TMF suppresses chondrocyte hypertrophy in osteoarthritic cartilage by mediating the FOXO3a/BMPER pathway

JISHANG HUANG[1], QUN REN[2], LINHUI JIAO[2], SHUO NIU[1], CHENGHONG LIU[1], JUAN ZHOU[3], LONGHUO WU[2] and YADONG YANG[1]

[1]Department of Orthopedics, First Affiliated Hospital of Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China; [2]College of Pharmacy, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China; [3]School of Basic Medicine, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China

Received November 21, 2023; Accepted April 26, 2024

DOI: 10.3892/etm.2024.12571

Abstract. Osteoarthritis (OA) is a disease of the joints, characterized by chronic inflammation, cartilage destruction and extracellular matrix (ECM) remodeling. Aberrant chondrocyte hypertrophy promotes cartilage destruction and OA development. Collagen X, the biomarker of chondrocyte hypertrophy, is upregulated by runt-related transcription factor 2 (Runx2), which is mediated by the bone morphogenetic protein 4 (BMP4)/Smad1 signaling pathway. BMP binding endothelial regulator (BMPER), a secreted glycoprotein, acts as an agonist of BMP4. 5,7,3',4'-tetramethoxyflavone (TMF) is a natural flavonoid derived from *Murraya exotica* L. Results of our previous study demonstrated that TMF exhibits chondroprotective effects against OA development through the activation of Forkhead box protein O3a (FOXO3a) expression. However, whether TMF suppresses chondrocyte hypertrophy through activation of FOXO3a expression and inhibition of BMPER/BMP4/Smad1 signaling remains unknown. Results of the present study revealed that TMF inhibited collagen X and Runx2 expression, inhibited BMPER/BMP4/Smad1 signaling, and activated FOXO3a expression; thus, protecting against chondrocyte hypertrophy and OA development. However, BMPER overexpression and FOXO3a knockdown impacted the protective effects of TMF. Thus, TMF inhibited chondrocyte hypertrophy in OA cartilage through mediating the FOXO3a/BMPER signaling pathway.

Correspondence to: Dr Yadong Yang, Department of Orthopedics, First Affiliated Hospital of Gannan Medical University, 128 Jinling Road, Ganzhou, Jiangxi 341000, P.R. China
E-mail: ydgogai@163.com

Key words: osteoarthritis, chondrocyte hypertrophy, BMP-binding endothelial regulatorbone morphogenetic protein 4/Smad1 signaling, Forkhead box protein O3a, 5,7,3',4'-tetramethoxyflavone

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by articular surface lesions, cartilage degeneration, chronic inflammation, subchondral bone alterations and osteophyte formation (1,2). Chronic inflammation, excessive mechanical loading, aging, obesity and trauma are all risk factors for OA (3). However, the pathological development of OA may be a result of numerous factors, including those at the cellular, ultra-structural and tissue levels (4). Current treatment options for OA in clinical practice include non-steroidal anti-inflammatory drugs and analgesics, which are the first-line treatment option for alleviating the symptoms of pain associated with OA (5). However, the aforementioned drugs do not have an effect on the progression of OA, and may lead to gastrointestinal and cardiovascular adverse events (6). Thus, novel therapeutic strategies that effectively inhibit the pathogenesis of OA are required.

OA may result due to imbalances in joint cartilage homeostasis, and a loss of articular cartilage may be facilitated by the hypertrophy-like phenotype switch of chondrocytes (7). Hypertrophic chondrocytes act as markers for numerous degenerative disorders, including OA (8). In hypertrophic chondrocytes, the expression of collagen X and runt-related transcription factor 2 (Runx2) is significantly increased (9). Collagen X is a protein marker for hypertrophic chondrocytes, and the presence of collagen X in the extracellular matrix (ECM) may be an indicator of progressive alterations in the growth plate during endochondral ossification (10). Runx2, a transcription factor expressed in pre-hypertrophic and hypertrophic chondrocytes, promotes the differentiation of proliferative chondrocytes into hypertrophic chondrocytes (11). Inactivation of Runx2 leads to decreased chondrocyte hypertrophy and reduced collagen X expression (12). This suggests that collagen X and Runx2 play key roles in chondrocyte hypertrophy and bone diseases. Moreover, the increased expression levels of collagen X and Runx2 are associated with cartilage destruction during OA development (13).

Bone morphogenetic protein (BMP)/Smads signaling may induce chondrocyte hypertrophy (14). Following treatment with BMP4, human chondrocytes exhibit an increase in the nuclear translocation of phosphorylated (p-)Smad1/5/8,

resulting in the increased expression of collagen X (15). Runx2 is also a downstream factor of the BMP4/Smad1/5/8 signaling pathway. Results of a previous study revealed that BMP4 promotes endochondral bone formation by increasing Runx2 expression (16). Inhibition of the BMP4/Smad/Runx2 pathway is associated with the suppression of osteogenic differentiation in bone marrow mesenchymal stem cells (BMSCs) (17). BMP-binding endothelial regulator (BMPER), a secreted glycoprotein, directly interacts with BMP4 to modulate its functions (18). BMPER acts as an agonist of BMP4 and mediates angiogenesis in endothelial cells (19). However, whether BMPER mediates chondrocyte hypertrophy and endochondral ossification remains unknown.

Forkhead box O (FOXO), a member of the FOX family, is a transcription factor with a highly conserved DNA-binding domain. There are four elements in the FOXO sub-family: Namely, FOXO1, FOXO3a, FOXO4 and FOXO6. FOXO3a activity is regulated by post-translational modifications, such as acetylation, phosphorylation and ubiquitination (20). The results of a previous study revealed that histone deacetylases, such as Sirt1 and Sirt3, deacetylate FOXO3a and mediate its transcriptional activity (21).

5,7,3',4'-tetramethoxyflavone (TMF) is a natural flavonoid derived from *Murraya exotica* L. The results of our previous study revealed that TMF exerts protective effects against the development of OA through activation of the Sirt1/FOXO3a signaling pathway (22). However, the molecular mechanism underlying TMF in the inhibition of OA development remains to be fully elucidated. Notably, BMPER is a downstream factor of FOXO3a (19). Thus, the present study aimed to determine whether TMF inhibited chondrocyte hypertrophy and OA progression by mediating FOXO3a/BMPER signaling.

Materials and methods

Animals. The present study (approval no. 2020365) was approved by The Institutional Animal Care and Use Committee of Gannan Medical University (Ganzhou, China), according to the principles of the Declaration of Helsinki. A total of 24 male rats (age, 8 weeks; weight, 220±20 g), provided by the Experimental Animal Center of Gannan Medical University (Ganzou, China), were housed in an SPF-grade room with a 12/12 h light/dark cycle, a temperature of 21-23˚C and a humidity of 45-55%. All animals had free access to food and water, and were left to acclimate to the conditions for 7 days.

Establishment of rat OA models. Rats were anesthetized with 3% sodium pentobarbitone (40 mg/kg) via an intraperitoneal injection (i.p.). After routine shaving and disinfection in the right knee, 10 μl of sterile saline with 1 mg of monosodium iodoacetate (MIA; Sigma-Aldrich; Merck KGaA) was intra-articularly injected (23). Rats in the negative control group received the same volume of vehicle. Rats were divided into four groups (n=6) as follows: Negative control group, MIA-induced model group, model group intragastrically treated with 25 mg/kg TMF and model group intragastrically treated with 100 mg/kg TMF (24). Signs in rats, such as excessive fur grooming, 20% loss of body weight and difficulty breathing during the experiment were considered humane endpoints, requiring immediate intervention. However, no cases required euthanasia due to observation of a humane endpoint. After 8 weeks, rats were sacrificed via cervical dislocation after euthanasia by sodium pentobarbitone (40 mg/kg; i.p.). Cartilage tissues were obtained from the joints for further examination.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). Cartilage tissues were fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology) for 48 h at 4˚C and subsequently decalcified in 10% EDTA solution for 25 days at 4˚C. Decalcified cartilage was embedded in paraffin and samples were cut into 4-μm slices. Subsequently, all samples were pre-heated for 20 min at 60˚C. The samples were then deparaffinized in xylene-I for 20 min at 60˚C and then in xylene-II for 10 min at room temperature, and rehydrated with 100, 95, 80 and 75% ethanol. H&E (Beijing Solarbio Science & Technology Co., Ltd.) staining was performed and histological evaluation was carried out. Briefly, sections were stained with hematoxylin for 5 min at 35˚C. Sections were then rinsed with running water, immersed in 1% hydrochloric acid alcohol for 5 sec, rinsed with running water, immersed in 1% aqueous ammonia for 5 sec, and rinsed with running water again. After soaking in 80, 90 and 95% alcohol for 5 min each, sections were stained with eosin for 5 min at 35˚C. Next, the stained sections were dehydrated using ascending ethanol solutions before sealing. The sections were analyzed using a confocal laser scanning microscope (LSM880; Carl Zeiss AG).

For immunohistochemical analysis, deparaffinized slices were eluted in 3% H_2O_2 to eliminate endogenous peroxidase activity. Samples were treated with goat serum (10%) for 30 min at room temperature, and subsequently incubated with the following primary antibodies: Anti-collagen X (1:200; cat. no. DF13214; Affinity Biosciences, Ltd.), anti-Runx2 (1:200; cat. no. AF5186; Affinity Biosciences, Ltd.), anti-BMPER (1:200; cat. no. AV52569; Sigma-Aldrich; Merck KGaA) or anti-FOXO3a (1:200; cat. no. AF6020; Affinity Biosciences, Ltd.) overnight at 4˚C. Following primary incubation, samples were incubated with the goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. BA1039; Boster Biological Technology) for 30 min at 37˚C. Samples were subsequently stained with DAB at room temperature for 10 min and analyzed using a confocal laser scanning microscope (LSM880; Carl Zeiss AG) and ImageJ software (version 1.51r; National Institutes of Health).

Cell culture. Human immortal C28/I2 cells (Procell Life Science & Technology Co., Ltd.) were cultured in low-glucose DMEM (Thermo Fisher Scientific, Inc.) supplemented with penicillin, streptomycin and 10% fetal bovine serum at 37˚C in an incubator with 5% CO_2. Recombinant human interleukin 1β (IL-1β; cat. no. IL038; Sigma-Aldrich; Merck KGaA) was used to establish OA-like chondrocyte models at 37˚C for 24 h. Samples were treated with TMF at concentrations of 5 and 20 μg/ml at 37˚C for 24 h (22,24,25).

Lentivirus (LV) infection. LV-BMPER and LV-short hairpin RNA (sh/shRNA)-FOXO3a lentiviral particles were constructed, screened and verified by OBiO Technology Corp. Ltd. (Shanghai, China). The coding sequence of

BMPER was cloned into the pLV-CMV-MCS-EF1-ZsGreen1-T2A-Puro vector. The FOXO3a short hairpin RNA (shRNA) was cloned into the pSLenti-U6-shRNA-CMV-E GFP-F2A-Puro-WPRE vector. The 2nd generation system was used to package the lentivirus. The lentiviral particles were prepared by transfecting 293T cells (Cell Bank of Type Culture Collection of The Chinese Academy of Science) with 12 μg of either a plasmid containing the coding sequence or an shRNA plasmid along with lentiviral packaging plasmid psPAX2 and the envelope plasmid pMD2.G at a ratio of 4:3:1 at 37˚C for 72 h. The 293T cells were cultured with DMEM containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 U/ml) at 37˚C for 48 h. The supernatant was collected by centrifugation (3,000 x g) at 4˚C for 10 min. The lentiviral particles were harvested after centrifugation (100,000 x g) at 4˚C for 120 min. Chondrocytes (5×10^4 cells/ml) were incubated into 6-well plates at 37˚C for 12-24 h to 20-30% confluency. Then, cells were infected with LV-BMPER and LV-sh-FOXO3a, according to the instructions recommended by manufacturer (OBiO Technology Corp. Ltd.). Simply, cells were transferred to serum-free media containing 5 mg/ml polybrene and lentiviral particles at a multiplicity of infection (MOI) of 10.0. The cells were cultured at 37˚C for 12 h, and the medium was replaced with a fresh complete medium. After 72 h of infection, cells were selected using puromycin-containing medium (1.5 μg/ml puromycin; Sigma-Aldrich; Merck KGaA) for 14 days, and 0.625 μg/ml puromycin was maintained for the subsequent experiments. The cells were observed under a fluorescence microscope. The infection efficiency was >90%.

Western blot analysis. Total protein was extracted from harvested C28/I2 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology), and 25 μg/lane of each sample was separated by SDS-PAGE on a 10% gel. The separated proteins were subsequently transferred onto a PVDF membrane and blocked for 1 h at 37˚C with 10% BSA Tris-buffered saline (TBS) containing 5% non-fat milk, followed by washing three times with TBS-Tween-20 (containing 0.05% Tween-20). Membranes were incubated with the following primary antibodies: Anti-collagen X (1:1,000; cat. no. DF13214; Affinity Biosciences, Ltd.), anti-Runx2 (1:1,000; cat. no. AF5186; Affinity Biosciences, Ltd.), anti-BMP4 (1:1,000; cat. no. AF5175; Affinity Biosciences, Ltd.), anti-Smad1 (1:1,000; cat. no. AF0614; Affinity Biosciences, Ltd.), anti-p-Smad1 (1:1,000; cat. no. AF8313; Affinity Biosciences, Ltd.), anti-FOXO3a (1:1,000; cat. no. AF6020; Affinity Biosciences, Ltd.), anti-BMPER (1:1,000; cat. no. AV52569; Sigma-Aldrich; Merck KGaA) and anti-β-actin (1:1,000; cat. no. AF7018; Affinity Biosciences, Ltd.) overnight at room temperature. Following primary incubation, membranes were incubated with the HRP-labeled goat anti-rabbit secondary antibody (1:5,000; cat. no. A0208; Beyotime Institute of Biotechnology) at room temperature for 1 h. Protein expression was analyzed using enhanced chemiluminescence Omni-ECL™ (cat. no. SQ101L; Epizyme; Ipsen Biopharmaceuticals, Inc.) and ImageJ software (version, 1.51r; National Institutes of Health).

Immunofluorescence assay. C28/I2 cells on glass coverslips were fixed with 4% paraformaldehyde at 37˚C for 30 min, and subsequently treated with 0.1% Triton X-100 for membrane infiltration at 37˚C for 10 min. Following blocking with 5% BSA at 37˚C for 30 min, C28/I2 cells were incubated with anti-collagen X (1:200; cat. no. DF13214; Affinity Biosciences, Ltd.) and anti-Runx2 (1:200; cat. no. AF5186; Affinity Biosciences, Ltd.) overnight at 4˚C. Following primary incubation, cells were incubated with goat anti-rabbit secondary IgG antibody (1:500; cat. no. S0001; Affinity Biosciences, Ltd.) for 1 h at room temperature. DAPI was added to cells at 37˚C for 20 min, and immunofluorescent intensity was analyzed using a confocal laser scanning microscope (Zeiss GmbH) and ImageJ software (National Institutes of Health).

Statistical analysis. Experiments were independently performed three times, and data are expressed as the mean ± standard deviation. GraphPad Prism 8 (GraphPad Software, Inc.) was used for statistical analysis. Comparisons between multiple groups were carried out using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TMF protects against chondrocyte hypertrophy in rat OA models. To explore the effects of TMF on chondrocyte hypertrophy, rat OA models were established (Fig. 1A). Results of the present study demonstrated that the cartilage in the model group was markedly damaged (Fig. 1A and B) and TMF exerted protective effects. In addition, the expression levels of collagen X (Fig. 1C and D) and Runx2 (Fig. 1E and F) were significantly increased in the cartilage of the OA group compared with those in the NC group. Consistently, TMF suppressed the expression of collagen X and Runx2, and these are biomarkers of chondrocyte hypertrophy. Thus, TMF may protect against chondrocyte hypertrophy in OA rats.

TMF inhibits chondrocyte hypertrophy in IL-1β-treated C28/I2 cells. To investigate the effects of TMF on chondrocyte hypertrophy *in vitro*, C28/I2 cells were treated with IL-1β (10 ng/ml) to establish OA-like chondrocytes. Results of the present study demonstrated that, compared with the NC group, treatment with IL-1β significantly increased the expression of collagen X (Fig. 2A and B) and Runx2 (Fig. 2A and C) in C28/I2 cells, indicating the induction of the hypertrophy-like phenotype of chondrocytes. However, treatment with TMF reversed the effects of IL-1β on C28/I2 cells. In addition, BMPER, BMP4 and p-Smad1/Smad1 ratio protein expression levels were significantly reduced following treatment with TMF, indicating that IL-1β-induced BMPER/BMP4/Smad1 signaling was suppressed in C28/I2 cells (Fig. 2A and D-F). Results of the immunofluorescent assay also demonstrated that TMF reduced the expression of collagen X and Runx2 in a concentration-dependent manner in IL-1β-treated C28/I2 cells (Fig. 2G and H). Thus, the inhibitory activity of TMF against chondrocyte hypertrophy may be associated with suppression of the BMPER/BMP4/Smad1 signaling pathway in C28/I2 cells. TMF at 20 μg/ml exhibited higher activity in protecting against IL-1β-induced chondrocyte hypertrophy than that at

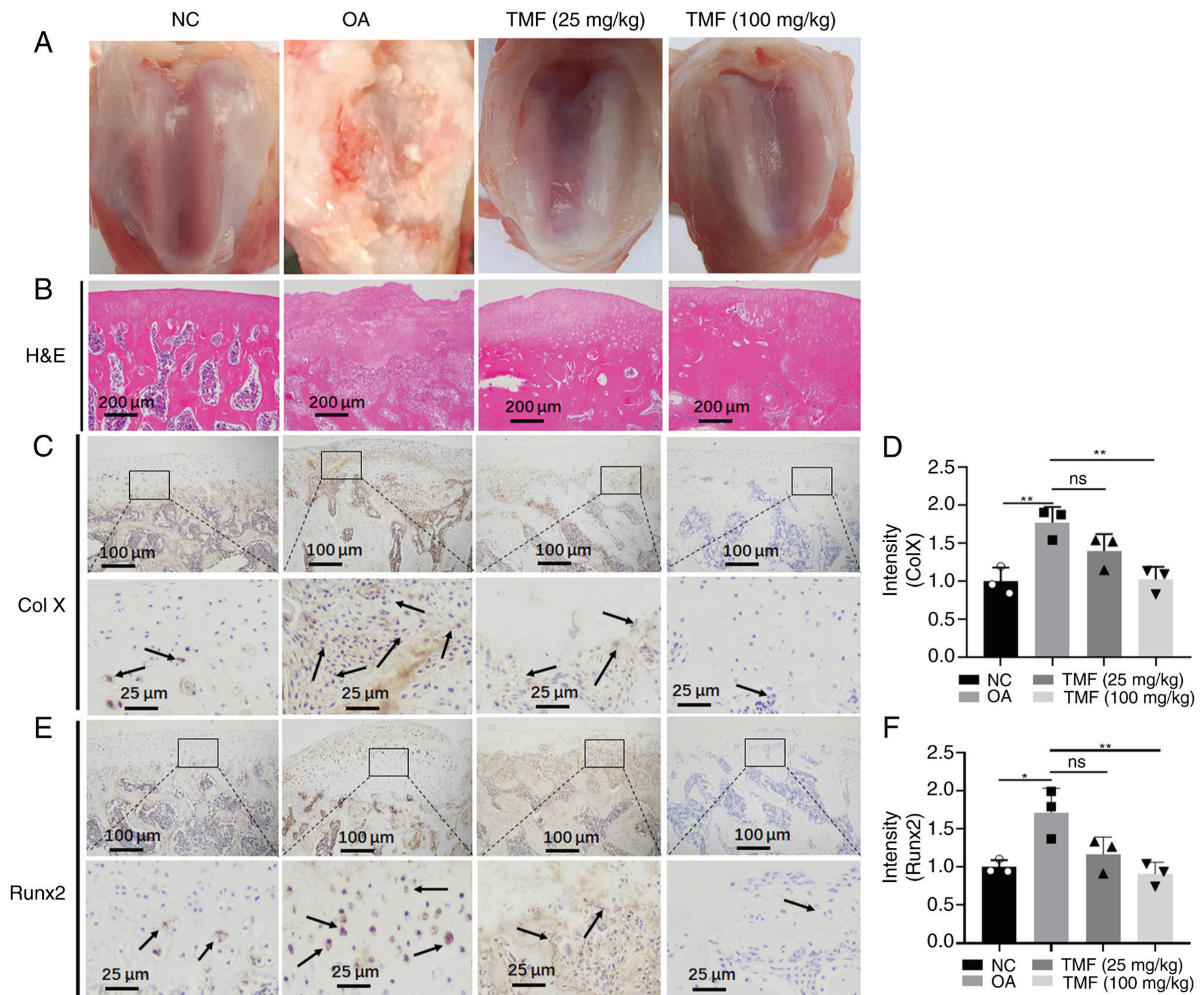

Figure 1. TMF exerts protective effects against OA development. (A) Overall observation of joint cartilage in rats. (B) H&E staining of cartilage. Immunohistochemical analysis of (C) Col X and (E) Runx2 expression levels. Immunofluorescent intensity of (D) collagen X and (F) Runx2. The arrows indicate positive staining. *P<0.05 and **P<0.01. TMF, 5,7,3',4'-tetramethoxyflavone; OA, osteoarthritis; Runx2, runt-related transcription factor 2; H&E, hematoxylin and eosin; NC, negative control; Col X, collagen X.

5 μg/ml. To better understand the protective effects of TMF, the 20 μg/ml concentration was selected for use in subsequent experiments instead of 5 μg/ml.

TMF inhibits chondrocyte hypertrophy by suppressing BMPER/BMP4 signaling. Expression levels of BMPER were significantly increased in rat OA models. However, TMF exhibited inhibitory activity against BMPER expression in the cartilage of rat OA models (Fig. 3A and B). Similar results are also displayed in Fig. 2A and D. To explore whether TMF inhibited chondrocyte hypertrophy through suppression of BMPER/BMP4 signaling, BMPER was overexpressed in C28/I2 cells. The significantly increased expression of BMPER detected by western blot analysis indicated successful infection (Fig. 3C and D). Results of the present study demonstrated that BMPER overexpression may reverse the effects of TMF on chondrocyte hypertrophy, indicated by the increased protein expression of collagen X (Fig. 3E and F) and Runx2 (Fig. 3E and G) in the BMPER-OE group compared with the TMF (20 μg/ml) group. In addition, BMPER overexpression significantly increased BMPER (Fig. 3E and H), BMP4 (Fig. 3E and I) and p-Smad1/Smad1 (Fig. 3E and J) expression levels in C28/I2 cells compared with the TMF (20 μg/ml) group. Results of the immunofluorescent assay also indicated that BMPER overexpression reversed the effects of TMF on collagen X and Runx2 expression (Fig. 3K and L). Thus, TMF inhibited chondrocyte hypertrophy through the suppression of BMPER/BMP4 signaling in C28/I2 cells.

TMF inhibits BMPER/BMP4 signaling-mediated chondrocyte hypertrophy through increasing FOXO3a expression. FOXO3a expression levels were reduced in the cartilage of rat OA models, and treatment with TMF increased FOXO3a expression (Fig. 4A and B). To further investigate the mechanisms underlying TMF-mediated inhibition of the BMPER/BMP4 signaling pathway, FOXO3a expression was silenced following the transfection of LV-sh-FOXO3a into C28/I2 cells. The significantly decreased expression of FOXO3a detected by western blot analysis indicated successful infection (Fig. 4C and D). Results of the present study demonstrated that

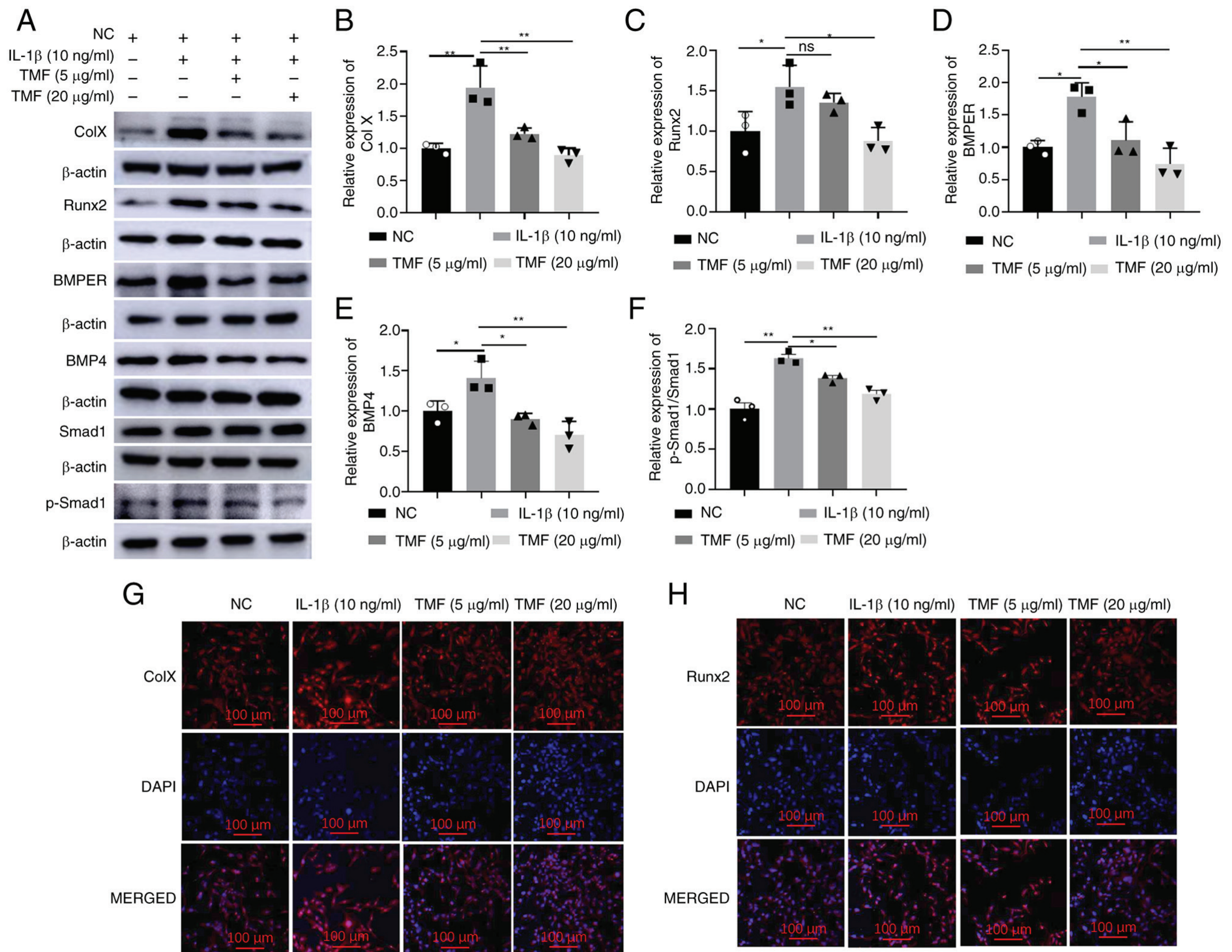

Figure 2. Effects of TMF on chondrocyte hypertrophy *in vitro*. Protein expression levels of (A and B) Col X, (A and C) Runx2, (A and D) BMPER, (A and E) BMP4 and (A and F) p-Smad1/Smad1. Immunofluorescent intensity of (G) Col X and (H) Runx2. *P<0.05 and **P<0.01. TMF, 5,7,3',4'-tetramethoxyflavone; BMPER, BMP-binding endothelial regulator; BMP4, bone morphogenetic protein 4; p-, phosphorylated; Runx2, runt-related transcription factor 2; Col X, collagen X; NC, negative control.

collagen X (Fig. 4E and F) and Runx2 (Fig. 4E and G) expression levels were significantly increased following FOXO3a knockdown compared with those in the TMF (20 μg/ml) group, highlighting that FOXO3a knockdown reversed the protective effects of TMF on chondrocyte hypertrophy in C28/I2 cells. In addition, FOXO3a knockdown also significantly increased BMPER, BMP4 and p-Smad1/Smad1 expression levels in LV-sh-FOXO3a-transfected C28/I2 cells compared with the TMF (20 μg/ml) group (Fig. 4E and H-K). Results of the immunofluorescent assay also indicated that FOXO3a knockdown reversed the effects of TMF on collagen X and Runx2 expression (Fig. 4L and M). Thus, TMF inhibited BMPER/BMP4 signaling-mediated chondrocyte hypertrophy through increasing FOXO3a expression.

Discussion

Due to the aberrant terminal hypertrophic differentiation of quiescent articular chondrocytes, chondrocyte hypertrophy is a key feature of OA (26). The development of the hypertrophic phenotype is associated with pathological ECM remodeling and cartilage destruction (27). The results of the present study revealed that MIA may severely damage rat knee joint cartilage and promote the expression of collagen X and Runx2, which are biomarkers of chondrocyte hypertrophy. *In vitro*, the BMPER/BMP4/Smad1 signaling pathway was activated, and treatment with TMF (20 μg/ml) inhibited chondrocyte hypertrophy by suppressing the BMPER/BMP4/Smad1 signaling pathway. Notably, BMPER overexpression or FOXO3a knockdown reversed the protective effects of TMF on chondrocyte hypertrophy in IL-1β-treated C28/I2 cells.

In addition to the inflammatory responses and degeneration in joint cartilage, hypertrophic phenotype alterations of chondrocytes in cartilage are key hallmarks of OA (28). Hypertrophic chondrocytes secrete catabolic enzymes, such as matrix metalloproteinase 13 (MMP-13) and a disintegrin and metalloproteinase with thrombospondin 4/5 (ADAMTS4/5), which promote cartilage destruction and OA development (29). Chondrocytes in the non-calcified zones of joint cartilage do not undergo hypertrophic phenotype alterations under normal conditions. However, OA-associated hypertrophic phenotype alterations in joint cartilage enhance pathological damage, such as calcification and vascularization (30,31). Inhibition of chondrocyte hypertrophy in joint

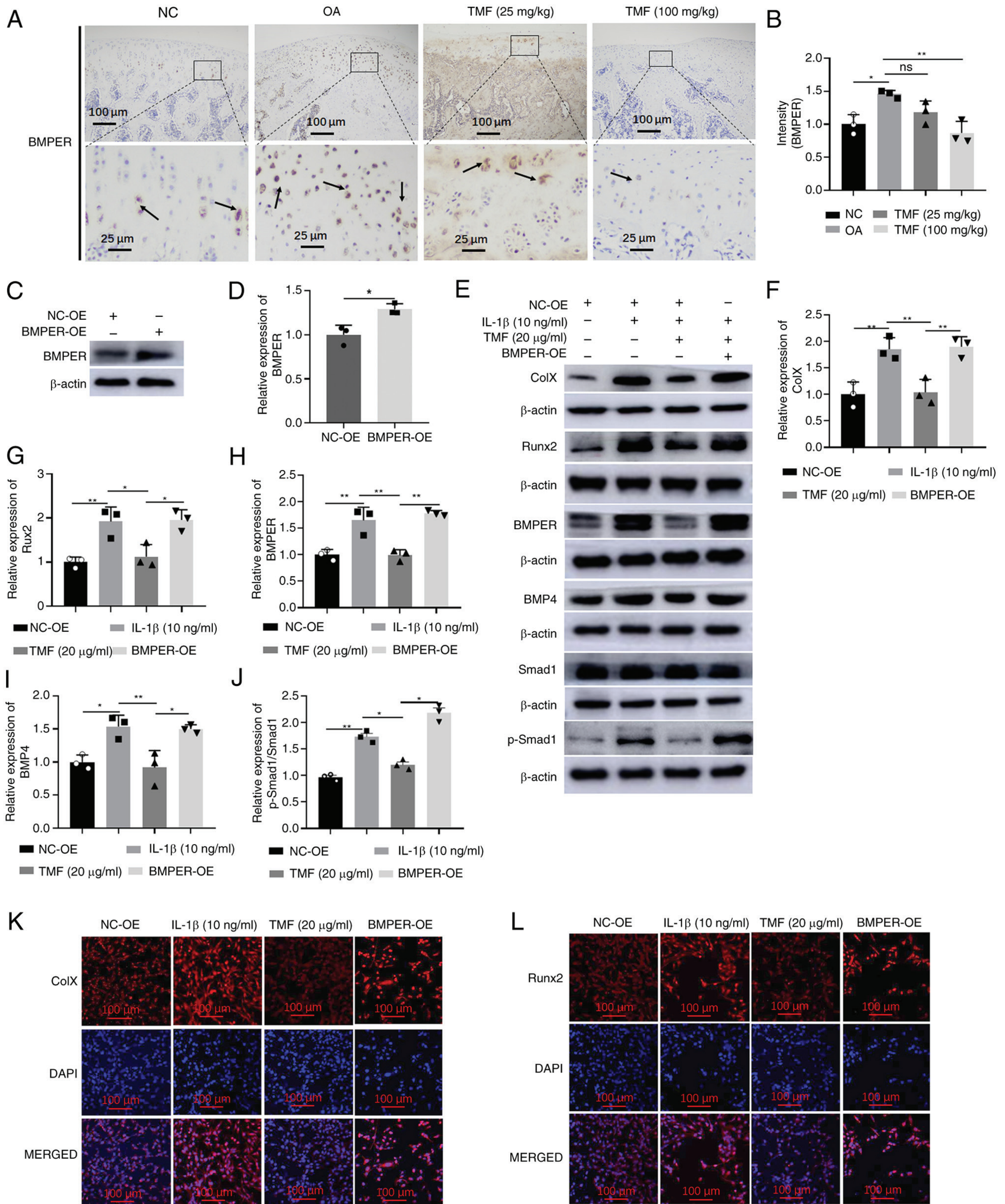

Figure 3. TMF inhibits chondrocyte hypertrophy by suppressing BMPER/BMP4 signaling. (A and B) Immunohistochemical analysis of BMPER in rat OA cartilage. The arrows indicate positive staining. (C and D) The BMPER protein expression was detected by western blot in LV-BMPER-infected C28/I2 cells. Protein expression levels of (E and F) collagen X, (E and G) Runx2, (E and H) BMPER, (E and I) BMP4 and (E and J) p-Smad1/Smad1. Immunofluorescent intensity of (K) collagen X and (L) Runx2. *P<0.05 and **P<0.01. TMF, 5,7,3',4'-tetramethoxyflavone; BMPER, BMP binding endothelial regulator; BMP4, bone morphogenetic protein 4; OA, osteoarthritis; p-, phosphorylated; NC, negative control; OE, overexpression; LV, lentivirus; Runx2, runt-related transcription factor 2.

cartilage exhibits potential as a therapeutic strategy for OA management (31). At present, numerous studies on the pathogenesis of OA are focused on inflammatory responses. Thus, further investigations are required to understand the mechanisms underlying chondrocyte hypertrophy in OA pathogenesis.

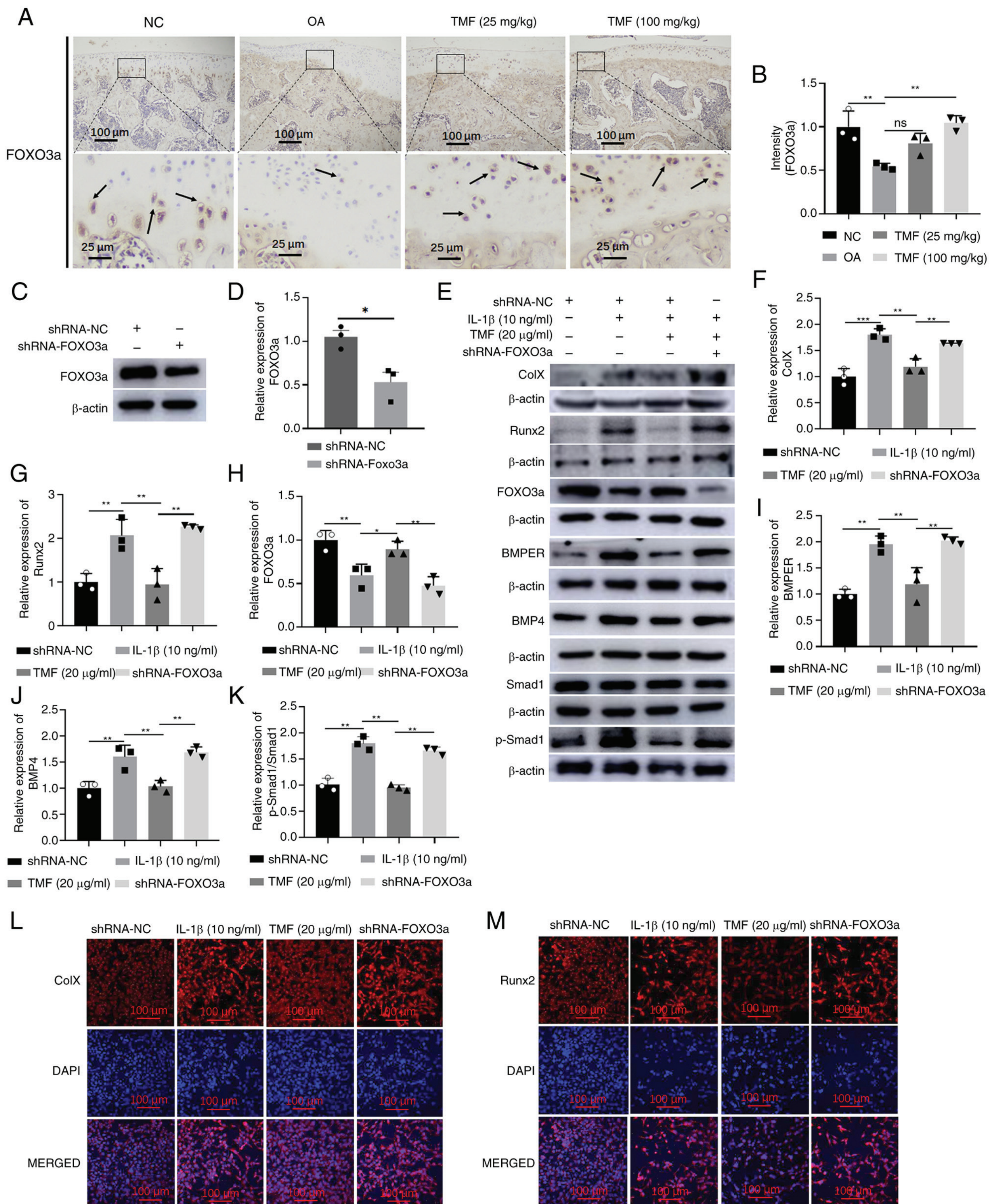

Figure 4. FOXO3a knockdown decreases the inhibitory activity of TMF against BMPER/BMP4 signaling-mediated chondrocyte hypertrophy. (A and B) Immunohistochemical analysis of FOXO3a in the cartilage of rat OA models. The arrows indicate positive staining. (C and D) The FOXO3a protein expression was detected by western blot in shRNA-FOXO3a-infected C28/I2 cells. Protein expression levels of (E and F) collagen X, (E and G) Runx2, (E and H) FOXO3a, (E and I) BMPER, (E and J) BMP4 and (E and K) p-Smad1/Smad1. Immunofluorescent intensity of (L) collagen X and (M) Runx2. *P<0.05, **P<0.01 and ***P<0.001. FOXO3a, forkhead box O 3a; TMF, 5,7,3',4'-tetramethoxyflavone; BMPER, BMP binding endothelial regulator; BMP4, bone morphogenetic protein 4; OA, osteoarthritis; p-, phosphorylated; shRNA, short hairpin RNA; Runx2, runt-related transcription factor 2.

Healthy levels of chondrocyte hypertrophy are essential for musculoskeletal development. However, OA-associated chondrocyte hypertrophy may be facilitated by the aberrant expression of BMP (32), TGFβ1 (33) and Indian Hedgehog (34) signaling pathways. Collagen X is minimally expressed in healthy joint cartilage, and increased collagen X expression

in hypertrophic chondrocytes indicates the initiation of endochondral bone formation (35). Runx2 expression is aberrantly increased in OA chondrocytes, and this promotes OA pathogenesis through increasing several target factors, such as COL10A1 (encoding collagen X), MMPs, alkaline phosphatase (ALP), osteopontin and vascular endothelial growth factor (VEGF) (36). Runx2 is mediated by various signaling pathways, such as BMP, TGFβ1 and Indian Hedgehog (37,38). Runx2 plays an important role in chondrocyte hypertrophy (38). Results of the present study revealed that collagen X and Runx2 expression levels were significantly increased *in vivo* and *in vitro*, highlighting the hypertrophic alterations of OA chondrocytes.

The BMP signaling pathway is an important mediator of chondrocyte hypertrophy and endochondral ossification. Activation of BMP signaling may result in phosphorylation of Smad proteins, such as Smad-1, -5 and -8, which are transcriptional factors that mediate the expression of downstream factors (39). Exogenous BMP4 increases the chondrocyte differentiation of bone marrow stem cells and promotes endochondral bone formation (40). Exogenous BMP4 promotes cartilage maturation and induces chondrocyte hypertrophy (41). Results of a previous study have revealed that exogenous BMP4 decreases the expression of collagen II, and increases the expression of collagen X and ALP in juvenile idiopathic arthritis synovial fibroblasts (42). Results of another previous study have revealed that treatment with the antagonist Noggin inhibits BMP4, which ultimately inhibits the late differentiation of mesenchymal stem cells and chondrocyte hypertrophy (43). Results of the present study revealed that IL-1β increased the expression of the BMP4/Smad1 signaling pathway and its downstream factors, such as collagen X and Runx2. Collectively, these results indicated that activation of the BMP4/Smad1 signaling pathway was associated with hypertrophic phenotype alterations of OA chondrocytes.

BMPER, also known as the vertebrate homolog of Drosophila cross-veinless 2, was originally identified in a screening assay for differentially expressed proteins in embryonic endothelial precursor cells (44). BMPER interacts with BMP-2, -4, -6, -7, -9 and BMP receptor Ia/b (45,46), and activates the BMP signaling pathway in a concentration-dependent manner (47). Results of a previous study have revealed that exogenous BMPER at low molar concentrations enhances Smad activation (47). However, at higher molar concentrations, BMPER may act as an inhibitor of BMP4 (47). BMPER overexpression increases BMP-2-induced osteogenic differentiation, VEGF expression and vascularization (48). BMPER knockdown inhibits the osteogenic differentiation of cyclic tensile strain-induced ossification in posterior longitudinal ligament cells (49). In human vascular smooth muscle cells, BMPER knockdown significantly decreases the expression of ALP and Runx-2, both of which are regulated by BMP2/Smad1/5/8 signaling (50). However, results of a previous study revealed that the absence of BMPER in the atrioventricular cushions increases BMP2/Smad1/5/8 signaling and SOX9 expression (51). In addition, in the presence of twisted gastrulation, full-length BMPER inhibits BMP-signaling in C2C12 cells (52). The results of a previous study have also demonstrated that BMPER exerts positive and negative effects on BMP activity, and these effects are context-dependent (53). At present, the specific association between BMPER and BMP4 remains controversial. The results of the present study revealed that the expression of BMPER in OA chondrocytes was increased. In addition, BMPER overexpression activated the BMP4/Smad1 signaling pathway and increased the expression of collagen X and Runx2. These results suggested that activation of the BMPER/BMP4 signaling pathway promoted chondrocyte hypertrophy in OA cartilage.

Results of a previous study demonstrated that increased FOXO3a expression or inhibited FOXO3a degradation may inhibit cell apoptosis (54). DL-3-n-butylphthalide inhibits PI3K/AKT signaling and increases FOXO3a expression, leading to the inhibition of human OA chondrocyte apoptosis and increased ECM synthesis (55). The results of a previous study have revealed that FOXO3a and peroxisome proliferators-activated receptor γ coactivator 1α (PGC-1α) increase AMPK activity and inhibit catabolic responses in chondrocytes by suppressing oxidative stress (56). Notably, the results of a previous study have revealed the positive role of FOXO3a in OA chondrocytes (57). FOXO3a interacts with BMPER and negatively modulates its activity, leading to suppression of BMP4/Smad1/5 signaling (19). In addition, deletion of FOXO3a results in increased BMPER/BMP4 signaling (19). The results of a previous study have revealed that BMP4 activates FOXO3a by regulating PI3K/AKT signaling in glioma stem cells (58). In the present study, FOXO3a knockdown increased activation of BMPER/BMP4/Smad1 signaling and promoted chondrocyte hypertrophy.

Moreover, the results of previous studies have demonstrated that Sirt1 acts as a positive upstream factor of FOXO3a (59,60). Results of our previous study revealed that activation of Sirt1 increases the expression of FOXO3a (22). TMF is a main bioactive component derived from *M. exotica*, which exerts multiple pharmacological activities, including anti-inflammatory, antioxidant, analgesic, anti-diabetes and antinociception properties (61,62). TMF may exhibit potential as an activator of Sirt1, enhancing the expression of FOXO3a and mediating cholesterol metabolism in OA chondrocytes (22). TMF also decreases the production of pro-inflammatory cytokines and exhibits chondroprotective activity by inhibiting the EP/cAMP/PKA and the β-catenin signaling pathways in OA chondrocytes (24,63). In the present study, TMF activated FOXO3a expression, inhibited BMPER/BMP4/Smad1 signaling and suppressed chondrocyte hypertrophy in OA chondrocytes.

The present study has a number of limitations. In the IHC assays, three samples (six in total) were randomly selected for analysis. Further studies on the total samples are still needed. It is essential to explore the dose response and time-dependence of TMF on chondrocyte hypertrophy in OA cartilage, and these were not determined in the present study. *In vitro* study, two concentrations of TMF were studied to inhibit the expression of ColX and Runx-2, which are the biomarkers of OA chondrocyte hypertrophy (64). However, the absence of more concentrations to validate the concentration-dependent TMF inhibition of OA chondrocyte hypertrophy was also a limitation of the present study. In addition, the association between FOXO3a and BMPER was not verified, and the cellular distribution of FOXO3a and BMPER was also not detected. The effects of BMPER overexpression and FOXO3a

knockdown on OA chondrocytes were investigated; however, further investigations into the effects of BMPER knockdown and FOXO3a overexpression on OA chondrocytes are required. The study on BMPER gene knockout mice will be explored in our future plans. In addition, the effects of exogenous BMPER and BMP4 on TMF-protected chondrocyte hypertrophy requires further validation. The nuclear translocation of p-Smad1 and the mechanisms underlying FOXO3a mediation of BMP2/4 were also not determined in the present study, highlighting the requirement for further investigations. The impact of TMF on the activity of BMP receptors remains unknown, and the protective effects of TMF on chondrocytes derived from patients with OA require further validation.

In conclusion, the results of the present study revealed that collagen X and Runx2 expression levels and the BMPER/BMP4/Smad1 signaling pathway were increased in the cartilage of rat OA models. TMF exhibited protective activities against chondrocyte hypertrophy by activating FOXO3 expression and inhibiting the BMPER/BMP4/Smad1 signaling pathway. The results of the present study also demonstrated that BMPER overexpression and FOXO3a knockdown reversed the protective effects of TMF. Thus, TMF inhibited chondrocyte hypertrophy by mediating the FOXO3a/BMPER/BMP4/Smad1 signaling pathway.

Acknowledgements

Not applicable.

Funding

The present study was supported by Jiangxi Provincial Natural Science (grant no. 20212ACB206002).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YY and LW designed and conceptualized the study. JH, QR, LJ, SN, CL and JZ conducted the experiments and revised the manuscript. JH and YY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study (approval no. 2020365) was approved by The Institutional Animal Care and Use Committee of Gannan Medical University (Ganzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Wang CJ, Cheng JH, Chou WY, Hsu SL, Chen JH and Huang CY: Changes of articular cartilage and subchondral bone after extracorporeal shockwave therapy in osteoarthritis of the knee. Int J Med Sci 14: 213-223, 2017.
2. Li X, Chen W, Liu D, Chen P, Wang S, Li F, Chen Q, Lv S, Li F, Chen C, *et al*: Pathological progression of osteoarthritis: A perspective on subchondral bone. Front Med: Apr 15, 2024 (Epub ahead of print).
3. Wojdasiewicz P, Poniatowski ŁA and Szukiewicz D: The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. Mediators Inflamm 2014: 561459, 2014.
4. Goldring MB and Otero M: Inflammation in osteoarthritis. Curr Opin Rheumatol 23: 471-478, 2011.
5. Kong H, Han JJ, Dmitrii G and Zhang XA: Phytochemicals against osteoarthritis by inhibiting apoptosis. Molecules 29: 1487, 2024.
6. Pelletier JP, Martel-Pelletier J, Rannou F and Cooper C: Efficacy and safety of oral NSAIDs and analgesics in the management of osteoarthritis: Evidence from real-life setting trials and surveys. Semin Arthritis Rheum 45 (4 Suppl): S22-S27, 2016.
7. Thielen NGM, Neefjes M, Vitters EL, van Beuningen HM, Blom AB, Koenders MI, van Lent PLEM, van de Loo FAJ, Blaney Davidson EN, van Caam APM and van der Kraan PM: Identification of transcription factors responsible for a transforming growth factor-β-driven hypertrophy-like phenotype in human osteoarthritic chondrocytes. Cells 11: 1232, 2022.
8. Park S, Bello A, Arai Y, Ahn J, Kim D, Cha KY, Baek I, Park H and Lee SH: Functional duality of chondrocyte hypertrophy and biomedical application trends in osteoarthritis. Pharmaceutics 13: 1139, 2021.
9. van der Kraan PM and van den Berg WB: Chondrocyte hypertrophy and osteoarthritis: Role in initiation and progression of cartilage degeneration? Osteoarthritis Cartilage 20: 223-232, 2012.
10. von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Glückert K and Stöss H: Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 35: 806-811, 1992.
11. Ferrao Blanco MN, Bastiaansen-Jenniskens YM, Chambers MG, Pitsillides AA, Narcisi R and van Osch GJVM: Effect of inflammatory signaling on human articular chondrocyte hypertrophy: Potential involvement of tissue repair macrophages. Cartilage 13 (2 Suppl): 168S-174S, 2021.
12. Yoshida CA, Yamamoto H, Fujita T, Furuichi T, Ito K, Inoue K, Yamana K, Zanma A, Takada K, Ito Y and Komori T: Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Genes Dev 18: 952-963, 2004.
13. Chen N, Wu RWH, Lam Y, Chan WCW and Chan D: Hypertrophic chondrocytes at the junction of musculoskeletal structures. Bone Rep 19: 101698, 2023.
14. Kawashima K, Ogawa H, Komura S, Ishihara T, Yamaguchi Y, Akiyama H and Matsumoto K: Heparan sulfate deficiency leads to hypertrophic chondrocytes by increasing bone morphogenetic protein signaling. Osteoarthritis Cartilage 28: 1459-1470, 2020.
15. Saitta B, Elphingstone J, Limfat S, Shkhyan R and Evseenko D: CaMKII inhibition in human primary and pluripotent stem cell-derived chondrocytes modulates effects of TGFβ and BMP through SMAD signaling. Osteoarthritis Cartilage 27: 158-171, 2019.
16. Li G, Peng H, Corsi K, Usas A, Olshanski A and Huard J: Differential effect of BMP4 on NIH/3T3 and C2C12 cells: Implications for endochondral bone formation. J Bone Miner Res 20: 1611-1623, 2005.
17. Liang C, Sun R, Xu Y, Geng W and Li J: Effect of the abnormal expression of BMP-4 in the blood of diabetic patients on the osteogenic differentiation potential of alveolar BMSCs and the rescue effect of metformin: A bioinformatics-based study. Biomed Res Int 2020: 7626215, 2020.
18. Helbing T, Wiltgen G, Hornstein A, Brauers EZ, Arnold L, Bauer A, Esser JS, Diehl P, Grundmann S, Fink K, *et al*: Bone morphogenetic protein-modulator BMPER regulates endothelial barrier function. Inflammation 40: 442-453, 2017.
19. Heinke J, Wehofsits L, Zhou Q, Zoeller C, Baar KM, Helbing T, Laib A, Augustin H, Bode C, Patterson C and Moser M: BMPER is an endothelial cell regulator and controls bone morphogenetic protein-4-dependent angiogenesis. Circ Res 103: 804-812, 2008.

20. Jin H, Zhang L, He J, Wu M, Jia L and Guo J: Role of FOXO3a transcription factor in the regulation of liver oxidative injury. Antioxidants (Basel) 11: 2478, 2022.
21. Zhao X, Liu Y, Zhu G, Liang Y, Liu B, Wu Y, Han M, Sun W, Han Y, Chen G and Jiang J: SIRT1 downregulation mediated Manganese-induced neuronal apoptosis through activation of FOXO3a-Bim/PUMA axis. Sci Total Environ 646: 1047-1055, 2019.
22. Peng F, Huang X, Shi W, Xiao Y, Jin Q, Li L, Xu D and Wu L: 5,7,3',4'-Tetramethoxyflavone ameliorates cholesterol dysregulation by mediating SIRT1/FOXO3a/ABCA1 signaling in osteoarthritis chondrocytes. Future Med Chem 13: 2153-2166, 2021.
23. Sakamoto J, Miyahara S, Motokawa S, Takahashi A, Sasaki R, Honda Y and Okita M: Regular walking exercise prior to knee osteoarthritis reduces joint pain in an animal model. PLoS One 18: e0289765, 2023.
24. Wu L, Liu H, Li L, Liu H, Yang K, Liu Z and Huang H: 5,7,3',4'-Tetramethoxyflavone exhibits chondroprotective activity by targeting β-catenin signaling in vivo and in vitro. Biochem Biophys Res Commun 452: 682-688, 2014.
25. Yang J, Liu H, Li L, Liu H, Shi W and Wu L: The chondroprotective role of TMF in PGE2-induced apoptosis associating with endoplasmic reticulum stress. Evid Based Complement Alternat Med 2015: 297423, 2015.
26. Horváth E, Sólyom Á, Székely J, Nagy EE and Popoviciu H: Inflammatory and metabolic signaling interfaces of the hypertrophic and senescent chondrocyte phenotypes associated with osteoarthritis. Int J Mol Sci 24: 16468, 2023.
27. Abou-Jaoude A, Courtes M, Badique L, Elhaj Mahmoud D, Abboud C, Mlih M, Justiniano H, Milbach M, Lambert M, Lemle A, *et al*: ShcA promotes chondrocyte hypertrophic commitment and osteoarthritis in mice through RunX2 nuclear translocation and YAP1 inactivation. Osteoarthritis Cartilage 30: 1365-1375, 2022.
28. Yoon DS, Kim EJ, Cho S, Jung S, Lee KM, Park KH, Lee JW and Kim SH: RUNX2 stabilization by long non-coding RNAs contributes to hypertrophic changes in human chondrocytes. Int J Biol Sci 19: 13-33, 2023.
29. Hu Q and Ecker M: Overview of MMP-13 as a promising target for the treatment of osteoarthritis. Int J Mol Sci 22: 1742, 2021.
30. Dreier R: Hypertrophic differentiation of chondrocytes in osteoarthritis: The developmental aspect of degenerative joint disorders. Arthritis Res Ther 12: 216, 2010.
31. Shigley C, Trivedi J, Meghani O, Owens BD and Jayasuriya CT: Suppressing chondrocyte hypertrophy to build better cartilage. Bioengineering (Basel) 10: 741, 2023.
32. Dicks AR, Maksaev GI, Harissa Z, Savadipour A, Tang R, Steward N, Liedtke W, Nichols CG, Wu CL and Guilak F: Skeletal dysplasia-causing TRPV4 mutations suppress the hypertrophic differentiation of human iPSC-derived chondrocytes. Elife 12: e71154, 2023.
33. Lian C, Tao T, Su P, Liao Z, Wang X, Lei Y, Zhao P and Liu L: Targeting miR-18a sensitizes chondrocytes to anticytokine therapy to prevent osteoarthritis progression. Cell Death Dis 11: 947, 2020.
34. Cong L, Jiang P, Wang H, Huang L, Wu G, Che X, Wang C, Li P, Duan Q, Guo X and Li P: MiR-1 is a critical regulator of chondrocyte proliferation and hypertrophy by inhibiting Indian hedgehog pathway during postnatal endochondral ossification in miR-1 overexpression transgenic mice. Bone 165: 116566, 2022.
35. Hoyland JA, Thomas JT, Donn R, Marriott A, Ayad S, Boot-Handford RP, Grant ME and Freemont AJ: Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. Bone Miner 15: 151-163, 1991.
36. Chawla S, Mainardi A, Majumder N, Dönges L, Kumar B, Occhetta P, Martin I, Egloff C, Ghosh S, Bandyopadhyay A and Barbero A: Chondrocyte hypertrophy in osteoarthritis: Mechanistic studies and models for the identification of new therapeutic strategies. Cells 11: 4034, 2022.
37. Bae SC, Lee KS, Zhang YW and Ito Y: Intimate relationship between TGF-beta/BMP signaling and runt domain transcription factor, PEBP2/CBF. J Bone Joint Surg Am 83-A (Suppl 1): S48-S55, 2001.
38. Nishimura R, Hata K, Takahata Y, Murakami T, Nakamura E and Yagi H: Regulation of cartilage development and diseases by transcription factors. J Bone Metab 24: 147-153, 2017.
39. Nordin K and LaBonne C: Sox5 is a DNA-binding cofactor for BMP R-Smads that directs target specificity during patterning of the early ectoderm. Dev Cell 31: 374-382, 2014.
40. Simonds MM, Schlefman AR, McCahan SM, Sullivan KE, Rose CD and Brescia AMC: The culture microenvironment of juvenile idiopathic arthritis synovial fibroblasts is favorable for endochondral bone formation through BMP4 and repressed by chondrocytes. Pediatr Rheumatol Online J 19: 72, 2021.
41. Shum L, Wang X, Kane AA and Nuckolls GH: BMP4 promotes chondrocyte proliferation and hypertrophy in the endochondral cranial base. Int J Dev Biol 47: 423-431, 2003.
42. Simonds MM, Schlefman AR, McCahan SM, Sullivan KE, Rose CD and Brescia AC: Juvenile idiopathic arthritis fibroblast-like synoviocytes influence chondrocytes to alter BMP antagonist expression demonstrating an interaction between the two prominent cell types involved in endochondral bone formation. Pediatr Rheumatol Online J 18: 89, 2020.
43. Karl A, Olbrich N, Pfeifer C, Berner A, Zellner J, Kujat R, Angele P, Nerlich M and Mueller MB: Thyroid hormone-induced hypertrophy in mesenchymal stem cell chondrogenesis is mediated by bone morphogenetic protein-4. Tissue Eng Part A 20: 178-188, 2014.
44. Moser M, Binder O, Wu Y, Aitsebaomo J, Ren R, Bode C, Bautch VL, Conlon FL and Patterson C: BMPER, a novel endothelial cell precursor-derived protein, antagonizes bone morphogenetic protein signaling and endothelial cell differentiation. Mol Cell Biol 23: 5664-5679, 2003.
45. Yao Y, Jumabay M, Ly A, Radparvar M, Wang AH, Abdmaulen R and Boström KI: Crossveinless 2 regulates bone morphogenetic protein 9 in human and mouse vascular endothelium. Blood 119: 5037-5047, 2012.
46. Pankratz F, Maksudova A, Goesele R, Meier L, Proelss K, Marenne K, Thut AK, Sengle G, Correns A, Begelspacher J, *et al*: BMPER improves vascular remodeling and the contractile vascular SMC phenotype. Int J Mol Sci 24: 4950, 2023.
47. Kelley R, Ren R, Pi X, Wu Y, Moreno I, Willis M, Moser M, Ross M, Podkowa M, Attisano L and Patterson C: A concentration-dependent endocytic trap and sink mechanism converts Bmper from an activator to an inhibitor of Bmp signaling. J Cell Biol 184: 597-609, 2009.
48. Xiao F, Wang C, Wang C, Gao Y, Zhang X and Chen X: BMPER enhances bone formation by promoting the osteogenesis-angiogenesis coupling process in mesenchymal stem cells. Cell Physiol Biochem 45: 1927-1939, 2018.
49. Ji N and Yu Z: IL-6/Stat3 suppresses osteogenic differentiation in ossification of the posterior longitudinal ligament via miR-135b-mediated BMPER reduction. Cell Tissue Res 391: 145-157, 2023.
50. Satomi-Kobayashi S, Kinugasa M, Kobayashi R, Hatakeyama K, Kurogane Y, Ishida T, Emoto N, Asada Y, Takai Y, Hirata K and Rikitake Y: Osteoblast-like differentiation of cultured human coronary artery smooth muscle cells by bone morphogenetic protein endothelial cell precursor-derived regulator (BMPER). J Biol Chem 287: 30336-30345, 2012.
51. Dyer L, Lockyer P, Wu Y, Saha A, Cyr C, Moser M, Pi X and Patterson C: BMPER promotes epithelial-mesenchymal transition in the developing cardiac cushions. PLoS One 10: e0139209, 2015.
52. Lockhart-Cairns MP, Lim KTW, Zuk A, Godwin ARF, Cain SA, Sengle G and Baldock C: Internal cleavage and synergy with twisted gastrulation enhance BMP inhibition by BMPER. Matrix Biol 77: 73-86, 2019.
53. He 何璇 XA, Berenson A, Bernard M, Weber C, Cook L, Visel A, Fuxman Bass JI and Fisher S: Identification of conserved skeletal enhancers associated with craniosynostosis risk genes. Hum Mol Genet: ddad182, 2023 (Epub ahead of print).
54. Chen L, Li S, Zhu J, You A, Huang X, Yi X and Xue M: Mangiferin prevents myocardial infarction-induced apoptosis and heart failure in mice by activating the Sirt1/FoxO3a pathway. J Cell Mol Med 25: 2944-2955, 2021.
55. Zhang Y, Dai J, Yan L, Lin Q, Miao H, Wang X, Wang J and Sun Y: DL-3-N-butylphthalide promotes cartilage extracellular matrix synthesis and inhibits osteoarthritis development by regulating FoxO3a. Oxid Med Cell Longev 2022: 9468040, 2022.
56. Zhao X, Petursson F, Viollet B, Lotz M, Terkeltaub R and Liu-Bryan R: Peroxisome proliferator-activated receptor γ coactivator 1α and FoxO3A mediate chondroprotection by AMP-activated protein kinase. Arthritis Rheumatol 66: 3073-3082, 2014.
57. Jiang A, Xu P, Yang Z, Zhao Z, Tan Q, Li W, Song C, Dai H and Leng H: Increased Sparc release from subchondral osteoblasts promotes articular chondrocyte degeneration under estrogen withdrawal. Osteoarthritis Cartilage 31: 26-38, 2023.

58. Ciechomska IA, Gielniewski B, Wojtas B, Kaminska B and Mieczkowski J: EGFR/FOXO3a/BIM signaling pathway determines chemosensitivity of BMP4-differentiated glioma stem cells to temozolomide. Exp Mol Med 52: 1326-1340, 2020.
59. Wan Q, Tang L, Jin K, Chen X, Li Y and Xu X: Quercetin and tanshinone prevent mitochondria from oxidation and autophagy to inhibit KGN cell apoptosis through the SIRT1/SIRT3-FOXO3a axis. Cell Mol Biol (Noisy-le-grand) 70: 257-263, 2024.
60. Qiu CW, Chen B, Zhu HF, Liang YL and Mao LS: Gastrodin alleviates cisplatin nephrotoxicity by inhibiting ferroptosis via the SIRT1/FOXO3A/GPX4 signaling pathway. J Ethnopharmacol 319: 117282, 2024.
61. Wu L, Li P, Wang X, Zhuang Z, Farzaneh F and Xu R: Evaluation of anti-inflammatory and antinociceptive activities of *Murraya exotica*. Pharm Biol 48: 1344-1353, 2010.
62. Rehman R, Anila, Muzaffar R, Arshad F, Hussain R and Altaf AA: Diversity in phytochemical composition and medicinal value of Murraya paniculata. Chem Biodivers 20: e202200396, 2023.
63. Wu L, Liu H, Zhang R, Li L, Li J, Hu H and Huang H: Chondroprotective activity of *Murraya exotica* through inhibiting β-catenin signaling pathway. Evid Based Complement Alternat Med 2013: 752150, 2013.
64. Liu W, Feng M and Xu P: From regeneration to osteoarthritis in the knee joint: The role shift of cartilage-derived progenitor cells. Front Cell Dev Biol 10: 1010818, 2022.

Copyright © 2024 Huang et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.