuke Maruyama³, Mika Ikegame⁴, Atsuhiko Hattori³,
Satoshi Kubota¹ and Takako Hattori^{1*}

¹Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of
Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8525, Japan

²Department of Preventive Dentistry, Okayama University Graduate School of Medicine,
Dentistry and Pharmaceutical Sciences, Okayama 700-8525, Japan

³Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental
University, Ichikawa, Chiba 272-0827, Japan

⁴Department of Oral Morphology, Okayama University Graduate School of Medicine, Dentistry
and Pharmaceutical Sciences, Okayama 700-8525, Japan

***Corresponding author:**

Takako Hattori

Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of
Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama City
700-8525, Japan.

Tel: +81-86-235-6646

Fax: +81-86-235-6649

E-mail: hattorit@md.okayama-u.ac.jp

Short title: Cyclic melatonin expression in chondrocytes

Keywords: melatonin, chondrocyte, skeletal growth, circadian rhythm, cell growth, mass
spectrometry (MS), arylalkylamine N-acetyltransferase (AANAT)

Abstract

Endochondral ossification, including bone growth and other metabolic events, is regulated by circadian rhythms. Herein, we provide evidence that melatonin has a direct effect on the circadian rhythm of chondrocytes. We detected mRNA expression of the genes which encode the melatonin-synthesizing enzymes AANAT (arylalkylamine N-acetyltransferase) and HIOMT (hydroxyindole O-methyltransferase), as well as the melatonin receptors MT1 and MT2 in mouse primary chondrocytes and cartilage. Production of melatonin was confirmed by mass spectrometric analysis of primary rat and chick chondrocytes. Addition of melatonin to primary mouse chondrocytes caused enhanced cell growth and increased expression of *Col2a1*, *Aggrecan*, and *Sox9*, but inhibited *Col10a1* expression in primary BALB/c mouse chondrocytes. Addition of luzindole, an MT1 and MT2 antagonist, abolished these effects. These data indicate that chondrocytes produce melatonin, which regulates cartilage growth and maturation via the MT1 and MT2 receptors. Kinetic analysis showed that melatonin caused rapid upregulation of *Aanat*, *Mt1*, *Mt2*, and *Pthrp* expression, followed by *Sox9* and *Ihh*. Furthermore, expression of the clock gene *Bmal1* was induced, while that of *Per1* was downregulated. Chronobiological analysis of synchronized C3H mouse chondrocytes revealed that melatonin induced the cyclic expression of *Aanat* and modified the cyclic rhythm of *Bmal1*, *Mt1*, and *Mt2*. In contrast, *Mt1* and *Mt2* showed different rhythms from *Bmal1* and *Aanat*, indicating the existence of different regulatory genes. Our results indicate that exogenous and endogenous melatonin work in synergy in chondrocytes to adjust rhythmic expression to the central suprachiasmatic nucleus clock.

INTRODUCTION

The rate of fetal and juvenile skeletal growth is controlled, to a large extent, by the development and expansion of cartilaginous growth plates in the long bones, ribs, and vertebrae. Parameters controlling the rate of chondrocyte proliferation, matrix deposition, and differentiation events vary along a spatial gradient. These begin with high rates of cell division and hyaline cartilage matrix deposition in the proliferating zone, and end after several steps of chondrocyte differentiation to form hypertrophic cells in the hypertrophic zone. The complex regulation of chondrocyte proliferation and hypertrophy during endochondral ossification involves numerous

antagonistically- and synergistically-acting hormones, growth factors and their receptors, and developmental control genes (Goldring *et al.* 2006, Liu *et al.* 2016, Reddi 1994, Shao *et al.* 2006).

There is considerable evidence to suggest that cartilage and bone growth in vertebrates oscillate in a circadian rhythm, but the exact mechanisms underpinning the circadian regulation of cartilage growth remain largely unknown. Russell *et al.* (1984) showed that the mineralization of rat growth plate cartilage occurs at night. Chondrocyte proliferation, however, occurs mostly in the early morning. The proliferative phase is followed by expansion of the growth plate, which peaks at noon in rats (Stevenson *et al.* 1990), coinciding with the highest rate of collagen matrix synthesis (Igarashi 2013).

The central mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and synchronizes the light/dark cycles of peripheral organs via hormonal and neuronal mechanisms. Cycle periods are regulated by a transcriptional/translational feedback loop consisting of the transcriptional activators BMAL1/CLOCK and a repressor complex formed by PER1/PER2 and CRY1/CRY2 (Dudek and Meng 2014). However, peripheral tissues are also capable of self-sustained circadian oscillation for >20 cycles when isolated from the central control, indicating the existence of organ-specific synchronizers of circadian rhythm at the cellular and tissue levels (Yoo *et al.* 2004). A long-term mouse *ex vivo* organ culture study revealed a tissue-autonomous circadian rhythm that persisted for several months in both articular and growth plate cartilage (Okubo *et al.* 2013). Recently, several studies have demonstrated the rhythmic expression of clock genes in cartilage; indicating that, similar to the central SCN, the circadian clock in cartilage is regulated by a BMAL1/CLOCK-PER/CRY feedback loop (Dudek *et al.* 2016, Gossan *et al.* 2013, Okubo *et al.* 2013, Takarada *et al.* 2012).

The circadian clock of murine chondrocytes also regulates a number of catabolic and anabolic genes that control key aspects of cartilage homeostasis. For example, BMAL1 stimulates *Ihh* expression (Takarada *et al.* 2012). Accordingly, in the growth plates of BMAL1-null mice, expression of *Ihh* is decreased and the rhythmic expression patterns of both *Per1* and *Ihh* are disrupted (Takarada *et al.* 2012).

In cartilage, the cyclic expression of *Bmal1* and *Per1* can be modulated by PTH (1–34), the active peptide of parathyroid hormone (PTH) (Kunimoto *et al.* 2016, Okubo *et al.* 2015). Both PTH and the related cartilage-derived parathyroid-related peptide (PTHrP) signal through the PTH receptor PPR1, and are key factors in the control of chondrocyte differentiation (Chung *et al.* 1998). It has been shown that PTH (1–34) induces *Per1* expression in osteoblasts and chondrocytes (Hanyu *et al.* 2011, Hinoi *et al.* 2006), thereby inducing phase shifts in the circadian

rhythm during femoral growth (Okubo *et al.* 2015) and bone fracture healing (Kunimoto *et al.* 2016).

While PTH (1–34) is a humoral factor that controls the circadian clock in cartilage; melatonin, an indole primarily synthesized in the pineal gland during darkness, is a primary factor in the regulation of circadian rhythm throughout the entire body. In addition to the pineal gland, many other tissues and organs produce melatonin (Acuna-Castroviejo *et al.* 2014). Melatonin has also been reported to affect the chondrogenesis of mesenchymal stem cells (Gao *et al.* 2014) and stimulate cartilage matrix synthesis (Pei *et al.* 2009). Therefore, we hypothesized that melatonin might be an integral part of the autonomous circadian cycle in cartilage. Articular cartilage and a large portion of embryonic cartilage are not vascularized, meaning that chondrocytes are shielded from humoral influences. This raises questions about whether chondrocytes are able to respond to circulating melatonin or, alternatively, able to synthesize melatonin themselves as part of the cyclic feedback of chondrocyte metabolism.

We show, for the first time, that chondrocytes from several species express the melatonin-synthesizing enzymes arylalkylamine N-acetyltransferase (Aanat) and N-Acetylserotonin O-methyltransferase (Hiomt), as well as the melatonin receptors Mt1 and Mt2 in peripheral regions of cartilage and hypertrophic cartilage in the growth plate, which are in close proximity to well-vascularized tissues. Furthermore, we demonstrate that melatonin can stimulate chondrocyte growth and regulate expression of cartilage-associated genes including *Ihh*, *Col2a1*, *Acan*, *Sox9*, and *Coll0a1*. Expression of melatonin in rat and chick chondrocytes was further confirmed by mass spectrometry analysis. We show that melatonin modifies the rhythmic expression of *Bmal1*, *Mt1*, and *Mt2*; and enhances the rhythmic expression of *Aanat* mRNA in primary chondrocytes, supporting the involvement of melatonin in the regulation of cartilage growth and homeostasis.

MATERIALS AND METHODS

Primary cell cultures

Chondrocytes were isolated from the ribcages of either embryonic day 18.5 (E18.5) or E19.5 BALB/c and C3H mouse embryos, as previously described (Tomita *et al.* 2013, Hattori *et al.* 2010). Primary chick (*Gallus gallus*) chondrocytes were isolated from the knee cartilage of E17.5 chick embryos. Primary rat chondrocytes were isolated from the ribcage cartilage of E20 rat embryos (Sprague Dawley). For all experiments, cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) in dishes covered with aluminum foil

to avoid light contamination, and passage was performed in a darkened room with minimum light. To synchronize circadian rhythm, serum concentration in the medium was reduced to 1% for 24 hr, 50% for 2 hr, then increased back to 10% for the duration of culture, as indicated in individual experiments. All animal experiments were conducted in accordance with the institutional rules following approval from the Animal Care and Use Committee of Okayama University in Japan (OKU-2018544).

Isolation of total RNA

Tissues from one or two mice at postnatal day 0 (P0) or chicks at E17.5 were harvested and homogenized using Isogen reagent (Nippon Gene, Tokyo, Japan). Total RNA was collected and further purified using an RNeasy kit (Qiagen, Hilden, Germany).

Real-time polymerase chain reaction

Resting/growing and hypertrophic cartilage samples from the ribcages of postnatal day 0 (P0) mice were prepared as described above (Hattori *et al.* 2010, Tomita *et al.* 2013). Chondrocytes were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing 10% FCS for various lengths of time as indicated under dark conditions before the total RNA was harvested using an RNeasy kit. Primers used are listed in Table 1. Expression of *GAPDH* was used to standardize total amounts of cDNA; however, as *GAPDH* expression varied among samples, plasmids in which the amplified cDNA fragments had been cloned were used to generate standard curves for calculation of gene copy numbers/ μ g RNA.

***In situ* hybridization**

In situ hybridization was performed on paraffin-embedded sections as previously described (Hattori *et al.* 2010, Schmidl *et al.* 2006), using digoxigenin-labeled mouse antisense RNA probes with anti-digoxigenin-labeled alkaline phosphatase and BM purple as a detection system. The probe for mouse *Col2a1* was kindly supplied by Dr. K. von der Mark (University of Erlangen, Erlangen, Germany) (Hattori *et al.* 2010, Schmidl *et al.* 2006). Probes specific for mouse *Mt1*, *Mt2*, and *Aanat* were generated by PCR amplification and cloned into a pGEM-T Easy vector (Promega; Madison, WI). Primers used for PCR amplification were as follows: *Mt1*, 5'-agtacgatccccggatctactctgtacct-3' and 5'-acatcttggtgtgcacacgagacaataa-3'; *Mt2*, 5'-taattgtttgtggtgagctcggccttggc-3' and 5'-ttggccttccttcgggcctggagcaccagt-3'; *Aanat*, 5'-

tgttgaacatcaactccctgaaacctgagg-3' and 5'-cagcccgccgcacggccgctgactgccc-3'.

Western blots

Cultured cells were washed with phosphate-buffered saline (PBS) and harvested with 10 mM Tris-HCl (pH 8.0) in 150 mM NaCl, 1% Triton X-100, and 0.1 mM phenylmethylsulphonyl fluoride. We used 66 µg of total protein for western blot analysis. Primary antibodies against MT2 (LSBio, Seattle, USA) and β-actin (Sigma, St. Louis, USA) were used.

Cell proliferation assay

Mouse primary chondrocytes were seeded into 96-well plates at a density of 0.8×10^4 cells/well and incubated for 24 hr. The medium was changed, then various concentrations of melatonin (N-acetyl-5-methoxytryptamine; Wako, Osaka, Japan) were added to wells. Cells were further incubated in the dark for 5 days, and cell density was measured with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based CellTiter 96 aqueous non-radioactive cell proliferation assay kit (Promega, Fitchburg, USA). To assess the inhibitory effects of luzindole (N-acetyl-2-benzyltryptamine, Wako) on melatonin signaling, cells were treated with 1×10^{-4} M luzindole for 1 hr, prior to melatonin exposure.

Quantification of melatonin and related molecules

Melatonin biosynthesis pathway products were monitored in primary chondrocytes harvested from E19.5 Sprague Dawley rats by triple mass spectrometric analysis. Conditioned media were collected after 3 days of culture, and cells were washed with PBS and harvested in 20 mM Tris-HCl (pH 8.0) in 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1 mM phenylmethylsulphonyl fluoride. After centrifugation, small aliquots of cell lysates were used for determination of protein concentration. The remaining supernatants were precipitated with acetone, and analyzed using an LCMS-8050 Triple Quadrupole Liquid Chromatograph mass spectrometer coupled to an LC-30AD high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) with an octadecylsilyl (ODS) column (2.0 mm × 150 mm, 3.0 µm particle size; Tosoh Bioscience, King of Prussia, PA).

For determination of AANAT activity, primary chondrocyte lysates from chick embryos were harvested in 10 mM PBS (pH 6.5) and incubated with 1 mM tryptamine-HCl and 0.1 mM acetyl-CoA at 37 °C for 60 min. The reaction was stopped with concentrated acetic acid, and the concentration of N-acetyltryptamine (NATP) produced was monitored as described for rat

chondrocyte lysate.

Samples for analysis were loaded onto the HPLC column, which had been pre-equilibrated with 10 mM ammonium acetate in 0.05% acetic acid at 25 °C, and separated using a gradient of buffer A (10 mM ammonium acetate and 0.05% acetic acid in water) and buffer B (100% methanol) starting at A95:B5 (vol:vol) and increasing to A50:B50. Melatonin, serotonin, N-acetylserotonin (in rat chondrocyte lysates), and NATP (in chick chondrocyte lysates) were quantified using mass spectrometry with electro-spray ionization in multiple reaction monitoring mode. Transition values were confirmed by analysis of the four molecules as markers. Control of the instrument and data acquisition were carried out using LC solution software (Shimadzu). An external standard curve was run on the day of analysis. Assay sensitivity limits for melatonin, serotonin, N-acetylserotonin, and NATP were as low as 11.1, 82.3, 61.2, and 274.1 fg, respectively, with a 2:1 signal-to-noise ratio. Intra- and inter-assay variation coefficients were 3.94% and 4.60% for melatonin, 3.60% and 9.05% for serotonin, 3.98% and 6.14% for N-acetylserotonin, and 2.99% and 4.21% for NATP, respectively.

Statistical analysis

With the exception of circadian rhythmicity, all statistical analyses were performed using SPSS 22.0 software (IBM Japan, Tokyo, Japan). Data are expressed as mean \pm standard deviation (SD), with $P < 0.05$ considered statistically significant. Circadian rhythmicity was analyzed using a program provided by Dr. Ziyi Wang (https://github.com/wong-ziyi/Cosinor_Analysis_of_Rhythms) that was modified from the Cosinor2 package of R (<https://cran.r-project.org/web/packages/cosinor2/index.html>) for the calculation of parameters (Bingham *et al.* 1982, Cornelissen 2014, Hessen *et al.* 2015). As we performed replicates for each experimental group, differences of τ between experimental groups were assessed using the Student's t-test. Statistical significance was accepted at $P < 0.05$ and all analyses were performed using R programming language with open source packages.

RESULTS

Quantitative analysis of *Mt1* and *Mt2* expression in mouse tissues

Real-time PCR analysis of RNA from several tissues of P0 BALB/c mice revealed that *Mt1* mRNA expression levels were highest in brain and liver tissues, while *Mt2* mRNA levels were

highest in kidney and skin tissues. Cartilage was found to contain substantial levels of mRNA (copies/ μ g RNA) of both *Mt1* (Figure 1A, $1.77 \pm 0.32 \times 10^4$) and *Mt2* (Figure 1B, $0.52 \pm 0.20 \times 10^4$). For comparison, mRNA expression of the cartilage marker gene, *Sox9*, was highest in cartilage (Figure 2E, $2.27 \pm 0.014 \times 10^7$).

Localization of *Mt1* and *Mt2* mRNA in mouse cartilage

Localization of melatonin receptor was examined in cartilage tissues of BALB/c mice *in vivo*. *In situ* hybridization analysis indicated relatively strong expression of *Mt1* and *Mt2* mRNA along the peripheral zone of cartilage and in the pre-hypertrophic and hypertrophic zones of the growth plate (Figure 2A). To examine whether melatonin receptor expression levels exhibited a gradient from the vascularized cartilage-bone border and lower hypertrophic zone to the avascular zones of proliferating and resting cartilage, chondrocytes were separately collected from hypertrophic and non-hypertrophic ribcage cartilage. Analysis of gene expression revealed that *Mt1* and *Mt2* mRNA levels were higher in *Col10a1*, *VEGF*, and *MMP13* mRNA-expressing hypertrophic chondrocytes compared with cells isolated from resting and proliferating cartilage (Figure 2B). This could indicate that the response to melatonin is high in the vascularized cartilage-bone border. Resting/proliferating chondrocytes and hypertrophic chondrocytes were also found to contain MT2 protein, with relatively high levels detected in hypertrophic chondrocytes (Figure 2C).

Effects of melatonin on chondrocyte growth and gene expression

To analyze the effects of melatonin on chondrocytes, primary chondrocyte cultures were prepared from the ribcage of E18.5 BALB/c mice. Melatonin was added to media at various concentrations, with or without luzindole (N-acetyl-2-benzyltryptamine, an antagonist of MT1 and MT2 receptors), and cells were cultured for 5 days. Analysis of cell density using an MTS-based assay revealed that melatonin at a final concentration of 1×10^{-8} M or 1×10^{-6} M stimulated cell growth by up to 20%. Addition of luzindole at a 100-fold molar excess (1×10^{-4} M) compared with melatonin (1×10^{-6} M) abolished the effect (Figure 3A). Luzindole (1×10^{-4} M) itself was not cytotoxic, as indicated by the presence of lactate dehydrogenase in the conditioned medium (data not shown).

The effect of melatonin on expression of chondrocyte differentiation markers was monitored. Mouse primary chondrocytes were cultured in various melatonin concentrations for 5 days before total RNA was purified and analyzed. Expression of *Aggrecan* and *Ccn2*, which encode enhancers of endochondral ossification, and *Sox9*, which encodes a strong inhibitor of chondrocyte

hypertrophy, were upregulated by melatonin in a concentration-dependent manner (Figure 3B). In contrast, expression of *Col10a1* (which encodes a marker of chondrocyte hypertrophy) was downregulated and *Ihh* (which encodes a regulator of hypertrophic chondrocyte differentiation) was only weakly upregulated by melatonin (Figure 3B). These results indicate that melatonin stimulates chondrocyte growth through MT1 and/or MT2 receptors, but inhibits chondrocyte maturation, possibly through induction of Sox9.

Detection of melatonin-synthesizing enzymes in cartilage

Analysis of *Aanat* and acetylserotonin O-methyltransferase (*Asmt*) or *Hiomt* mRNA levels in the rib cartilage of newborn BALB/c mice revealed high enough levels for detection (Figure 4A; *Aanat* [copies/ μ g RNA]: cartilage, $2.30 \pm 1.62 \times 10^3$; brain, $6.61 \pm 0.04 \times 10^3$; *Hiomt*: cartilage, 30.7 ± 30 ; brain: 25.1 ± 11.3). *In situ* hybridization indicated relatively strong expression of *Aanat* mRNA along the peripheral zone of cartilage, as well as in the pre-hypertrophic and hypertrophic zones of the growth plate (Figure 4B).

Melatonin production by chondrocytes

Although expression of *Aanat*, *Mt1*, and *Mt2* was detected at the level of transcription in chondrocytes from BALB/c mice, the levels of synthesis were too low to be detected at the protein level, consistent with a report by Ebihara et al. (1986). Therefore, we analyzed melatonin production by rat chondrocytes using mass spectrometry. Melatonin was detected in the conditioned medium collected after 3 days of culture (0.94 ± 0.66 pg/mL), but not in fresh medium that had not been exposed to chondrocytes (control). Intermediates of melatonin synthesis from tryptophan, serotonin and N-acetylserotonin, were observed in the control media and in the conditioned media (data not shown). Melatonin was present in chondrocyte lysates (37.79 ± 25.74 pg/mg protein), but serotonin and N-acetylserotonin were not detected.

Detection of melatonin-synthesizing enzymes and receptors in chick chondrocytes

In line with the results from mouse tissue, mRNA expression (copies/ μ g RNA) of *Mt1* ($1.06 \pm 0.011 \times 10^4$), *Mt2* ($2.2 \pm 0.14 \times 10^4$), *Aanat* ($3.19 \pm 0.11 \times 10^4$), and *Hiomt* ($7.27 \pm 0.62 \times 10^4$) was observed in chick cartilage (Figure S1).

Accumulation of melatonin and enhanced AANAT activity in chick chondrocytes

Chick chondrocytes were cultured in the dark, and total RNA and cell lysates were collected and

evaluated every 4 hr. The mRNA expression of *Aanat* peaked 18 hr after the medium change (Figure S2A), which was followed by melatonin accumulation, peaking at 22 hr after the medium change (Figure S2B). Using tryptamine as a substrate, cellular AANAT activity was monitored as NATP production by cell lysates. Maximum AANAT activity was observed 22 hr after the medium change (Figure S2C). These results demonstrate that cyclic expression of *AANAT* mRNA reflects cycles of enzymatic activity.

Kinetic analysis of melatonin activity in chondrocytes

Although chondrocytes are able to produce endogenous melatonin, they may still respond to circulating melatonin originating from the pineal gland. To investigate whether chondrocytes respond to melatonin in a time-dependent manner, melatonin was added to cultured BALB/c chondrocytes, and total RNA was collected at regular intervals. Surprisingly, the addition of melatonin increased levels of *Aanat*, *Mt1*, and *Mt2* mRNA after 30 min (Fig 5A), indicating that chondrocytes exhibit a rapid response to melatonin stimulation, and demonstrating the autoinduction of melatonin synthesis in these cells. Furthermore, melatonin-stimulated expression of *Pthrp* and *Ihh*, which encode key factors in the regulation of chondrocyte differentiation in the growth plate, was observed. Melatonin was found to affect expression of the transcription factor and clock gene, *Bmal1*, and inhibit *Per1* expression (Figure 5A). These results suggest that chondrocytes may themselves produce melatonin in response to circulating melatonin, and also upregulate melatonin receptor expression, eventually synchronizing clock gene expression in chondrocytes with the central circadian clock.

The enhancement of *Aanat* and *Ihh* gene expression by melatonin in chondrocytes was abolished by exposure to luzindole (Figure 5B).

Melatonin modifies the circadian expression of *Bmal1* and melatonin receptors in mouse primary chondrocytes and induces cyclic expression of *Aanat*

As melatonin both enhanced mRNA expression of *Aanat*, *Mt1*, and *Mt2*, and altered *Bmal1* and *Per1* gene expression, we next examined the possibility that exogenous melatonin could affect also the cycle expression or period of BMAL1 (a master regulator), AANAT (a rate-limiting enzyme for melatonin synthesis), and melatonin receptor MT1 and MT2 genes. Primary BALB/c mouse chondrocytes were synchronized by culturing in DMEM containing 1% serum for 24 hr, followed by DMEM containing 50% serum for 2 hr. Subsequently, the media was changed to DMEM containing 10% serum (normal condition, time point zero). Control chondrocytes were

cultured continuously in DMEM containing 10% serum. Analysis of *Bmal1* mRNA that was isolated from synchronized cultures every 4 hr revealed cyclic expression (22.63 hr period, $P = 9.7 \times 10^{-17}$; Figure 6A) with significantly higher amplitudes than was observed for unsynchronized cells. The addition of melatonin to synchronized BALB/c primary chondrocytes at different time points (9 and 13 hr) after synchronization moved the *Bmal1* mRNA expression peak to 17 hr after melatonin addition (Figure 6B), indicating that exogenous melatonin modulates circadian expression of *Bmal1* and other rhythmic gene expressions in BALB/c chondrocytes. To confirm the cyclic expression of *Bmal1*, melatonin was added to BALB/c chondrocytes 13 hours after synchronization and RNA was collected every 4 hr interval for over 58 hours. The addition of melatonin did not affect the rhythmic expression of *Bmal1* (without melatonin: 23.28-hr period, $P = 0.04$; +melatonin: 22.68-hr period, $P = 0.04$, significantly fitted to cosinor model, Fig. 6C). Rhythmic expression of *Mt1* mRNA was observed only after the addition of exogenous melatonin (17.03-hr period, $P = 3.88 \times 10^{-9}$, Fig. 6D). The *Mt2* mRNA expression pattern was similar to that of *Mt1*; however, no significant rhythmic expression was observed ($P = 0.07$, Figure 6E). Analysis of *Aanat* mRNA in BALB/c chondrocytes did not show rhythmic expression either with or without the addition of melatonin (data not shown).

To analyze the effects of melatonin on chondrocytes which express higher levels of endogenous melatonin than BALB/c chondrocytes, C3H mouse primary chondrocytes were used and synchronized as described above. In C3H mouse primary chondrocytes, exogenous melatonin induced the cyclic expression of *Aanat* mRNA with a 23.27-hr period ($P = 0.03$, Figure 7A). Simultaneous analysis of *Bmal1* mRNA revealed an 18.67 hr cycle (Figure 7B), while melatonin changed the *Bmal1* expression rhythm from an 18.67-hr period ($P = 0.04$) in control cells to a 23.34-hr period ($P = 2.6 \times 10^{-5}$), similar to the melatonin-stimulated expression rhythm of *Aanat* mRNA (Figure 7B). Of note, the copy number of *Bmal1* mRNA in C3H chondrocytes was almost 10 times lower ($0.3\text{--}1.0 \times 10^5$ copies/ μg RNA) than in BALB/c chondrocytes ($0.5\text{--}2.0 \times 10^6$ copies/ μg RNA). In contrast, *H1omt* mRNA did not exhibit a cyclic expression pattern (data not shown). Interestingly, exogenous melatonin also caused an 8-hr shift in the cyclic expression peaks of *Mt1* and *Mt2* mRNA in C3H chondrocytes, without changing their circadian periods of 16.96 hr (*Mt1*, +melatonin: $P = 0.02$) and 16.93 hr (*Mt2*, +melatonin: $P = 0.007$), or 17.42 hr (*Mt1*, -melatonin: $P = 0.08$) and 17.14 hr (*Mt2*, -melatonin: $P = 0.02$), respectively (Figure 7C and D). These results indicate that rhythmic expression of melatonin receptors is controlled by other regulators in addition to *Bmal1*.

DISCUSSION

To the best of our knowledge, this is the first study to show that chondrocytes produce melatonin and express the melatonin-synthesizing enzyme AANAT and melatonin receptors MT1 and MT2. Melatonin production by chondrocytes was verified by mass spectrometric analysis of rat and chick chondrocyte lysates. We also demonstrate that mouse growth plate chondrocytes respond to melatonin via the MT1 and MT2 melatonin receptors. Melatonin-stimulated chondrocyte growth and expression of endochondral ossification markers such as CCN2, aggrecan, IHH, and SOX9, the latter being a strong promoter of chondrogenesis (Tomita et al. 2013) and inhibitor of chondrocyte maturation (Hattori *et al.* 2010). However, expression of *Col10a1*, which encodes a marker of chondrocyte hypertrophy, was suppressed. This suggests that melatonin accelerates chondrocyte proliferation but inhibits terminal differentiation and hypertrophy.

Many mouse lines that have been established for research purposes do not produce melatonin in the pineal gland because they lack AANAT activity (Ebihara *et al.* 1986). Our experiments show that BALB/c mouse chondrocytes express the MT1 and MT2 receptors, as well as AANAT and HIOMT. These findings suggest that this mouse strain retains a melatonin signaling system. Although melatonin biosynthesis was below the detection level, it may have been decreased during strain establishment.

We also measured the synthesis of N-acetyltryptamine by tryptamine, a serotonin analog, as an indicator of AANAT enzymatic activity in chick chondrocytes. The enzyme is particularly abundant in the pineal gland, which is the major site of melatonin synthesis (Stehle *et al.* 2011). This is consistent with the notion that melatonin may be produced in most cells that contain mitochondria, since melatonin is a mitochondria-targeted antioxidant (Reiter *et al.* 2016). Surprisingly, kinetic analysis of the effects of melatonin on chondrocyte gene expression revealed rapid increases in *Aanat*, *Mt1*, *Mt2*, and *Pthrp* mRNA levels, indicating a rapid and enhanced response to melatonin. The increase in expression of *Ihh* mRNA in response to melatonin was delayed compared with other genes which were analyzed. This may indicate that the upregulation of *Ihh* by melatonin was modified through upregulation of *Pthrp*. Luzindole, an antagonist of melatonin receptors, abolished the enhancement of *Aanat* and *Ihh* mRNA production, confirming that the enhanced expression of these genes by melatonin is at least partially mediated by the MT1 and MT2 receptors. The addition of Luzindole repressed *Ihh* expression, indicating an MT1/MT2-independent effect of luzindole.

Because relatively high levels of *Aanat*, *Mt1*, and *Mt2* mRNA were observed in the peripheral

regions and the hypertrophic zone of cartilage, avascular cartilage may respond differently to melatonin in an endocrine manner. Moreover, melatonin produced in cartilage may affect gene expression in a para/autocrine manner. Several *in vitro* studies have reported the effects of melatonin on chondrocyte metabolism (Gao *et al.* 2014, Pei *et al.* 2009), but it is difficult to compare results: For example, Gao *et al.* used relatively large melatonin concentrations to show that, at 5×10^{-5} M, melatonin enhanced chondrogenic differentiation of human mesenchymal stem cells (Gao *et al.* 2014), while Pei *et al.* (Pei *et al.* 2009) showed that the mRNA and protein expression levels of type II collagen, SOX9, and aggrecan were enhanced by the addition of 4×10^{-6} M melatonin to porcine articular chondrocytes. Our results are largely consistent with these reports, confirming that melatonin enhances chondrocyte proliferation and the expression of cartilage-associated genes. However, a study using cultured chondrocytes from rat vertebral body growth plates showed that high concentrations of melatonin (4×10^{-4} M) caused inhibition of cell proliferation and mRNA expression of *Col2a1*, *Aggrecan*, *Sox9*, and *Smad4* (Zhong *et al.* 2013). We used rib cartilage as a chondrocyte source, however, the effects of melatonin may vary among cartilage types (e.g. hyaline, fibrous, and elastin cartilage) or, even among hyaline cartilages and permanent cartilages (e.g. articular or trachea cartilage). Also hypertrophic chondrocytes from growth plates may show different responses to melatonin. This needs to be further analyzed.

The concept of a tissue-autonomous circadian clock in cartilage has been described in previous studies, and the rhythmic expression of clock genes in articular and growth plate cartilage has been demonstrated: Several studies have shown that the circadian clock in cartilage is regulated by a BMAL1/CLOCK and PER/CRY feedback loop (Dudek *et al.* 2016, Gossan *et al.* 2013, Okubo *et al.* 2013, Takarada *et al.* 2012). The key focus of our study was the effect of melatonin on the circadian cycle of cartilage growth. Our results demonstrate that exogenous melatonin upregulates *Bmal1*, but downregulates *Per1*, in chondrocytes; suggesting that melatonin modifies rhythmic gene expression. To analyze the effects of melatonin on rhythmic gene expression, primary BALB/c mouse chondrocytes were synchronized, and this synchronization enhanced the amplitude of *Bmal1* mRNA expression, and the addition of melatonin changed the cyclic *Bmal1* expression peaking 17 hr after melatonin addition. Analyzing rhythmic gene expression using RNA collected every 4 hours over 58 hours, however, the rhythmic expression of *Bmal1* did not change following the addition of melatonin. Induction of the rhythmic expressions of *Mt1* and *Mt2* (not significant, but similar to *Mt1* mRNA) was observed after addition of melatonin.

To investigate the effects of exogenous melatonin on melatonin synthesis, and to investigate

the endogenous melatonin effects in chondrocytes, we compared C3H mouse primary chondrocytes with BALB/c. In C3H chondrocytes, exogenous melatonin enhanced the amplitude of *Aanat* mRNA expression and altered the rhythmic expression of *Aanat* and *Bmal1* compared with the control group. This could be explained by the fact that melatonin modulates rhythmic gene expression by regulating *Bmal1*, a master regulator, and induces endogenous melatonin expression in cartilage. The cartilaginous melatonin could enhance rhythmic gene expression in a para/autocrine manner. Upregulated endogenous melatonin further regulates *Bmal1* rhythmic expression in C3H chondrocytes, and changes the *Bmal1* rhythmic interval from ~17 to ~24 hr. In contrast, BALB/c chondrocytes without endogenous melatonin show a ~24 hr *Bmal1* expression cycle which is not affected by melatonin addition, because *Bmal1* levels in BALB/c cells is sufficiently high, whereas C3H chondrocytes with a lower *Bmal1* expression undergo an ~18 hr *Bmal1* expression cycle despite endogenous melatonin; only after addition of exogenous melatonin, *Bmal1* rhythmic expression cycle extends to ~24 hrs as in Balb/c cells. This indicates that *Bmal1* is the key factor in determining the circadian cycle in chondrocytes, but it is largely controlled by melatonin.

The *Mt1* and *Mt2* receptors showed different expression patterns in BALB/c and C3H cells. Our data indicate that *Mt1* and *Mt2* expression is induced in BALB/c chondrocytes only after melatonin stimulation. In synchronized cultures, addition of melatonin induced *Mt1* and *Mt2* rhythmic expression with a ~17 hr period, because BALB/c chondrocytes retain the ability to respond to melatonin (via receptors, signal transduction, and transcription control). In contrast to BALB/c chondrocytes, C3H chondrocytes showed rhythmic expression of *Mt1* and *Mt2* with a ~17 hr period in the absence of exogenous melatonin, due to higher levels of endogenous melatonin. The addition of melatonin to C3H chondrocytes changed the cyclic expression peak by a 13 hr shift, but the cycle period was unchanged, indicating that melatonin receptors may be regulated by rhythmic regulators other than BMAL1.

We were not able to detect rhythmic expression of *aanat* in BALB/c chondrocytes, although we did detect temporary induction of *aanat* by melatonin. This may be due to the discontinuation of rhythmic induction of *Aanat* in BALB/c chondrocytes.

Kunimoto et al. (Kunimoto *et al.* 2016) have shown that PTH (1–34) is able to reset the cartilage circadian clock in murine femur organ culture, while a lack of BMAL1 caused disruption of the cyclic expression of *Ihh* and *Per1* in cartilage (Takarada *et al.* 2012). Notably, our results show that melatonin stimulates *Pthrp*, *Ihh*, and *Bmal1* expression, but inhibits *Per1* expression, suggesting that melatonin directly or indirectly regulates the expression of circadian clock-

associated genes.

DECLARATION OF INTEREST

No competing financial interests exist.

FUNDING

This work was supported by JSPS KAKENHI (grant number JP16K11476) and by funding from the Foundation for Growth Science, Ryobi Teien Foundation, by a SHISEIKAI Scholarship Fund for basic medical science researchers (Keiko Watanabe Award), and by China Scholarship Council to S. Fu.

AUTHOR CONTRIBUTIONS

T.H., M.I., and A.H. defined the research theme, designed the experimental approach, and critically revised the manuscript. T.H., S.F., M.K., S.K., D.H., Y.S., A.T., and Y.M. designed the methods and experiments, and carried out most experiments. T.H., S.F., T.N., Y.U., Y.M., M.I., A.H., and S.K. analyzed data and interpreted the results. T.H. wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Dr. Kenji Tomioka, Dr. Takeshi Takarada, and Dr. Masaharu Takigawa for useful suggestions, and Yoshiko Miyake and Tomoko Yamamoto for technical and secretarial assistance. We also thank Dr. Daisuke Ekuni, Dr. Manabu Morita (Department of Preventive Dentistry), and Dr. Ziyi Wang (Department of Orthodontics) for help with statistical analysis.

REFERENCES

- Acuna-Castroviejo D, Escames G, Venegas C, Diaz-Casado ME, Lima-Cabello E, Lopez LC, Rosales-Corral S, Tan DX and Reiter RJ 2014 Extrapineal melatonin: sources, regulation, and potential functions. *Cell Mol Life Sci* **71(16)** 2997-3025.
- Bingham C, Arbogast B, Guillaume GC, Lee JK and Halberg F 1982 Inferential statistical methods for estimating and comparing cosinor parameters. *Chronobiologia* **9(4)** 397-439.
- Chung UI, Lanske B, Lee K, Li E and Kronenberg H 1998 The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. *Proc Natl Acad Sci U S A* **95(22)** 13030-5.
- Cornelissen G 2014 Cosinor-based rhythmometry. *Theor Biol Med Model* **11** 16.
- Dudek M, Gossan N, Yang N, Im HJ, Ruckshanthi JP, Yoshitane H, Li X, Jin D, Wang P, Boudiffa M, Bellantuono I, Fukada Y, Boot-Handford RP and Meng QJ 2016 The chondrocyte clock gene *Bmal1* controls cartilage homeostasis and integrity. *J Clin Invest* **126(1)** 365-76.
- Dudek M and Meng QJ 2014 Running on time: the role of circadian clocks in the musculoskeletal system. *Biochem J* **463(1)** 1-8.
- Ebihara S, Marks T, Hudson DJ and Menaker M 1986 Genetic control of melatonin synthesis in the pineal gland of the mouse. *Science* **231(4737)** 491-3.
- Gao W, Lin M, Liang A, Zhang L, Chen C, Liang G, Xu C, Peng Y, Chen C, Huang D and Su P 2014 Melatonin enhances chondrogenic differentiation of human mesenchymal stem cells. *J Pineal Res* **56(1)** 62-70.

518 Goldring MB, Tsuchimochi K and Ijiri K 2006 The control of chondrogenesis. *J Cell*
519 *Biochem* **97(1)** 33-44.
520

521 Gossan N, Zeef L, Hensman J, Hughes A, Bateman JF, Rowley L, Little CB, Piggins
522 HD, Rattray M, Boot-Handford RP and Meng QJ 2013 The circadian clock in
523 murine chondrocytes regulates genes controlling key aspects of cartilage
524 homeostasis. *Arthritis Rheum* **65(9)** 2334-45.
525

526 Hanyu R, Hayata T, Nagao M, Saita Y, Hemmi H, Notomi T, Nakamoto T, Schipani
527 E, Knönenberg H, Kaneko K, Kurosawa H, Ezura Y and Noda M 2011 Per-1
528 is a specific clock gene regulated by parathyroid hormone (PTH) signaling in
529 osteoblasts and is functional for the transcriptional events induced by PTH.
530 *J Cell Biochem* **112(2)** 433-8.
531

532 Hattori T, Muller C, Gebhard S, Bauer E, Pausch F, Schlund B, Bosl MR, Hess A,
533 Surmann-Schmitt C, von der Mark H, de Crombrughe B and von der Mark
534 K 2010 SOX9 is a major negative regulator of cartilage vascularization, bone
535 marrow formation and endochondral ossification. *Development* **137(6)** 901-11.
536

537 Hessen E, Nordlund A, Stalhammar J, Eckerstrom M, Bjerke M, Eckerstrom C,
538 Gothlin M, Fladby T, Reinvang I and Wallin A 2015 T-Tau is Associated with
539 Objective Memory Decline Over Two Years in Persons Seeking Help for
540 Subjective Cognitive Decline: A Report from the Gothenburg-Oslo MCI Study.
541 *J Alzheimers Dis* **47(3)** 619-28.
542

543 Hinoi E, Ueshima T, Hojo H, Iemata M, Takarada T and Yoneda Y 2006 Up-
544 regulation of per mRNA expression by parathyroid hormone through a
545 protein kinase A-CREB-dependent mechanism in chondrocytes. *J Biol Chem*
546 **281(33)** 23632-42.
547

548 Igarashi K, Saeki, S., Shinoda, H. 2013 Diurnal rhythms in the incorporation and
549 secretion of 3H-proline and 3H-galactose by cartilage cells and osteoblasts in
550 various bone-forming sites in growing rats. *Orthodontic Waves* **72(1)** 11-15.

551
552 Kunimoto T, Okubo N, Minami Y, Fujiwara H, Hosokawa T, Asada M, Oda R, Kubo
553 T and Yagita K 2016 A PTH-responsive circadian clock operates in ex vivo
554 mouse femur fracture healing site. *Sci Rep* **6** 22409.
555
556 Liu CF, Samsa WE, Zhou G and Lefebvre V 2016 Transcriptional control of
557 chondrocyte specification and differentiation. *Semin Cell Dev Biol*.
558
559 Okubo N, Fujiwara H, Minami Y, Kunimoto T, Hosokawa T, Umemura Y, Inokawa
560 H, Asada M, Oda R, Kubo T and Yagita K 2015 Parathyroid hormone resets
561 the cartilage circadian clock of the organ-cultured murine femur. *Acta Orthop*
562 **86(5)** 627-31.
563
564 Okubo N, Minami Y, Fujiwara H, Umemura Y, Tsuchiya Y, Shirai T, Oda R, Inokawa
565 H, Kubo T and Yagita K 2013 Prolonged bioluminescence monitoring in
566 mouse ex vivo bone culture revealed persistent circadian rhythms in articular
567 cartilages and growth plates. *PLoS One* **8(11)** e78306.
568
569 Pei M, He F, Wei L and Rawson A 2009 Melatonin enhances cartilage matrix
570 synthesis by porcine articular chondrocytes. *J Pineal Res* **46(2)** 181-7.
571
572 Reddi AH 1994 Bone and cartilage differentiation. *Curr Opin Genet Dev* **4(5)** 737-44.
573
574 Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M and Qin L 2016
575 Melatonin as an antioxidant: under promises but over delivers. *J Pineal Res*
576 **61(3)** 253-78.
577
578 Russell JE, Grazman B and Simmons DJ 1984 Mineralization in rat metaphyseal
579 bone exhibits a circadian stage dependency. *Proc Soc Exp Biol Med* **176(4)**
580 342-5.
581
582 Schmidl M, Adam N, Surmann-Schmitt C, Hattori T, Stock M, Dietz U, de
583 Crombrughe B, Poschl E and von der Mark K 2006 Twisted gastrulation

modulates bone morphogenetic protein-induced collagen II and X expression
in chondrocytes in vitro and in vivo. *J Biol Chem* **281(42)** 31790-800.

Shao YY, Wang L and Ballock RT 2006 Thyroid hormone and the growth plate. *Rev
Endocr Metab Disord* **7(4)** 265-71.

Stehle JH, Saade A, Rawashdeh O, Ackermann K, Jilg A, Sebesteny T and Maronde
E 2011 A survey of molecular details in the human pineal gland in the light
of phylogeny, structure, function and chronobiological diseases. *J Pineal Res*
51(1) 17-43.

Stevenson S, Hunziker EB, Herrmann W and Schenk RK 1990 Is longitudinal bone
growth influenced by diurnal variation in the mitotic activity of chondrocytes
of the growth plate? *J Orthop Res* **8(1)** 132-5.

Takarada T, Kodama A, Hotta S, Mieda M, Shimba S, Hinoi E and Yoneda Y 2012
Clock genes influence gene expression in growth plate and endochondral
ossification in mice. *J Biol Chem* **287(43)** 36081-95.

Tomita N, Hattori T, Itoh S, Aoyama E, Yao M, Yamashiro T and Takigawa M 2013
Cartilage-specific over-expression of CCN family member 2/connective tissue
growth factor (CCN2/CTGF) stimulates insulin-like growth factor expression
and bone growth. *PLoS One* **8(3)** e59226.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong
HK, Oh WJ, Yoo OJ, Menaker M and Takahashi JS 2004
PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals
persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad
Sci U S A* **101(15)** 5339-46.

Zhong ZM, Li T, Xu ZX, Meng TT, Zeng JH, Zheng S, Ye WB, Wu Q and Chen JT
2013 Effect of melatonin on the proliferation and differentiation of
chondrocytes from rat vertebral body growth plate in vitro. *Int J Med Sci*

617 **10(10)** 1392-8.

618

619

620

Figure legends

FIGURE 1. Expression of *Mt1*, *Mt2*, and *Sox9* mRNA in cartilage and other tissues of BALB/c mice. Tissues from newborn (postnatal day 0, P0) BALB/c mice were harvested and total RNA was isolated. Expression of *Mt1*, *Mt2*, and *Sox9* mRNA was detected in cartilage and other tissues, including brain. Data are shown as mean \pm standard deviation (n = 3).

FIGURE 2. Detection of *Mt1* and *Mt2* mRNA and MT2 protein in cartilage. (A) *In situ* hybridization. Sections of tibiae from BALB/c mice at embryonic day 19.5 (E19.5) were fixed at 03:00 a.m. A *Col2a1* probe was used as a marker of cartilaginous tissue. Key: AS, antisense probe; S, sense probe. Boxes in upper panels indicate the magnified regions shown in the lower panels. Scale bars: 200 μ m and 20 μ m. (B) Comparison of gene expression in hypertrophic versus resting/proliferating chondrocytes. Hypertrophic cartilage and resting/proliferating cartilage were separately collected from BALB/c mouse ribcages at E18.5 and cultured. Hypertrophic chondrocytes were characterized by low expression of *Sox9* mRNA and high expression of *Col10a1*, *Vegf*, and *Mmp13* mRNA. Expression levels of *Mt1* and *Mt2* mRNA were also higher in hypertrophic chondrocytes than in resting/proliferating chondrocytes. Data are shown as mean \pm standard deviation (n = 3). *P < 0.05, as determined by Student's t-tests. (C) Protein expression. Western blot analysis showed slightly enhanced MT2 protein levels in cultured hypertrophic chondrocytes compared with resting/proliferating chondrocytes. Actin was used as a loading control.

FIGURE 3. Melatonin stimulates chondrocyte proliferation and induces changes in gene expression. (A) Primary chondrocytes were isolated from the ribcages of BALB/c mice at embryonic day 18.5 (E18.5) and cultured for 23 hr. Luzindole or a vehicle control (dimethylsulfoxide) was added to the media, followed 1 hr later by melatonin at the indicated concentrations. Afterwards, cells were cultured for an additional 5 days in the dark. Cell density was measured with an MTS-based assay. Melatonin caused increased cell numbers with the greatest effect at 1×10^{-8} M, while luzindole abolished melatonin-induced enhancement of cell proliferation. Data are shown as mean \pm standard deviation (n = 12). *P < 0.05 compared with melatonin (-)/luzindole (-) group; ^{##}P < 0.05 compared with 10^{-6} M melatonin, as determined by the Bonferroni test. (B) Mouse primary chondrocytes were plated, and 24 hr later melatonin was added at the indicated concentrations. Measurement of mRNA expression by real-time

polymerase chain reaction revealed that *Ccn2*, *Aggrecan*, *Sox9*, and *Ihh* were upregulated by melatonin, while *Col10a1* mRNA levels decreased. Data are shown as mean \pm standard deviation (n = 3). *P < 0.05 compared with dimethylsulfoxide, as determined by one-way analysis of variance followed by Dunnett's *post hoc* test.

FIGURE 4. Detection of melatonin-synthesizing enzyme mRNA in cartilage and other tissues of BALB/c mice. (A) Tissues from newborn BALB/c mice were harvested and total RNA was isolated. Both *Aanat* and *Hiomt* mRNA were detected in cartilage and other tissues, including brain. Data are presented as mean \pm standard deviation (n = 3). (B) *In situ* hybridization analysis of *Aanat* mRNA. Sections of tibiae from BALB/c mice at embryonic day 19.5 were fixed at 03:00 a.m., as performed for Figure 2A. Key: AS, antisense probe; S, sense probe. Boxes in upper panels indicate magnified regions shown in lower panels. Scale bars: 200 μ m and 20 μ m.

FIGURE 5. Effect of melatonin on gene expression in mouse primary chondrocytes. (A) Melatonin (at 1×10^{-6} M) or dimethyl sulfoxide (control) was added to cells and total RNA was harvested at the indicated time points. Expression of *Aanat*, *Mt1*, *Mt2*, *Bmall*, and *Pthrp* mRNA as significantly induced 0.5 hr after the addition of melatonin, while *Ihh* mRNA expression was induced after 2 hr. Expression of *Per1* was inhibited at 0.5 hr. Data are presented as mean \pm standard deviation (n = 3). *P < 0.05 compared with dimethyl sulfoxide, as determined by two-way analysis of variance and Tukey's *post hoc* test. (B) Inhibitory effects of luzindole, an MT1 and MT2 melatonin receptor antagonist, in mouse primary chondrocytes. Luzindole (1×10^{-4} M) was added to cultures 1 hr before the addition of melatonin. Total RNA was collected 2 hr after melatonin addition. Upregulation of *Aanat* and *Ihh* mRNA by melatonin was abolished by luzindole. Data are presented as mean \pm standard deviation (n = 3). *P < 0.05 compared with control; **P < 0.05 compared with melatonin (+) group; as determined by one-way analysis of variance and Tukey's *post hoc* test.

FIGURE 6. Cyclic expression and rhythm modification of *Bmall*, *Aanat*, and melatonin receptor mRNA by melatonin. (A) Primary chondrocytes isolated from BALB/c mice were plated on 3.5-cm dishes at a density of 4×10^5 cells/dish cultured for 48 hr in the dark. For synchronization, half of the cells were placed in Dulbecco's Modified Eagle Medium containing 1% serum for 24 hr after reaching subconfluence, followed by 2 hr in 50% serum. Subsequent passage into 10% serum-containing media was set as the 0 hr timepoint. Solid line: synchronized

cells. Dashed line: control cells (not synchronized, cultured continuously in 10% serum). Red line: fitting curve from the calculated formula. Total RNA was harvested every 4 hr. All experiments were performed more than four times and real-time polymerase chain reaction analysis using a cosinor2 package in *R*, statistical significance was accepted at $P < 0.05$. (A) After synchronization, the amplitude of the rhythmic expression of *Bmal1* mRNA was strongly enhanced. (B) Addition of melatonin (at 1×10^{-6} M) at various time points indicated by ▲ to BALB/c chondrocytes synchronized and cultured as in (A) had no effect on the expression of *Bmal1*. ▲+mel(9) and ▲+mel(13): melatonin was added 9 or 13 hr, respectively, after synchronization. Period time between melatonin addition and peak point is indicated. (C) BALB/c mouse primary chondrocytes were cultured and synchronized as described above. In the (+mel) group, melatonin was added at final concentration of 10^{-6} M after 13 hr from synchronization as indicated by ▲ and total RNA was collected at each indicated time point. *Bmal1* expression did not show large changes in the circadian interval (23.47 hr, -melatonin; 22.23 hr, +melatonin) or peak time point. (D) *Mt1* mRNA showed circadian expression after the addition of melatonin with a 17.03 hr circadian interval. (E) Although the expression pattern of *Mt2* mRNA was similar to that of *Mt1*, no significant circadian expression was observed for either (+melatonin and -melatonin) groups.

FIGURE 7. C3H mouse primary chondrocytes were synchronized as described above and melatonin was added to a final concentration of 10^{-6} M 13 hr after synchronization (indicated by ▲), and total RNA was collected at each indicated time point. (A) In control cultures without melatonin, *Aanat* mRNA did not show a clear circadian rhythm; however, melatonin stimulation induced strong circadian expression ($P < 0.05$; circadian interval was 23.27 hr). (B) In contrast to BALB/c chondrocytes, *Bmal1* mRNA expression in C3H chondrocytes responded to melatonin with a change the circadian interval from 18.67 hr to 23.34 hr. (C and D) Expression of *Mt1* and *Mt2* mRNA showed similar circadian expression; intervals were 17.42 hr (-melatonin) and 16.96 hr (+melatonin) for *Mt1*, and 17.14 hr (-melatonin) and 16.93 hr (+melatonin) for *Mt2*. Red line: fitting curve from the calculated formula. All experiments were performed more than four times and real-time polymerase chain reaction analysis carried out with the cosinor2 package in *R*. Statistical significance was accepted at $P < 0.05$.

720

721

722

Tables

Table 1. Primers used for real-time PCR analysis.

genes	mouse (5'-3') mouse (3'-5')	chick (5'-3') chick (3'-5')
<i>MT1</i>	TCCTGTCTGTGTACCGCAAC CGAGGTCTGCCACAGCTAAA	AGCCACCATCCTCATCTTCAC TATATTTCCCGCGTTCCGCA
<i>MT2</i>	GAAGGGCTCTTTGTCACCAAGTTAC GGTTCAGGAGCCCATAAACAAT	GCAAGCTCAGGAACTCAGGTAA GCATTTACCCAAAGTCCATCC
<i>AANAT</i>	ForBALB/c:AGGGTTAGGAAGTGCCGGAT and AGTGCGTTTGAGATTGAGCG	TAGCAGGAAACACCCAGCCT TCCCTAACAGATCCCCTGGA
	for C3H: CAAGTGCAGAGCAAGCAACC and CTCAAACCAGCCCAGTGACA	
<i>HIOMT</i>	GCCATCTACAGGTCGGAGG GAAGGGCGAGAGGTGGAAG	GAGTGCTGCTGGTTGAATCG CTGCTGTTGCTCCTTTCT
<i>SOX9</i>	ATCTGAAGAAGGAGAGCGAG TCAGAAGTCTCCAGAGCTTG	CGTTCTTCACCGACTTCCTC AGGAAGCTGGCTGACCAGTA
<i>AggreCAN</i>	GAGGAGAGAACTGGAGAAG GGCGATAGTGAATACAA	
<i>IHH</i>	CTGGCTGCGATTCTTCACAC ACTGAGGTGCAAGCCCATCT	
<i>ColXa1</i>	TGCTGCCTCAAATACCCTTTCT TGCGTATGGGATGAAGTATTG	
<i>VEGF</i>	CCTGGTAATGGCCCCTCCTC CCCCATTGCTCTGTGCCTTG	
<i>MMP13</i>	TCCTCGGAGACTGGTAATGG TGATGAAACCTGGACAAGCA	
<i>GAPDH</i>	CAATGACCCCTTCATTGACC GACAAGCTTCCCGTTCTCAG	ACTTTGGCATTGTGGAGGGT ACGCTGGGATGATGTTCTGG

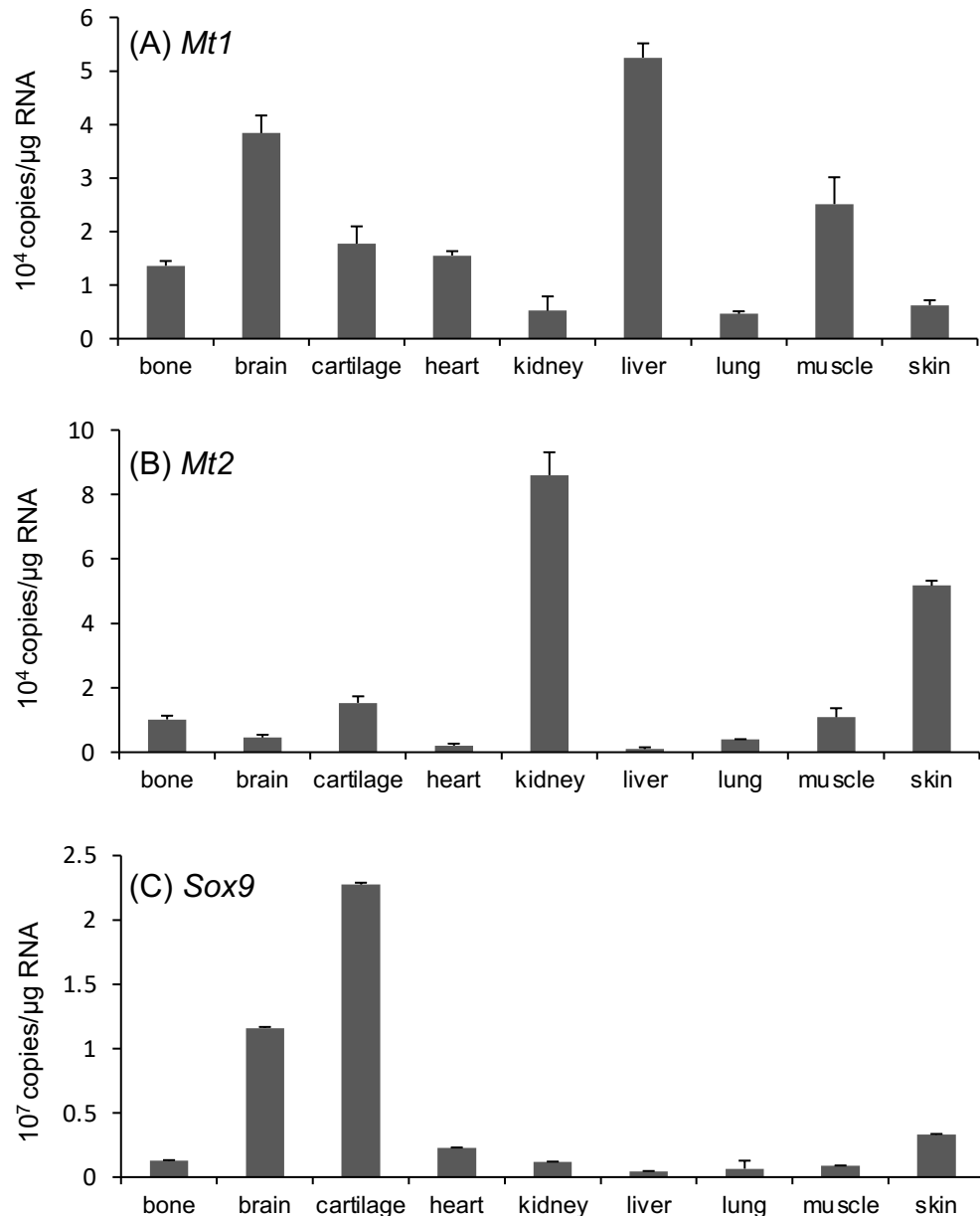


FIGURE 1. Expression of *Mt1*, *Mt2*, and *Sox9* mRNA in cartilage and other tissues of BALB/c mice. Tissues from newborn (P0) BALB/c mice were harvested and total RNA was isolated. *Mt1*, *Mt2*, and *Sox9* mRNA expression was detected in cartilage and other tissues, including brain. Data represent mean \pm SD (n = 3).

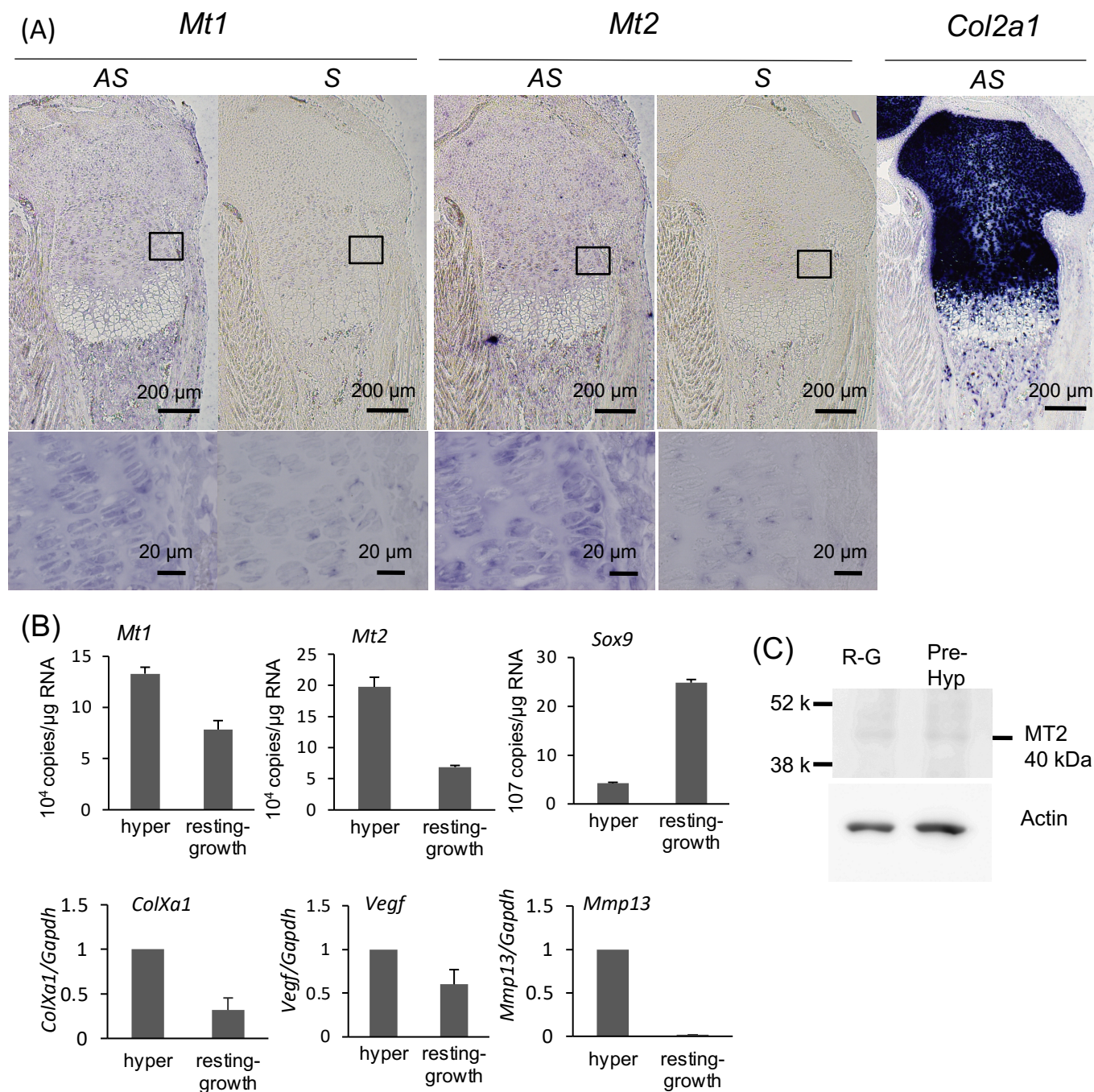


Figure 2

FIGURE 2. Detection of *Mt1* and *Mt2* mRNA and MT2 protein in cartilage. (A) *In situ* hybridization. Sections of tibiae from BALB/c mice at embryonic day 19.5 (E19.5) were fixed at 03:00 a.m. A *Col2a1* probe was used as a marker of cartilaginous tissue. AS, antisense probe; S, sense probe. Boxes in upper panels indicate magnified region shown in lower panels. Scale bars: 200 μ m and 20 μ m. (B) Comparison of gene expression in hypertrophic versus resting/proliferating chondrocytes. Hypertrophic cartilage and resting/proliferating cartilage were separately collected from BALB/c mouse ribcages at E18.5 and cultured. Hypertrophic chondrocytes were characterized by low expression of *Sox9* mRNA and high expression of *Col10a1*, *Vegf*, and *Mmp13* mRNA. *Mt1* and *Mt2* expression levels were also higher in hypertrophic chondrocytes than in resting/proliferating chondrocytes. Data represent mean \pm SD (n = 3). *p < 0.05, determined by Student's t-tests. (C) Protein expression. Western blot analysis showing slightly enhanced MT2 protein levels in cultured hypertrophic chondrocytes compared with resting/proliferating chondrocytes. Actin was used as a loading control.

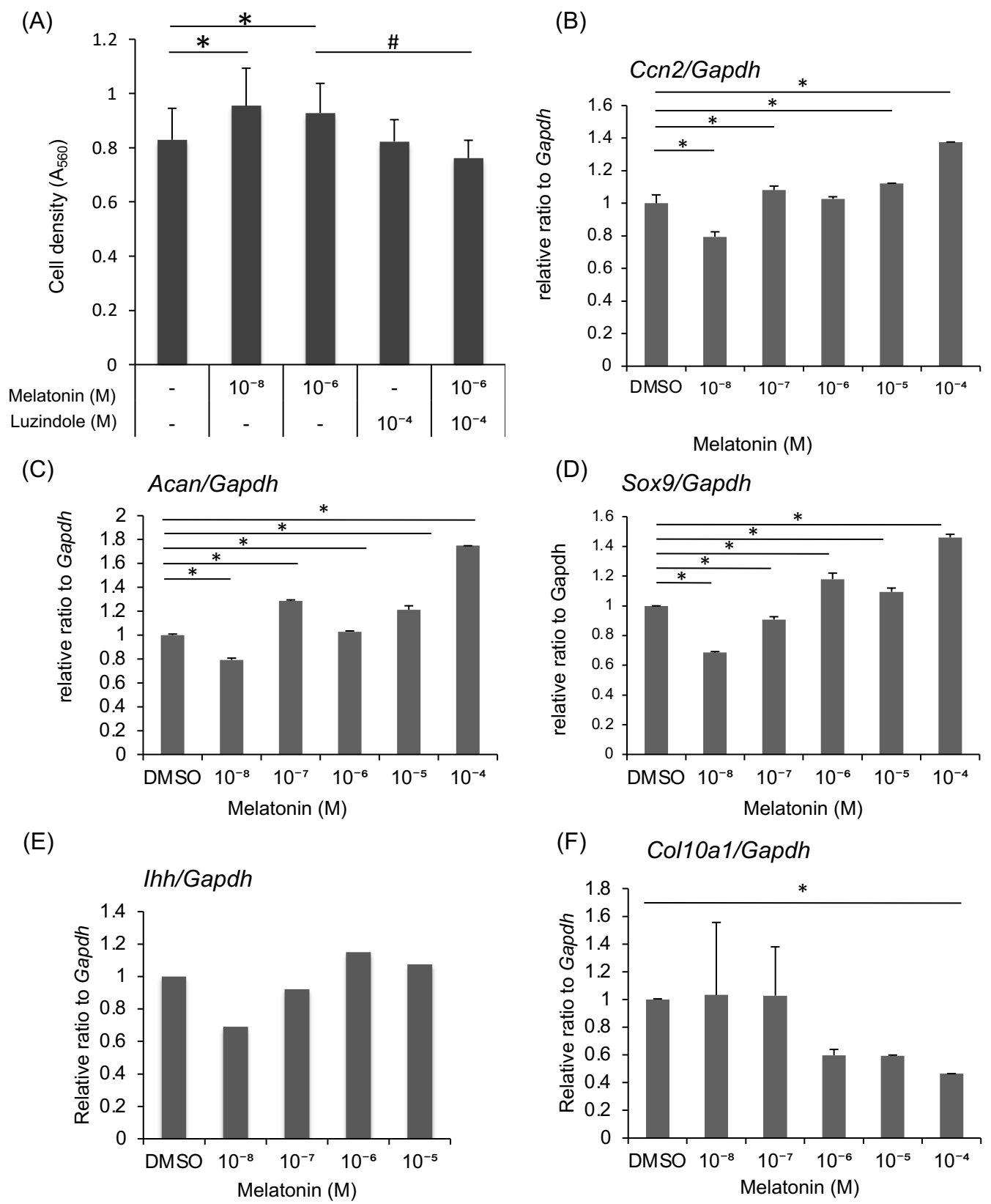


Figure 3

FIGURE 3. Melatonin stimulates chondrocyte proliferation and induces changes in gene expression. (A) Primary chondrocytes were isolated from the ribcages of BALB/c mice at embryonic day 18.5 and cultured for 23 hr. Luzindole or vehicle control (dimethylsulfoxide, DMSO) was added to the medium, followed 1 hr later by melatonin at the indicated concentrations; afterwards, cells were cultured for an additional 5 days. During the culture period, cells were kept in the dark. Cell density was measured with an MTS-based assay. Melatonin increased cell number with the greatest effect at 1×10^{-8} M, while luzindole abolished melatonin-induced enhancement of cell proliferation. Data represent mean \pm SD (n=12). * $p < 0.05$ compared with melatonin (-)/luzindole (-) group; ## $p < 0.05$ compared with 10^{-6} M melatonin, as determined by Bonferroni test. (B) Mouse primary chondrocytes were plated, and 24 hr later melatonin was added at the indicated concentrations. Measurement of mRNA expression by real-time PCR revealed that *Ccn2*, *Aggrecan*, *Sox9*, and *Ihh* were increased by melatonin, while *Col10a1* mRNA levels were decreased. Data represent mean \pm SD (n=3). * $p < 0.05$ compared with DMSO, as determined by one-way ANOVA followed by Dunnett's *post hoc* tests.

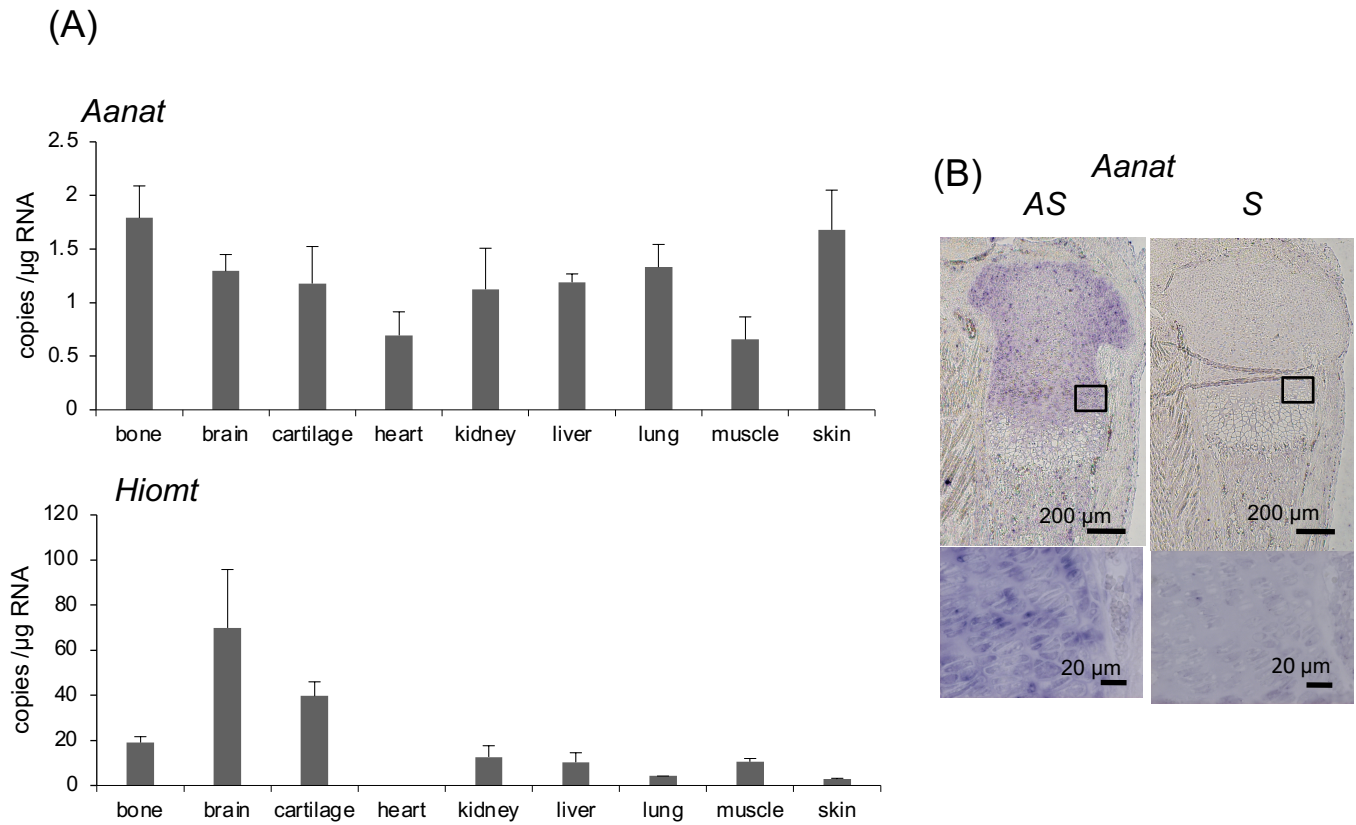


FIGURE 4. Detection of melatonin-synthesizing enzyme mRNA in cartilage and other tissues of BALB/c mice (A) Tissues from newborn BALB/c mice were harvested and total RNA was isolated. Both *Aanat* and *Hiomt* mRNA were detected in cartilage and other tissues, including brain. Data represent mean \pm SD ($n = 3$). (B) *In situ* hybridization analysis of *Aanat* mRNA. Sections of tibiae from BALB/c mice at embryonic day 19.5 were fixed at 03:00 a.m., as performed for Figure 2A. AS, antisense probe; S, sense probe. Boxes in upper panels indicate magnified region shown in lower panels. Scale bars: 200 μ m and 20 μ m.

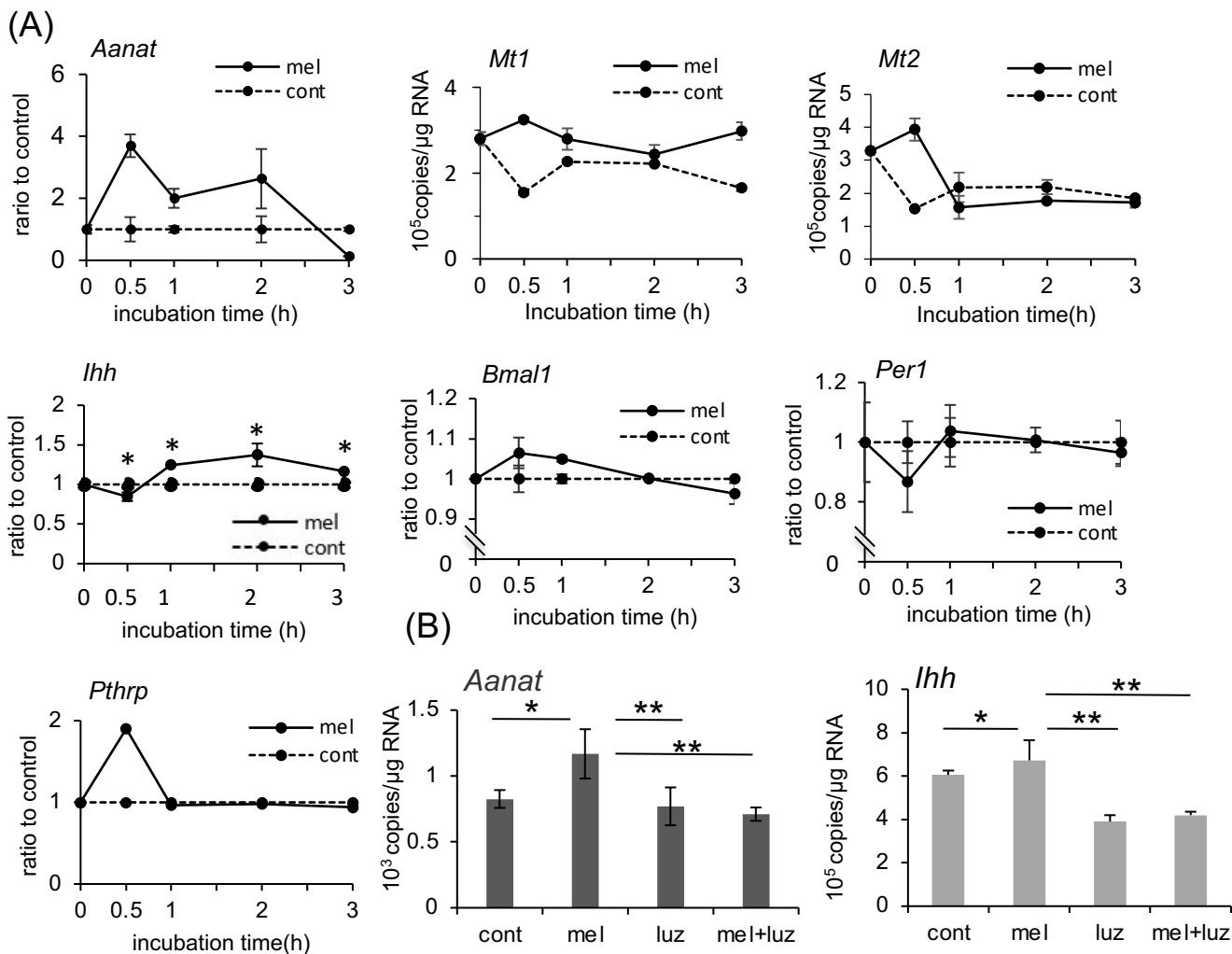


FIGURE 5. Effect of melatonin on gene expression in mouse primary chondrocytes. (A) Melatonin (1×10^{-6} M) or DMSO (control) was added to cells and total RNA was harvested at the indicated time points. *Aanat*, *Mt1*, *Mt2*, *Bmal1*, and *Pthrp* mRNA were significantly induced 0.5 hr after adding melatonin, while, *Ihh* mRNA was induced after 2 hr. *Per1* expression was inhibited at 0.5 hr. Data represent mean \pm SD ($n = 3$ individuals). * $p < 0.05$ compared with DMSO, as determined by two-way ANOVA and Tukey's *post hoc* tests. (B) Inhibitory effects of luzindole, a MT1 and MT2 melatonin receptor antagonist, in mouse primary chondrocytes. Luzindole (1×10^{-4} M) was added to cultures 1 hr before melatonin. Total RNA was collected 2 hr after melatonin addition. Upregulation of *Aanat* and *Ihh* mRNA by melatonin was abolished by luzindole. Data represent mean \pm SD ($n = 3$). * $p < 0.05$ compared with control; ** $p < 0.05$ compared with melatonin (+) group; as determined by one-way ANOVA and Tukey's *post hoc* tests.

Figure 5

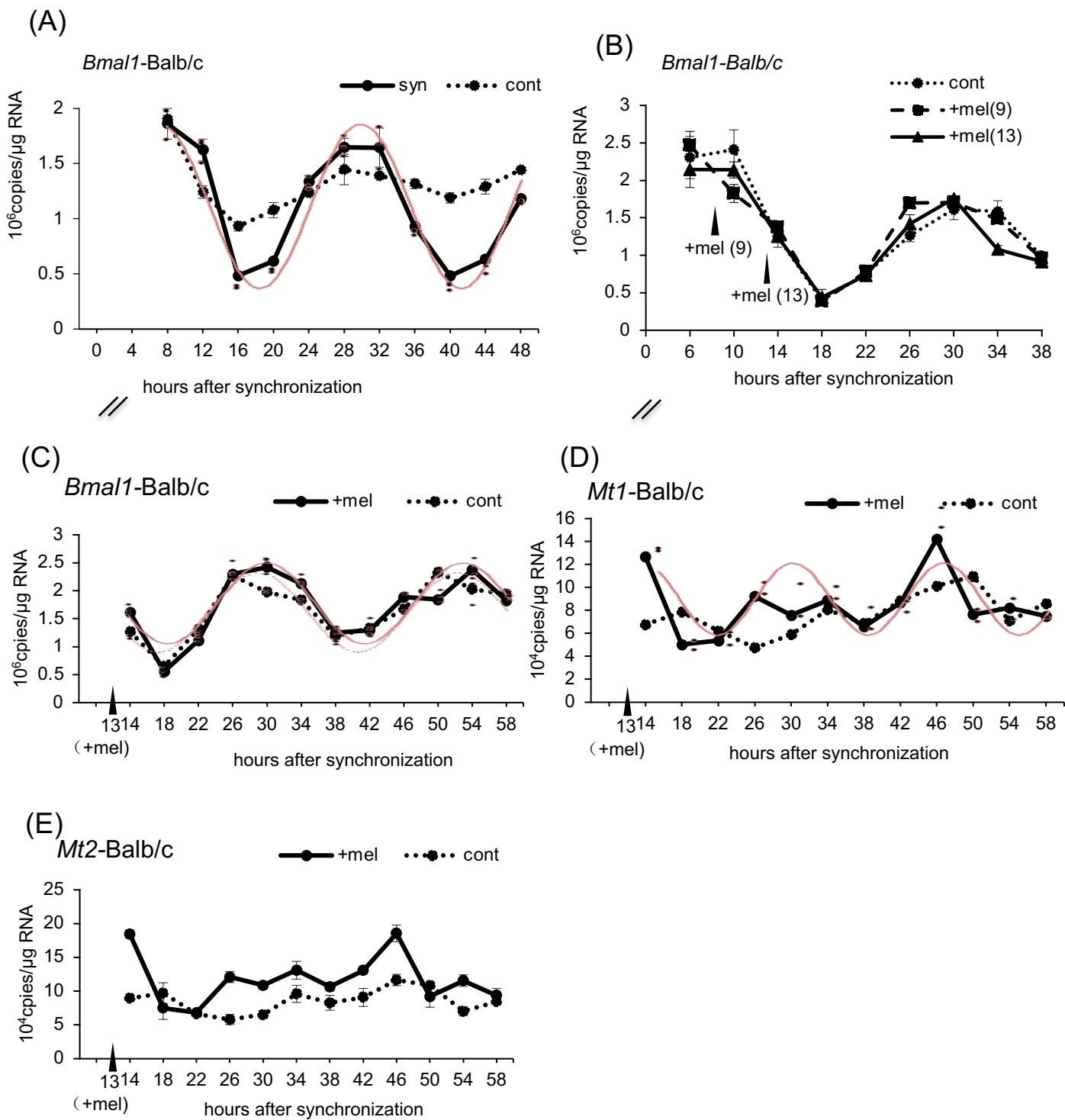


Figure 6

FIGURE6. (A) Primary chondrocytes isolated from BALB/c mice were plated on 3.5-cm dishes at a density of 4×10^5 cells/dish cultured for 48 hrs in the dark. For synchronization, , half of the cells after reaching subconfluence were placed in DMEM containing 1% serum for 24 hrs, followed by 2hrs in, 50% serum,. Subsequent change into 10% serum-containing media was set as 0 hr timepoint). Solid line: synchronized cells. Dashed line: control cells (not synchronized, cultured continuously in 10% serum. Red line: fitting curve from the calculated formula. Total RNA was harvested every 4 hr. All experiments were performed more than four times and real-time PCR analysis using a cosinor2 package in *R*, statistical significance was accepted at $P < 0.05$ (A)After synchronization, the amplitude of the rhythmic expression of *Bmal1* mRNA was strongly enhanced as analyzed. (B) Addition of melatonin (1×10^{-6} M) at various time points to BALB/c chondrocytes synchronized and cultured as in (A) had no effect on the expression of *Bmal1*. (C) BALB/c mouse primary chondrocytes, which do not express endogenous melatonin, were cultured using same method as (Figure 7A). Compared with C3H chondrocytes, *Bmal1* expression did not show large changes of circadian interval (23.47 hr, -melatonin; 22.23 hr, +melatonin) (D)*Mt1* mRNA showed circadian expression only after the addition of melatonin with a 17-hr circadian interval.

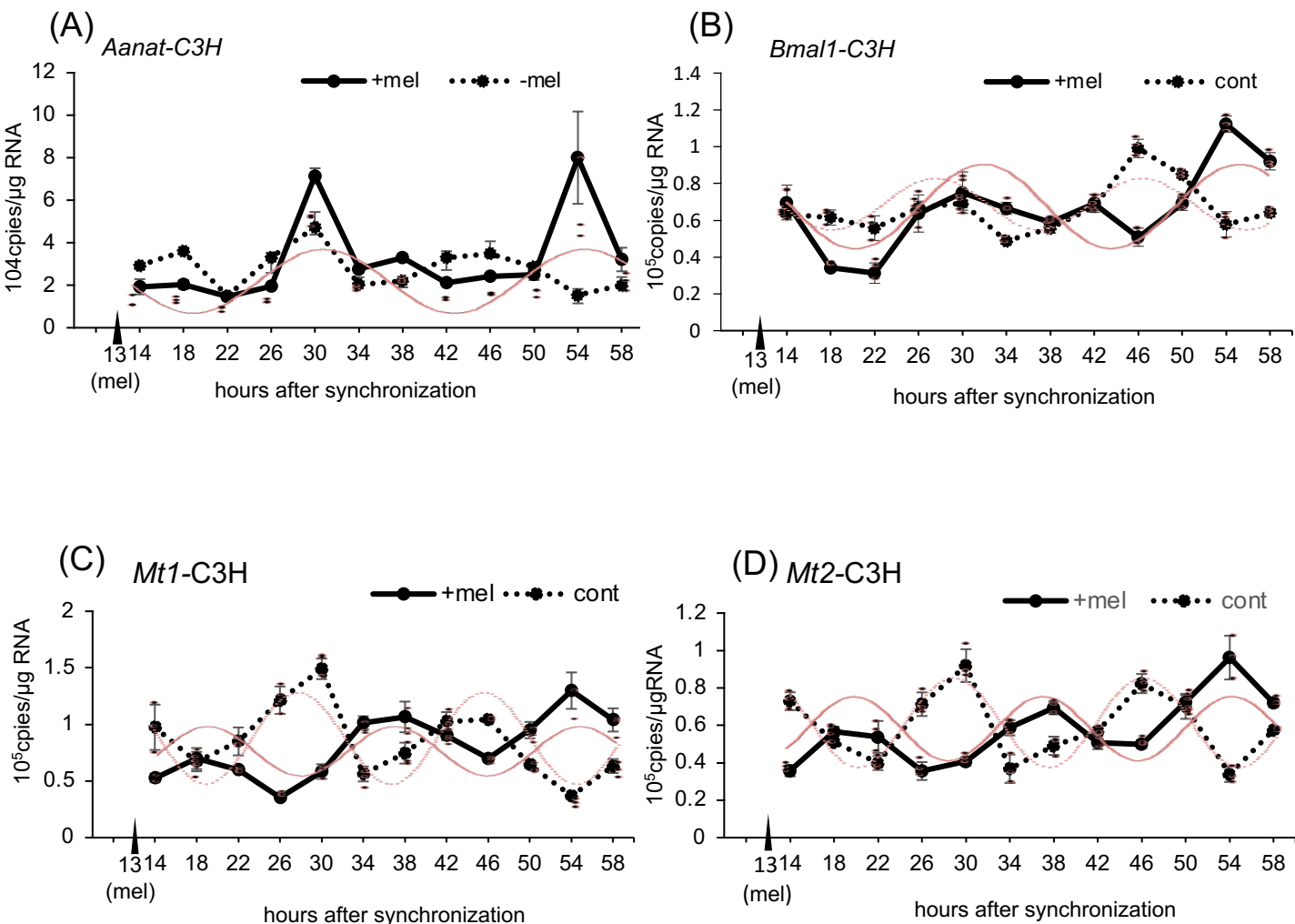


FIGURE 7. Cyclic expression and rhythm modification of *Bmal1*, *Aanat*, and melatonin receptor mRNA by melatonin. (A) C3H mouse primary chondrocytes synchronized as described above received melatonin final conc. 10^{-6} M) for 13 hr and total RNA was collected at each indicated time point. In control cultures without melatonin, *Aanat* mRNA did not show a clear circadian rhythm; however, melatonin stimulation induced strong circadian expression ($p < 0.05$; circadian interval was ~ 24 hr). (B) In contrast to BALB/c chondrocytes which did not respond to melatonin, *Bmal1* mRNA expression in C3H chondrocytes responded to melatonin with a change the circadian interval from 18.67 hr to 23.34 hr. (C and D) *Mt1* and *Mt2* mRNA showed similar circadian expression; however, circadian intervals were 16.96 hr (-melatonin) and 17.42 hr (+melatonin), respectively.