The GSK-3β/β-catenin signaling pathway is involved in HMGB1-induced chondrocyte apoptosis and cartilage matrix degradation

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Abstract. Knee osteoarthritis (KOA) is a common joint disease with a high incidence rate among middle-aged and elderly individuals. However, the precise underlying pathological mechanisms and effective treatment of this disease remain to be determined. To explore the effect of high mobility group box 1 (HMGB1) on chondrocyte apoptosis and catabolism, the ATDC5 cell line was cultured as an *in vitro* model for cartilage research. Cultured cells were treated with recombinant HMGB1 at different concentrations. Hoechst staining and flow cytometry demonstrated that HMGB1 administration significantly induced apoptosis of ATDC5 cells, which was the same as the effect of interleukin-1β treatment. HMGB1 also induced cartilage matrix degradation, as shown by Alcian blue staining. Moreover, HMGB1 markedly upregulated the expression levels of matrix metallopeptidases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), while genetic silencing of HMGB1 significantly suppressed their expressions. The glycogen synthase kinase (GSK)-3β/β-catenin pathway was activated upon HMGB1 treatment. Pharmacological inhibitors or HMGB1 knockdown inactivated the GSK-3β/β-catenin pathway, inhibited the expression levels of downstream genes, including MMPs and ADAMTS, and attenuated the apoptosis of ATDC5 cells. Furthermore, the data demonstrated that HMGB1 promoted chondrocyte dysfunction via the regulation of estrogen sulfotransferase and Runt-related transcription factor 2. Thus, the findings of the present study demonstrated that HMGB1 induces chondrocyte cell apoptosis via activation of GSK-3β/β-catenin and the subsequent expression of multiple targeted genes.

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Introduction

Osteoarthritis (OA) is a joint disease commonly occurring in middle-aged and elderly individuals (1,2), in which the bones, synovium, joint capsule and other structures develop chronic aseptic inflammation due to degenerative changes in the joint. OA can affect multiple joints of the body, particularly the joints of the lower limb, among which knee OA (KOA) has the highest incidence rate, with ~250 million patients with KOA reported in 2010, accounting for 3.6% of the total world population (1). Among individuals aged >65 years, this percentage may be as high as 60%. The main symptoms of KOA are joint pain, loss of function and stiffness, which can cause inconvenience or even disability, resulting in the loss of the ability to work and a significant socioeconomic burden. Multiple causes of KOA have been identified, including joint injuries, abnormal joint development and genetic factors. Previous studies have demonstrated that obesity, high joint pressure or mild inflammation in the joints are risk factors for the development of KOA (3). The molecular mechanism of KOA is complicated, involving a variety of cell types and signaling pathways. Thus, the mechanism underlying the occurrence and development of KOA requires further investigation (4).

High mobility group box protein 1 (HMGB1) is a non-histone in the nucleus, the biological activity of which varies with its location and post-translational modifications. Accumulating evidence suggests that the mRNA expression levels of HMGB1 and receptor for advanced glycation end products (RAGE) are increased in patients with KOA. Cartilage is mainly composed of chondrocytes and various extracellular matrix proteins, including highly sulfated polymeric protein polysaccharides, small proteoglycans and collagen (3). During the development of OA, the synthetic catabolic balance of the cartilage matrix is dysregulated. Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), which are secreted by chondrocytes, are the major extracellular matrix-degrading enzymes. In rheumatoid arthritis and synovial fibroblasts from patients with KOA, HMGB1 forms a complex with lipopolysaccharide, interleukin (IL)-1α or IL-1β, promoting the production of pro-inflammatory cytokines and MMPs (5). Another study has demonstrated that HMGB1 increases the expression level of MMP3 and MMP13 in wild-type mouse chondrocytes, whereas this effect was not

observed in the knee joint chondrocytes of toll-like receptor (TLR)2/TLR4$^{-/-}$ mice (6). In addition, HMGB1 promotes chondrocyte apoptosis (7). These results suggest that HMGB1 may promote the production of MMPs in chondrocytes through the TLR2/TLR4 receptor pathway and that HMGB1 may promote the apoptosis of chondrocytes and the deterioration of KOA. However, the specific underlying molecular mechanism remains unclear.

Numerous signaling pathways are involved in the process of OA, including bone morphogenetic proteins (8), Indian hedgehog (9), hypoxia-induced and Wnt signaling pathways (10,11). As an important component of the Wnt signaling pathway, β-catenin is activated by Wnt family proteins and participates in the pathological process of KOA (12). Previous studies have also demonstrated that the Wnt signaling pathway is involved in the homeostasis and degradation of the cartilage matrix by regulating the expression of anabolic or catabolic genes, such as increased expression of MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS4 and ADAMTS5, which promote the degradation of the extracellular matrix and trigger KOA (13). Thus, the Wnt/β-catenin signaling pathway may enhance the degradation of articular cartilage matrix and promote the occurrence and development of KOA by increasing the expression of MMPs and ADAMTs.

Recent studies have revealed that, during epithelial-to-mesenchymal transition (EMT), overexpression of HMGB1 promotes AKT phosphorylation and triggers the inactivation of glycogen synthase kinase (GSK)-3β, leading to the intracellular aggregation of GSK-3β and the transfer of β-catenin into the nucleus; HMGB1 also affects the expression of the corresponding EMT marker gene β-catenin, and RAGE is involved in this process, suggest that HMGB1 may activate the Wnt signaling pathway by binding to RAGE to activate intracellular phosphoinositide 3-kinase (PI3K)/AKT, thus inducing EMT (14).

HMGB1 has been previously studied in OA; it is highly expressed in the articular cartilage and synovium and plays an important role in the process of KOA (15,16). However, the specific mechanism underlying the activity of HMGB1 in KOA has not been fully elucidated. The aim of the present study was to determine how HMGB1 regulates the PI3K/AKT/GSK-3β/β-catenin signaling pathway.

Materials and methods

Cell line and manipulation. The ATDC5 mouse pre-chondral cell line is a universal cell model for extracellular study of cartilage derived from mouse teratoma cells. The ATDC5 cell line was obtained from ATCC and was cultured according to the ATCC protocol. Cells were seeded into a 96-well plate at $7x10^3$ cells/well and cultured in a humidified incubator with 5% CO_2 at 37˚C overnight in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Subsequently, ATDC5 cells were cultured in ITS (containing $3x10^{-8}$ mol/l sodium selenite, 5.5 μM/ml human transferrin and 10 μM/ml bovine insulin) for 14 days and allowed to differentiate into chondrocytes. To study the effects of HMGB1 on chondrocyte apoptosis and catabolism, the chondrocytes were divided into two groups and treated with HMGB1 [0, 10, 50, 100 or 300 ng/ml recombinant HMGB1 protein, with purity >95% (SDS-PAGE), purchased from Abcam (cat. no. ab167718)] or IL-1β (5 ng/ml) and PBS. To study the mechanism of KOA, the chondrocytes were divided into four groups: i) Chondrocytes + HMGB1; ii) chondrocytes + HMGB1 + LY294002 [10 μM (17); PI3K inhibitor]; iii) chondrocytes + HMGB1 + MK-2206 [1 μM (18); AKT inhibitor]; iv) chondrocytes + SB415286 [10 μM (19); GSK-3β inhibitor]. The concentration of HMGB1 used was 100 ng/ml. Cells were harvested at 48 h for further analysis. The experiment was performed in triplicate.

Hoechst 33342 staining assay. Hoechst 33342 staining was used to study the apoptosis of ATDC5 cells. Cells were seeded into a 96-well plate at a density of $7x10^3$ cells/well, cultured in 100 μl medium and treated with HMGB1 (0, 10, 50, 100 and 300 ng/ml) or IL-1β (5 ng/ml) in a humidified incubator with 5% CO_2 at 37˚C overnight. Subsequently, the medium was removed, and the cells were fixed at 4˚C in 4% paraformaldehyde for 1 h and permeabilized in saponin (0.1% v/v in PBS-BSA). Hoechst 33342 (1 μg/ml; Promega Corporation) was added to each well, and the cells were further incubated in the dark for 30 min at 37˚C. To assess specific apoptosis, apoptotic and living cells with condensed and fragmented nuclei were visualized using a blue filter of a Nikon TE2000-U inverted fluorescence microscope (Nikon Corporation) at x200 magnification. The experiments were repeated three times.

Flow cytometry. ATDC5 cells were seeded into a 96-well plate at a density of $7x10^3$ cells/well, and subjected to different treatments. Subsequently, the cells were digested and harvested, then fixed with ethanol overnight at 4˚C. Apoptosis was examined using the Annexin V-FITC/propidium iodide (PI) kit (Multisciences Lianke Biotech) according to the manufacturer's protocol. Cell apoptosis rate was measured by a FACSCalibur flow cytometer (BD Biosciences) with Cell Quest software version 5.1 (BD Biosciences). The experiments were repeated three times.

siRNA and cell transfection. ATDC5 cells were seeded in 6-well plates and transfected with 100 pmol negative control (NC) or siRNA fragments against HMGB1 (RiboBio) by using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The siRNA sequences for NC and siHMGB1 were as follows: siHMGB1-#1, 5'-GGAATAACACTGCTGCAGA-3'; siHMGB1-#2, 5'-CTGCGAAGCTGAAGGAAAA-3'; NC, 5'-GCCAGATTTCTCAGGTGATAA-3'. The experiments were repeated three times.

Alcian blue staining. Alcian blue staining was performed on ATDC5 cells. Briefly, cells were fixed with 100% methanol and stained with 0.1% Alcian blue 8GS (Sigma-Aldrich; Merck KGaA) in 0.1 N HCl for 4 h at room temperature and images were captured. The dye was quantified by solubilizing the sample in 6M guanidine HCL overnight. The absorbance at 620 nm was measured by a spectrophotometer. The experiments were repeated three times.

Western blotting. Western blotting was performed according to the standard procedure using polyclonal antibodies specific

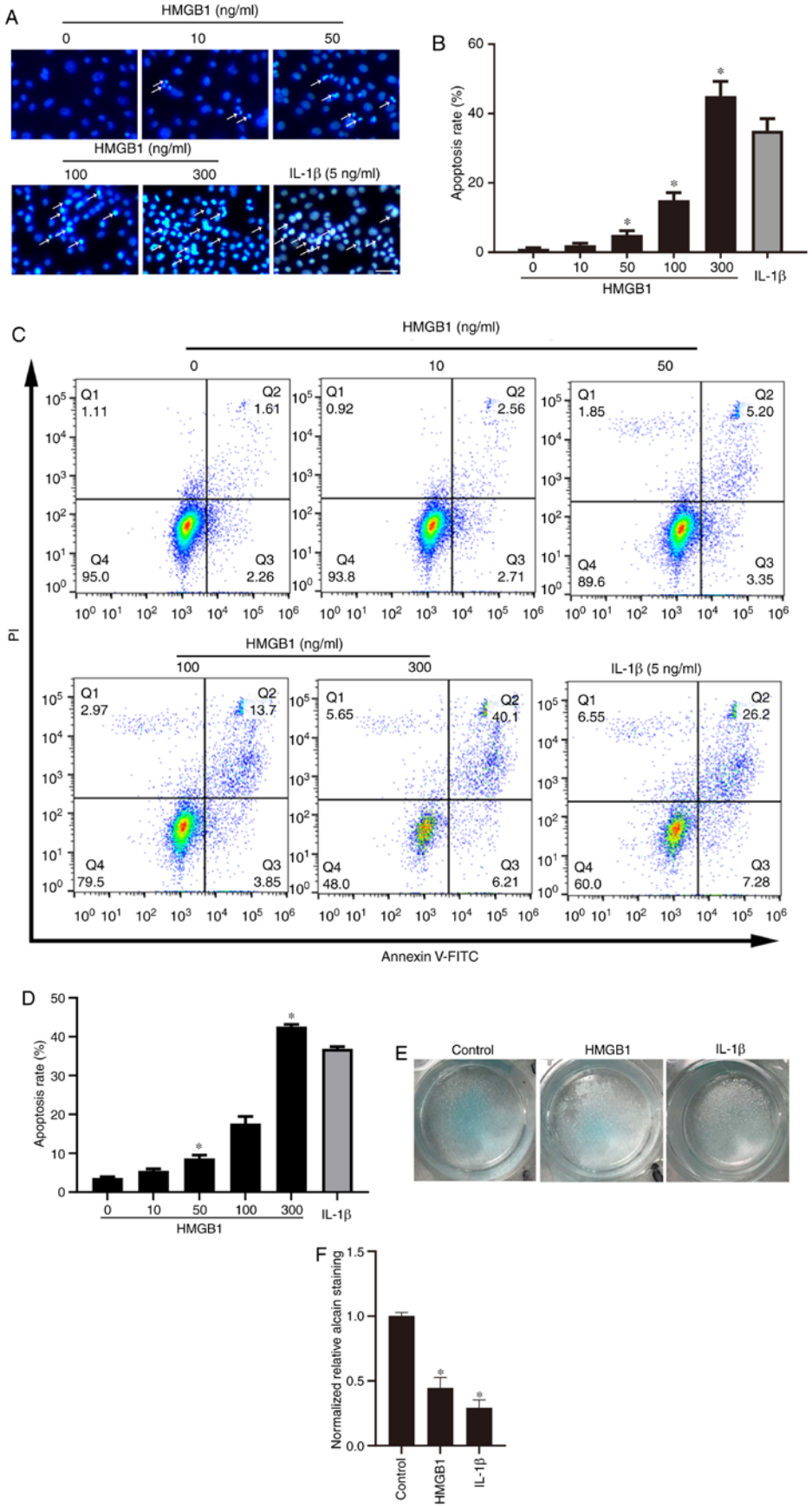

Figure 1. Exposure to HMGB1 promotes chondrocyte apoptosis. (A) Hoechst 33342 staining was performed on ATDC5 cells treated with HMGB1 (0, 10, 50, 100 and 300 ng/ml) or IL-1β (positive control group). Apoptotic cells exhibited morphological changes in the nuclei typical of apoptosis. Images were captured under a fluorescence microscope. Arrows, apoptotic ATDC5 cells. Scale bar, 20 μm. The quantified data are shown in (B) *P<0.05 compared with the 0 concentration group. (C) Cells were treated as in (A) and flow cytometry assay was performed; the quantified data are shown in (D). *P<0.05 compared with the 0 concentration group. (E and F) Cells were treated with HMGB1 (100 ng/ml) or IL-1β (5 ng/ml). Alcian blue staining was performed on ATDC5 cells and the stained area was quantified. *P<0.05 compared with control. HMGB1, high mobility group box 1 protein; IL-1β, interleukin 1β.

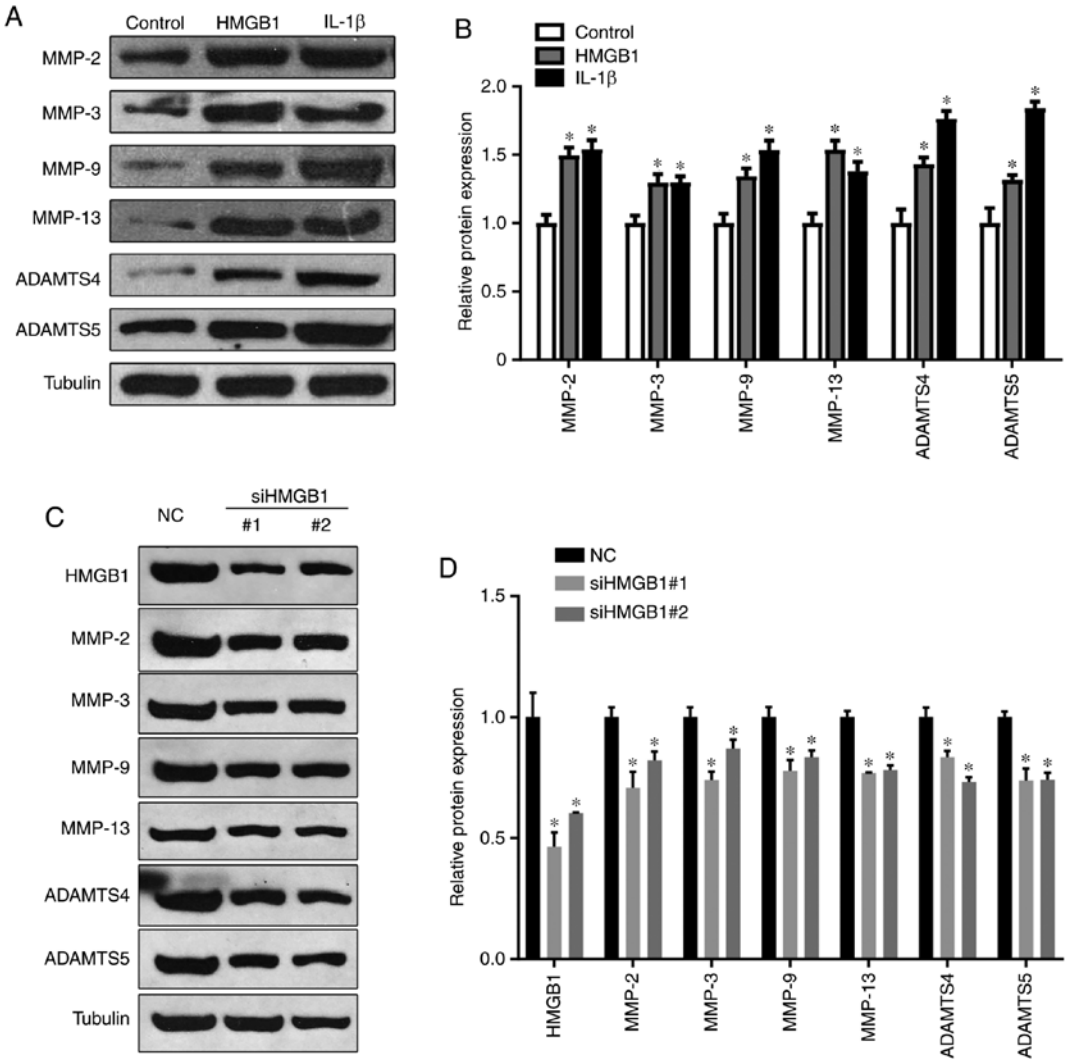

Figure 2. HMGB1 regulates the expression of cartilage matrix degradation genes. (A and B) Cells were treated with HMGB1 (100 ng/ml) or IL-1β (5 ng/ml) and western blotting demonstrated the expression levels of MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS4 and ADAMTS5 protein expression in ATDC5 cells. *P<0.05 compared with control group. (C and D) Cells were transfected with siRNA fragments against HMGB1, and the expression levels of the indicated proteins were detected by western blotting; the relative expression levels are shown in (D). *P<0.05 compared with the NC group. HMGB1, high mobility group box 1 protein; IL-1β, interleukin 1β; MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; NC, negative control.

for MMPs, ADAMTs, pAKT, GSK-3β, pGSK-3β, β-catenin, estrogen sulfotransferase (EST-1) and Runt-related transcription factor 2 (Runx2). Immunoblotting and protein extraction were performed as previously described (20,21). Total protein was extracted from ATDC5 cells using a radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) supplemented with protease and phosphatase inhibitors. Protein concentration was quantified by the BCA assay. A total of 30 μg proteins were loaded per lane and separated by 10% SDS-PAGE and a transferred to a PVDF membrane (EMD Millipore). The membrane was blocked with 5% non-fat milk and incubated with primary antibodies (all from Cell Signaling Technology, Inc., except where otherwise indicated) against MMPs (1:1,000, MMP-2 cat. no. 40994, MMP-3 cat. no. 14351, MMP-9 cat. no. 13667 and MMP-13 cat. no. 69926), ADAMTs (Abcam, 1:1,000, ADAMT-4 cat. no. ab185722 and ADAMT-5 cat. no. ab41037), GSK-3β (1:2,000, cat. no. 12456), pGSK-3β (1:1,000, cat. no. 5558), β-catenin (1:1,000, cat. no. 9582), EST-1 (Abcam, 1:1,000, cat. no. ab63877) and Runx2 (1:1,000, cat. no. 8486) in TBS buffer at 4˚C overnight. Tubulin was used as a loading control (1:3,000; cat. no. 60008-1-Ig; Proteintech Group, Inc.). The membranes were subsequently incubated with the secondary antibodies of horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (1:3,000; cat. no. 7076S; EarthOx, Life Sciences). SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) and Gel Doc™ XR+ System (Bio-Rad Laboratories, Inc.) were used to develop the blots. The intensity of the bands was analyzed using Quantity One software (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. The results represented at least three independent experiments.

Statistical analysis. GraphPad Prism 5 software (GraphPad Software, Inc.) was used to analyze and plot the data. The results are presented as the mean ± standard error of the mean. To determine the significance of the differences between the control and treatment groups, Student's t-test and ANOVA

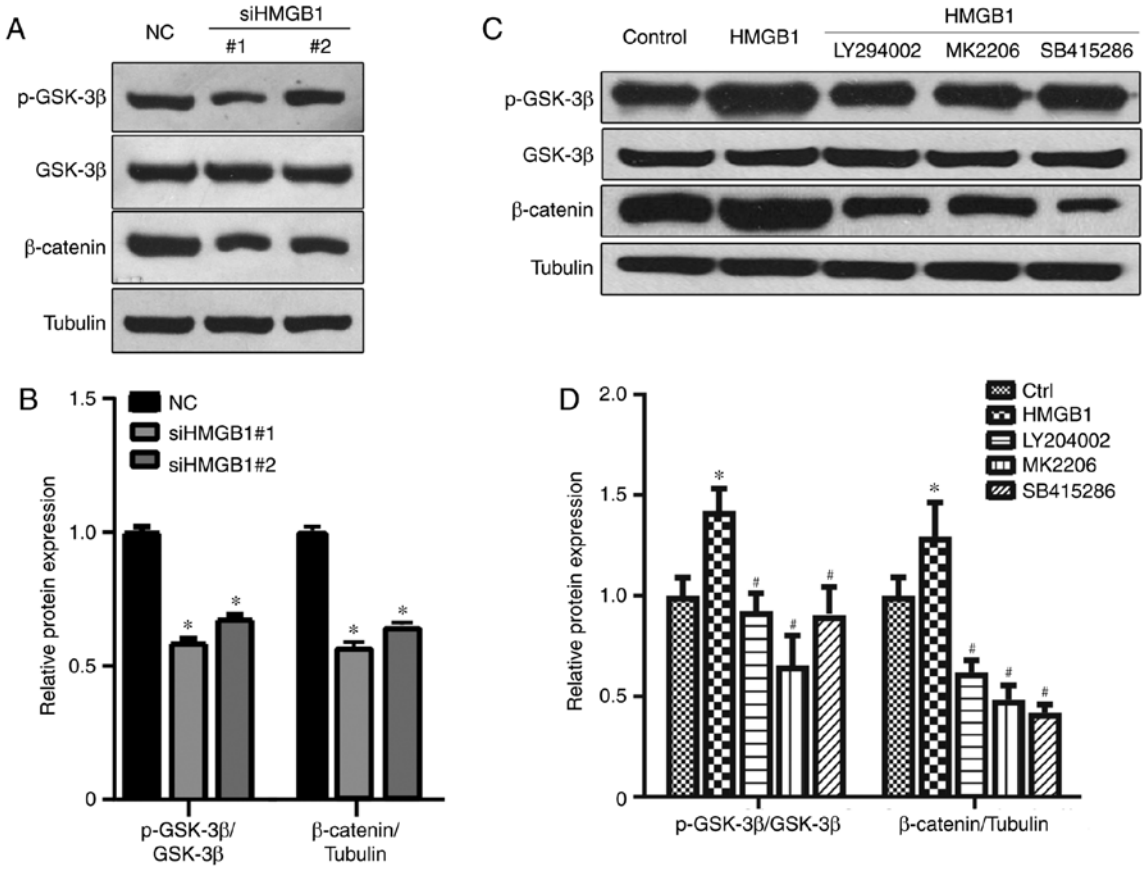

Figure 3. HMGB1 is involved in the regulation of the GSK-3β/β-catenin pathway. (A and B) Cells were transfected with siRNA fragments against HMGB1, the expression levels of phosphorylated GSK-3β (p-GSK-3β), total GSK-3β and β-catenin were detected by western blotting and the relative expression levels (phosphorylated GSK-3 was compared with total GSK-3; β-catenin was compared with tubulin, then the control groups were set to 100% for normalization) are shown in (B). *P<0.05 compared with the NC group. (C and D) Cells were treated with HMGB1 with or without the PI3K/AKT/GSK-3β kinase inhibitors LY294002, MK-2206 or SB415286. The protein expression levels were (C) examined by western blotting and (D) quantified. *P<0.05 compared with the control group. #P<0.05 compared with the HMGB1 group. p, phosphorylated; HMGB1, high mobility group box 1 protein; GSK, glycogen synthase kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B.

followed by Bonferroni's post-hoc test were used. P<0.05 was considered to indicate a statistically significant difference.

Results

HMGB1 promotes chondrocyte apoptosis and cartilage matrix degradation. To investigate whether HMGB1 promoted apoptosis and participates in the development of KOA, ATDC5 cells were cultured and treated with recombinant HMGB1 t different concentrations. IL-1β was used as inflammatory control. Hoechst 33342 staining was performed to detect apoptosis (Fig. 1A and B). Representative images of Hoechst 33342 staining demonstrated that the number of apoptotic ATDC5 cells (white arrows) increased with increasing dose of HMGB1. Apoptotic ATDC5 cells displayed a round and shrunken cell body with condensed or fragile nuclei. To further confirm the extent of apoptosis, cells were subjected to flow cytometry detection. The results also demonstrated that HMGB1 induced apoptosis in a concentration-dependent manner (Fig. 1C and D). The positive control IL-1β induced apoptosis of ATDC5 cells. In order to lower the toxicity, the concentration of 100 ng/ml HMGB1 was selected for the following experiments. The major pathological change in KOA is cartilage degeneration and necrosis, and cartilage proteoglycan is the main component of normal cartilage tissue. Therefore, the changes in proteoglycanase and its cleavage products associated with cartilage proteoglycan metabolism during the progression of KOA were investigated. To evaluate the effects of HMGB1 on the endochondral ossification of ATDC5 cells, the deposition of sulfated glycosaminoglycans was determined using Alcian blue staining. ATDC5 cells treated with HMGB1 and IL-1β exhibited decreased Alcian Blue staining (Fig. 1E and F). These results suggested that HMGB1 induces chondrocyte apoptosis and promotes cartilage matrix degradation.

HMGB1 promotes the expression of cartilage matrix degradation genes. To further determine whether HMGB1 promotes cartilage matrix degradation, the expressions of cartilage matrix degradation genes, including MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS4 and ADAMTS5, were examined by western blotting. The results revealed that, compared with the control group, the expression levels of cartilage matrix degradation genes were significantly upregulated in ATDC5 cells treated with HMGB1 (Fig. 2A and B). In the positive control group treated with IL-1β, the expression levels of these genes were also upregulated. Moreover, siRNA fragments against endogenous HMGB1 were synthesized. The transfection

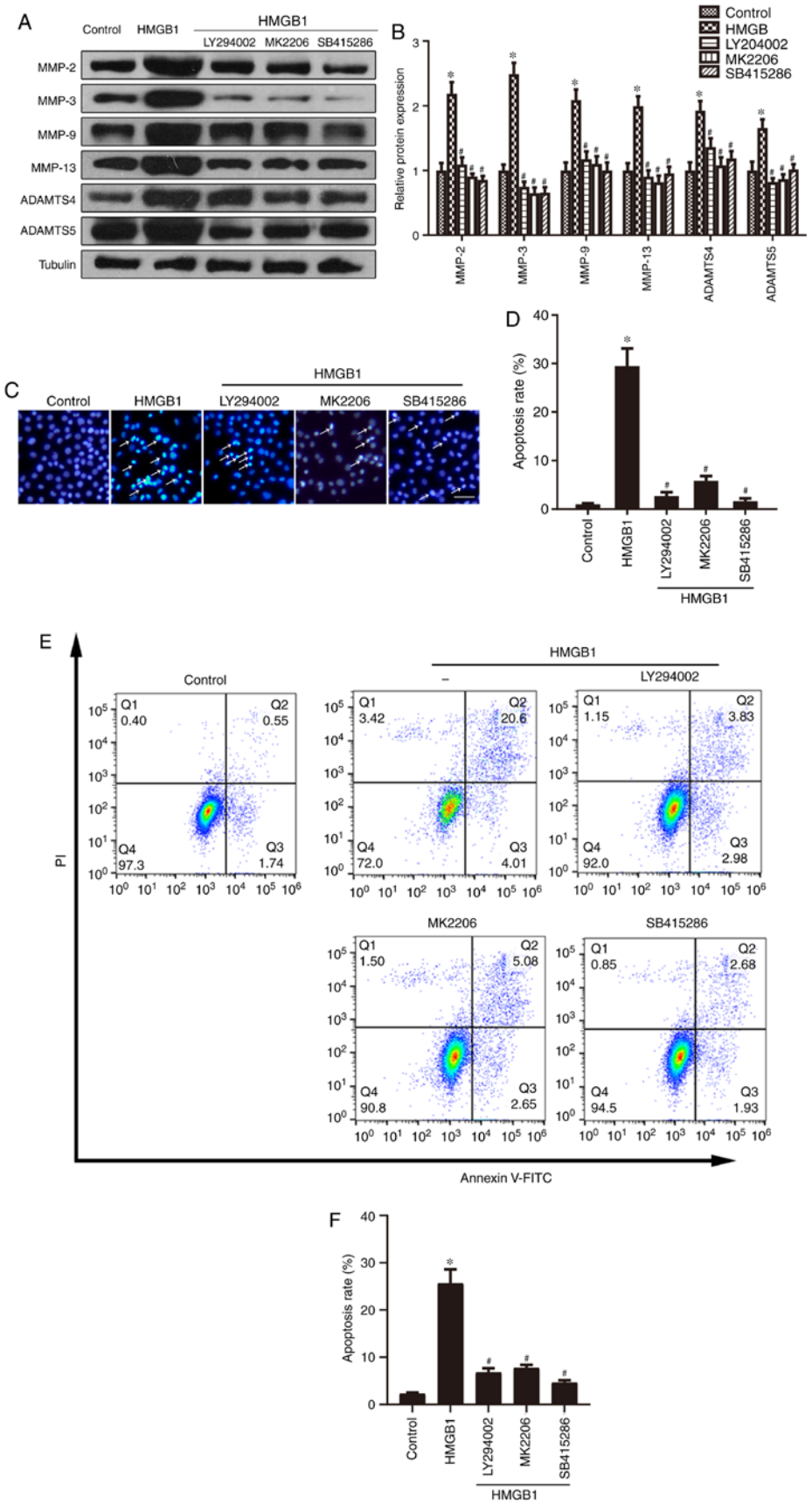

Figure 4. The GSK-3β/β-catenin pathway contributes to the HMGB1-induced expression of cartilage matrix degradation genes and cell apoptosis. (A and B) Western blotting revealed increased protein expression of MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS4 and ADAMTS5 in ATDC5 cells following HMGB1 treatment, which was reversed by LY294002, MK-2206 and SB415286. (C and D) Hoechst 33342 staining was performed on ATDC5 cells and the stained cells were quantified. The number of apoptotic ATDC5C cells (arrows) increased in cells treated with HMGB1, which was reversed by LY294002, MK-2206 and SB415286. Scale bar, 20 μm. (E and F) Cells subjected to the indicated treatments were subjected to flow cytometry. *P<0.05 compared with the control group. $^{\#}$P<0.05 compared with the HMGB1 group. HMGB1, high mobility group box 1 protein; MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; GSK, glycogen synthase kinase.

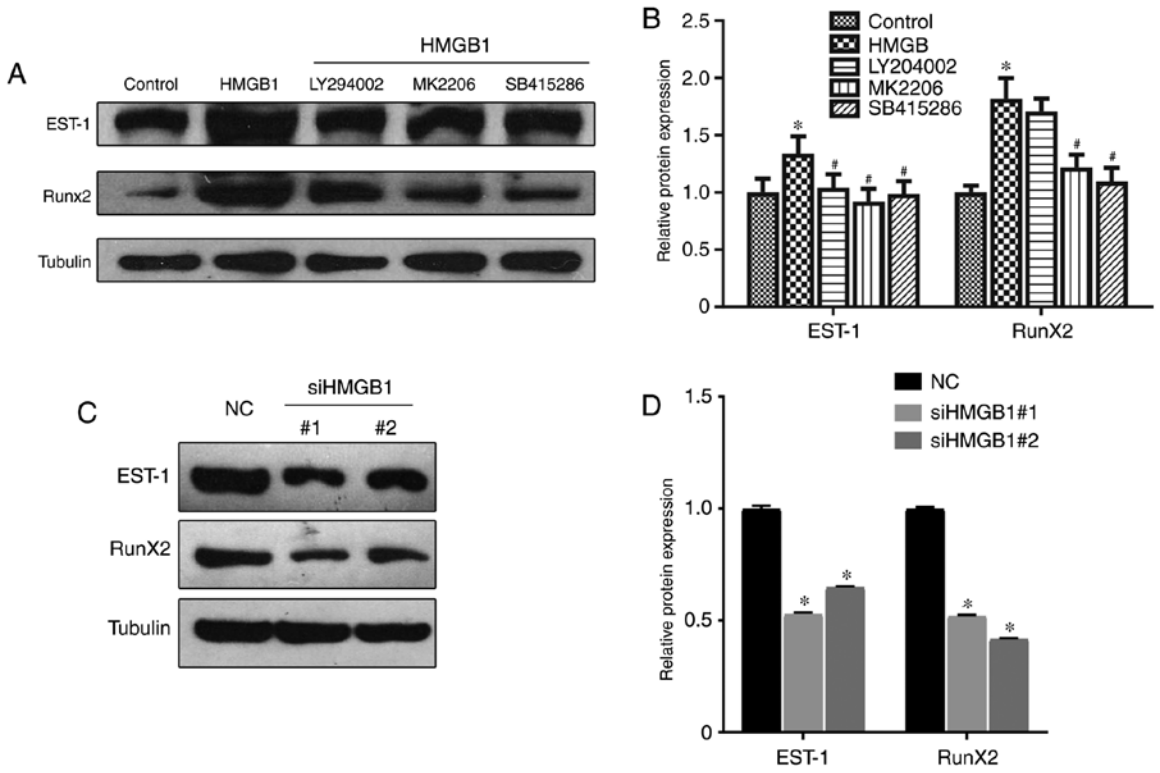

Figure 5. HMGB1 promotes chondrocyte dysfunction through the overexpression of EST-1 and Runx2. (A and B) Western blotting revealed increased protein expression of EST-1 and Runx2 in ATDC5 cells following HMGB1 treatment, which was reversed by LY294002, MK-2206 and SB415286. *P<0.05 compared with the control group. #P<0.05 compared with the HMGB1 group. (C and D) Cells were transfected with siRNA fragments against HMGB1 and subjected to western blotting. The expression levels of EST-1 and Runx2 were shown and quantified. *P<0.05 compared with the NC group. HMGB1, high mobility group box 1 protein; EST-1, estrogen sulfotransferase; Runx2, Runt-related transcription factor 2; NC, negative control.

significantly decreased the expression levels of HMGB1 along with the downregulated expression of MMPs and ADAMTS (Fig. 2C and D). These data further demonstrate that HMGB1 exposure promotes cartilage matrix degradation and apoptosis via the regulation of matrix degradation genes.

HMGB1 is involved in the regulation of the GSK-3β/β-catenin pathway. β-Catenin is an important component of the Wnt signaling pathway that can be activated by Wnt family proteins and participates in the pathological process of KOA. However, whether the GSK-3β/β-catenin pathway is also involved in the pathological process of HMGB1-induced KOA remains elusive. As shown in Fig. 3A and B, cells with HMGB1 knockdown exhibited a decrease in the expression levels of phosphorylated GSK-3β (p-GSK-3β) and β-catenin. Following HMGB1 treatment, significant upregulation of pGSK-3β and β-catenin expression levels was observed, which was reversed by the addition of the GSK-3 upstream kinase PI3K inhibitor LY294002, the AKT inhibitor MK-2206 and the GSK-3β inhibitor SB415286 (Fig. 3C and D). These results suggest that HMGB1 may induce the activation of the GSK-3β/β-catenin pathway, which may subsequently lead to the abnormal regulation of chondrocytes.

Regulation of the GSK-3β/β-catenin pathway contributes to HMGB1-induced expression of cartilage matrix degradation genes and apoptosis. Increased GSK-3 phosphorylation (inactivation) leads to the cytoplasmic accumulation and translocation of β-catenin into the nucleus, leading to the expressions of downstream target genes (22). To further explore the mechanisms underlying HMGB1-induced cartilage matrix degradation, the expression levels of cartilage matrix degradation genes targeted by β-catenin accumulation were determined by western blotting. As shown in Fig. 4A and B, compared with the control group, the expression levels of MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS4 and ADAMTS5 were significantly increased following treatment with HMGB1, an effect that was partially reversed by blocking the PI3K/AKT/GSK-3β/β-catenin pathway with SB415286, LY294002 and MK-2206. To explore the potential involvement of the GSK-3β/β-catenin pathway in HMGB1-induced chondrocyte apoptosis, Hoechst 33342 staining was performed. Compared with the control group, the number of apoptotic ATDC5 cells (white arrows) was significantly increased following treatment with HMGB1. However, this effect was reversed by the application of LY294002, MK-2206 and SB415286 (Fig. 4C and D). In addition, the cytometry assay revealed the same trend (Fig. 4E and F). These results indicated that the activation of the GSK-3β/β-catenin pathway may contribute to HMGB1-induced expression of cartilage matrix degradation genes and chondrocyte apoptosis.

HMGB1 promotes chondrocyte dysfunction through the upregulation of EST-1 and Runx2. EST-1 and Runx2 regulate the transcription of multiple genes and play key roles in the differentiation and maturation of chondrocytes. Recently, Runx2 was found to be regulated by β-catenin accumulation (23,24). To determine whether HMGB1 may regulate

chondrocyte differentiation and maturation through EST-1 and Runx2, the expression levels of EST-1 and Runx2 were analyzed by western blotting. As shown in Fig. 5A and B, HMGB1 significantly increased the protein expression levels of EST-1 and Runx2, which was partially reversed following treatment with LY294002, MK-2206 and SB415286 (Fig. 5A and B). Furthermore, genetic knockdown of HMGB1 markedly downregulated the expression levels of EST-1 and Runx2 (Fig. 5C and D). These results suggest that HMGB1 may promote chondrocyte differentiation via the regulation of EST-1 and Runx2 in OA.

Discussion

KOA is a common joint disease with a high incidence rate among middle-aged and elderly individuals; however, the underlying molecular mechanisms have yet to be fully elucidated. The results of the present study demonstrated that HMGB1 may promote chondrocyte apoptosis and the expression of cartilage matrix degradation genes, such as MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS4 and ADAMTS5, which may induce the occurrence and progression of KOA. In addition, HMGB1 upregulated the expression levels of p-GSK-3β and β-catenin; when the GSK-3β/β-catenin pathway was inhibited, the number of apoptotic cells and the expression of MMPs and ADAMTs were decreased, which indicated that HMGB1 may induce the occurrence and development of KOA by activating the GSK-3β/β-catenin pathway. The results also demonstrated that HMGB1 may promote chondrocyte dysfunction by upregulating EST-1 and Runx2.

HMGB1 was firstly identified as a nuclear protein regulating DNA replication and chromatin function (25). Despite its role in the pathogenesis of inflammatory diseases (26) and rheumatoid arthritis (27), accumulating evidence suggests that HMGB1 also plays an important role in OA. HMGB1 is mainly distributed in the cytoplasm and matrix of chondrocytes in OA cartilage (28). The expression levels of HMGB1 have been associated with the degree of synovitis, pain and daily activity in patients with KOA (29). Inhibition of HMGB1 suppressed the inflammatory symptoms (30). In a rat model or collagen-induced arthritis, treatment with HMGB1 antibody alleviated the arthritis symptoms (14). Recently, HMGB1 was reported to be secreted in the extracellular environment and act as a cytokine (31). HMGB1 can be released by osteoarthritic synoviocytes by IL-1β stimuli (32,33). However, the detailed mechanisms of action of secreted HMGB1 in OA remain to be fully elucidated. In the present study, recombinant HMGB1 was applied to treat the cultured chondrogenic cells, and the effect was compared with that of IL-1β. The results demonstrated that HMGB1 induced cell apoptosis and the expression of various cartilage matrix degradation-related genes, including MMPs and ADAMTS. Although the pro-inflammatory effects were not revealed by observing the expression of inflammatory factors, the data suggested that HMGB1 acted as a ligase to affect the surrounding cells, mediating inflammation and cell death during OA.

The pathogenesis of OA is complex, and the major cause of OA is cartilage degradation (34). The extracellular matrix components, such as aggrecan and collagen II, are mainly secreted by chondrocytes (35). In normal cartilage tissue, the extracellular matrix synthesis and catabolic processes are balanced, and the thickness and properties of the cartilage matrix tend to be stable (36,37). During the process of OA, the synthetic catabolic balance of the cartilage matrix is disturbed, the cartilage matrix synthesis is insufficient, catabolism increases, and the cartilage matrix is degraded, which results in the thinning of the cartilage layer and degeneration. It is well known that proinflammatory factors, such as IL-1β, promote cartilage degradation by stimulating the production of MMPs (38). MMPs degrade matrix components, including collagen and other basement membrane proteins, resulting in cartilage matrix degradation (39). It has been reported that HMGB1 treatment of OA chondrocytes results in the phosphorylation of nuclear factor-κB (NF-κB) and MMP expression (40). The results of the present study demonstrated that HMGB1 promoted chondrocyte apoptosis and upregulated the expression levels of the cartilage matrix degradation genes MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS4 and ADAMTS5. Although changes in the mRNA levels were not detected, the data are consistent with those of previous studies.

Wnt signaling regulation of β-catenin plays critical role in variety of physiological processes (41). Previous studies have demonstrated that the expression level of β-catenin was upregulated in degenerative chondrocytes, resulting in loss of cartilage (42,43). In addition, conditional activation of β-catenin led to the abnormal differentiation of adult mouse chondrocytes and induced an OA-associated phenotype in mice (44). Takamatsu *et al* (29) demonstrated that verapamil inhibited the Wnt signaling pathway and the expression of the Wnt response gene MMP3 by increasing the expression of the antagonistic protein secreted frizzled-related protein 3, thus inhibiting the progression of KOA. The results of the present study revealed that HMGB1 upregulated the expression levels of p-GSK-3β and β-catenin; blocking the PI3K/AKT/GSK-3 signaling pathway decreased the cell apoptosis rate and the expression levels of MMPs and ADAMTs, indicating that HMGB1 may induce the occurrence and development of KOA by activating the GSK-3β/β-catenin pathway. EST-1 and Runx2 are osteogenic differentiation-specific transcription factors that regulate the transcription of multiple genes (45,46) that play important roles in the formation and differentiation of osteoblasts, differentiation and maturation of chondrocytes, formation and absorption of osteoclasts and production of bone matrix proteins (47,48). In addition, in rheumatoid arthritis, a number of other signaling pathways that may regulate MMPs have also been identified, such as Nrf2/HO-1 signaling (49), NF-κB inflammatory signaling (50,51), mitochondrial/caspase-mediated pathways (52) or mitogen-activated protein kinase signaling pathway (53); however, its role in HMGB1-induced chondrocyte apoptosis requires further investigation. The results of the present study also indicated that HMGB1 may promote chondrocyte dysfunction by upregulating EST-1 and Runx2.

In conclusion, the results of the present study preliminarily demonstrated the effects of HMGB1 on KOA, including changes in the GSK-3β/β-catenin pathway, and explored the possible mechanism underlying the role of HMGB1 in KOA, providing a theoretical basis and new evidence for the in-depth study of the molecular mechanism of KOA pathogenesis. The findings of the present study may aid in identifying molecular targets for

the development of new drugs and the establishment of novel treatment options for KOA. However, the precise molecular biological mechanisms remain to be fully elucidated.

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Availability of data and materials

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZS, XM, TT, PZ and LZ performed and experiments analyzed the results. XM and LZ revised and analyzed the data. TT and PZ contributed to the revised experiment. ZS, XM and YJ wrote the manuscript and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent to publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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