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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin enhances chondrogenic differentiation of human mesenchymal stem cells

Abstract: Intramembranous ossification and endochondral ossification are two ways through which bone formation and fracture healing occur. Accumulating amounts of evidence suggests that melatonin affects osteoblast differentiation, but little is known about the effects of melatonin on the process of chondrogenic differentiation. In this study, the effects of melatonin on human mesenchymal stem cells (MSCs) undergoing chondrogenic differentiation were investigated. Cells were induced along chondrogenic differentiation via high-density micromass culture in chondrogenic medium containing vehicle or 50 nM melatonin. Histological study and quantitative analysis of glycosaminoglycan (GAG) showed induced cartilage tissues to be larger and richer in GAG, collagen type II and collagen type X in the melatonin group than in the untreated controls. Real-time RT-PCR analysis demonstrated that melatonin treatment significantly up-regulated the expression of the genes involved in chondrogenic differentiation, including aggrecan (*ACAN*), collagen type II (*COL2A1*), collagen type X (*COL10A1*), SRY (sex-determining region Y)-box 9 (*SOX9*), runt-related transcription factor 2 (*RUNX2*) and the potent inducer of chondrogenic differentiation, bone morphogenetic protein 2 (*BMP2*). And the expression of melatonin membrane receptors (MT) MT1 and MT2 were detected in the chondrogenic-induced-MSCs by immunofluorescence staining. Luzindole, a melatonin receptor antagonist, was found to partially block the ability of melatonin to increase the size and GAG synthesis of the induced cartilage tissues, as well as to completely reverse the effect of melatonin on the gene expression of *ACAN*, *COL2A1*, *COL10A1*, *SOX9* and *BMP2* after 7 days of differentiation. These findings demonstrate that melatonin enhances chondrogenic differentiation of human MSCs at least partially through melatonin receptors.

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Introduction

The methoxyindole melatonin (N-acetyl-5-methoxytryptamine), which was first discovered in the 1950s, is an uncommonly widely distributed molecule [1, 2]. It has been shown to be involved in a number of physiological processes, such as circadian rhythm, sleep, inhibition of tumor growth, immune response, and reproduction [3–7].

Intramembranous ossification and endochondral ossification are two ways through which bone formation and fracture healing occur, and both processes begin with MSCs proliferation and condensation [8]. Recent studies have shown that melatonin affects bone formation through enhancing osteogenic differentiation. Melatonin treatment can prevent osteoporosis in ovariectomized rats and increase the volume of newly formed cortical bone of femora in mice [9–13]. Topical application of melatonin was found to accelerate osteointegration of dental implants and bone implants in Beagle dog and rabbit models [14–16]. In vitro, melatonin has been reported to promote osteogenic differentiation in several kinds of cells, including MC3T3-E1 preosteoblasts, rat osteoblast-like osteosarcoma 17/2.8 cells, human bone cells, human osteoblasts, and bone marrow mesenchymal stem cells (MSCs) [9, 17–21]. Regarding chondrogenic differentiation, only one relevant study, conducted by Pei et al. [22], reported that melatonin can enhance cartilage matrix synthesis of porcine articular chondrocytes, which are terminally differentiated chondrocytes. The data regarding the effects of melatonin on chondrogenic differentiation of precommitted progenitor cells are limited.

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of unique mesenchymal cell types, such as osteoblasts, chondrocytes, and adipocytes [23]. Previous studies have shown that melatonin plays an important role in regulating the osteogenic differentiation and adipogenic differentiation of MSCs [17, 24]. However, the effects of melatonin on

chondrogenic differentiation of human MSCs have not yet been studied.

In the present study, melatonin was evaluated for its ability to affect chondrogenic differentiation of human MSCs, as well as the potential underlying mechanism. The goals of this study are to further determine the important role of melatonin in the regulation of the differentiation of MSCs and in bone formation and to provide further evidence for the use of melatonin as a drug in orthopedics, especially in the enhancement of fracture healing.

Materials and methods

Isolation and culture of human bone marrow–derived mesenchymal stem cells (MSCs)

The study was approved by the Ethical Committee of Sun Yat-sen University, and written informed consent was obtained from all subjects included in the study. Human MSCs were isolated from bone marrow obtained from healthy volunteer donors as described previously [17, 24, 25]. Briefly, the bone marrow samples were diluted with PBS. Cells were then fractionated on a lymphoprep density gradient by centrifugation at 500 g for 20 min. Interfacial mononuclear cells were collected, resuspended in low-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, seeded and incubated at 37°C/5% CO_2. After 48 hr, nonadherent cells were removed by changing the medium. Thereafter, the medium was changed every 3 days. When the cells reached 80–90% confluence, they were trypsinized, counted and plated again. Cells from passages 3–6 were used for the experiments.

Chondrogenic differentiation

High-density micromass culture system was applied for the chondrogenic differentiation of human MSCs as described previously [25]. Briefly, culture-expanded MSCs were trypsinized, washed and then resuspended at 2×10^7 cells/mL in a chemically defined chondrogenic medium consisting of high-glucose DMEM supplemented with 10 ng/mL recombinant human transforming growth factor-β3 (TGF-β3; Peprotech, Rocky Hill, NJ, USA), 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/mL ascorbic acid 2-phosphate (Sigma–Aldrich), 1 mM sodium pyruvate (Sigma–Aldrich), 40 μg/mL proline (Sigma–Aldrich) and ITS+ Universal Culture Supplement Premix (BD Biosciences, Bedford, MA, USA; final concentrations: 6.25 μg/mL bovine insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenous acid, 5.33 μg/mL linoleic acid and 1.25 mg/mL bovine serum albumin). Droplets (12.5 μL) were carefully placed in each interior well of a 24-well plate. Cells were allowed to adhere at 37°C for 2 hr, followed by addition of 500 μL chondrogenic medium containing vehicle or 50 nM melatonin (Sigma–Aldrich). The medium was changed every 3 days, and induced-cartilage tissues were harvested on days 7, 14, and 21. To assess the involvement of melatonin receptors, cells were also exposed to chondrogenic medium in the presence of 5 μM luzindole (Sigma–Aldrich), which is a melatonin receptor antagonist.

Quantitative analysis of glycosaminoglycan (GAG)

Micromasses were washed and digested in PBS containing 0.03% papain (Merck, Darmstadt, Germany), 5 mM cysteine hydrochloride (Sigma–Aldrich) and 10 mM EDTA (Sigma–Aldrich) for 16 hr at 65°C. The GAG concentration was measured using the 1, 9-dimethylmethylene blue (DMMB; Sigma–Aldrich) dye binding assay. Briefly, an aliquot of the lysate was reacted with DMMB solution for 10 min, and the absorbance at 525 nm was measured using Varioskan Flash (Thermo Scientific, Waltham, MA, USA). DNA concentration was calculated by fluorescent dye Hoechst 33258 (Sigma–Aldrich) binding assay using SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). For comparison, GAG content was normalized by DNA content.

Histology and immunohistochemistry

Micromasses were fixed in 4% paraformaldehyde for 3 hr, then dehydrated with ethanol, washed with xylene, and embedded in paraffin. Sections with a thickness of 5 μm were cut and coated on the glass slides. Safranin O staining was used to assess the proteoglycan level, and immunohistochemistry was used to assess the levels of collagen type II and collagen type X. Hematoxylin (Sigma–Aldrich) served as a counterstain. Briefly, for Safranin O staining, sections were deparaffinized and exposed to 0.1% Safranin O solution for 5 min. For immunohistochemistry, the Histostain-Plus kit (ZSGB-BIO, Beijing, China) was used. After deparaffinization, tissue sections were treated with pepsin at 37°C for 10 min, incubated with peroxidase-blocking solution for 10 min, and then allowed to react with the appropriate primary antibodies overnight at 4°C [rabbit anti-human collagen type II polyclonal antibody (Abzoom Biolabs, Dallas, TX, USA) diluted at 1:100 and rabbit anti-human collagen type X polyclonal antibody (Millipore, Billerica, MA, USA) diluted at 1:50]. Detection was conducted with a DAB Horseradish Peroxidase Color Development Kits (ZSGB-BIO) according to the manufacturer's protocols. Finally, the sections were photographed with an Olympus BX51 microscope (Olympus, Tokyo, Japan).

Immunofluorescence staining

After deparaffinization, tissue sections were microwaved in 10 mM citrate buffer, incubated with blocking solution containing 1% BSA in PBS for 1 hr, and reacted with the appropriate primary antibodies overnight at 4°C [goat anti-human melatonin receptor 1A (MT1) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:50 and goat anti-human melatonin receptor 1B (MT2) polyclonal antibody (Santa Cruz Biotechnology) diluted at 1:50]. Then, samples were immunostained with FITC-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hr at room temperature. Finally, sections were stained with DAPI, covered with glycerol, and

examined with confocal microscope Zeiss LSM 710 (Carl Zeiss, Heidelberg, Germany).

Reverse transcription and real-time PCR analysis

Total RNA was extracted from micromasses using RNA-iso Plus reagent (TaKaRa, Dalian, China) and then converted to cDNA using PrimeScript® RT Master Mix (TaKaRa) according to the manufacturer's protocols. Real-time PCR was performed on iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green I Master Mix (TOYOBO, Osaka, Japan). Expression of the following genes was examined: aggrecan (*ACAN*), collagen type II (*COL2A1*), collagen type X (*COL10A1*), SRY (sex determining region Y)-box 9 (*SOX9*), runt-related transcription factor 2 (*RUNX2*) and bone morphogenetic protein 2 (*BMP2*). The level of expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene served as reference. The primer sequences used are listed in Table 1. Each PCR was processed in triplicate. The C_t value of the *GAPDH* was subtracted from the C_t value of the target gene ($^{\triangle}C_t$), and the average $^{\triangle}C_t$ value of the triplicates was recorded. The relative expression levels of each gene were determined using the $2^{-\Delta\Delta^{C_t}}$ method.

Statistical analysis

All quantitative data were presented as mean ± standard error (S.E.). Statistical analysis was performed using one-way ANOVA followed by a Newman–Keuls post hoc *t*-test with the SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). The level of statistical significance was set at $P < 0.05$.

Results

To determine the effects of melatonin on chondrogenic differentiation of human MSCs, 50 nM melatonin was added to chondrogenic medium. As shown in Fig. 1A, the induced cartilage tissues treated with melatonin were larger than those of the controls, especially on the early stage of differentiation. The quantitative analysis of glycosaminoglycan (GAG) showed that the GAG synthesis increased gradually as incubation continued and was significantly elevated by melatonin treatment on days 7 and 21 ($P < 0.05$), as well as slightly elevated on day 14, compared with the untreated controls ($P > 0.05$) (Fig. 1B). After 21 days of chondrogenic differentiation, in the induced cartilage tissues treated with melatonin, Safranin O staining showed a higher and more homogenous GAG distribution, and immunohistochemical staining showed more accumulations of collagen type II and collagen type X than in the controls (Fig. 1C).

Real-time RT-PCR was used to evaluate the effects of melatonin on the expression of genes involved in chondrogenic differentiation of human MSCs. As shown in Fig. 2, consistent with the results of quantitative GAG analysis and histological findings (Fig. 1B–C), melatonin significantly up-regulated the gene expression of *ACAN*, *COL2A1* and *COL10A1* during chondrogenic differentiation, as well as the gene expression of transcription factors *SOX9* and *RUNX2*, which are critical to chondrogenic differentiation. Of note, the expression of *BMP2*, which is a potent inducer of bone and cartilage development, was detected and found to be significantly elevated by melatonin during chondrogenic differentiation of human MSCs.

The involvement of melatonin receptors in chondrogenic differentiation of human MSCs was here evaluated. The expression of melatonin receptors (MT) MT1 and MT2 were detected in the induced chondrogenic MSCs by immunofluorescence staining (Fig. 3). Then, the ability of luzindole, a melatonin receptor antagonist, to inhibit the effects of melatonin on chondrogenic differentiation was evaluated. 5 μM luzindole was added to chondrogenic medium containing vehicle or 50 nM melatonin. Luzindole alone (CHO/LUZ group) was not found to affect the chondrogenic differentiation of human MSCs (Fig. 4). However, the enhancement of melatonin on the size and GAG synthesis of the induced cartilage tissues (CHO/MEL group) was partially reversed by luzindole (CHO/MEL/LUZ group) (Fig. 4A–B). Real-time

Table 1. Primers used for real-time PCR

Gene (Accession no.)	Primer sequence	Product size (bp)
GAPDH (NM_002046)	5′-AGAAAAACCTGCCAAATATGATGAC-3′ 5′-TGGGTGTCGCTGTTGAAGTC-3′	126
ACAN (NM_001135)	5′-TGCATTCCACGAAGCTAACCTT-3′ 5′-GACGCCTCGCCTTCTTGAA-3′	84
COL2A1 (NM_001844)	5′-GGCAATAGCAGGTTCACGTACA-3′ 5′-CGATAACAGTCTTGCCCCACTT-3′	79
COL10A1 (NM_000493)	5′-CAAGGCACCATCTCCAGGAA-3′ 5′-AAAGGGTATTTGTGGCAGCATATT-3′	70
SOX9 (NM_000346)	5′-AGCGAACGCACATCAAGAC-3′ 5′-GCTGTAGTGTGGGAGGTTGAA-3′	110
RUNX2 (NM_001024630)	5′-AGAAGGCACAGACAGAAGCTTGA-3′ 5′-AGGAATGCGCCCTAAATCACT-3′	78
BMP2 (NM_001200)	5′-ACTACCAGAAACGAGTGGGAA-3′ 5′-GCATCTGTTCTCGGAAAACCT-3′	113

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACAN, aggrecan; COL2A1, collagen type II; COL10A1, collagen type X; SOX9, SRY (sex determining region Y)-box 9; RUNX2, runt-related transcription factor 2; BMP2, bone morphogenetic protein 2.

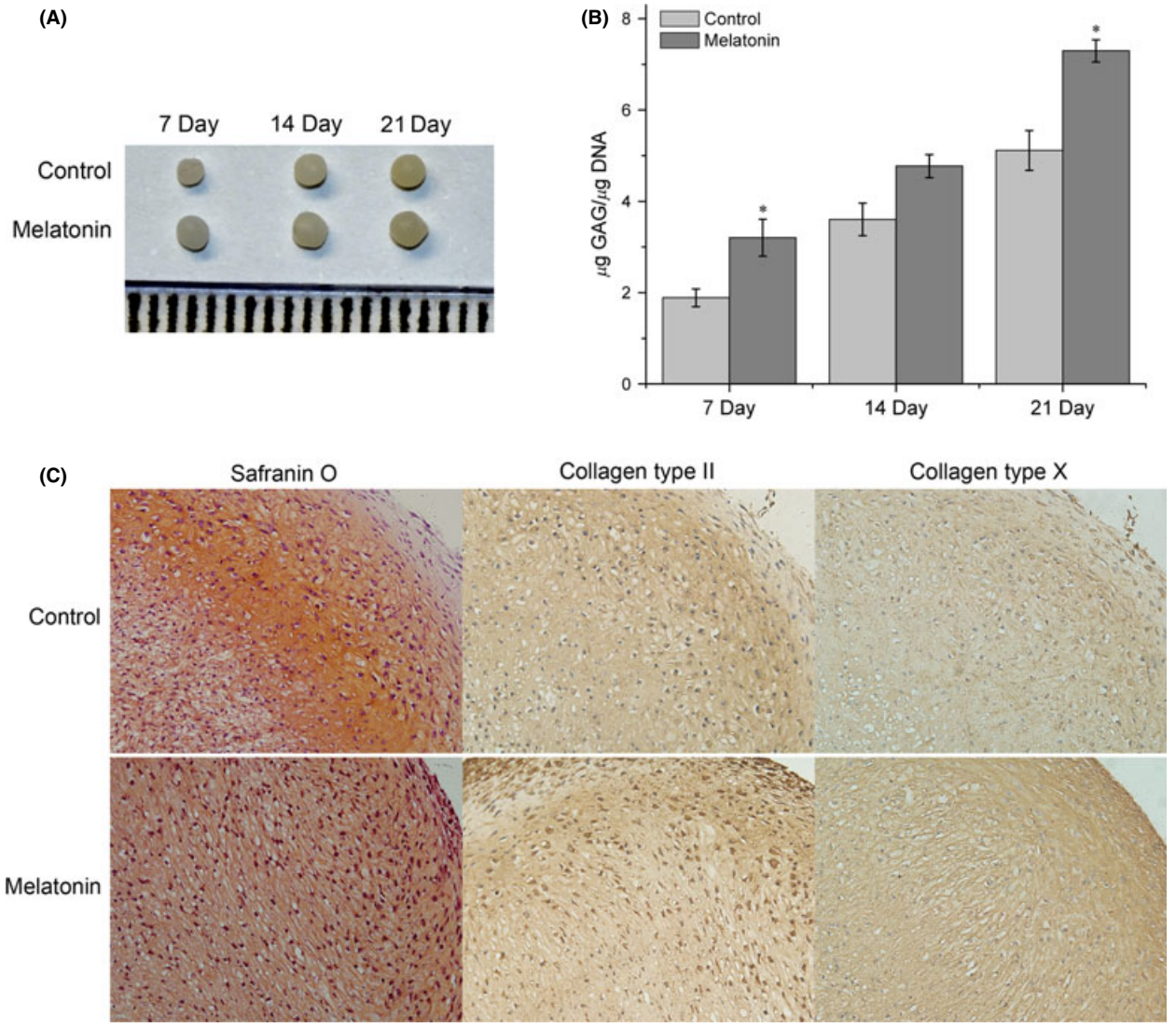

Fig. 1. Effects of melatonin on chondrogenic differentiation of human MSCs. Cells were seeded in 24-well plates via high-density micromass culture and induced along chondrogenic differentiation in chondrogenic medium containing vehicle or 50 nM melatonin. (A) Macroimages of induced cartilage tissues were taken at days 7, 14, and 21. Scale bar = 1 mm. (B) Glycosaminoglycan (GAG) content was quantitatively analyzed and normalized by DNA content at days 7, 14, and 21. The results are representative of three independent experiments. $*P < 0.05$ versus the control. (C) Safranin O staining for proteoglycan and immunohistochemistry for collagen type II and collagen type X were performed after 21 days of differentiation. Magnification: ×200.

RT-PCR was also conducted to investigate the effects of luzindole on melatoin-induced mRNA expression of the genes involved in chondrogenic differentiation. As shown in Fig. 4C, after 7 days of differentiation, the effects of melatonin to enhance the expression of *ACAN*, *COL2A1*, *COL10A1*, *SOX9* and *BMP2* (CHO/MEL group) was blocked by luzindole (CHO/MEL/LUZ group).

Discussion

In the present study, the effects of melatonin on chondrogenic differentiation of human MSCs were investigated. MSCs were induced along chondrogenic differentiation via high-density micromass culture in chondrogenic medium supplemented with vehicle or 50 nM melatonin. Results showed that melatonin treatment led to increased expression of chondrocyte differentiation markers as evidenced by quantitative GAG analysis, histological finding and real-time RT-PCR analysis, all of which indicated that melatonin promoted chondrogenic differentiation of human MSCs. In the previous studies, we found that melatonin can enhance osteogenesis and inhibit adipogenesis of MSCs [17, 24]. And thus we suggested that melatonin is an important regulator of MSCs differentiation and may have potential applications in the promotion of bone formation and fracture healing.

Pei et al. [22] reported that melatonin can enhance cartilage matrix synthesis of procine articular chondrocytes including aggrecan and collagen type II but had no effect on the expression of collagen type X, which is a hypertrophic marker. In the present study, we found during chondrogenic differentiation of human MSCs, melatonin

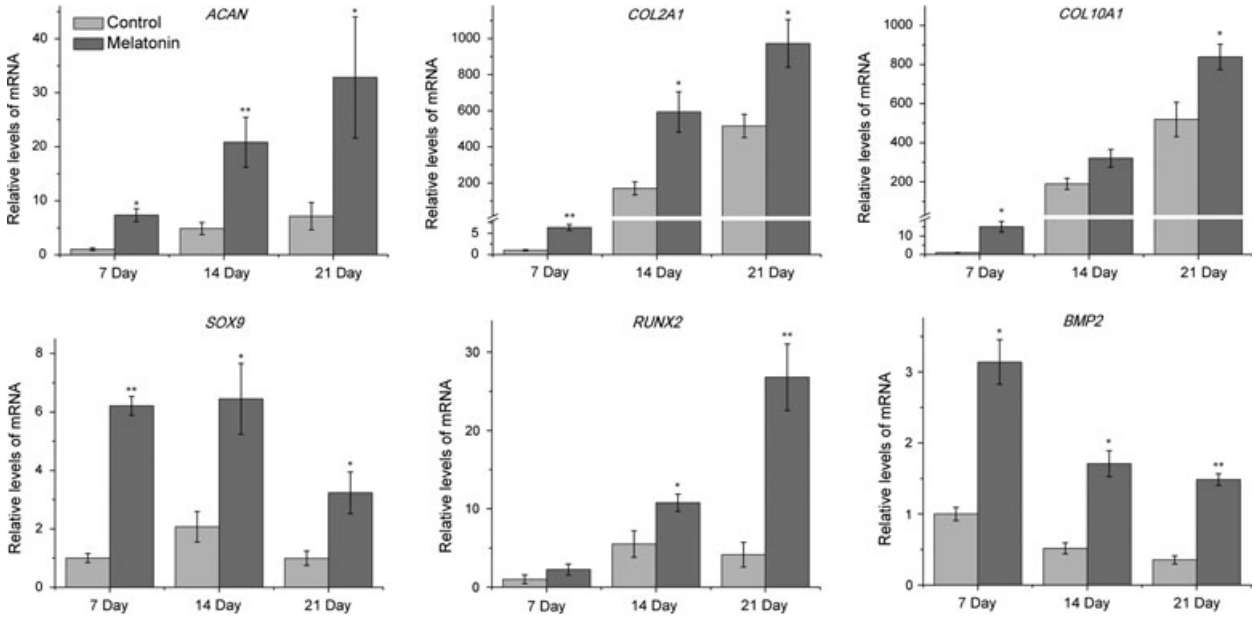

Fig. 2. Effects of melatonin on the expression of genes involved in chondrogenic differentiation. Cells were seeded in 24-well plates via high-density micromass culture and induced along chondrogenic differentiation in chondrogenic medium containing vehicle or 50 nM melatonin. The mRNA expression of aggrecan (*ACAN*), collagen type II (*COL2A1*), collagen type X (*COL10A1*), SRY (sex determining region Y)-box 9 (*SOX9*), runt-related transcription factor 2 (*RUNX2*), and bone morphogenetic protein 2 (*BMP2*) was measured using quantitative real-time PCR and normalized to *GAPDH* expression. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method. The results are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$ versus the control.

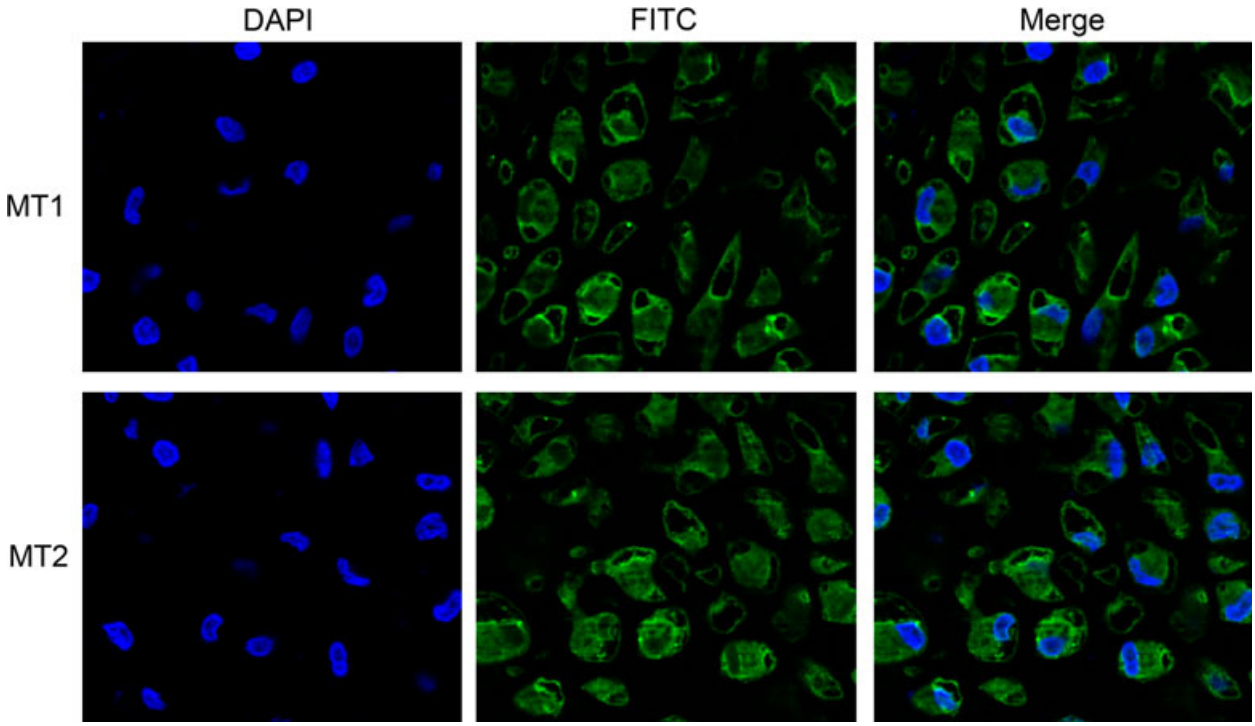

Fig. 3. Expression of melatonin receptors in chondrogenic differentiation of human MSCs as indicated by immunofluorescence staining. Cells were seeded in 24-well plates via high-density micromass culture and induced along chondrogenic differentiation in chondrogenic medium. Samples of induced cartilage tissues were harvested on day 7. The expressions of melatonin receptors (MT) MT1 and MT2 were visualized by immunofluorescence staining using anti-MT1, MT2 antibodies and FITC-conjugated secondary antibodies (green). Nuclei were counterstained using DAPI (blue). The far right panels show merged green and blue images. Magnification: ×630.

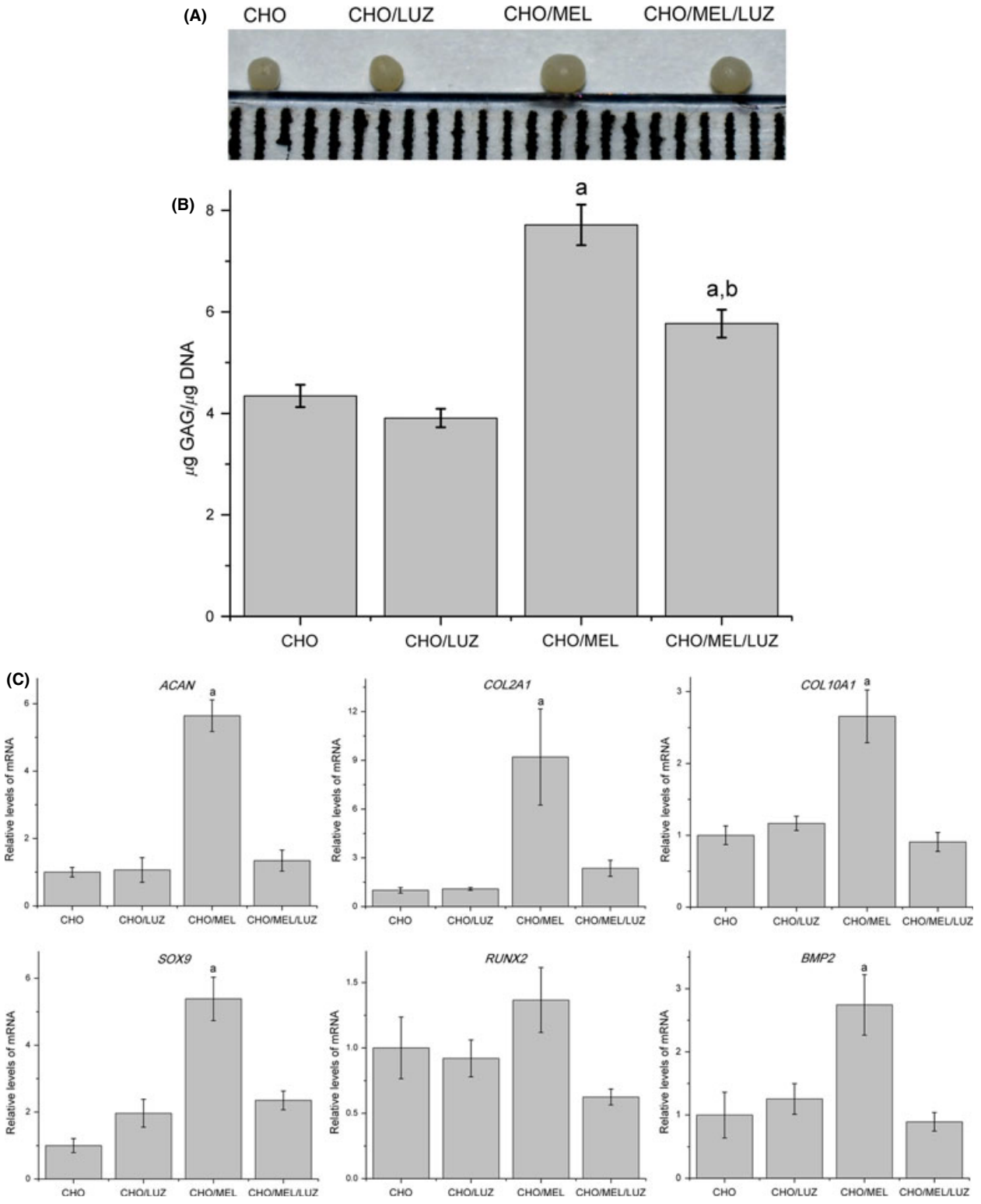

Fig. 4. Effects of melatonin receptor antagonist luzindole on human MSCs undergoing chondrogenic differentiation in chondrogenic medium supplemented with melatonin. Cells were seeded in 24-well plates via high-density micromass culture and induced along chondrogenic differentiation in chondrogenic medium supplemented with vehicle (CHO), with 5 μM luzindole (CHO/LUZ), with 50 nM melatonin (CHO/MEL) or with both melatonin and luzindole (CHO/MEL/LUZ). (A) Macroimages of induced cartilage tissues were taken after 7 days of differentiation. Scale bar = 1 mm. (B) Glycosaminoglycan (GAG) content was quantitatively analyzed and normalized by DNA content after 21 days of differentiation. The results are representative of three independent experiments. (C) The mRNA expression of *ACAN*, *COL2A1*, *COL10A1*, *SOX9*, *RUNX2* and *BMP2* was measured using quantitative real-time PCR and normalized to *GAPDH* expression after 7 days of differentiation. The relative expression level of these genes was calculated using the $2^{-\Delta\Delta C_t}$ method. The results are representative of three independent experiments. [a]$P < 0.05$ versus CHO group, [b]$P < 0.05$ versus CHO/MEL group.

treatment yielded chondrocyte-micromasses with larger size and more accumulations of GAG, collagen type II and collagen type X. Real-time RT-PCR analysis showed that melatonin significantly up-regulated not only the gene expression of the chondrogenic markers including *ACAN*, *COL2A1*, *COL10A1,* and *SOX9*, but also *RUNX2*, a transcription factor critical to chondrocyte maturation and osteogenesis [26]. The different effects of melatonin observed in the present and previous studies may be attributed to the heterogeneity of the cells studied that articular chondrocytes are terminally differentiated chondrocytes, while MSCs are multipotent precursor cells. Current studies also indicated that different signal pathways were involved in melatonin-induced differentiation of articular chondrocytes and MSCs. In the melatonin-treated articular chondrocytes, up-regulation of internal transforming growth factor beta1 (TGF-β1) expression was observed [22], while in our study, melatonin was found to enhance the gene expression of *BMP2* during chondrogenic differentiation of MSCs. And further study is needed to identify the specific molecular mechanisms. Similarly, the diverse effects of melatonin have also been observed in the regulation of adipogenic differentiation. Gonzalez et al. [27] reported that melatonin can promote adipocyte differentiation of 3T3L1 murine preadipocytes through melatonin receptors. However, in our previous study, melatonin was found to inhibit adipogenesis of MSCs and luzindole cannot block the inhibitory effects [17].

In mammals, there are two subtypes of melatonin membrane receptors (MT), MT1 and MT2, both of which are members of the seven transmembrane G protein-coupled receptor family [28]. In the present study, MT1 and MT2 membrane receptors were detected in the chondrogenic-induced-MSCs using immunofluorescence staining. Luzindole, a melatonin receptor antagonist, was used to determine whether melatonin receptors were involved in chondrogenic differentiation of MSCs. Findings showed that luzindole can partially block the effects of melatonin on the size and GAG synthesis of the induced cartilage tissues, and completely reverse melatonin-induced gene expression of *ACAN*, *COL2A1*, *COL10A1*, *SOX9,* and *BMP2*, all of which indicated that the ability of melatonin to enhance chondrogenic differentiation was at least partially dependent upon melatonin membrane receptors. Recent studies have reported that melatonin is a ligand for the ROR/RZR family of orphan nuclear receptors and that it can interact with intracellular proteins, such as calmodulin- and tubulin-associated proteins [29, 30]. Melatonin can also exert direct antioxidant effects [31]. Further study is needed to identify other mechanisms by which melatonin affects chondrogenic differentiation. The MT2 melatonin receptor has been reported to be involved in melatonin-induced osteogenic differentiation of MSCs [20,21], and our data showed that melatonin can inhibit adipogenic differentiation of MSCs likely via orphan nuclear receptor RORα (L. Zhang, W. Gao, P. Su, D. Huang, unpublished data). Therefore, it is here speculated that melatonin's effects on adipogenic, osteogenic, and chondrogenic differentiation of human MSCs may act through different pathways.

Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor β (TGFβ) superfamily, play crucial roles in the development of bone and cartilage [32, 33]. Recent studies have shown that in vitro BMPs can induce chondrogenic differentiation of human MSCs [34–38]. Sethi et al. [21] reported that during osteogenic differentiation of human MSCs, melatonin can enhance the expression of BMP2 through the MT2 melatonin receptor. Similarly, Park et al. [39] reported that melatonin can up-regulate the expression of BMP2 and BMP4 during the differentiation of mouse osteoblastic MC3T3-E1 cells and that the BMP-dependent signaling pathway is involved in the melatonin-induced differentiation of MC3T3-E1 cells. In the present study, melatonin was found to significantly elevate the expression of BMP2 during chondrogenic differentiation of human MSCs, and the melatonin receptor antagonist luzindole can reverse the enhancing effect, indicating that melatonin-induced expression of BMP2 is mediated by melatonin receptors. The mechanism through which activation of melatonin receptors stimulated the expression of BMP2 in chondrogenic differentiation remains unknown. It has been reported that activation of ERK1/2 can contribute to BMP2 transcription during osteoblast differentiation [40], and melatonin has been shown to stimulate phosphorylation of ERK1/2 via melatonin receptors [20, 21, 39]. Further study is needed to identify the exact mechanism by which melatonin-induced chondrogenic differentiation takes place. In addition, smads 1, 5, and 8, which are the immediate downstream molecules of BMP receptors, play a central role in BMP signal transduction downstream. There is some evidence that phosphorylation of smad1/5/8 is positively involved in cartilage development [41]. Combined deletions of smads 1, 5, and 8 has been shown to cause severe chondrodysplasia in mice [42], and in vitro blockage of smad1/5/8 phosphorylation can significantly inhibit chondrogenic differentiation of human MSCs [43]. Our data showed that melatonin treatment can stimulate phosphorylation of smad1/5/8 during chondrogenic differentiation of human MSCs (data not shown). In this way, we speculated that the BMP/smad signal pathway is involved in melatonin-induced chondrogenic differentiation of human MSCs, and further study is needed to confirm this hypothesis.

In conclusion, the present study demonstrates that melatonin can enhance chondrogenic differentiation of human MSCs at least partially through melatonin receptors. It also provides further evidence for the use of melatonin as a drug in orthopedics, especially in the enhancement of fracture healing. However, the underlying signal transduction mechanism requires further elucidation. New studies are planned to assess the role of the BMP/smad signal pathway in the effects of melatonin on chondrogenic differentiation of human MSCs.

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