Original Article

MELATONIN PROMOTES TENDON-DERIVED STEM CELLS DIFFERENTIATION AND INHIBITS OXIDATIVE STRESS IN TRAUMA-INDUCED HETEROTOPIC OSSIFICATION

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Abstract

Background: Heterotopic ossification (HO) is a common complication of muscle and tendon injury, but its underlying mechanism is currently unclear. Melatonin (MT), a hormone mainly secreted by the pineal gland, plays a key role in the pathogenesis of skeletal diseases. This study aimed to investigate the effects and potential mechanisms of melatonin on the differentiation of tendon-derived stem cells (TDSC) and development of tendon HO. **Methods:** We employed *in vivo* and *in vitro* assays to assess the effects of melatonin and its receptors on TDSC differentiation, specifically focusing on chondrogenic and osteogenic pathways involved in ectopic ossification. Additionally, we investigated the activation of the mTOR complex 1 (mTORC1) pathway by melatonin and its impact on chondrogenesis and osteogenesis in TDSC. The influence of antioxidant enzyme expression and reactive oxygen species (ROS) modulation was analyzed, with a particular focus on Sirtuin 1 (SIRT1) activation, as a mechanism to mediate antioxidative effects and regulate osteogenic differentiation. **Results:** Our findings demonstrate that melatonin and its receptors significantly contribute to chondrogenesis and osteogenesis during ectopic ossification. Melatonin was observed to activate the mTORC1 pathway, which promoted chondrogenic and osteogenic differentiation in TDSC, accelerating the progression of HO. Inhibiting the mTORC1 pathway reduced melatonin-induced HO in a mouse model, indicating the pathway's essential role in this process. Furthermore, melatonin enhanced the expression of antioxidant enzymes, thereby reducing ROS levels and enhancing TDSC osteogenic differentiation potential through SIRT1 activation. Suppression of SIRT1 activation *in vivo* mitigated HO progression, highlighting its role in oxidative stress regulation and osteogenic differentiation. **Conclusions:** These findings suggest that melatonin accelerates HO by activating the mTORC1 pathway to promote chondrogenic and osteogenic differentiation of TDSC. Additionally, melatonin's antioxidative effect, mediated through SIRT1 activation, preserves TDSC osteogenic potential in the early stages of injury. This study identifies mTORC1 and SIRT1 as potential therapeutic targets for the prevention and management of HO, offering new insights into the molecular mechanisms of HO development and treatment strategies.

Keywords: Melatonin, mTORC1 pathway, oxidative stress, SIRT1.

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Introduction

Heterotopic ossification (HO) is a pathological process characterized by increased osteogenesis of soft tissues outside the normal bone. It is often caused by trauma, burns, tissue injuries and joint replacement [1]. HO often involves muscles, tendons, ligaments and soft tissues around joints, causing chronic pain, reducing range of motion of joints, and even leading to disability [2]. Due to the lack of understanding of its pathogenesis, there is no reliable treatment strategy to inhibit ectopic ossification onset. Currently, non-steroidal anti-inflammatory drugs and low-dose radiotherapy are the commonly used methods for preventing HO. However, they increase the risk of bone nonunion and cancer [3]. Although the only effective treatment option for mature ectopic bone tissue is surgical resection, it is associated with surgical trauma, postoperative recurrence and other problems, making it a less-effective approach [4].

Therefore, there is an urgent need to understand the pathogenesis of HO and develop new prevention and treatment strategies.

Recently, a new kind of tendon cell, referred to as tendon-derived stem cells (TDSC), has been discovered [5,6]. TDSC has a potential for self-replication and multidirectional differentiation. Previous data have shown that the TDSC can differentiate into adipocytes, osteocytes and chondrocytes under suitable conditions [7]. The formation of ectopic ossification has been shown to be closely related to abnormal differentiation of mesenchymal stem cells (MSCs) into osteocytes and chondrocytes rather than muscle cells or tenocytes under regulation of various signals [8,9].

Therefore, disruption of neuroendocrine signals in bone metabolism is thought to be a new starting point for occurrence of ectopic ossification [10]. Many studies have reported increased expression of substance P, leptin, neurotrophic factor 3 and other neuropeptides during the development of ectopic ossification, and these factors influence its progression [11–13]. Melatonin (MT) is mainly synthesized at night in the pineal gland and participates in various physiological functions, including cell apoptosis, anti-aging and other physiological functions [14–16]. Recent data have demonstrated that melatonin can modulate osteogenic differentiation of MSCs and contribute to osteoporosis and fracture healing [17,18]. In our earlier studies, we demonstrated that melatonin promotes ectopic osteogenesis by regulating endothelial-mesenchymal transformation after tendon injury [19]. However, the effect of melatonin on osteogenic and chondro-genic differentiation of TDSC and its mechanism remain poorly defined. mTOR complex 1 (mTORC1) has been reported to play an important role in postnatal osteogenesis and bone remodeling [20]. Activation of mTORC1 promotes the differentiation of recruited mesenchymal stem cells into mature osteoblasts, whereas inhibition of mTORC1 diminishes osteogenesis [21]. Our previous study demonstrated that mTORC1 signaling facilitates the chondrogenic differentiation of TDSC in HO [22]. This mechanism may elucidate the role of melatonin in the pathogenesis of HO.

It has been suggested that oxidative stress participates in cell senescence and apoptosis in skeletal diseases [23]. Oxidative stress at the injured site is an early feature of HO [24]. Accumulation of inflammatory mediators inevitably leads to local oxidative stress in the very early stages of injury [25]. Increased oxidative stress leads to osteoblast inhibition and disruption of the bone formation process [26]. Sirtuin 1 (SIRT1) is a nicotinamide (NAM) adenine dinucleotide-dependent sirtuin involved in many important cellular and physiological processes. It deacetylates key transcription factors, enzymes and proteins [27]. Previous data showed that SIRT1 exerts cell antioxidation effects [28–30]. A recent study demonstrated that activation of SIRT1 attenuated injury-induced HO by suppressing the inflammatory response of macrophages [31]. Moreover, it has been suggested that SIRT1 may be involved in melatonin-mediated determination of stem cell fate in osteoporosis bone marrow adiposity [32]. However, the antioxidant role of SIRT1 in melatonin-regulated HO formation is still not fully understood.

In this study, we investigated the effect of melatonin on the chondrogenic and osteogenic differentiation of TDSC *in vitro*. *In vivo* experiments were conducted to explore its pathogenesis and investigate the antioxidant effect of SIRT1 during heterotopic ossification and the role of melatonin.

Materials and Methods

Animals and Treatment

A group of 6-week-old male C57/B6 mice were obtained from Guangdong Experimental Animal Center (Guangzhou, China). After 8-week-old mice were anesthetized, bilateral calves of the mice were disinfected, and then the medial skin of the Achilles tendon was cut longitudinally to separate the tissue around the tendon to avoid damaging important nerves and vessels. The midpoint of the Achilles tendon tissue was completely severed under direct vision. The skin was sutured, and then the mice were resuscitated in a metal pot at 30 °C after the operation. The mice were returned to their cages after successful revival. After surgery, the mice were randomly divided into 4 groups: HO group (ip: 0.2 mL saline), MT group (ip: MT (0.05 mg/kg/day, MCE, Shanghai, China, HY-B0075)), luzindole (LUZ) group (ip: MT (0.05 mg/kg/day) + LUZ (0.01 mg/kg/day, MCE, HY-101254)), and rapamycin (RA) group (ip: MT (0.05 mg/kg/day) + RA (0.1 mg/kg/day, MCE, HY-10219)), 18 in each group only. Six mice from each group were collected at intervals of 4, 8, and 12 weeks.

Cell Culture

Achilles tendon tissue from 4-week-old mice was excised and incubated with collagenase type I (3 mg/mL, Gibco, NY, USA, 17100017) at 37 °C for 3 hours, followed by isolation of tenocytes. A 7-day culture of TDSC was performed in DMEM (Dulbecco's modified Eagle's medium, Gibco, NY, USA, C11995500BT) containing 100 units/mL penicillin and 100 g/mL streptomycin with 20 % fetal bovine serum (Gibco, NY, USA, 10099141C). These cells were used in P3.

Osteogenesis In Vitro

A 14-day incubation period in osteogenic induction medium (DMEM medium supplemented with 50 μmol/L ascorbic acid, 0.1 μmol/L dexamethasone, and 10 mmol/L β-glycerol phosphate) was used to induce osteogenic differentiation. The medium was supplemented with melatonin (1, 10 and 100 ng/mL) while control cells were grown in medium containing dimethyl sulfoxide (DMSO).

Chondrogenesis

TDSC (2.5×10^5) were placed in a 15 mL polypropylene tube and centrifuged to pellet. The pellets were cultured for 21 days in chondrogenic medium (low-glucose DMEM supplemented 1 % Insulin-Transferrin-Selenium (ITS, BD, Franklin Lakes, NJ, USA, 354351), 50 μg/mL vitamin C (Sigma-Aldrich, St. Louis, MO, USA, A5960), 10 nM dexamethasone (Wako, Tokyo, Japan, 041-18861), 1 mM pyruvate, and 10 ng/mL TGF-β3 (NovoProtein, Suzhou, China, CJ44-50) to achieve chondrogenic differentiation. The medium was changed every 2–3 days.

Cell Activity Analysis

We analyzed the cytotoxicity of melatonin on TDSC using the CCK-8 (Biosharp, Beijing, China, BS350C) method. TDSC (1×10^4) were inoculated into 96-well plates and treated with melatonin for 48 hours. We performed the CCK-8 assay following the manufacturer's recommendations to ascertain the cell survival rate.

ALP and Alizarin Red S (ARS) Cell Staining Assay

After induction of osteogenesis, TDSC were washed with PBS and fixed with 4 % paraformaldehyde for 30 min. They were then grown in osteogenic medium for 14 days and stained with ALP for osteogenesis. After 21 days of culture, for 40 min at 37 °C, 2 % alizarin red S dye solution (Solarbio, Beijing, China, G8550) was used to assess matrix mineralization.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Quantitative RT-PCR was performed to determine the effect of different treatments on gene expression of Achilles tendon and cultured cells. Briefly, total RNA was extracted from tissues using TRIZOL reagent (Sigma-Aldrich, St. Louis, MO, USA, T9424), followed by cDNA synthesis using Prime Kit (TaKaRa, Osaka, Japan, RR037A). Quantitative RT-PCR was performed using the Takara ExTaqII kit according to the manufacturer's instructions. Primers were purchased from Beijing Tsingke Biotech Co., Ltd. (Guangzhou, China), and the primer sequences of mRNAs used are shown in **Supplementary Table 1**. Expression levels were normalised to endogenous GAPDH, and fold changes in relative gene levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

To extract proteins from TDSC, cells were lysed for 30 min using a Radio-Immunoprecipitation Assay (RIPA) Lysis Buffer containing protease and phosphatase inhibitors. Protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the membrane was blocked in skim milk and then incubated overnight at 4 °C with the following monoclonal antibodies: COL2A1 (1:1000, Servicebio, Wuhan, China, sc377386), SOX9 (1:1000, abcam, Cambridge, MA, USA, ab185230), ACTB (1:50000, ABclonal, Wuhan, China, AC026), pS6 (1:1000, CST, Boston, MA, USA, #4858), S6 (1:1000, ABclonal, Wuhan, China, A6058), pS6K1 (1:1000, ABclonal, Wuhan, China, AP0253), S6K1 (1:1000, ABclonal, Wuhan, China, A6058), RUNX2 (1:1000, AffinityBiosciences, SanAntonio, TX, USA, AF5186), ALP (1:1000, ABclonal, Wuhan, China, A1239), OSX (1:1000, ABclonal, Wuhan, China, A18699) or OPN (1:1000, ABclonal, Wuhan, China, A1361). The membrane was exposed to chemiluminescence using enhanced chemiluminescence (Tanon, Shanghai, China, 5200) system. Gray values of Western blot bands for control and experimental groups were measured using Image-J software (NIH Image J system, Bethesda, MD, USA, V1.8.0). Protein levels were estimated by dividing the gray values of the target protein by those of the internal reference protein, with results for the experimental group expressed as fold change relative to the control group.

Assessment and Measurement of ROS

The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a fluorescence probe to evaluate reactive oxygen species (ROS) production in TDSC. TDSC were cultured in 24-well plates, and then the medium was replaced with α-MEM enriched with or without H_2O_2 (10 μM) or melatonin (100 ng/mL). Cells were incubated in the dark with DCFH-DA diluted to 10 μm for 25 min at 37 °C. They were then washed 3 times in PBS, and the fluorescence intensity was recorded at 488 nm.

Immunostaining Assay

Tissue samples from the Achilles tendon were fixed with 0.1 M phosphate buffer containing 4 % paraformaldehyde. The fixed tendon tissue was soaked in 10 % ethylenediamine tetra acetic acid (pH7.0) for 25 days, decalcified, embedded in paraffin. It was then sectioned with paraffin (4 μm thick). To perform immunohistochemical staining, the tendon segments were rehydrated and then incubated for 10 min in 3 % H_2O_2 to suppress endogenous peroxidase activity. Sections were subsequently treated with primary antibodies: RUNX2 (1:100, ABclonal, Wuhan, China, A2851), SOX9 (1:50, ABclonal, Wuhan, China, A19710), MTR1 (1:100, Huabio, Hangzhou, China, ER191296), MTR2 (1:200, abcam, Cambridge, MA, USA, ab203346), pS6 (1:100, CST, Boston, MA, USA, #4858) and kept at 4 °C overnight. This was followed by three washes with tris-buffered saline (TBS). Next, the secondary antibody and enzyme-labeled secondary antibody were applied at room temperature. Finally, the sections were visualized using Diaminobenzidine (DAB, Proteintech, Wuhan, China, PR30010) and results were observed under a light microscope.

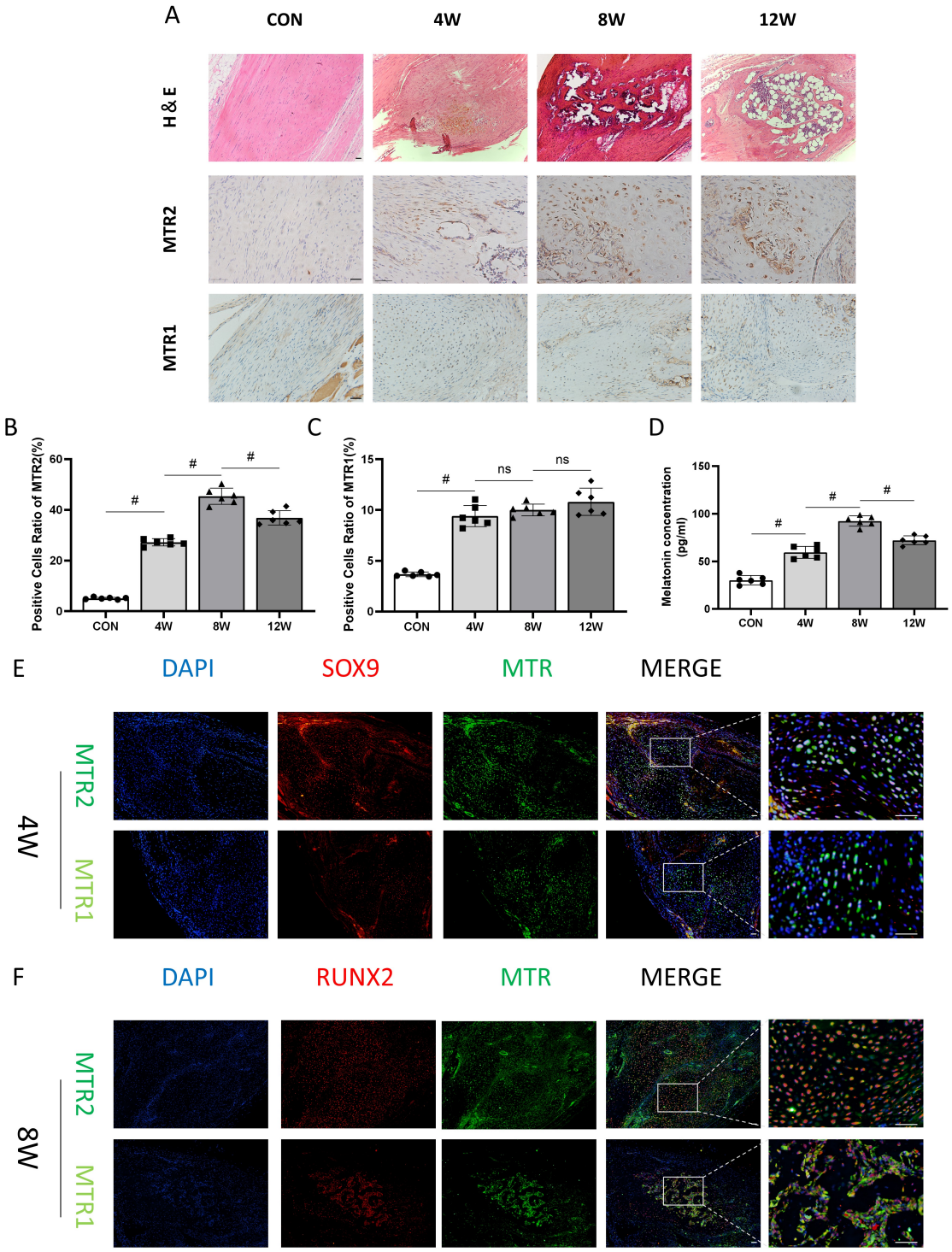

Fig. 1. The role of melatonin in HO formation. (**A**) HE staining images showing morphological changes at 4, 8, and 12 weeks after surgery. IHC images showing the expression of melatonin receptor (MTR1 and MTR2) at 4, 8, and 12 weeks after surgery. (**B**) Quantitative analysis of MTR2-positive cells at each time point after the operation. (**C**) Quantitative analysis of MTR1-positive cells at each time point after the operation. (**D**) Changes of serum melatonin concentrations in mice in the control and experimental groups. (**E**) Confocal images showing immunostaining of SOX9 (red) and MTR (green) merged with DAPI (blue) for adjacent tissue sections. (**F**) Confocal images showing immunostaining images for RUNX2 (red) and MTR (green) merged with DAPI (blue) in adjacent tissue sections. Scale bar = 50 μm. All data represent the mean ± SD. n = 6. ns, not significant, #$p < 0.01$. MTR, melatonin receptor; HO, heterotopic ossification; SD, standard deviation; IHC, immunohistochemistry; HE, hematoxylin-eosin.

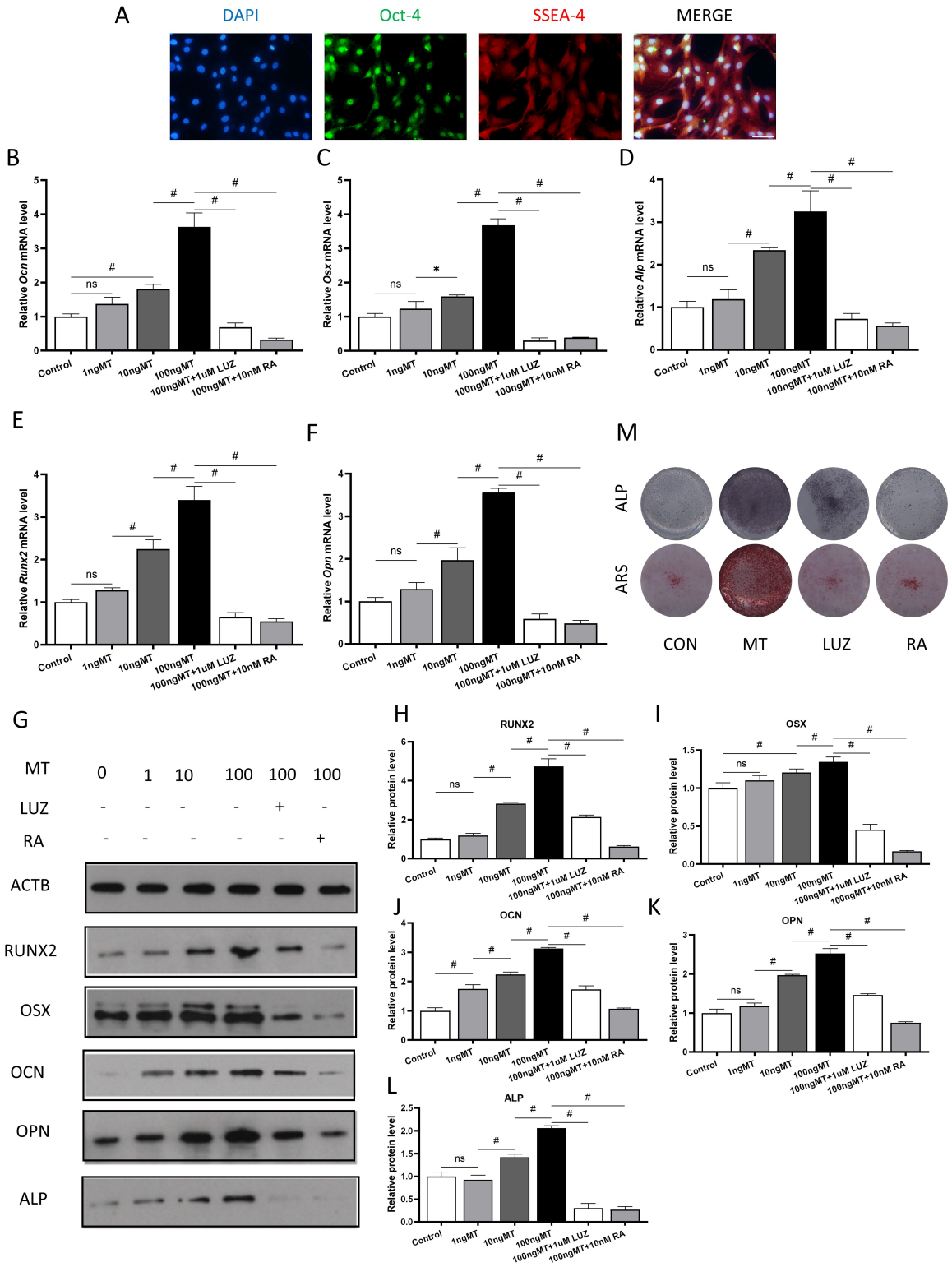

Fig. 2. Melatonin promotes the osteogenic differentiation of TDSC *in vitro*. (**A**) Identification of TDSC. Blue fluorescence shows nuclei; Green fluorescence shows OCT-4; Red fluorescence shows SSEA-4; Merge image shows cells co-expressing OCT-4 and SSEA-4. Scale bar = 50 μm. (**B–F**) Quantitative RT-PCR assays for expression of osteogenesis-specific genes *Ocn*, *Osx*, *Alp*, *Runx2*, and *Opn*. (**G-L**) Western blot analysis of OCN, OSX, ALP, RUNX2, and OPN. (**M**) Alizarin red and ALP staining of TDSC. Scale bar = 50 μm. All data represent the mean ± SD. n = 3. ns, not significant, *$p < 0.05$ and #$p < 0.01$. MT, melatonin; LUZ, luzindole; RA, rapamycin; TDSC, tendon-derived stem cells; RT-PCR, reverse transcription-polymerase chain reaction; ARS, alizarin red S.

The Analysis of Micro-Computed Tomography (Micro-CT)

Limbs of animals from the experimental and control groups were scanned using a Micro-CT (San-coMedical, Brutiseren, Switzerland, CT-80), and then the volume of HO was measured. The specimens were divided into sections with an average thickness of 20 microns. The scanning voltage was 60 kV while the current was 150 μA. The volume of HO was calculated by 3D reconstruction using a Micro-CT system software.

Statistical Analysis

All results were analyzed with GraphPad Prism 8.0.2 software (Graph-Pad Software, San Diego, CA, USA). Two-tailed Student's *t*-test was applied to the comparison between the two groups. One-way analysis of variance (One-way ANOVA) was used for multiple group comparisons. The statistically significant differences were shown, and differences at $p < 0.05$ were considered significant. The data are presented as the means ± standard deviation (SD).

Results

Roles of the Melatonin Receptors in HO Formation

The histological staining of the mice specimens at 4, 8 and 12 weeks after Achilles tendon injury (Fig. 1**A**) showed that formation of HO after Achilles tendon injury was an endochondral ossification process. In the early stage of ectopic ossification (4 weeks), cartilage was formed mainly in the Achilles tendon. In the middle stage of ectopic ossification (4–8 weeks), the cartilage was gradually replaced by bone, while in the late stage of ectopic ossification (8–12 weeks), the bone matured and gradually became enlarged. To determine the effect of melatonin and its receptors on the formation of HO after Achilles tendon injury, different melatonin receptors (MTR) were stained with anti-MTR1 and MTR2 antibodies at 4, 8 and 12 weeks after the injury. The data demonstrated significant increase in the expression of MTR1 and MTR2 receptors after the Achilles tendon injury (Fig. 1**A–C**), suggesting that melatonin receptor may play important roles in the formation of HO. Besides, the concentration of melatonin in the serum of the experimental and control groups was analyzed by enzyme linked immunosorbent assay (ELISA). The immunohistochemical staining results were in line with the findings related to HO. They demonstrated substantially elevated levels of melatonin in the experimental group compared to the control group. Moreover, the concentration of melatonin was significantly higher in the 8th week as opposed to the 4th and 12th weeks (Fig. 1**D**). These observations indicate a significant surge in melatonin levels in mice during the HO process following Achilles tendon amputation, particularly at the 8-week point after model establishment. In addition, to define the relationship between melatonin receptor in chondrogenesis and osteogenic differentiation, we performed immunofluorescence (IF) double labeling staining on the Achilles tendon injured mice (Fig. 1**E–F**). The results showed that SOX9, a chondrogenic marker, and OCN, an osteogenic marker, were co-localized with melatonin receptors. These data suggested that melatonin receptor might participate in endochondral ossification during HO.

Isolation and Identification of TDSC

TDSC were cultured in DMEM media. Initially, as shown in Fig. 2**A**, morphological analysis and IF staining with OCT-4 and SSEA-4 antibodies were performed to identify primary cells.

Melatonin Promotes the Osteogenic Differentiation of TDSC

The expression of osteogenic markers OCN, OSX, ALP, RUNX2 and OPN *in vitro* following melatonin stimulation was examined. We observed that melatonin significantly increased *Ocn*, *Osx*, *Alp*, *Runx2*, and *Opn* expression as indicated by the RT-PCR results (Fig. 2**B–F**). Western blot assays showed that the protein levels of OCN, OSX, ALP, RUNX2 and OPN were comparable to those obtained by RT-PCR (Fig. 2**G–L**). Furthermore, a staining with alizarin red and alkaline phosphatase revealed that melatonin enhanced osteogenic differentiation of the TDSC (Fig. 2**M**).

Melatonin Promotes the Chondrogenic Differentiation of TDSC

TDSC were subjected to chondrogenic induction for 7, 14, and 21 days at varying melatonin concentrations (0, 1, 10, and 100 ng/mL). We then analyzed genes associated with cartilage differentiation markers using RT-PCR. The results shown in Fig. 3**A–E** revealed that after 21 days of chondrogenic differentiation, 1 ng/mL melatonin had no discernible effect on TDSC chondrogenesis compared to the control group. Conversely, 10 ng/mL melatonin significantly augmented chondrogenic differentiation in TDSC. The expression of chondroblast marker genes—*Sox9* and *Col2a1* mRNA—was all up-regulated under melatonin treatment. Western blot analysis also demonstrated increased levels of SOX9 and COL2A1 proteins, with a more pronounced effect observed at higher melatonin concentrations (100 ng/mL). This indicates that melatonin promotes chondrogenic differentiation in TDSC in a concentration-dependent manner.

Role of the Melatonin Receptor in Chondrogenic and Osteogenic Differentiation

We employed luzindole (LUZ), a melatonin receptor antagonist, to investigate whether melatonin's effect on promoting chondrogenic and osteogenic differentiation in TDSC is mediated through the receptor pathway. The administration of LUZ resulted in a reversal of the effects induced by melatonin, indicating the involvement of receptor-

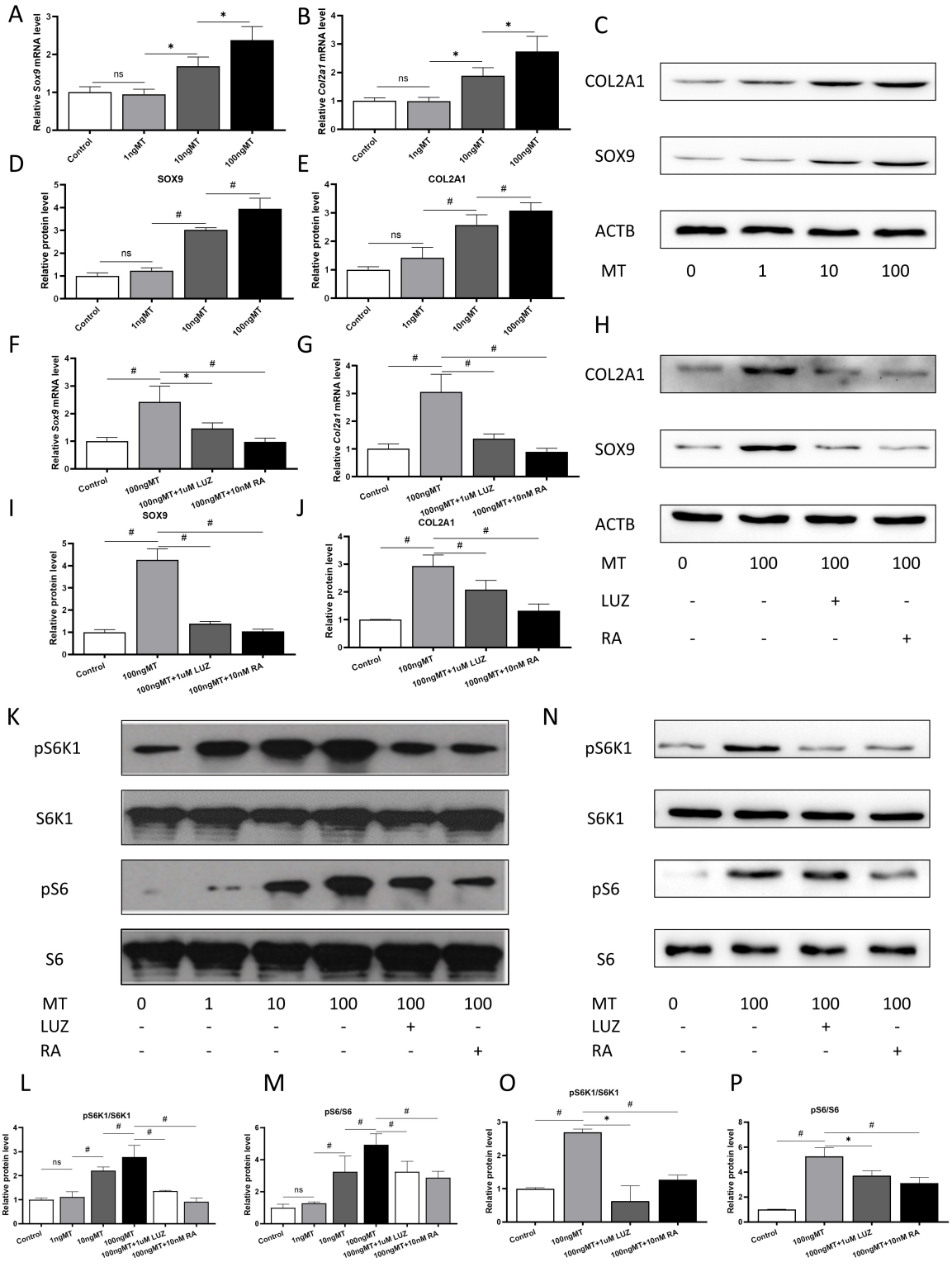

Fig. 3. Melatonin promotes the chondrogenic differentiation of TDSC and activates the mTORC1 pathway *in vitro*. (**A,B**) Chondrogenic markers mRNA were upregulated in response to MT. (**C–E**) Western blot analysis of SOX9 and COL2A1 protein expression. (**F,G**) Quantitative RT-PCR assays for expression of chondrogenesis—specific genes *Sox9* and *Col2a1*. (**H–J**) Western blot analysis of SOX9 and COL2A1 protein expression. (**K–M**) After osteogenic induction, western blot analysis of pS6K1, S6K1, pS6 and S6 protein expression. (**N–P**) After chondrogenic induction, western blot analysis of pS6K1, S6K1, pS6 and S6 protein expression. All data represent the mean ± SD. n = 3. ns, not significant, *$p < 0.05$ and #$p < 0.01$. MT, melatonin; LUZ, luzindole; RA, rapamycin; mTORC1, mTOR complex 1.

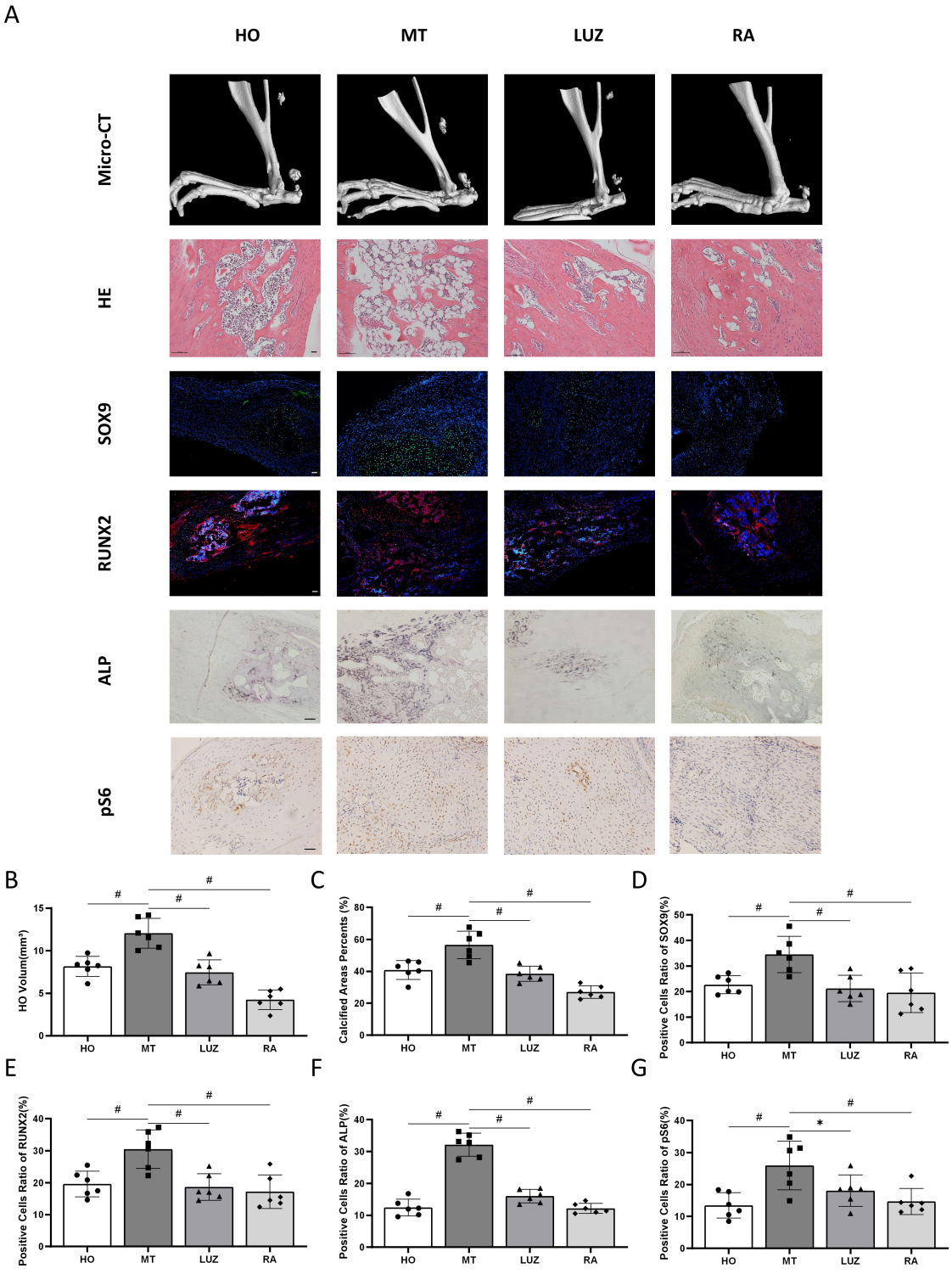

Fig. 4. Melatonin accelerates the pathogenesis of heterotopic ossification through the mTORC1 signaling pathway *in vivo*. (**A**) The representative images of Micro-CT, HE, IF and IHC staining. (**B**) Quantitative analysis of heterotopic ossification bone volume. (**C**) Quantitative histological analysis of the proportion (%) of calcified areas. (**D–G**) Quantitative analysis of the positive cell ratio for SOX9, RUNX2, ALP and pS6. HO group: ip-0.2 mL saline; MT group: ip-MT (0.05 mg/kg/day); LUZ group: ip-(MT (0.05 mg/kg/day) + LUZ (0.01 mg/kg/day)), RA group: ip-(MT (0.05 mg/kg/day) + RA (0.1 mg/kg/day)). Scale bar = 50 μm. All data represent the mean ± SD. n = 6. ns, not significant, *$p < 0.05$ and #$p < 0.01$. MT, melatonin; LUZ, luzindole; RA, rapamycin; IF, immunofluorescence; Micro-CT, Micro-computed tomography.

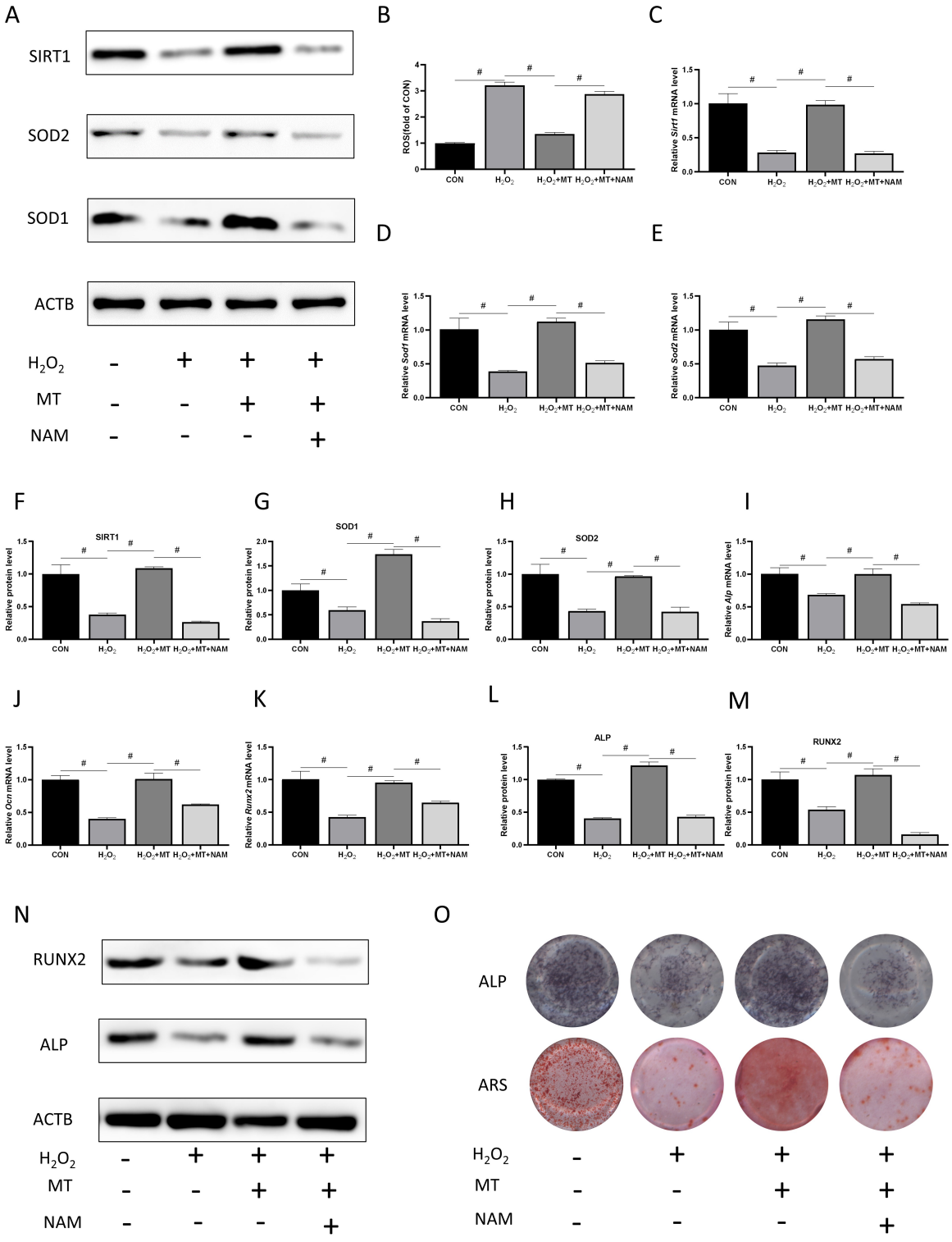

Fig. 5. Melatonin and NAM affect the osteogenic potential of TDSC under oxidative stress. (**A**) The protein levels of SOD1, SOD2, and SIRT1 were determined by Western blotting. (**B**) ROS levels in the TDSC were measured by the DCFH-DA. (**C–E**) Quantitative RT-PCR analysis of expression levels of *Sod1*, *Sod2* and *Sirt1*. (**F–H**) Densitometry of western blots was quantified by the ratios to ACTB. (**I–K**) Quantitative RT-PCR analysis of expression levels of osteogenesis markers (*Runx2*, *Alp*, and *Ocn*) expression. (**L–N**) The protein levels of osteogenesis markers were determined using Western blot assays. (**O**) Alizarin red staining and the ALP staining. All data represent the mean ± SD. n = 3. ns, not significant, #$p < 0.01$. MT, melatonin; NAM, nicotinamide; SIRT1, Sirtuin 1; ROS; reactive oxygen species; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate.

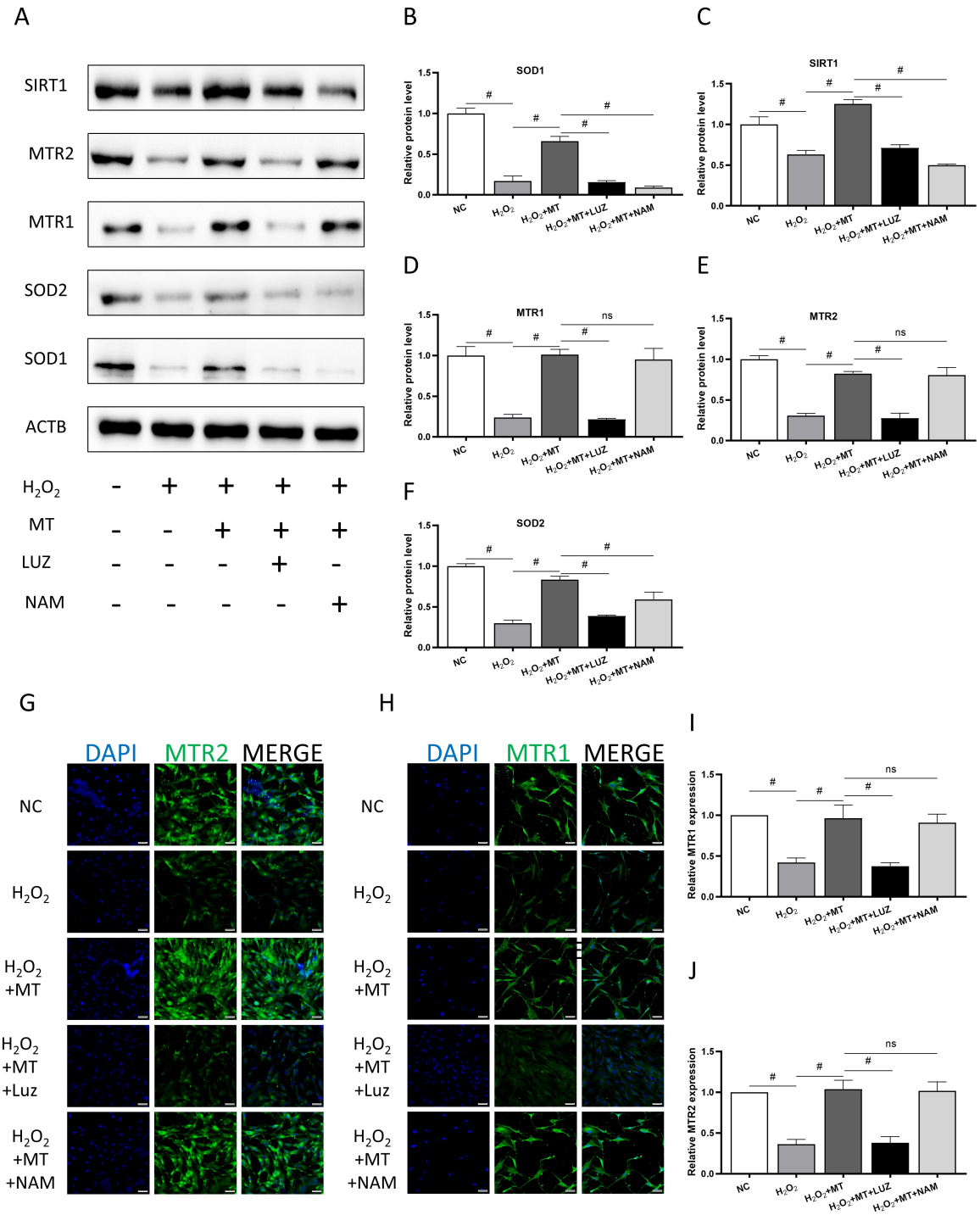

Fig. 6. Melatonin activated SIRT1 pathway through MTR in response to H_2O_2-induced damage. (**A–F**) The protein levels of antioxidant enzymes, melatonin receptors and SIRT1 in TDSC were determined using Western blot assays. (**G**) MTR1 immunofluorescence in TDSC. MTR1 was tagged with green fluorescence. The nucleus was labeled with DAPI. (**H**) MTR2 immunofluorescence in TDSC. MTR2 was tagged with green fluorescence. The nucleus was labeled with DAPI. (**I**) MTR1 fluorescence quantitative analysis. (**J**) MTR2 fluorescence quantitative analysis. Scale bar = 50 μm. All data represent the mean ± SD. n = 3. ns, not significant, #$p <$ 0.01. MTR, melatonin receptor; MT, melatonin; LUZ, luzindole; NAM, nicotinamide.

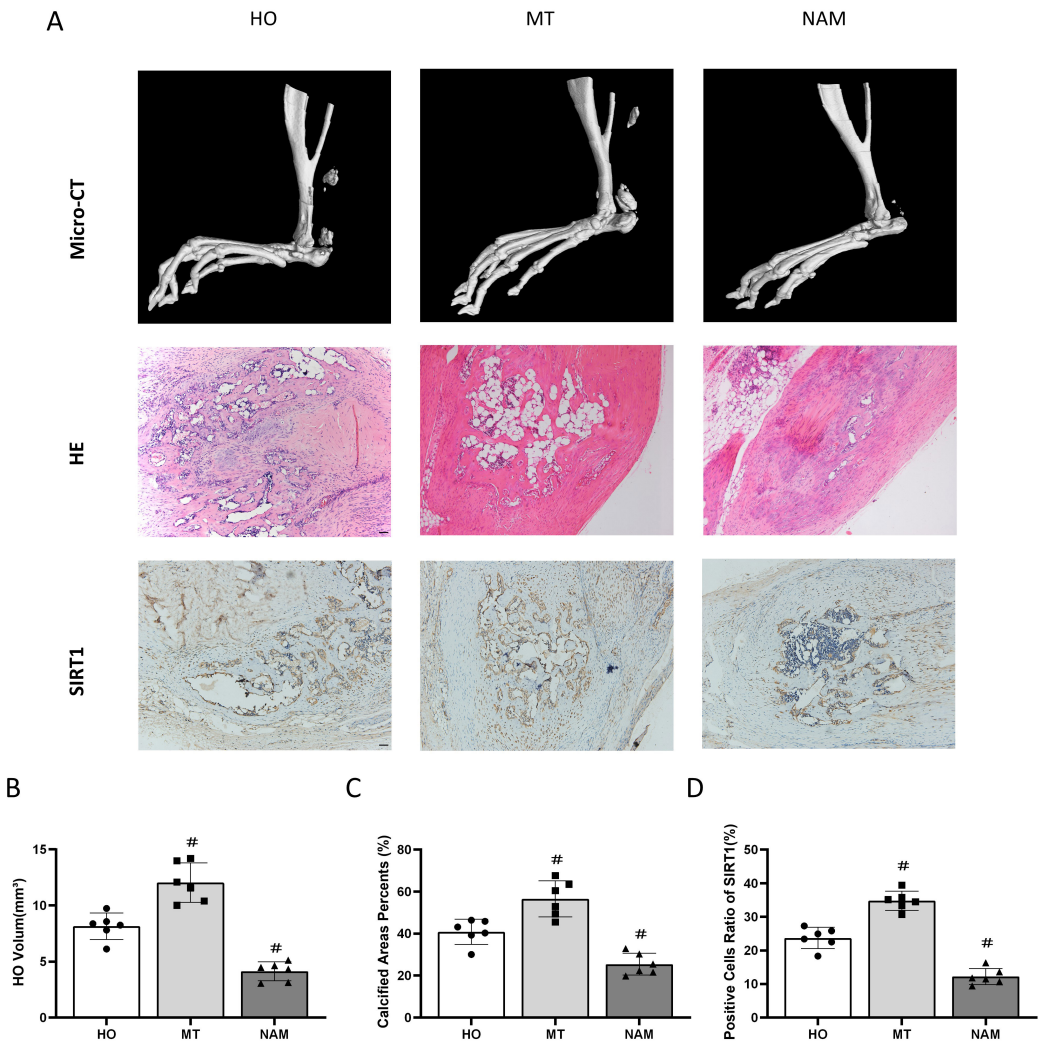

Fig. 7. The effect of NAM on heterotopic ossification. (**A**) Representative images of Micro-CT, HE and IHC staining. (**B**) Quantitative analysis of heterotopic ossification bone volume. (**C**) Quantitative histological analysis of the proportion (%) of calcified areas. (**D**) Quantitative assessment of the proportion of cells expressing SIRT1. HO group: ip-0.2 mL saline; MT group: ip-MT (0.05 mg/kg/day); NAM group: ip-NAM (100 mg/kg/day). Scale bar = 50 μm. All data represent the mean ± SD. n = 6. ns, not significant, #$p < 0.01$. MT, melatonin; NAM, nicotinamide.

mediated pathways. Correspondingly, during the osteogenesis process, the mRNA and protein expression levels of OCN, OSX, ALP, RUNX2 and OPN were significantly diminished in the LUZ group compared to the MT group. These findings provide compelling evidence that the receptor pathway plays a crucial role in the melatonin-induced chondrogenic and osteogenic differentiation of TDSC.

Melatonin Accelerates Chondrogenic and Osteogenic Differentiation via the mTORC1 Pathway

Rapamycin, inhibitor of the mTORC1 signaling pathway, was used to investigate the mechanism by which melatonin regulates TDSC differentiation *in vitro*. After melatonin treatment for 14 days, western blots analysis showed that melatonin significantly increased S6K1 and S6 phosphorylation in a dose-dependent manner (Fig. 3**K–M**). Our data showed that the mTORC1 pathway was activated by melatonin during osteogenic differentiation of TDSC. Furthermore, rapamycin (10 nmol/mL) significantly suppressed the melatonin-induced enhancement of gene and protein expression of OCN, OSX, ALP, RUNX2 and OPN (Fig. 2**B–K**). Consistent with its positive role in osteogenesis, melatonin also activated the mTORC1 pathway during cartilage differentiation (Fig. 3**N–P**). Compared to the MT group, the incorporation of RA resulted in a reduction in both gene and protein expression levels of cartilage markers (Fig. 3**F–J**), as well as a decrease in the relative expression of pS6K1 and pS6 during chondrogenic differentia-

tion (Fig. 3**N–P**). These observations strongly indicate that melatonin activates the mTORC1 pathway, thereby enhancing osteogenic and chondrogenic differentiation in TDSC.

Melatonin Accelerates HO Generation through mTORC1 Pathway

A Micro-CT analysis of melatonin-induced heterotopic bone formation in tendons was performed. There were differences in the sizes of these heterotopic bones (Fig. 4**A–B**). There was a significant difference in the size of heterotopic bone between the MT group and the HO group. In contrast, LUZ group heterotopic bones were smaller than MT. In addition, according to hematoxylin-eosin (HE) staining, melatonin treatment resulted in visible disorder of fibroblasts and collagen fibers. The calcified area was larger in the MT group than in the HO group. LUZ treatment reversed melatonin-induced increase in the calcified area (Fig. 4**A,C**). Furthermore, we investigated the *in vivo* expression levels of SOX9, RUNX2, and ALP to determine the impact of melatonin on heterotopic ossification. The findings indicated that melatonin upregulated the expression levels of SOX9, RUNX2 and ALP. Conversely, the expression levels of these markers were reduced in the LUZ group compared to the MT group (Fig. 4**A,D–F**). We examined the activation of the mTORC1 signaling pathway by evaluating the downstream marker p-S6 (Fig. 4**A,G**). The results indicated that melatonin treatment led to an upregulation of p-S6 expression. In further tests, we identified potential signaling pathways involved in the regulatory effects of melatonin on HO formation using rapamycin, a specific inhibitor of mTORC1 signaling. Our study demonstrated that rapamycin treatment led to a reduction in the number of $SOX9^+$, $RUNX2^+$, and ALP^+ cells in the injured Achilles tendon, thereby inhibiting ectopic bone formation at these sites (Fig. 4**A,D–F**). Consequently, rapamycin treatment reversed melatonin-induced chondrogenic and osteogenic differentiation *in vivo*.

Melatonin Alleviates H2O2-Induced Oxidative Stress through SIRT1 Signaling Pathway

The oxidative status of the TDSC was assessed by evaluating the superoxide dismutase (SOD) activity and ROS production. After TDSC were treated with 10 μM H_2O_2 for 24 hours, the SOD activity decreased, indicating that the cellular antioxidant system was impaired under high ROS conditions. However, after treatment with H_2O_2 and melatonin together, SOD increased significantly (Fig. 5**A–H**). The SOD activity was significantly rescued by pre-treatment with 100 ng/mL melatonin for 24 hours before exposure. Changes in ROS production showed a similar trend. Flow cytometry analysis indicated that melatonin pretreated cells showed a significant difference from non-pretreated cells (Fig. 5**B**).

To investigate whether the SIRT1 pathway mediates TDSC responses to melatonin treatment, we analyzed SIRT1 protein levels. Western blot analysis results demonstrated that SIRT1 expression was significantly lower in H_2O_2-treated cells than in the control group. As expected, melatonin reversed the inhibitory effect of SIRT1 expression in H_2O_2-treated cells. Subsequently, we applied the SIRT1 specific inhibitor nicotinamide (NAM) to determine whether the antioxidant effects of melatonin depend on SIRT1. Interestingly, co-culture with NAM abolished the upregulation of SIRT1 by melatonin but abolished the increased antioxidant effect of melatonin after treatment with NAM (Fig. 5**A–H**). These results confirm that melatonin promotes SIRT1-dependent antioxidant signaling.

Melatonin Restores the Osteogenic Potential of TDSC by Preserving SIRT1-Mediated Intracellular Antioxidant Properties

Osteogenic induction of TDSC was performed to assess the effect of melatonin on osteogenesis under oxidative stress. Following the induction of TDSC, both ALP and alizarin red staining demonstrated that exposure to 10 μM H_2O_2 impaired the osteogenic potential of TDSC (Fig. 5**O**). However, pretreatment with melatonin restored the osteogenic capacity of these cells. Consistent findings were observed through RT-PCR and Western blot analyses, which examined osteogenesis markers such as RUNX2, ALP, and OCN (Fig. 5**I–N**). Thus, melatonin treatment effectively reinstated the osteogenic potential of TDSC under conditions of H_2O_2-induced oxidative stress. Next, we used NAM, a specific inhibitor of the SIRT1 signaling pathway, to determine whether melatonin could protect the osteogenesis ability of TDSC by activating SIRT1 signaling. The TDSC were cotreated with NAM (5 μM) with melatonin before H_2O_2 exposure. Following osteogenic induction, the expression levels of RUNX2, ALP, and OCN in the (H_2O_2 + MT + NAM) group were significantly reduced compared to those in the (H_2O_2 + MT) group. Based on these results, NAM appears to abolish the protective effect of melatonin under H_2O_2-induced oxidative stress conditions. These results suggest that melatonin can stimulate the osteogenic differentiation of TDSC under oxidative stress through the SIRT1 activation.

Melatonin Protects TDSC from H2O2-Induces Oxidative Damage via MTR1/MTR2-Mediated SIRT1 Activation

In addition, we investigated the expression of MTR1/MTR2 under oxidative stress conditions and demonstrated that H_2O_2 can reduce the expression of MTR1 and MTR2 in TDSC. Our experimental data showed that melatonin increased MTR1 and MTR2 protein expression and fluorescence signal (Fig. 6). To further demonstrate that melatonin induces SIRT1 signaling through melatonin receptors, TDSC were treated with luzindole, NAM, and melatonin. As shown in Fig. 6, LUZ inhibited the expression of MTR1, MTR2, SIRT1, SOD1 and SOD2. However, NAM did not reverse the melatonin-induced increase in

MTR1/MTR2. Taken together, our results further confirm that SIRT1 activation is mediated by MTR1 and MTR2.

In Vivo Administration of NAM Alleviates HO

To determine the effect of NAM on HO, histopathological analysis and Micro-CT analysis were performed on Achilles tendon tissues in different treatment groups. As shown in the Fig. 7, after prolonged administration of NAM (12 weeks), ectopic bone mass decreased and ossification area was significantly reduced in the NAM treated group compared with the control group. In addition, immunohistochemical results showed that melatonin significantly increased the expression of SIRT1 during heterotopic ossification, suggesting that melatonin can activate SIRT1 *in vivo*. Conversely, NAM markedly attenuated SIRT1 expression in the context of heterotopic ossification of the Achilles tendon.

Discussion

Heterotopic ossification, an abnormal pathological process, is defined as bone formation in extraosseous muscles and soft tissues, which forms an ectopic bone tissue that is composed of the endochondral bone [33]. Currently, the pathogenesis mechanisms of heterotopic ossification have not been fully established. Several neuroendocrine factors, including substance P [34], leptin [13] and neurotrophic factor 3 [11] are involved in pathogenesis of heterotopic ossification. In our previous study, we showed that melatonin promoted ectopic osteogenesis by enhancing endothelial mesenchymal transformation [19]. However, the effects of melatonin on TDSC differentiation are yet to be conclusively determined.

Heterotopic ossification is a typical endochondral ossification process, and the effects of chondrogenic differentiation of TDSC on ectopic ossification cannot be ignored. In this study, we confirmed that melatonin promoted the chondrogenic differentiation of TDSC as well as the expression of osteogenic markers to enhance its osteogenesis. Furthermore, melatonin promoted the formation of HO *in vivo*. Inactivation of the mTORC1 signaling by rapamycin counteracted the effects of melatonin on osteogenic and chondrogenic differentiations of TDSC *in vitro* and HO formation *in vivo*. This study reveals the positive regulatory effects of melatonin on mTORC1 activity during aberrant differentiation of TDSC in HO, which provides new ideas for its prevention and treatment.

Melatonin enhances MSC differentiation through its receptors [35]. Mesenchymal stem cells express melatonin receptors (MTR1 and MTR2) in bone [36] implying that melatonin has important roles in the differentiation behavior of the cells. Our findings indicate a significant upregulation of MT and its receptor levels during the *in vivo* formation of ectopic ossification, suggesting a contributory role of MT in the pathogenesis of this condition. Following an Achilles tendon injury, the expression levels of MTR1 and MTR2 were observed to increase. Notably, the expression levels of MTR2 were significantly higher compared to MTR1, suggesting that MTR2 may play a predominant role in the formation of HO.

The co-expression of melatonin receptors with SOX9 and RUNX2 suggests a potential role for melatonin receptors in endochondral osteogenesis. In both *in vivo* and *in vitro* settings, the melatonin receptor antagonist markedly reversed the aforementioned effects. These results indicate that melatonin and its receptors play a role in cartilage formation and the osteogenic differentiation of TDSC during ectopic osteogenesis. Consequently, melatonin and its receptors may serve as potential targets for the prevention of HO.

Oxidative stress in injured sites is among the early events of HO [25]. Xie *et al*. [37] demonstrated that prevention of early immune response-induced oxidative stress could effectively treat HO. Excessive ROS in cells indicates increased oxidative stress. At the physiological level, ROS regulates cell proliferation, differentiation, survival and apoptosis [38–40]. Excessive ROS can disrupt cellular redox homeostasis, promoting apoptosis, cellular damage, lipid peroxidation, and mitochondrial dysfunction [41]. Impaired osteogenic capacity of mesenchymal stem cells causes accumulation of ROS under oxidative stress [42]. In this study, melatonin treatment protected TDSC from oxidative stress, and the activities of SOD were increased while ROS levels were suppressed. Under oxidative stress conditions, melatonin stimulated osteogenic differentiation of TDSC, increased the expression of RUNX2, ALP, and OCN, and promoted calcified nodule deposition and ALP activity. In addition, *in vitro* results showed that oxidative stress reduced the expression of melatonin receptor on TDSC, and melatonin maintained the expression level of melatonin receptor on TDSC.

SIRT1 maintains a balance between bone formation and bone resorption by regulating the ratio of osteoblasts to osteoclasts [43]. Expression levels of SIRT1 are correlated with bone mineral density and bone fragility, and it is a potential biomarker of bone metabolism and bone-related diseases [44]. We found that expression level of SIRT1 was up-regulated during ectopic ossification, and melatonin treatment increased SIRT1 expression, implying that melatonin may interact with SIRT1 during ectopic ossification. *In vitro*, NAM, a SIRT1 antagonist, weakened the antioxidant effects of melatonin, resulting in loss of osteogenic differentiation potentials of TDSC. SIRT1 increased its transcriptional activity, and enhanced the synthesis of antioxidant enzymes such as catalase and SOD. It has been reported that deacetylation of lysine residues in FoxO1 promotes its nuclear retention [45]. Melatonin promotes SOD through a process mediated by SIRT1 activation. Moreover, *in vivo* and *in vitro,* melatonin treatment up-regulated SIRT1 levels leading to enhanced osteogenic capacity of TDSC exposed to oxidative stress, and pro-

moted HO formation. In this study, we found that NAM has therapeutic effects on heterotopic ossification. After daily administration of NAM, there was a reduction in the volume of ectopic bone, compared to the control group. Sun *et al*. [31] reported that SIRT1 activation can inhibit post-traumatic ectopic ossification by suppressing macrophage-mediated inflammation. In this study, we postulated that these divergent findings might be associated with the different duration of intervention. The transient activation of SIRT1 predominantly modulates the inflammatory response subsequent to injury, whereas its prolonged activation facilitates the differentiation of TDSC and their subsequent endochondral ossification. While the specific functions of SIRT1 in different stages are not yet fully understood, its activation is crucial in the development of HO. Notably, our findings revealed that the antioxidant effect of melatonin relies on the activation of SIRT1. Interestingly, the deactivation of SIRT1 does not impact the expression of melatonin receptors. However, it was observed that melatonin could enhance the expression of melatonin receptors under conditions of oxidative stress. The potential positive feedback loop in maintaining melatonin receptor levels warrants further investigation. In summary, our results show that melatonin accelerates heterotopic ossification by activating the mTORC1 pathway to promote chondrogenic and osteogenic differentiation of TDSC. Inhibition of the mTORC1 pathway suppresses the melatonin-induced differentiation of TDSC and HO development. Melatonin reduces oxidative stress damage of TDSC and enhances the osteogenic differentiation potential of TDSC via SIRT1-mediated antioxidation. Therefore, strategies inhibiting SIRT1 may be effective therapeutic approaches for the treatment of heterotopic ossification. This study elucidated the complex roles of melatonin in the pathogenesis of HO and its underlying mechanisms, thereby offering a valuable reference for the development of novel strategies for the prevention and treatment of HO.

Conclusions

These findings suggest that melatonin accelerates HO by activating the mTORC1 pathway to promote chondrogenic and osteogenic differentiation of TDSC. Additionally, melatonin's antioxidative effect, mediated through SIRT1 activation, preserves TDSC osteogenic potential in the early stages of injury. This study identifies mTORC1 and SIRT1 as potential therapeutic targets for the prevention and management of HO, offering new insights into the molecular mechanisms of HO development and treatment strategies.

List of Abbreviations

HO, heterotopic ossification; MT, melatonin; TDSC, tendon-derived stem cells; SIRT1, Sirtuin 1; mTORC1, mTOR complex 1; LUZ, luzindole; RA, rapamycin; MTR, melatonin receptor; NAM, nicotinamide; MSCs, mesenchymal stem cells; SD, standard deviation; ANOVA, analysis of variance; ROS; reactive oxygen species; IHC, immunohistochemistry; HE, hematoxylin-eosin; IF, immunofluorescence; RT-PCR, reverse transcription-polymerase chain reaction; Micro-CT, Micro-computed tomography; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; SOD, superoxide dismutase; ARS, alizarin red S.

Availability of Data and Materials

Requests for materials should be addressed to L. Wang.

Author Contributions

JL, SC and LW contributed to the design of this work. JL, QHC and WSZ contributed to the interpretation of data and drafted the manuscript. MYH, YYX and SYL analyzed the data. YTJ and JZ drafted the work. All authors read and approved the final manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Ethics Approval and Consent to Participate

These experiments were approved by the Ethical Committee for Animal Research (IAC(S)1901003-1).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.22203/eCM.v049a06.

References

[1] Cao G, Zhang S, Wang Y, Quan S, Yue C, Yao J, *et al*. Pathogenesis of acquired heterotopic ossification: Risk factors, cellular mechanisms, and therapeutic implications. Bone. 2023; 168: 116655. https://doi.org/10.1016/j.bone.2022.116655.

[2] Liu EY, Hildebrand A, Horner NS, Athwal GS, Khan M, Alolabi B. Heterotopic ossification after total elbow arthroplasty: a systematic review. Journal of Shoulder and Elbow Surgery/American Shoulder and Elbow Surgeons ... [*et al*.]. 2019; 28: 587–595. https://doi.org/10.1016/j.jse.2018.10.003.

[3] Wang X, Li F, Xie L, Crane J, Zhen G, Mishina Y, *et al*. Inhibition of overactive TGF-β attenuates progression of heterotopic ossification in mice. Nature Communications. 2018; 9: 551. https://doi.org/10.1038/s41467-018-02988-5.

[4] Pacifici M. Acquired and congenital forms of heterotopic ossification: new pathogenic insights and therapeutic opportunities. Current Opinion in Pharmacology. 2018; 40: 51–58. https://doi.org/10.1016/j.coph.2018.03.007.

[5] Zhang J, Wang JH. Production of PGE(2) increases in tendons subjected to repetitive mechanical loading and induces differentiation of tendon stem cells into non-tenocytes. Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society. 2010; 28: 198–203. https://doi.org/10.1002/jor.20962.

[6] Rui YF, Lui PP, Li G, Fu SC, Lee YW, Chan KM. Isolation and characterization of multipotent rat tendon-derived stem cells. Tissue Engineering. Part A. 2010; 16: 1549–1558. https://doi.org/10.1089/ten.TEA.2009.0529.

[7] Zhang J, Wang JH. Characterization of differential properties of rabbit tendon stem cells and tenocytes. BMC Musculoskeletal Disorders. 2010; 11: 10. https://doi.org/10.1186/1471-2474-11-10.

[8] Sorkin M, Huber AK, Hwang C, Carson WF 4th, Menon R, Li J, *et al*. Regulation of heterotopic ossification by monocytes in a mouse model of aberrant wound healing. Nature Communications. 2020; 11: 722. https://doi.org/10.1038/s41467-019-14172-4.

[9] Loder SJ, Agarwal S, Chung MT, Cholok D, Hwang C, Visser N, *et al*. Characterizing the Circulating Cell Populations in Traumatic Heterotopic Ossification. The American Journal of Pathology. 2018; 188: 2464–2473. https://doi.org/10.1016/j.ajpath.2018.07.014.

[10] Huang H, Cheng WX, Hu YP, Chen JH, Zheng ZT, Zhang P. Relationship between heterotopic ossification and traumatic brain injury: Why severe traumatic brain injury increases the risk of heterotopic ossification. Journal of Orthopaedic Translation. 2018; 12: 16–25. https://doi.org/10.1016/j.jot.2017.10.002.

[11] Zhang J, Wang L, Chu J, Ao X, Jiang T, Bin Yan, *et al*. Macrophage-derived neurotrophin-3 promotes heterotopic ossification in rats. Laboratory Investigation; A Journal of Technical Methods and Pathology. 2020; 100: 762–776. https://doi.org/10.1038/s41374-019-0367-x.

[12] Zhang J, Wang L, Cao H, Chen N, Yan B, Ao X, *et al*. Neurotrophin-3 acts on the endothelial-mesenchymal transition of heterotopic ossification in rats. Journal of Cellular and Molecular Medicine. 2019; 23: 2595–2609. https://doi.org/10.1111/jcmm.14150.

[13] Jiang H, Chen Y, Chen G, Tian X, Tang J, Luo L, *et al*. Leptin accelerates the pathogenesis of heterotopic ossification in rat tendon tissues via mTORC1 signaling. Journal of Cellular Physiology. 2018; 233: 1017–1028. https://doi.org/10.1002/jcp.25955.

[14] Claustrat B, Leston J. Melatonin: Physiological effects in humans. Neuro-Chirurgie. 2015; 61: 77–84. https://doi.org/10.1016/j.neuchi.2015.03.002.

[15] Talib WH. Melatonin and Cancer Hallmarks. Molecules: A Journal of Synthetic Chemistry and Natural Product Chemistry. 2018; 23: 518. https://doi.org/10.3390/molecules23030518.

[16] Cipolla-Neto J, Amaral FGD. Melatonin as a Hormone: New Physiological and Clinical Insights. Endocrine Reviews. 2018; 39: 990–1028. https://doi.org/10.1210/er.2018-00084.

[17] Majidinia M, Reiter RJ, Shakouri SK, Mohebbi I, Rastegar M, Kaviani M, *et al*. The multiple functions of melatonin in regenerative medicine. Ageing Research Reviews. 2018; 45: 33–52. https://doi.org/10.1016/j.arr.2018.04.003.

[18] Lee S, Le NH, Kang D. Melatonin alleviates oxidative stress-inhibited osteogenesis of human bone marrow-derived mesenchymal stem cells through AMPK activation. International Journal of Medical Sciences. 2018; 15: 1083–1091. https://doi.org/10.7150/ijms.26314.

[19] Zhang J, Tang J, Liu J, Yan B, Yan B, Huang M, *et al*. Melatonin Promotes Heterotopic Ossification Through Regulation of Endothelial-Mesenchymal Transition in Injured Achilles Tendons in Rats. Frontiers in Cell and Developmental Biology. 2021; 9: 629274. https://doi.org/10.3389/fcell.2021.629274.

[20] Chen J, Long F. mTORC1 signaling controls mammalian skeletal growth through stimulation of protein synthesis. Development. 2014; 141: 2848–2854. https://doi.org/10.1242/dev.108811.

[21] Isomoto S, Hattori K, Ohgushi H, Nakajima H, Tanaka Y, Takakura Y. Rapamycin as an inhibitor of osteogenic differentiation in bone marrow-derived mesenchymal stem cells. Journal of Orthopaedic Science: Official Journal of the Japanese Orthopaedic Association. 2007; 12: 83–88. https://doi.org/10.1007/s00776-006-1079-9.

[22] Fu J, Zhang J, Jiang T, Ao X, Li P, Lian Z, *et al*. mTORC1 coordinates NF-κB signaling pathway to promote chondrogenic differentiation of tendon cells in heterotopic ossification. Bone. 2022; 163: 116507. https://doi.org/10.1016/j.bone.2022.116507.

[23] Banfi G, Iorio EL, Corsi MM. Oxidative stress, free radicals and bone remodeling. Clinical Chemistry and Laboratory Medicine. 2008; 46: 1550–1555. https://doi.org/10.1515/cclm.2008.302.

[24] Huang Y, Wang X, Lin H. The hypoxic microenvironment: a driving force for heterotopic ossification progression. Cell Communication and Signaling: CCS. 2020; 18: 20. https://doi.org/10.1186/s12964-020-0509-1.

[25] Hu Y, Wang Z. Rapamycin prevents heterotopic ossification by inhibiting the mTOR pathway and oxidative stress. Biochemical and Biophysical Research Communications. 2021; 573: 171–178. https://doi.org/10.1016/j.bbrc.2021.07.060.

[26] Almeida M, Porter RM. Sirtuins and FoxOs in osteoporosis and osteoarthritis. Bone. 2019; 121: 284–292. https://doi.org/10.1016/j.bone.2019.01.018.

[27] Chen C, Zhou M, Ge Y, Wang X. SIRT1 and aging related signaling pathways. Mechanisms of Ageing and Development. 2020; 187: 111215. https://doi.org/10.1016/j.mad.2020.111215.

[28] Xu D, Liu L, Zhao Y, Yang L, Cheng J, Hua R, *et al*. Melatonin protects mouse testes from palmitic acid-induced lipotoxicity by attenuating oxidative stress and DNA damage in a SIRT1-dependent manner. Journal of Pineal Research. 2020; 69: e12690. https://doi.org/10.1111/jpi.12690.

[29] Hong Q, Zhang L, Das B, Li Z, Liu B, Cai G, *et al*. Increased podocyte Sirtuin-1 function attenuates diabetic kidney injury. Kidney International. 2018; 93: 1330–1343. https://doi.org/10.1016/j.kint.2017.12.008.

[30] Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, *et al*. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science. 2004; 303: 2011–2015. https://doi.org/10.1126/science.1094637.

[31] Sun Z, Li J, Luo G, Liu W, He Y, Wang F, *et al*. Pharmacological activation of SIRT1 by metformin prevented trauma-induced heterotopic ossification through inhibiting macrophage mediated inflammation. European Journal of Pharmacology. 2021; 909: 174386. https://doi.org/10.1016/j.ejphar.2021.174386.

[32] Huang X, Chen W, Gu C, Liu H, Hou M, Qin W, *et al*. Melatonin suppresses bone marrow adiposity in ovariectomized rats by rescuing the imbalance between osteogenesis and adipogenesis through SIRT1 activation. Journal of Orthopaedic Translation. 2023; 38: 84–97. https://doi.org/10.1016/j.jot.2022.10.002.

[33] Kaplan FS, Glaser DL, Hebela N, Shore EM. Heterotopic ossification. The Journal of the American Academy of Orthopaedic Surgeons. 2004; 12: 116–125. https://doi.org/10.5435/00124635-200403000-00007.

[34] Kan L, Lounev VY, Pignolo RJ, Duan L, Liu Y, Stock SR, *et al*. Substance P signaling mediates BMP-dependent heterotopic ossi-

fication. Journal of Cellular Biochemistry. 2011; 112: 2759–2772. https://doi.org/10.1002/jcb.23259.

[35] Xie Y, Han N, Li F, Wang L, Liu G, Hu M, *et al*. Melatonin enhances osteoblastogenesis of senescent bone marrow stromal cells through NSD2-mediated chromatin remodelling. Clinical and Translational Medicine. 2022; 12: e746. https://doi.org/10.1002/ctm2.746.

[36] Yang Y, Fan C, Deng C, Zhao L, Hu W, Di S, *et al*. Melatonin reverses flow shear stress-induced injury in bone marrow mesenchymal stem cells via activation of AMP-activated protein kinase signaling. Journal of Pineal Research. 2016; 60: 228–241. https://doi.org/10.1111/jpi.12306.

[37] Xie K, Chen L, Yang J. RNA Sequencing Evidences the Prevention of Oxidative Stress is Effective in Injury-Induced Heterotopic Ossification Treatment. Journal of Biomedical Nanotechnology. 2021; 17: 196–204. https://doi.org/10.1166/jbn.2021.3019.

[38] Janbandhu V, Tallapragada V, Patrick R, Li Y, Abeygunawardena D, Humphreys DT, *et al*. Hif-1a suppresses ROS-induced proliferation of cardiac fibroblasts following myocardial infarction. Cell Stem Cell. 2022; 29: 281–297.e12. https://doi.org/10.1016/j.stem.2021.10.009.

[39] Su LJ, Zhang JH, Gomez H, Murugan R, Hong X, Xu D, *et al*. Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis. Oxidative Medicine and Cellular Longevity. 2019; 2019: 5080843. https://doi.org/10.1155/2019/5080843.

[40] Shi L, Liang F, Zheng J, Zhou K, Chen S, Yu J, *et al*. Melatonin Regulates Apoptosis and Autophagy Via ROS-MST1 Pathway in Subarachnoid Hemorrhage. Frontiers in Molecular Neuroscience. 2018; 11: 93. https://doi.org/10.3389/fnmol.2018.00093.

[41] Sies H, Belousov VV, Chandel NS, Davies MJ, Jones DP, Mann GE, *et al*. Defining roles of specific reactive oxygen species (ROS) in cell biology and physiology. Nature Reviews. Molecular Cell Biology. 2022; 23: 499–515. https://doi.org/10.1038/s41580-022-00456-z.

[42] Denu RA, Hematti P. Effects of Oxidative Stress on Mesenchymal Stem Cell Biology. Oxidative Medicine and Cellular Longevity. 2016; 2016: 2989076. https://doi.org/10.1155/2016/2989076.

[43] Chen Y, Zhou F, Liu H, Li J, Che H, Shen J, *et al*. SIRT1, a promising regulator of bone homeostasis. Life Sciences. 2021; 269: 119041. https://doi.org/10.1016/j.lfs.2021.119041.

[44] Zhang W, Zhou X, Hou W, Chen E, Ye C, Chen M, *et al*. Reversing the imbalance in bone homeostasis via sustained release of SIRT-1 agonist to promote bone healing under osteoporotic condition. Bioactive Materials. 2023; 19: 429–443. https://doi.org/10.1016/j.bioactmat.2022.04.017.

[45] Jiang Y, Luo W, Wang B, Wang X, Gong P, Xiong Y. Resveratrol promotes osteogenesis via activating SIRT1/FoxO1 pathway in osteoporosis mice. Life Sciences. 2020; 246: 117422. https://doi.org/10.1016/j.lfs.2020.117422.

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