RESEARCH

Suppressive effect of melatonin on osteoclast function via osteocyte calcitonin

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Abstract

Many studies have investigated the actions of melatonin on osteoblasts and osteoclasts. However, the underlying mechanisms, especially regarding osteocyte function, remain largely unknown. Therefore, this study aimed to clarify the underlying mechanisms of melatonin action on bone tissue via osteocyte function. Chick calvariae were employed as a model. *In ovo* injection of melatonin (5, 50 and 500 µg) dose-dependently decreased the mRNA expression levels of cathepsin K and matrix metalloproteinase 9 (MMP9) in chick calvariae without affecting the expression levels of receptor activator of NF-κB ligand or osteoprotegerin. Surprisingly enough, the expression of calcitonin mRNA in chick calvariae was significantly raised. After 3 days of *in vitro* treatment of melatonin (10⁻⁷ and 10⁻⁵ M) on newly hatched chick calvariae, both calcitonin mRNA expression in calvariae and the concentration of calcitonin in cultured medium were augmented in a dose-dependent manner, coincident with the decreased mRNA expression levels of cathepsin K and MMP9. Immunohistochemical analyses revealed expression of melatonin receptors and calcitonin by osteocytes buried in bone matrix. Moreover, the mRNA expression levels of melatonin receptors, calcitonin and sclerostin (a marker of osteocyte), were strongly and positively correlated. In conclusion, we demonstrated the expression of melatonin receptors and calcitonin expression in osteocytes for the first time and suggest a new mechanism underlying the suppressive effect of melatonin on osteoclasts via upregulation of calcitonin secretion by osteocytes.

Key Words

- ▶ melatonin
- ▶ melatonin receptor
- ▶ calcitonin
- ▶ osteocyte
- ▶ osteoclast

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous hormone mainly synthesised in the vertebrate pineal gland in response to darkness (Reiter 1991) and circulating levels exhibit a conspicuous circadian rhythm (Reiter 1993). This amine hormone is known to be involved in several physiological functions, which include immune response (Szczepanik 2007,

Cardinali *et al.* 2008), energy metabolism (Srinivasan *et al.* 2013, Kumar Jha *et al.* 2015) and bone turnover (Amstrup *et al.* 2013). Another amine hormone, calcitonin, an osteoclast-suppressing hormone, is primarily secreted by C cells of the thyroid gland in mammals or the ultimobranchial gland in lower vertebrates, including chickens.

Bone tissue is composed of three types of component cells, osteoclasts, osteoblasts and osteocytes, and an intercellular matrix consisting of type I collagen and hydroxyapatite. Osteocytes are the predominant cell type in bone tissue, accounting for more than 90%, and are also very long-lived (Knothe Tate *et al.* 2004, Franz-Odenaal *et al.* 2006). The osteocytes are terminally differentiated osteoblasts that become trapped and buried in the bone matrix that is self-secreted. These cells possess a number of cellular processes in canaliculae that extend throughout the bone tissue and are connected with each other and/or with other cell types on the bone surface.

Many studies have investigated the action of melatonin on osteoblasts. In human mesenchymal stem cells and mouse pre-osteoblastic MC3T3-E1 cells, melatonin enhances the expression of runt-related transcription factor 2 and bone morphogenetic proteins 2 and 4 (Zhang *et al.* 2010, Park *et al.* 2011). Alkaline phosphatase (ALP) and osteocalcin synthesis is reportedly upregulated by melatonin treatment in several osteoblastic cell lines (Roth *et al.* 1999, Radio *et al.* 2006) and human primary osteoblasts (Satomura *et al.* 2007). Besides, melatonin treatment was found to decrease the expression of receptor activator of NF- κ B ligand (RANKL), while elevating osteoprotegerin (OPG) expression (Koyama *et al.* 2002, Histing *et al.* 2012). Thus, melatonin prevents osteoclast differentiation and activation, and may inhibit bone resorption. Moreover, it is also reported that knockout of MT₂, a melatonin membrane receptor, reduced the bone mass of mice, as compared to the wild type (Sharan *et al.* 2017). In addition, we demonstrate a suppressive effect of melatonin on the activity of osteoclasts using cultured fish scales as a model of membrane bone (Suzuki & Hattori 2002). This is the first report related to melatonin function directly on osteoclasts in any vertebrate species.

The relationships between the pineal gland and bone tissue are well documented. For instance, spinal malformations have been reported in pinealectomised chickens (Machida *et al.* 1993, Katoh & Hattori 1995, Turgut *et al.* 2005), rats (Machida *et al.* 1999) and Atlantic salmon (Fjelldal *et al.* 2004). An experimental chicken model of scoliosis was developed by surgical ablation of the pineal gland and displayed pathoanatomical features similar to those of human idiopathic scoliosis. Furthermore, the experimental model raised by pinealectomy of newly hatched chicks developed symptoms of osteoporosis characterised by bone loss associated with scoliosis (Turgut *et al.* 2005, Aota *et al.* 2013). On the other hand, studies in calcitonin of C cells reported that removal of the pineal gland in rats increased the number of C cells

(Csaba & Baráth 1974) and increased the content of endoplasmic reticulum and the number of secretory granules (Csaba & Baráth 1975). Hence, calcitonin may possibly be a mediator between melatonin and bone metabolism.

To date, calcitonin production has been validated in brain, pituitary, liver, lung and kidney of mammals including humans (Deftos *et al.* 1980, Flynn *et al.* 1981, Russwurm *et al.* 2001) and also in the brain and pituitary gland of avian species (Galan Galan *et al.* 1981, Maddineni *et al.* 2007). In the present study, we focused on calcitonin production in osteocytes and its regulation by melatonin. Therefore, we used chick calvariae, which are thought to be a more pure bone tissue with less bone marrow. Here we report the expression of the melatonin receptors and calcitonin in osteocytes for the first time and suggest a new mechanism for the suppressive effect of melatonin on osteoclast function via upregulation of calcitonin secretion by osteocytes.

Materials and methods

Animals

Inseminated eggs and newly hatched broiler chicks (*Gallus gallus domesticus*) were purchased from commercial sources. Inseminated eggs were incubated at 37.8°C with automatic turning (once per hour) and newly hatched chicks were housed in a cage maintained at 34 ± 1°C under constant light until euthanised. Calvariae at embryonic day 14–20 (E14–E20) after the start of incubation and those of newly hatched chicks were used for the experiments. The protocols of all animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Tokyo, Japan) and were conducted by authorised investigators.

Gene expression patterns in chick calvariae during embryonic development

Calvariae of E14, E16, E18 and E20 embryos were collected and the periosteum was removed in phosphate-buffered saline (pH 7.4). The obtained calvariae were crushed in Lysing Matrix K using a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). Then, total RNA was extracted using Isogen reagent (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's instructions.

Total RNA (1 µg) was reverse-transcribed using PrimeScript Reverse Transcriptase and oligo dT primers (Takara Bio). Reverse transcription-polymerase chain

Table 1 Oligonucleotide sequences of primers used for chick RT-PCR and real-time RT-PCR.

Name (accession no.)	Forward primer (5'–3')	Reverse primer (5'–3')
GAPDH (NM_204305)	ACTTTGGCATTGTGGAGGGT	ACGCTGGGATGATGTTCTGG
Mel1a (NM_205362)	AGCCACCATCCTCATCTTCAC	TATATTTCCCGCTTCCGCA
Mel1b (NM_001293103)	GCAAGCTCAGGAAGCTCAGGTAA	GCATTTACACCAAAGTCCATCC
Mel1c (NM_205361)	TGGCTCCTTGACAGTATGACC	AAGTCACGATGGACAGTGGG
Osteocalcin (NM_205387)	AAAGCCTTCATCTCCCACCG	CAGCTCACACCTCTCTGTT
ALP (NM_205360)	CCAAGAACCTCATCTCTTCC	GGAAGCTGTCCATCTCCAG
Sclerostin (XM_427339)	TCCCCGAGATCACCGAAAAC	AGTGTGTGGTGTATGTGCCTT
DMP1 (NM_206993)	ACACGCTTCTACACCTCTGC	TGCTGCATCCTCAGGCTCT
MEPE (NM_204569)	CAGGACGGTGAAACACATATCTC	TGACATTGGCCTCACTGGTAG
Cathepsin K (NM_204971)	GGTGTACTGCGTCTCCAACA	CATACAGCTCTCATCTGCC
MMP9 (NM_204667)	TCGTCCTTCTGGAAATCAGG	TCTTGGTGAGCACATCTTGG
RANKL (NM_001083361)	TGTTGGCTCTGATGCTTGTCT	GCTTCTGGCTCTCAATCTTCTC
OPG (NM_001033641)	GTTTGCAGTTCCTACTCGTTCC	TCAAAGCTGGAAAGTCTGC
Calcitonin (NM_001113708)	CCAGTCTGAGTACTTGTGTGCTG	AGGGTTTCCCATAGTTTGC

reaction (RT-PCR) amplifications were performed with Ex *Taq* DNA Polymerase (Takara Bio) and primers for chicken melatonin receptors (mel1a, mel1b and mel1c) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control (Table 1), using cDNA generated from the cerebral and calvarial tissues of E20 embryos as templates. The conditions for RT-PCR were 35 cycles of denaturation for 30s at 94°C, annealing for 30s at 60°C and extension for 1min at 72°C. PCR products were analysed by electrophoresis on 2% agarose gels, containing Tris base, acetic acid and ethylenediaminetetraacetic acid (EDTA), which were then stained with ethidium bromide. There were no amplified products when using the reverse-transcription-omitted samples as a negative control.

To investigate mRNA expression levels in chick calvariae during embryonic development, real-time RT-PCR was performed using primers for the melatonin receptors mel1a and mel1c, the bone-related genes osteocalcin, sclerostin and cathepsin K, and calcitonin (Table 1). The primary transcript of chicken calcitonin/CGRP gene (Gene ID: 396256) is alternatively spliced into exons 1, 2, 3 and 4 for calcitonin, and exons 1, 2, 3, 5 and 6 for CGRP. PCR primers for calcitonin were designed in exon 4, and the primer sequences were designed to not cross CGRP. PCR amplifications were performed using an Mx3000P qPCR System (Agilent Technologies) and SYBR Ex *Taq* DNA Polymerase (Takara Bio). The conditions for real-time RT-PCR consisted of 40 cycles of denaturation at 95°C for 10s and annealing/extension at 60°C for 40s, followed by melt curve analysis. The mRNA expression level of each gene of interest was normalised to that of GAPDH, as an internal standard. There were no changes and/or trends in GAPDH mRNA expression levels among embryonic ages. The correlations among melatonin receptors,

bone-related genes and calcitonin mRNA expression levels were determined from these data. The number of samples used for the correlation analyses was 32 in total, including E14 ($n=6$), E16 ($n=10$), E18 ($n=8$) and E20 ($n=8$).

In addition, histological analysis was performed with alkaline phosphatase (for osteoblasts; Burston's azo-dye method), tartrate-resistant acid phosphatase (for osteoclasts; Cole's azo-dye method) and methyl green (for nuclei) staining in E14 and E16 chick embryo calvariae.

In ovo injection of melatonin

To examine the action of melatonin on bone tissue, *in ovo* injection was performed. Melatonin (5, 50 and 500 µg; Wako Pure Chemical Industries, Osaka, Japan) or a vehicle (saline with 5% dimethyl sulfoxide) was repeatedly injected into the air cells of inseminated eggs at E15 and E16. Calvariae without the periosteum were sampled at E17, and the mRNA expression levels of functional factors of osteoblast (osteocalcin and ALP), osteocyte (sclerostin, dentin matrix protein 1; DMP1 and matrix extracellular phosphoglycoprotein; MEPE) and osteoclast (cathepsin K and matrix metalloproteinase 9; MMP9), as well as RANKL, OPG and calcitonin were measured by real-time RT-PCR. The PCR conditions were the same as those described above and the primer sequences are listed in Table 1. There were no changes and/or trends in the mRNA expression levels of GAPDH, an internal standard, among melatonin-treated groups vs control.

In vitro melatonin treatment

In order to investigate the direct action of melatonin on bone tissue, the calvariae of newly hatched chicks were cultured *in vitro*. Briefly, the calvariae of newly

hatched chicks without the periosteum were collected and cut midsagittally and then divided into control and experimental groups. The obtained hemi-calvariae were washed three times with phosphate-buffered saline and pre-cultured in 500 µL of BGJb medium (Thermo Fisher Scientific) supplemented with 0.1% bovine serum albumin for 24 h at 37°C under an atmosphere of 5% CO₂/95% air. After pre-culturing, the medium was replaced with fresh medium with or without the addition of melatonin (final concentration, 10⁻⁷ M or 10⁻⁵ M with 0.1% ethanol) followed by 72 h of culture. Thereafter, the cultured hemi-calvariae were salvaged and the mRNA expression levels of cathepsin K, MMP9 and calcitonin were determined by real-time RT-PCR in a similar manner. The cultured medium was also collected and stored at -80°C until assayed for calcitonin concentrations using a chicken calcitonin enzyme-linked immunosorbent assay (ELISA) kit (LifeSpan BioSciences, Seattle, WA, USA) in accordance with the manufacturer's instructions.

Immunohistochemical analysis of melatonin receptors and calcitonin in chick calvariae

For immunohistochemical analyses, calvariae of E20 embryos and newly hatched chicks without the periosteum were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and modestly decalcified with 5% EDTA (pH 7.4). For immunostaining of the melatonin receptors, calvariae were dehydrated in a graded series of ethanol, cleared with xylene, embedded in paraffin and sectioned at 5 µm. Cryosections of the calvariae, at a thickness of 5 µm, were used for calcitonin immunostaining. Antigen retrieval was performed by heating the specimens in citrate buffer solution (pH 6.0), followed by incubation at 4°C overnight with rabbit anti-human MT2 antibody (dilution, 1:500; Medical and Biological Laboratories, Nagoya, Japan) or rabbit anti-salmon calcitonin antibody (1:50,000; Okuda *et al.* 1999) as a primary antibody, respectively. For the calcitonin antibody, due to the amino acid sequence high similarity (84.4%) between salmon and chicken calcitonin, this antibody is able to cross-react with chicken calcitonin. Antibodies pre-absorbed with each antigenic peptide were used as a negative control. Immunopositive reactions were enhanced with the VECTASTAIN Elite ABC-Peroxidase Kit (Vector Laboratories, Burlingame, CA, USA) and visualised with 3,3'-diaminobenzidine. Finally, the slides were counterstained with methyl green or haematoxylin. The images were gained by microscopy observations,

and only minor adjustments in their brightness and contrast were made.

Statistical analysis

Data are presented as the mean ± standard error of the mean. Statistical analyses were performed using one-way analysis of variance followed by the Tukey–Kramer test or the paired *t*-test. A probability (*P*) value of <0.05 was considered statistically significant.

Results

mRNA expression levels of melatonin receptors and bone-related genes in chick calvariae during embryonic development

The mRNA expression patterns of the melatonin receptors (mel1a, mel1b and mel1c) were analysed by RT-PCR and agarose gel electrophoresis. As a result, mel1a and mel1c mRNA were detected in the calvariae of E20 chick embryos, while mel1b and mel1c were observed in the cerebrum (Fig. 1A). During embryonic development, mel1a and mel1c were markedly elevated in the calvariae from E16 onward (>500 and 470% vs E14, respectively) (Fig. 1B and C). For bone-related genes, the mRNA expression level of osteocalcin (a marker of osteoblast) was gradually increased during development (Fig. 1D) and that of sclerostin (a marker of osteocyte) was highly expressed from E16 (>450% vs E14), as was the melatonin receptors (Fig. 1E), whereas there was no significant change in mRNA expression of cathepsin K (a marker of osteoclast) (Fig. 1F). The data with individual values are shown in Supplementary Fig. 1 (see section on [supplementary data](#) given at the end of this article). In addition, histological analysis with triple-staining of E14 and E16 chick calvariae demonstrated a clear increase in the number of osteocytes in E16 compared with E14. Meanwhile, a small number of osteoclasts were observed, but the count did not appear to be different between E14 and E16 (Supplementary Fig. 2).

Effects of *in ovo* injection of melatonin on the expression of bone-related genes in chick calvariae

Repeated *in ovo* injection of melatonin (5, 50 and 500 µg) into the air cells of inseminated eggs at E15 and E16 did not affect mRNA expression of osteocalcin and ALP (functional factors of osteoblast) in the calvariae (Fig. 2A and B); however, the mRNA expression levels of sclerostin, DMP1 and MEPE (functional factors of

Figure 1

Changes in the mRNA expression levels of melatonin receptors and bone-related genes in chick calvariae during embryonic development. (A) mRNA expression patterns of melatonin receptors in the cerebrum and calvariae of E20 embryos. mRNA expression levels of the melatonin receptors (B) mel1a and (C) mel1c, and the bone-related genes (D) osteocalcin, (E) sclerostin and (F) cathepsin K in E14, 16, 18 and 20 embryo calvariae. Relative mRNA expression levels were determined by real-time RT-PCR and normalised to GAPDH mRNA levels. Columns with different letters indicate significant differences ($P < 0.05$; Tukey-Kramer test, $n = 6-10$).

osteocyte) (68.2–43.8%, 90.5–59.8% and 82.6–64.0%, respectively), as well as cathepsin K and MMP9 (functional factors of osteoclast) (86.1–59.2% and 87.9–59.6%, respectively) were dose-dependently decreased (Fig. 2C, D, E, F and G). Meanwhile, there were no significant changes in the mRNA expression levels of RANKL and OPG (Fig. 2H and I). The data with individual values are shown in Supplementary Fig. 3.

Expression analysis of melatonin receptors in chick osteocytes

The mRNA expression levels of the melatonin receptors mel1a and mel1c were weakly, but positively, correlated with that of osteocalcin ($r=0.396$ and 0.255 , respectively) (Fig. 3A and B), while strong positive correlations were observed with sclerostin mRNA expression ($r=0.822$ and 0.870) (Fig. 3C and D). However, there were no correlations between the mRNA expression levels of the melatonin receptors and cathepsin K ($r=-0.045$ and -0.043) (Fig. 3E and F). The number of samples used for the correlation analyses was 32 in total, including E14 ($n=6$), E16 ($n=10$), E18 ($n=8$) and E20 ($n=8$). Immunohistochemical analysis of calvariae showed immunopositive reactions for the melatonin receptors principally in osteocytes trapped in the bone matrix (Fig. 3G), while only slightly in osteoclasts (data not shown). These reactions were abolished with the use of peptide pre-absorbed primary antibody (Fig. 3H).

Expression of calcitonin in chick calvariae

The mRNA expression of calcitonin, an ultimobranchial gland hormone that suppresses osteoclastic activity, was noticed in chick embryo calvariae and the mRNA expression levels were elevated from E16 (>590% vs E14,

Fig. 4A and Supplementary Fig. 1F), as with those of the melatonin receptors and sclerostin (Fig. 1B, C and E). Meanwhile, the mRNA expression level of calcitonin was poorly, but positively, correlated with that of osteocalcin ($r=0.313$) (Fig. 4B), whereas a marked positive correlation was found with sclerostin mRNA expression ($r=0.896$) (Fig. 4C). However, there was no correlation between calcitonin and cathepsin K mRNA expression levels ($r=-0.074$) (Fig. 4D). Immunohistochemical analysis revealed the calcitonin expression in buried osteocytes and the cells on the surface of calvariae, which were thought to be osteoblast-lineage cells, either osteoblasts or bone lining cells (osteoblasts in a resting state) (Fig. 4E). The immunopositive reactions were dissipated when using the peptide pre-absorbed primary antibody (Fig. 4F).

Effects of melatonin treatment on calcitonin expression in chick calvariae and secretion of calcitonin in cultured medium

Repeated *in ovo* injection of melatonin (5, 50 and 500 μg) into the air cells of inseminated eggs significantly raised calcitonin mRNA expression levels in calvariae (112.7–140.0%) (Fig. 5A and Supplementary Fig. 3J). After 3 days of *in vitro* melatonin treatment (10^{-7}M and 10^{-5}M final conc.) of newly hatched chick calvariae, both calcitonin mRNA expression levels in calvariae (126.3–143.4%) and concentrations of calcitonin in the cultured medium (251.2–385.7%) were augmented in a dose-dependent manner (Fig. 5B and C). Besides *in ovo* injection, the mRNA expression levels of cathepsin K and MMP9 were significantly decreased (76.6–69.1% and 78.5–73.7%, respectively) in the organ culture system (Fig. 5D and E). The data with individual values are shown in Supplementary Fig. 4.

Figure 2

Effects of *in ovo* injection of melatonin on the expression levels of bone-related genes in chick calvariae. mRNA expression levels of functional factors of osteoblast ((A) osteocalcin and (B) ALP), osteocyte ((C) sclerostin, (D) DMP1 and (E) MEPE) and osteoclast ((F) cathepsin K and (G) MMP9), as well as those of osteoclast differentiation ((H) RANKL and (I) OPG) after *in ovo* injection of melatonin. Relative mRNA expression levels were determined by real-time RT-PCR and normalised to GAPDH mRNA levels. Columns with different letters indicate significant differences ($P < 0.05$; Tukey–Kramer test, $n = 6–8$).

In addition, calcitonin mRNA expression levels in chick embryo calvariae demonstrated intense positive correlations with melatonin receptor mRNA levels ($r=0.881$ vs mel1a and 0.966 vs mel1c) (Fig. 6A and B). A summarised image of the osteoclast suppressive effect of melatonin via osteocyte function is shown in Fig. 6C.

Discussion

Cell-to-cell interactions among osteoblasts, osteoclasts and osteocytes are thought to be very important for adequate bone modelling and remodelling. Thus, isolated and cultured cells may not be suitable to investigate the actual physiological activities and functions. In this respect, chick calvariae, which are composed of osteoblasts, osteocytes and osteoclasts with relatively low amounts of bone marrow, could be a good model system. In addition, an *in ovo* experiment system, which is scarcely influenced by ambient light conditions, is better suited to evaluate the *in vivo* effect of exogenous melatonin administration on bone formation. The use of chick calvariae as a model of bone demonstrated the expression of the melatonin receptors and calcitonin

expression of osteocytes for the first time and suggests a new mechanism underlying the suppressive effect of melatonin on osteoclasts via upregulation of calcitonin secretion by osteocytes (Fig. 6C).

The involvement of melatonin function in bone modelling and turnover seems to be conserved evolutionarily in vertebrates. In fishes and birds, pinealectomy causes spinal malformations with a reduction in vertebral mineral content (Fjelldal *et al.* 2004, Turgut *et al.* 2005). Likewise, patients with adolescent idiopathic scoliosis (AIS) have either low levels of circulating melatonin or impaired melatonin signalling (Sadat-Ali *et al.* 2000, Moreau *et al.* 2004, Azeddine *et al.* 2007, Man *et al.* 2011), which are often accompanied with juvenile osteoporosis (Burner *et al.* 1982, Cheng *et al.* 2000). Previously, we demonstrated a suppressive effect of melatonin on the activity of osteoclasts using fish scales as a bone model (Suzuki & Hattori 2002), and this observation was consistent with *in vivo* studies in mice (Koyama *et al.* 2002, Histing *et al.* 2012) and humans (Kotlarczyk *et al.* 2012, Amstrup *et al.* 2015). The results of the present study also showed an osteoclast suppressive effect of melatonin both *in ovo* and *in vitro* using chick calvariae.

Figure 3

Expression of melatonin receptors in chick osteocytes. Correlation coefficients between melatonin receptors (mel1a and mel1c) and the bone-related genes (A and B) osteocalcin, (C and D) sclerostin and (E and F) cathepsin K were determined from the mRNA expression levels in chick embryo calvariae ($n = 32$). Immunohistochemical staining of E20 embryo calvariae was performed using (G) anti-MT2 melatonin receptor antibody or (H) pre-absorbed antibody. Black and white arrowheads indicate osteocytes with immunopositive and -negative reactions, respectively (bars = 20 μ m).

Also, we confirmed the expression of calcitonin and melatonin receptor (MT2) in human bone tissue, as well as chick calvariae in the present study, and found strong positive correlations among mRNA expression levels of human calcitonin, MT2 melatonin receptor and sclerostin, a marker of osteocyte (Nakano M, Ikegame M, Yamamoto T & Hattori A, unpublished observations). Moreover, we found that melatonin treatment increased calcitonin secretion in cultured rat calvariae

and fish scales, which is homologous to a membrane bone (Ikegame M, Hattori A & Suzuki N, unpublished observations). Therefore, we concluded that melatonin could suppress osteoclastic activity via upregulation of calcitonin secretion by osteocytes, not only in chickens, but in other vertebrate species including humans.

In lower vertebrates, including chickens, three membrane receptors for melatonin, termed mel1a, mel1b and mel1c, have been identified. RT-PCR analysis revealed

Figure 4

Expression of calcitonin in chick calvariae. (A) mRNA expression levels of calcitonin in chick calvariae during embryonic development. Columns with different letters indicate significant differences ($P < 0.05$; Tukey-Kramer test, $n = 6-10$). Correlation coefficients between calcitonin and the bone-related genes (B) osteocalcin, (C) sclerostin and (D) cathepsin K were determined from the mRNA expression levels in chick embryo calvariae ($n = 32$). Immunohistochemical staining of newly hatched chick calvariae was performed using (E) anti-calcitonin antibody or (F) pre-absorbed antibody. Black and white arrowheads indicate osteocytes with immunopositive and -negative reactions, respectively (bars = 20 μ m).

Figure 5

Effects of *in ovo* and *in vitro* treatment of melatonin on calcitonin expression in chick calvariae. (A) mRNA expression levels of calcitonin were measured in E17 embryo calvariae after *in ovo* injection of melatonin. Columns with different letters indicate significant differences ($P < 0.05$; Tukey–Kramer test, $n = 6–8$). (B) mRNA expression levels of calcitonin and (C) concentrations of calcitonin in the medium were measured in newly hatched chick calvariae after 3 days of incubation with or without melatonin (10⁻⁷ M and 10⁻⁵ M) by real-time RT-PCR and specific ELISA kits, respectively ($n = 5–8$). mRNA expression levels of the functional factors of osteoclasts, (D) cathepsin K and (E) MMP9, in newly hatched chick calvariae were also measured after 3 days of incubation. * $P < 0.05$, ** $P < 0.01$, # $P < 0.05$ and ## $P < 0.01$ vs the control group (paired *t*-test).

the mRNA expression profiles of *mel1a* and *mel1c* in chick calvariae, while those of *mel1b* and *mel1c* were observed in the cerebrum. The expression patterns of melatonin receptors in the cerebrum were in agreement with previous reports (Liu *et al.* 1995, Reppert *et al.* 1995). To date, melatonin receptor expression in osteoblasts has been demonstrated in mammals, including humans (Satomura *et al.* 2007, Man *et al.* 2011). However, in this study, the mRNA expression levels of the melatonin receptors *mel1a* and *mel1c* were much more strongly and positively correlated with sclerostin mRNA expression than that of osteocalcin. Furthermore, immunopositive reactions of the melatonin receptors were detected in matured osteocytes buried in the bone matrix, rather than osteoblasts or bone lining cells on the surface of chick calvariae.

Although previous studies have shown that melatonin promotes osteoblast differentiation and enhances the functional activities of several cell lines and isolated primary osteoblasts (Roth *et al.* 1999, Radio *et al.* 2006, Satomura *et al.* 2007, Zhang *et al.* 2010, Park *et al.* 2011), we found that melatonin had no influence by *in ovo* injection on osteocalcin and ALP mRNA expression levels in chick embryo calvariae. This observation of osteoblastic activity is consistent with *in vivo* melatonin injection in mice (Koyama *et al.* 2002) and oral administration in rats (Ladizesky *et al.* 2003) or our former data from an *in vitro* culture system using fish scales, in which osteoblasts and osteoclasts co-existed (Suzuki & Hattori 2002). These findings may imply differences of melatonin action on osteoblasts between solely cultured and those co-existing with osteoclasts,

Figure 6

A possible mechanism for the osteoclast suppressive effect of melatonin via osteocyte secretion of calcitonin. (A and B) Correlation coefficients between melatonin receptors (*mel1a* and *mel1c*) and calcitonin were also calculated on the basis of mRNA expression levels in chick embryo calvariae ($n = 32$). (C) A summarised image of the osteoclast-suppressive effect of melatonin by osteocyte secretion of calcitonin. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0707>.

thereby reflecting physiological conditions. On the other hand, *in ovo* injection of melatonin decreased the mRNA expression levels of sclerostin, as well as DMP1 and MEPE in chick calvariae. To the best of our knowledge, this observation is the first to give the influence of melatonin on the expression of osteocyte marker genes. However, the mechanisms of melatonin action on osteocyte marker genes expression are unclear. Therefore, further studies to precisely investigate the effects of melatonin on osteocyte activities are required. Moreover, both *in ovo* and *in vitro* treatment of melatonin suppressed the functional activities of osteoclasts in a dose-dependent manner. Some functional factors of osteoclast such as cathepsin K and MMP9 are also expressed by osteocytes and are thought to be involved in osteocytic osteolysis. However, since there was no correlation between mRNA expression levels of cathepsin K and sclerostin, an osteocyte marker ($r=0.053$), we inferred that these factors were expressed principally by osteoclasts rather than osteocytes, at least in our study conditions. This suppressive effect of melatonin on osteoclastic activity is in agreement with previous reports (Koyama *et al.* 2002, Suzuki & Hattori 2002, Histing *et al.* 2012). However, unexpectedly, no significant changes in RANKL and OPG mRNA expression levels were found in chick embryo calvariae, despite the decrease in sclerostin expression. Instead, both calcitonin mRNA expression in calvariae and concentrations of calcitonin in cultured medium were dose-dependently increased by *in ovo* and *in vitro* melatonin treatment.

Calcitonin, an osteoclast-suppressing hormone, is primarily secreted by C cells of the thyroid gland in mammals or the ultimobranchial gland in lower vertebrates, including chickens. To date, calcitonin production has been validated in brain, pituitary, liver, lung and kidney of mammals including humans (Deftos *et al.* 1980, Flynn *et al.* 1981, Russwurm *et al.* 2001), and also in the brain and pituitary gland of avian species (Galan *et al.* 1981, Maddineni *et al.* 2007). In the present study, we found the calcitonin mRNA expression in chick calvariae and immunohistochemical analysis revealed the calcitonin expression in osteocytes and bone lining cells of chick calvariae. To the best of our knowledge, this is the first report to demonstrate calcitonin expression in bone tissue. Melatonin receptor expression by osteocytes together with the marked positive correlations of mRNA expression levels among sclerostin (a marker of osteocyte), melatonin receptors and calcitonin (Figs 3C, D, 4C and 6A, B) strongly suggests that melatonin affects osteocytes directly and suppresses osteoclastic activity via upregulation of calcitonin secretion by osteocytes (Fig. 6C).

Gooi *et al.* (2010) reported calcitonin receptor expression in rat calvariae osteocytes, as well as significant upregulation of sclerostin expression in osteocytes by calcitonin treatment. Secretion of calcitonin by osteocytes, as noted in our study, suggested that calcitonin may affect other osteocytes in a paracrine manner. However, expression of calcitonin receptor by osteocytes has not been identified in birds, including chickens. In the present study, melatonin treatment led to the downregulation of sclerostin expression concomitant with increased calcitonin expression. If calcitonin secreted by osteocytes in chick calvariae affected other osteocytes in a paracrine fashion, the results of the present study would conflict with those reported by Gooi *et al.* Therefore, it is worth studying calcitonin receptor expression in osteocytes and the paracrine action of calcitonin secreted by osteocytes in other vertebrate species including chickens.

Pinealectomy of newly hatched chicks causes scoliosis, which can be restored by pineal body autograft (Machida *et al.* 1993) or melatonin administration (Katoh & Hattori 1995, Kono *et al.* 2011). These findings point out the importance of melatonin signalling for appropriate bone development and modelling. The experimental induction of scoliosis by pinealectomy of newly hatched chick is often associated with osteoporosis, which is characterised by lowered bone mineral density (Turgut *et al.* 2005, Kono *et al.* 2011, Aota *et al.* 2013). As with the experimental chick model, the prevalence of juvenile osteoporosis has also been reported in AIS patients (Burner *et al.* 1982, Cheng *et al.* 2000). The pathological conditions of spinal deformity and osteoporosis might be due to an imbalance between bone formation and resorption. In the vertebrae of the chicken experimental scoliosis model, the abundance of activated osteoclasts was increased (Yoshihara *et al.* 2005, Aota *et al.* 2013), while the numbers of osteoblasts (Kono *et al.* 2011) and osteocytes (Turgut *et al.* 2005) were reduced. These factors are thought to contribute to the development of scoliotic and osteoporotic states. The results of the present study showed that melatonin treatment upregulated calcitonin secretion in chick calvariae. We therefore consider that decreased level of calcitonin, which is locally secreted by osteocytes, caused by a deficiency of melatonin signalling is an etiological factor of spinal deformity and osteoporosis in pinealectomised chickens.

So far, studies have investigated the possible effects of melatonin on the treatment of osteoporosis. Even though modest and limited effects have been reported, melatonin was found to enhance the potency of estradiol in the prevention of bone loss in rats caused by ovariectomy

(Ladizesky *et al.* 2001, 2003). Moreover, we also found significant increases in bone mineral density and bone strength in aged male mice following administration of melatonin in drinking water (Igarashi-Migitaka J, Suzuki N & Hattori A, unpublished observations). In addition, we recently developed a synthetic melatonin derivative, 1-benzyl-2,4,6-tribromomelatonin (bromomelatonin), and the oral administration of this agent augmented the total bone mineral density of the femoral metaphysis in both ovariectomised rats and those fed a low-calcium diet with more capability than melatonin (Suzuki *et al.* 2008a,b). More recently, the effects of melatonin treatment on human bone were examined in peri- and post-menopausal women. These studies demonstrated the beneficial effects of oral melatonin administration on bone health, as the imbalance of bone turnover and lowered bone mineral density were improved (Kotlarczyk *et al.* 2012, Amstrup *et al.* 2015). Nonetheless, further studies are required to determine whether the action of melatonin, which suppresses osteoclast function via upregulation of calcitonin secretion by osteocytes, is observed in both ovariectomised rats and peri- or post-menopausal women.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0707>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Study design: A H and M N. Study conduct and data collection: M N, M I, J I M and Y M. Data analysis: M N and M I. Data interpretation: A H, M N and N S. Drafting manuscript: M N and A H. Approval of final version of the manuscript: all authors.

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