

Excessive mechanical stretch-mediated osteoblasts promote the catabolism and apoptosis of chondrocytes via the Wnt/ β -catenin signaling pathway

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Abstract. Excessive biomechanical loading is considered an important cause of osteoarthritis. Although the mechanical responses of chondrocytes and osteoblasts have been investigated, their communication during mechanical loading and the underlying molecular mechanisms are not yet fully known. The present study investigated the effects of excessive mechanically stretched osteoblasts on the metabolism and apoptosis of chondrocytes, and also assessed the involvement of the Wnt/ β -catenin signaling pathway. In the present study, rat chondrocytes and osteoblasts were subjected to mechanical tensile strain, and an indirect chondrocyte-osteoblast co-culture model was established. Reverse transcription-quantitative PCR and western blotting were performed to determine the expression levels of genes and proteins of interest. An ELISA was performed to investigate the levels of cytokines, including matrix metalloproteinase (MMP) 13, MMP 3, interleukin-6 (IL-6) and prostaglandin E2 (PG E2), released from osteoblasts. Flow cytometry was performed to detect the apoptosis of chondrocytes exposed to stretched osteoblast conditioned culture medium. The levels of MMP 13, IL-6 and PG E2 increased significantly in the supernatants of stretched osteoblasts compared with the un-stretched group. By contrast, the mRNA expression levels of Collagen 1a and alkaline phosphatase were significantly decreased in

osteoblasts subjected to mechanical stretch compared with the un-stretched group. The mRNA expression level of Collagen 2a was significantly decreased, whereas the expression levels of MMP 13 and a disintegrin and metalloproteinase with thrombospondin-like motifs 5 were significantly increased in chondrocytes subjected to mechanical stretch compared with the un-stretched group. In the co-culture model, the results indicated that excessive mechanically stretched osteoblasts induced the catabolism and apoptosis of chondrocytes, which was partly inhibited by Wnt inhibitor XAV-939. The results of the present study demonstrated that excessive mechanical stretch led to chondrocyte degradation and inhibited osteoblast osteogenic differentiation; furthermore, excessive mechanically stretched osteoblasts induced the catabolism and apoptosis of chondrocytes via the Wnt/ β -catenin signaling pathway.

Introduction

Osteoarthritis (OA) is becoming an increasing burden to patients, communities and social care systems (1). Currently, effective therapy for OA is limited, although exercise has been recommended as an effective strategy for preventing or treating OA (2). Accumulating evidence indicates that the effect of exercise on joints is intensity-dependent; a moderate amount of exercise serves an important role in the prevention and treatment of OA, whereas high intensity exercise is hypothesized to induce OA (3,4).

OA is primarily characterized by progressive articular cartilage degradation. The subchondral bone has been reported to serve an important role in OA (5). Cartilage and its underlying subchondral bone act as a unit, affecting one another in OA (6). The effect of exercise has been investigated on cartilage and the subchondral bone (4,7); however, the interaction between cartilage and the subchondral bone with exercise remains unclear.

Both the chondrocytes in articular cartilage and the osteoblasts in subchondral bone are sensitive to mechanical

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stimuli and can transform mechanical signals into biological responses (8-10). It has been demonstrated that mechanical stretch influenced the biological behavior of osteoblasts, thereby profoundly affecting bone remodeling (8). Furthermore, Yan *et al* (9) demonstrated that the mechanical strains of 2,500 $\mu\epsilon$ at 0.5 Hz can induce osteoblast differentiation via the ERK signaling pathway. For chondrocyte responses to mechanical stimulation, Agarwal *et al* (10) indicated that the mechanical strain of 6% at 0.5 Hz directly suppresses the catabolism of chondrocytes, whereas the absence of mechanical stress or high-magnitude of strain (>10% at 0.5 Hz) causes chondrocyte catabolism and apoptosis (11,12). However, the interaction between chondrocytes and osteoblasts with excessive mechanical loading remains unknown. To the best of our knowledge, no previous study has investigated apoptosis in the communication of chondrocytes and osteoblasts in the mechanical environment, and the underlying mechanisms are also not completely understood. The best recognized biochemical hallmark of both early and late stages of apoptosis is the activation of cysteine proteases (caspases). Caspase-3 is the most important apoptotic agent in the caspase family and when activated by apoptotic signaling, caspase-3 is converted to cleaved caspase-3. Detection of caspase-3 and cleaved caspase-3 in cells or tissues is an key method for apoptosis (13).

High expression of collagen 2a (Col 2a) is a specific phenotype of chondrocytes, while alkaline phosphatase (ALP), collagen 1a (Col 1a) and osteocalcin (OCN) are mainly expressed by osteoblasts (14,15). SRY-related high mobility group-box 9 (SOX9) is a key transcription factor in chondrogenesis and serves an important role in the proliferation and differentiation of chondrocytes (16). Non-physiological mechanical stress can change the phenotype of chondrocytes, transforming chondrocytes into hypertrophic chondrocytes, secreting collagen X (Col X) and OCN, which are rarely expressed in normal chondrocytes (17). In addition to mechanical environmental changes, various inflammatory cytokines and proteases are reportedly involved in the initiation and progression of cartilage degeneration (18). Interleukin-6 (IL-6) and prostaglandin E2 (PG E2) serve an key role in the inflammatory response of OA. These inflammatory mediators enhance matrix degradation and inhibit the synthesis of matrix related proteins (19). Excessive production of cartilage degrading enzymes such as the aggrecanases and matrix metalloproteinases (MMPs), which are key in the degradation of aggrecan and Coll 2a, has been demonstrated in OA (20,21). The Wnt signaling pathway serves an important role in the development and maintenance of cartilage and is closely associated with OA (22). Up- or downregulation of the classical Wnt signaling pathway displays a negative impact on cartilage development and maintenance, eventually leading to OA-like features (23). On the one hand, Miclea *et al* (24) demonstrated that upregulation of β -catenin in chondrocytes induced matrix degradation and inhibited chondrocyte proliferation, leading to OA-like features. On the other hand, Chen *et al* (25) reported that ablation of β -catenin in transgenic mice decreased the proliferation of chondrocytes and promoted chondrocyte apoptosis. Glycogen synthase kinase 3 β (GSK-3 β) is another important protein in the Wnt signaling pathway. It can lead to the degradation of β -catenin when the Wnt signaling pathway is not activated. Inactivation of GSK-3 β leads to

the accumulation of β -catenin, thus mediating proliferation and apoptosis (26). The Wnt/ β -catenin signaling pathway is involved in transmitting the signals of mechanical loading to cells, including chondrocytes and osteoblasts (27-30). However, the role of Wnt/ β -catenin signaling in the interaction between chondrocytes and osteoblasts under the mechanical environment remains unknown.

Therefore, the present study investigated the effects of excessive mechanical stretch on chondrocytes and osteoblasts. The effects of stretched osteoblasts on the metabolism and apoptosis of chondrocytes were investigated, and the role of Wnt/ β -catenin signaling in the indirect chondrocyte-osteoblast co-culture model was assessed. The results of the present study may further the current understanding of the mechanisms underlying excessive loading in OA, thus aiding with improving the prevention and treatment of OA.

Materials and methods

Culture and mechanical stretching of osteoblasts. A total of 16 rats were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou, China), housed in rooms with a maintained temperature of 22-25°C and humidity of 50 \pm 5% under a 12-h light-dark cycle and provided food and water *ad libitum*. All experiments were performed in accordance with protocols approved by the Animal Ethics Committee of Nanfang Hospital, Southern Medical University (approval no. NFYY-2016-128; Guangzhou, China). Neonatal male Sprague-Dawley (SD) rats (age, 3-5 days; weight, 8-15 g, n=8) used for the isolation of primary osteoblasts were euthanized by decapitation. Male SD rats (age, 18-20 days; weight, 45-52 g, n=8) used for the isolation of articular chondrocytes were anesthetized by intraperitoneal injection of 7% chloral hydrate (350 mg/kg) followed by sacrifice by cervical dislocation. No signs of peritonitis were observed after the administration of chloral hydrate.

Primary osteoblasts were isolated from the cortical bone of the calvaria of neonatal SD rats by successive enzymatic digestion. Cells from passages 2-4 were used in the present study. Osteoblasts were subjected to mechanical stretching using a Flexcell FX-5000™ Flexercell Tension System (Flexcell International Corp.), as previously described (6,31), at a mechanical strain of 20% and a frequency of 1 Hz for 24 h. For the control group, osteoblasts were collected and placed onto plates without mechanical stretching (un-stretched group). Following mechanical stretching for 24 h, osteoblasts from stretched (Scm) and un-stretched (Ucm) conditioned medium were collected and centrifuged at 1,000 x g for 10 min at 37°C. Subsequently, the supernatant was transferred to a fresh tube and stored at -80°C to be used for subsequent experiments. Osteoblasts were collected for RNA extraction. The morphology of osteoblasts before and after stretching was observed using an inverted microscope (Olympus Corporation).

Culture and stimulation of chondrocytes. Rat articular chondrocytes were isolated by enzymatic digestion of articular cartilage from SD rats as previously described (22). Briefly, articular cartilage was cut into small pieces, then the pieces were digested with 0.25% Trypsin-EDTA solution and 0.2%

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Primer sequences (5'-3')
GAPDH	F: GGCACAGTCAAGGCTGAGAATG R: ATGGTGGTGAAGACGCCAGTA
Col 1a	F: CATGTTTCAGCTTTGTGGACC R: TTAGGGACCCCTTAGGCCATT
ALP	F: AACAACTGACTGACCCCTTC R: TCCACTAGCAAGAAGAAGCC
OCN	F: GACTGCATTCTGCCTCTCTG R: ATTCACCACCTTACTGCCCT
Cyclin D1	F: CGTACCCTGACACCAATCTC R: TGAAGTAAGAAACGGAGGGC
β -catenin	F: TAAATGACGAGGACCAGGTG R: CACTATGGCAGACACCATCT
MMP 13	F: CTCTTGAGCTGGACTCATTG R: CTGCAAACCTGGAAGTCTTCC
Col X	F: TGCTGCTATTGTCCTTGAAC R: ACCTTGCTCTCCTCTTAGTG
Col 2a	F: CCCAGAACATCACCTACCAC R: GGTACTCGATGATGGTCTTG
SOX 9	F: GGGCTCTGTGCTCTACTCCA R: AGGTCTGGTGAGCTGTGTGT
ADAMTS 5	F: TGTGCCGTGATTGAAGATGA R: TCATGAGAGAGGCCAAGGA

F, forward; R, reverse; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ALP, alkaline phosphatase; MMP 13, matrix metalloproteinase 13; SOX 9, SRY-related high mobility group-box gene 9; ADAMTS 5, a disintegrin and metalloproteinase with thrombospondin-like motifs 5; Col, collagen; OCN, osteocalcin.

collagenase type II (Sigma-Aldrich; Merck KGaA), respectively. To avoid phenotype loss, chondrocyte from passage 1-2 were used in the present study. Before applying the mechanical stretch, cells were starved by incubation in Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) containing 1% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) for 12 h, followed by incubation in culture medium supplemented with 0.1% FBS. Subsequently, a mechanical stretch of 20% elongation was applied to the chondrocytes at a frequency of 1 Hz for 24 h (stretched group for chondrocytes). For the co-culture, chondrocytes were stimulated with Scm or Ucm at 37°C for 24 h. Then, 10 μ M XAV-939 (Sigma-Aldrich; Merck KGaA) was added to the chondrocytes at 37°C for 24 h to assess the role of the Wnt/ β -catenin signaling pathway. After 24 h, stimulated chondrocytes were retained and harvested for extraction of RNA or proteins. The chondrocytes without co-culture were defined as the control group. The morphology of chondrocytes before and after stretching was observed using an inverted microscope (Olympus Corporation).

Cytokine assay. Levels of total rat MMP 13 (cat. no. NBP3-06931), MMP 3 (cat. no. NBP3-06894), IL-6 (cat.

no. R6000B) and PG E2 (cat. no. NBP3-00461) in supernatants were measured using ELISA kits (Novus Biologicals). Samples were analyzed in serial dilutions in duplicate and measured against standard curves according to the manufacturer's instructions.

Flow cytometric analysis of cell death. To determine early apoptosis/necroptosis, a fluorescent dye Annexin V-FITC/PI Apoptosis Detection kit (Abcam) was used according to the manufacturer's protocol. Briefly, treated chondrocytes were harvested and washed once in ice-cold PBS. After resuspension in 500 μ l binding buffer, 5 μ l Annexin V-FITC and 5 μ l PI solution were added for 30 min at 4°C in the dark. Subsequently, samples were analyzed by a BD FACSCanto™ II (BD Biosciences) flow cytometer using BD FACSDiva software (version 6.1.3; BD Biosciences).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from monolayer cultured chondrocytes and osteoblasts using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). First-strand cDNA synthesis was conducted using a PrimeScript RT Reagent kit according to the manufacturer's instructions (Takara Biotechnology Co., Ltd.). Subsequently, qPCR was performed using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) and the following thermocycling conditions: After an initial denaturation at 95°C for 30 sec, 40 cycles of a two-cycle procedure (denaturation at 95°C for 15 sec, annealing and extension at 60°C for 32 sec) were performed. mRNA expression levels were normalized to the internal reference gene GAPDH. The forward and reverse primer sequences are presented in Table I. The difference between the mean Cq values of the gene of interest and the housekeeping gene was labelled Δ Cq, and the difference between Δ Cq and the Cq value of the calibrator sample was labelled $\Delta\Delta$ Cq. The $2^{-\Delta\Delta$ Cq method was used to determine relative mRNA expression levels (32).

Western blotting. Total protein was isolated from chondrocytes using lysis buffer containing 1 M Tris HCl (pH 8), 5 M NaCl, 20% Triton X-100, 0.5 M EDTA and a protease inhibitor cocktail (Roche Diagnostics). The cell lysate was clarified by centrifugation (12,000 x g for 10 min at 4°C) and protein concentrations were determined using a bicinchoninic acid protein assay (Sigma-Aldrich; Merck KGaA). Protein (10 μ g) was separated via SDS-PAGE (12% gel), and the separated proteins were subsequently transferred to a nitrocellulose membrane. Then, the membrane was blocked in TBS with 0.1% Tween-20 containing 5% non-fat milk at 37°C for 1 h. The membranes were incubated overnight at 4°C with primary antibodies targeted against: Caspase 3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), cleaved caspase 3 (1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), β -catenin (1:1,000; cat. no. sc-7963; Santa Cruz Biotechnology, Inc.), GSK-3 β (1:1,000; cat. no. 9315; Cell Signaling Technology, Inc.) and β -actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody at 37°C for 2 h. (Goat Anti-Mouse IgG; 1:3,000; cat. no. CW0102; CoWin Biosciences; Goat Anti-Rabbit IgG;

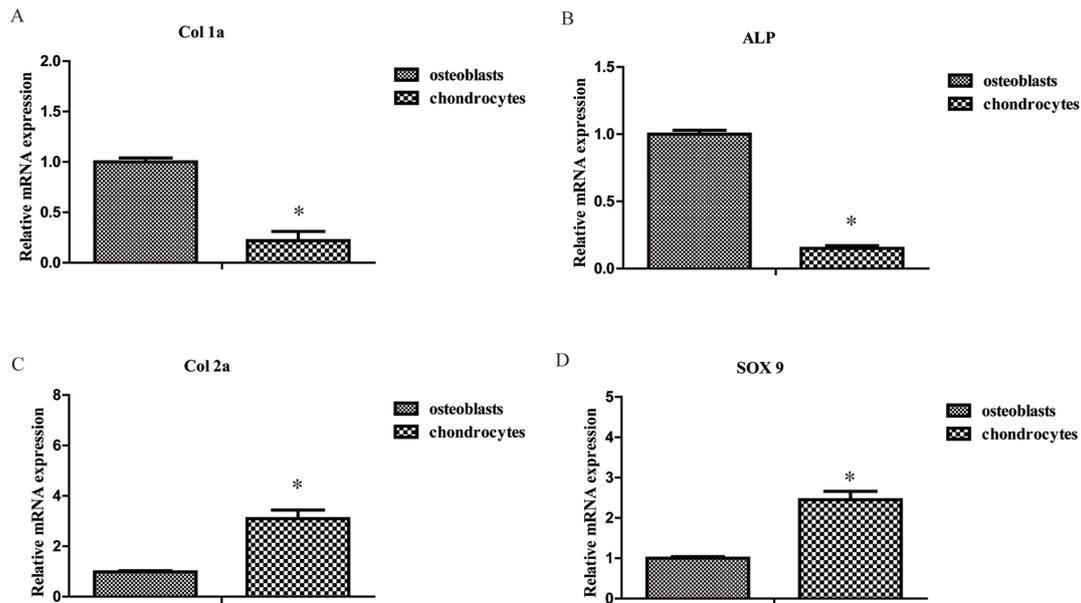


Figure 1. Comparison of relative expression of bone and cartilage-associated genes in osteoblasts and chondrocytes. Compared with osteoblasts, relative expression levels of (A) Col 1a and (B) ALP were significantly lower in chondrocytes, whereas significantly higher (C) Col 2a and (D) SOX 9 expression levels were observed in chondrocytes. Data were analyzed using the unpaired Student's t-test. * $P < 0.05$ vs. osteoblasts. Col, Collagen; ALP, alkaline phosphatase; SOX 9, SRY-associated high mobility group-box gene 9.

1:3,000; CW0103; CoWin Biosciences) Proteins bands were developed by enhanced chemiluminescence (EMD Millipore) and visualized by exposure to X-ray film. The bands were quantified by the densitometry with ImageJ software (version 1.51; National Institutes of Health). β -actin was used as the loading control.

Statistical analysis. To confirm the reproducibility of the results, the experiments were repeated three times. The results are presented as the mean \pm standard deviation. Statistical analyses were performed using SPSS software (version 16.0; SPSS, Inc.). Data were analyzed using the unpaired Student's t-test or one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cellular phenotypes of osteoblasts and chondrocytes.

To compare the cellular phenotypes of osteoblasts and chondrocytes, RT-qPCR was performed to detect the expression levels of bone and cartilage-associated genes. The expression levels of Col 1a and ALP mRNA were lower in chondrocytes compared with osteoblasts, respectively (1.00 ± 0.04 vs. 0.22 ± 0.09 and 1.00 ± 0.03 vs. 0.15 ± 0.02 , both $P < 0.05$; Fig. 1A and B). However, the expression levels of Col 2a and SOX 9 mRNA were significantly higher in chondrocytes compared with osteoblasts, respectively (1.00 ± 0.05 vs. 3.10 ± 0.34 and 1.00 ± 0.04 vs. 2.45 ± 0.21 , $P < 0.05$; Fig. 1C and D). Prior to mechanical stretching, chondrocytes and osteoblasts were cobblestone-like and fibroblast-like, respectively (Fig. 2A and C). Cells became notably longer and were oriented perpendicularly to the axis of the external strain following mechanical stretch (Fig. 2B and D).

Excessive mechanical stretch inhibits osteoblast osteogenesis.

In order to investigate the effect of excessive mechanical stretch on osteoblasts, the present study analyzed alterations in osteogenesis- and proliferation-associated genes of osteoblasts. The RT-qPCR results revealed that Col 1a and ALP expression levels in the stretched group were significantly decreased compared with the un-stretched group, respectively (1.00 ± 0.03 vs. 0.58 ± 0.09 and 1.00 ± 0.08 vs. 0.45 ± 0.55 , both $P < 0.05$; Fig. 3A and B). However, no significant difference was observed between the stretched and un-stretched groups for Cyclin D1 and β -catenin gene expression levels (1.00 ± 0.04 vs. 0.86 ± 0.11 and 1.00 ± 0.05 vs. 0.93 ± 0.07 , both $P > 0.05$; Fig. 3C and D). These results indicated that excessive mechanical stretch inhibited osteoblast osteogenesis.

Excessive mechanical stretch increases the levels of inflammatory cytokines in the supernatants of osteoblasts.

The present study investigated the mechanism underlying the effect of stretch-stimulated osteoblasts on chondrocytes. ELISAs were performed to assess the levels of inflammatory cytokines released from osteoblasts. The results revealed that MMP 13 (140.5 ± 11.2 vs. 169.7 ± 21.1 pg/ml), IL-6 (322.2 ± 23.6 vs. 367.2 ± 35.4 pg/ml) and PG E2 (239.7 ± 17.6 vs. 278.1 ± 21.5 pg/ml) levels were significantly upregulated in the supernatants of osteoblasts subjected to mechanical stretch compared with the un-stretched group ($P < 0.05$; Fig. 3E, G and H). By contrast, no significant difference between the stretched and un-stretched groups was observed for MMP 3 (156.2 ± 17.1 vs. 165.1 ± 16.3 pg/ml; $P > 0.05$; Fig. 3F).

Excessive mechanical stretch induces chondrocyte catabolism.

Compared with the un-stretched group, the mRNA expression levels of Col 2a and SOX 9 were significantly decreased in the stretched group, respectively (1.00 ± 0.01 vs. 0.34 ± 0.08 and 1.00 ± 0.06 vs. 0.48 ± 0.12 , $P < 0.05$; Fig. 4A and B). By

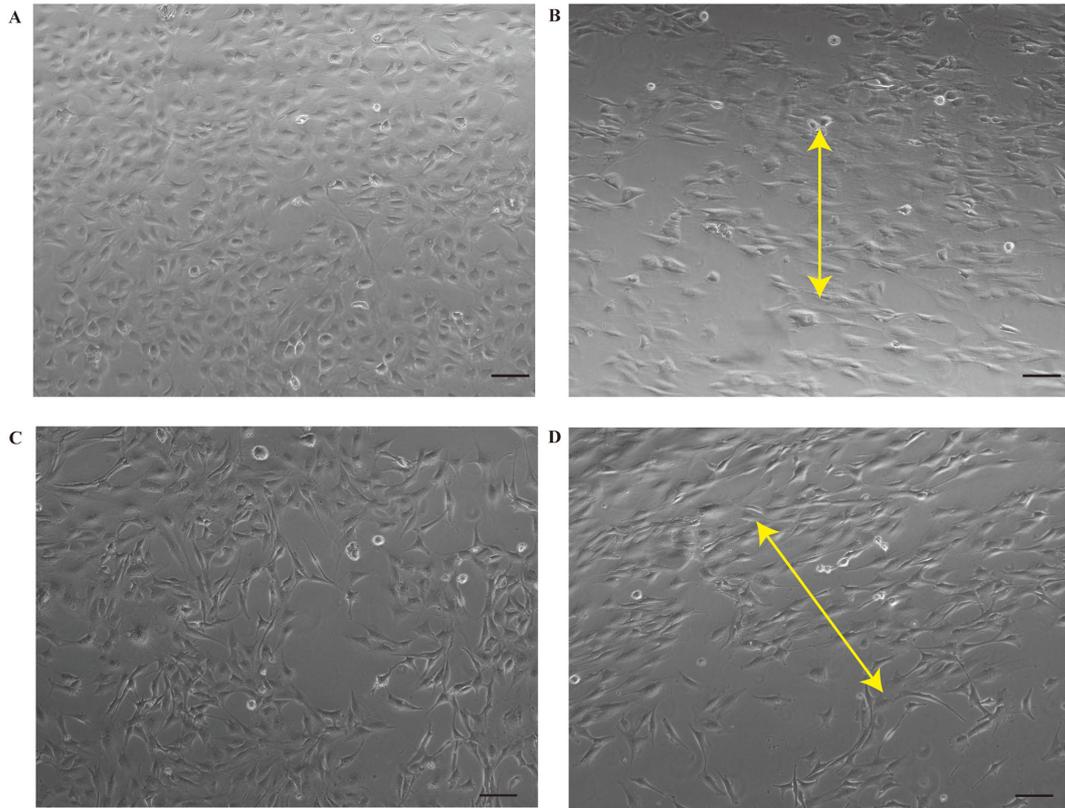


Figure 2. Representative images of chondrocytes and osteoblasts before and after mechanical stretch. (A) Before mechanical stretch, chondrocytes were cobblestone-like. (B) After mechanical stretch, chondrocytes became longer and perpendicular to the axis of the external strain. (C) Before mechanical stretch, osteoblasts were fibroblast-like and randomly oriented. (D) After mechanical stretch, osteoblasts were notably thinner compared with non-stretched cells. Magnification, x100. Scale bar, 100 μ m. The yellow arrow indicates direction of stretch field.

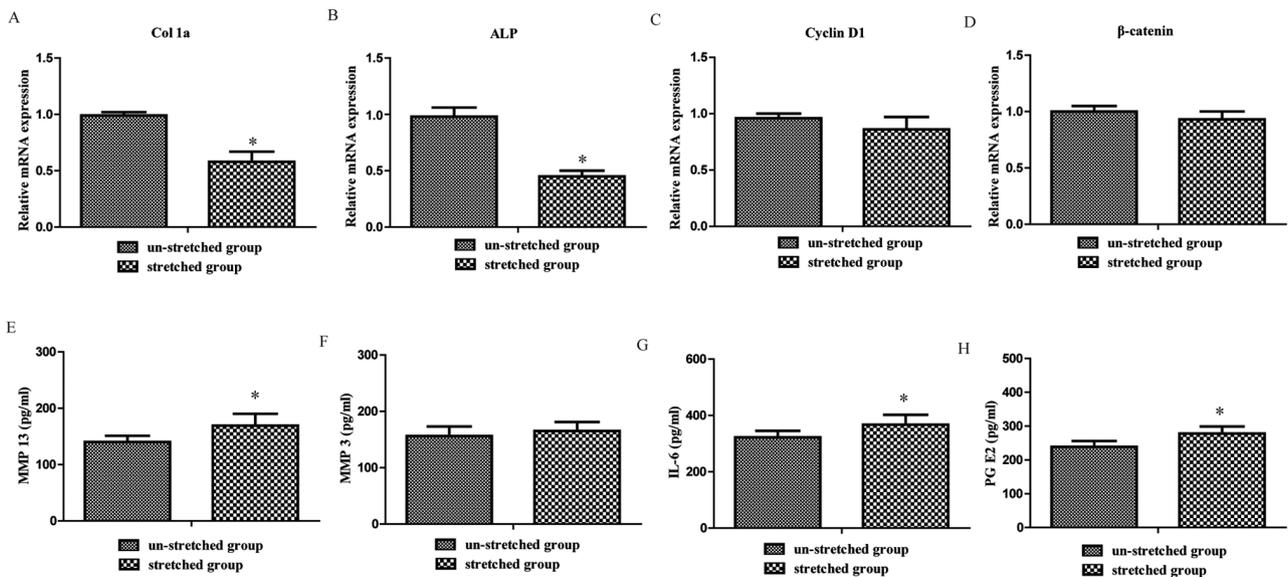


Figure 3. Excessive mechanical stretch inhibits osteoblast differentiation and increases the levels of cytokines in the supernatant of osteoblasts. mRNA expression levels of (A) Col 1a and (B) ALP in the stretched group were significantly decreased compared with the un-stretched group. There were no significant differences in (C) Cyclin D1 and (D) β -catenin expression levels between the un-stretched and stretched groups. Compared with the un-stretched group, the levels of (E) MMP 13, (G) IL-6 and (H) PG E2 were significantly increased in the stretched group, but there was no difference in the levels of (F) MMP 3. Data were analyzed using an unpaired Student's t-test. * $P < 0.05$ vs. un-stretched group. Col 1a, Collagen 1a; ALP, alkaline phosphatase; MMP, matrix metalloproteinase; IL-6, interleukin-6; PG E2, prostaglandin E2.

contrast, the expression levels of MMP 13 and a disintegrin and metalloproteinase with thrombospondin-like motifs 5

(ADAMTS 5) were significantly increased in the stretched group compared with the un-stretched group, respectively

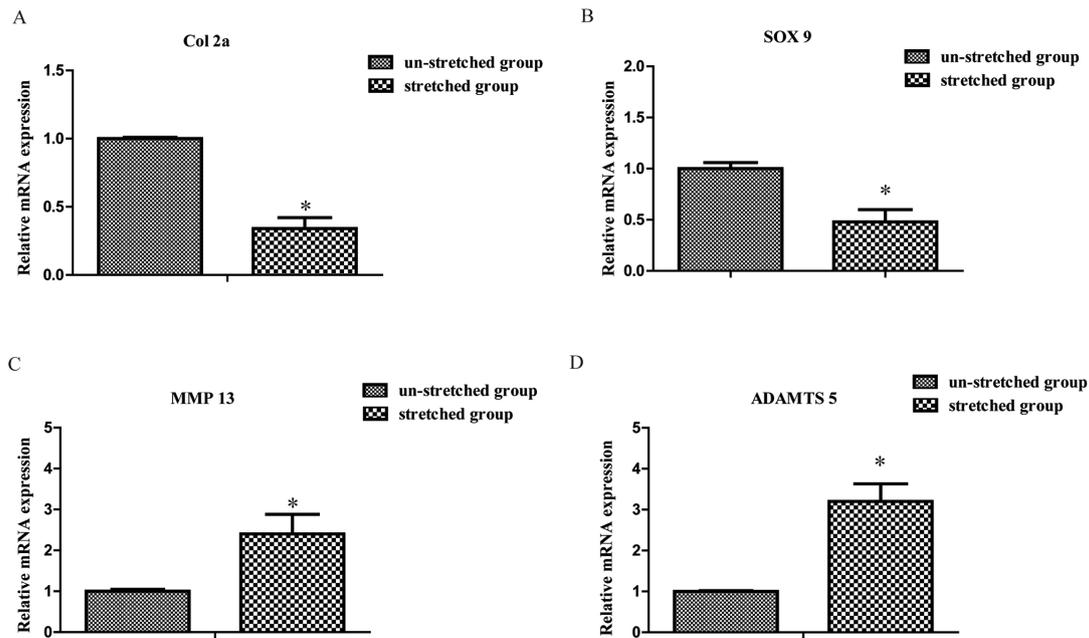


Figure 4. Excessive mechanical stretch induces chondrocyte catabolism. mRNA expression levels of (A) Col 2a, (B) SOX 9, (C) MMP 13 and (D) ADAMTS 5 in the un-stretched and stretched groups. Compared with the un-stretched group, the expression levels of Col 2a and SOX 9 were significantly decreased in the stretched group, whereas the expression levels of MMP 13 and ADAMTS 5 were significantly increased. Data were analyzed using an unpaired Student's t-test. *P<0.05 vs. un-stretched group. Col 2a, Collagen 1a; SOX 9, SRY-associated high mobility group-box gene 9; MMP, matrix metalloproteinase; ADAMTS 5, a disintegrin and metalloproteinase with thrombospondin-like motifs 5.

(1.00 ± 0.05 vs. 2.40 ± 0.48 and 1.00 ± 0.02 vs. 3.20 ± 0.43 , both $P < 0.05$; Fig. 4C and D).

Stretch-stimulated osteoblasts induce the catabolism and apoptosis of co-cultured chondrocytes. In order to investigate the effects of excessively stressed osteoblasts on the metabolism and apoptosis of chondrocytes, the present study established an indirect osteoblast-chondrocyte co-culture model. As presented in Fig. 5B and C, the mRNA expression levels of Col 2a and SOX 9 in the Scm group were significantly decreased compared with the control group, respectively (1.00 ± 0.01 vs. 0.36 ± 0.03 and 1.00 ± 0.02 vs. 0.34 ± 0.03 , both $P < 0.05$). Moreover, the mRNA expression levels of MMP 13 (Fig. 5A), Col X (Fig. 5D), OCN (Fig. 5E) and ADAMTS 5 (Fig. 5F) were significantly increased in the Scm group compared with the control group, respectively (1.00 ± 0.02 vs. 2.20 ± 0.21 and 1.00 ± 0.03 vs. 1.78 ± 0.12 , 1.00 ± 0.03 vs. 1.95 ± 0.11 and 1.00 ± 0.01 vs. 1.86 ± 0.11 , all $P < 0.05$). Compared with the control group, the flow cytometry results revealed that the apoptosis rate was significantly increased in the Scm group ($4.50 \pm 0.33\%$ vs. $14.30 \pm 0.90\%$, $P < 0.05$; Fig. 5G and H). Furthermore, compared with the control group, the protein expression levels of cleaved caspase 3/caspase 3 were significantly upregulated in the Scm group (1.00 ± 0.01 vs. 2.78 ± 0.22 , $P < 0.05$; Fig. 5I and J).

Wnt/ β -catenin signaling pathway is involved in the mechanism underlying stretched osteoblast-mediated alterations to the metabolism and apoptosis of chondrocytes. The present study further investigated the effect of stretched osteoblasts on the metabolism and apoptosis of chondrocytes. The western blotting results demonstrated that the protein expression levels of β -catenin in the Scm group were significantly higher compared with the control group (1.00 ± 0.02 vs. 1.86 ± 0.11 , $P < 0.05$; Fig. 5I and L), whereas the

protein expression levels of GSK-3 β in the Scm group were significantly lower in the Scm group compared with the control group (1.00 ± 0.03 vs. 0.48 ± 0.05 , $P < 0.05$; Fig. 5I and K). By using the Wnt inhibitor XAV-939, the present study also investigated whether the Wnt/ β -catenin signaling pathway was involved in stretched osteoblast-mediated effects on chondrocytes. The results indicated that the Wnt inhibitor notably mitigated stretched osteoblast-mediated effects on chondrocytes (all $P < 0.05$; Fig. 5A-L), suggesting that the Wnt/ β -catenin signaling pathway was involved in stretched osteoblast-mediated effects on the metabolism and apoptosis of the chondrocytes.

Discussion

Articular cartilage exists in a complex environment with various mechanical stresses that serve important roles in regulating the metabolism of articular chondrocytes (11,33). OA induced by mechanical force is characterized by decreased chondrocyte proliferation and degradation of the extracellular matrix (10-11). Bleuel *et al* (17) reported that a mechanical stretch of 3-10% protects chondrocytes from catabolism, whereas excessive mechanical stretch causes chondrocyte catabolism. Consistent with these previous results, the results of the present study demonstrated that a mechanical stretch of 20% induced chondrocyte catabolism, demonstrating that excessive mechanical stretch resulted in the catabolism of chondrocytes.

Subchondral bone serves an important role in the development of OA. A moderate amount of mechanical load has been reported to serve an important role in maintaining bone balance and bone mass (5-6). However, bone cells are not always in a state of physiological stress; in some cases,

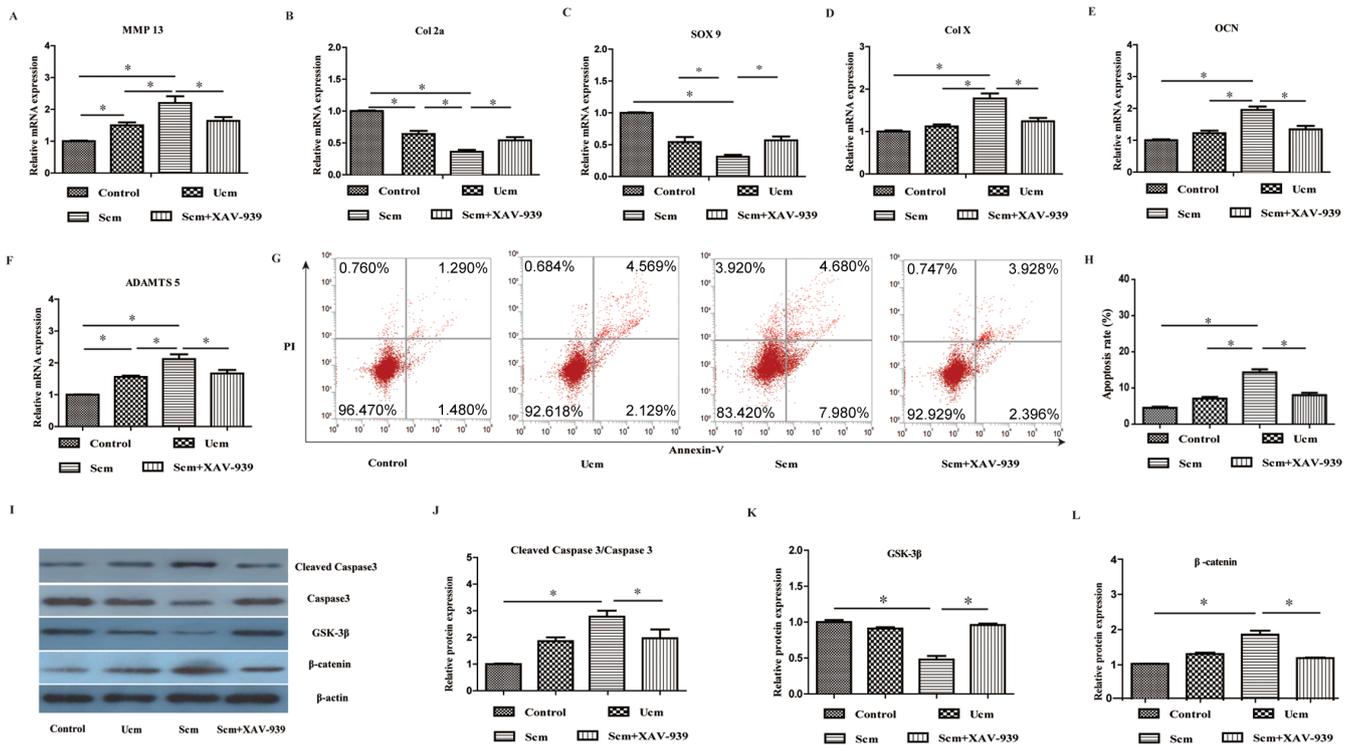


Figure 5. Inhibition of the Wnt signaling pathway mitigates mechanically stretched osteoblast-mediated effects on the catabolism and apoptosis of chondrocytes. mRNA expression levels of (A) MMP 13, (B) Col 2a, (C) SOX 9, (D) Col X, (E) OCN and (F) ADAMTS 5 were determined. mRNA expression levels of MMP 13, Col X, OCN and ADAMTS 5 in the Scm group were significantly increased compared with the control group (chondrocytes without co-culture). Expression levels of Col 2a and SOX 9 were significantly lower in the Scm group compared with the control group. Scm-mediated effects were significantly inhibited by XAV-939. Cell apoptosis was (G) assessed by flow cytometry and (H) quantified by FACS analysis after staining with Annexin V and PI. The results demonstrated that the rate of apoptosis in the Scm group was significantly increased compared with the control group, which was significantly reversed by XAV-939. Protein expression levels were (I) determined via western blotting and semi-quantified for (J) cleaved caspase 3/caspase 3, (K) GSK-3 β and (L) β -catenin. Protein expression levels of β -catenin and cleaved caspase 3/caspase 3 in the Scm group were significantly higher compared with the control group, whereas GSK-3 β protein expression levels in the Scm group were significantly lower compared with the control group. Scm-mediated effects were partly inhibited by XAV-939. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. * P <0.05. MMP, matrix metalloproteinase; Col, collagen; OCN, osteocalcin; ADAMTS 5, a disintegrin and metalloproteinase with thrombospondin-like motifs 5; Scm, stretched conditioned medium; Ucm, un-stretched conditioned medium; PI, propidium iodide; SOX 9, SRY-associated high mobility group-box gene 9.

the bone tissue is subjected to the overloaded mechanical environment (34,35). Our previous studies demonstrated that high intensity exercise induced decreased mineralization of subchondral bone and stiffer trabecular bone, which adversely affected the overlying articular cartilage (36,37). In addition, Tang *et al* (38) suggested that the effect of mechanical stretch (0, 6, 12 and 18%) on osteoblasts occurred in a magnitude-dependent manner; proper mechanical stretch promotes osteoblast proliferation and differentiation, whereas high mechanical stretch inhibits osteoblast proliferation and differentiation. Fushiki *et al* (31) considered 18% mechanical stretch as high magnitude stretch, Lin *et al* (39) considered 23% mechanical stretch as excessive mechanical stretch for osteoblasts, Zhang *et al* (40) used 20% mechanical stretch as excessive mechanical stretch for smooth muscle cells, and Yao *et al* (7) used 20% mechanical stretch as excessive mechanical stretch for osteoblasts. According to the experimental design in the present study, 20% mechanical stretch was selected as excessive mechanical stretch. The results of the present study demonstrated that an excessive mechanical stretch of 20% inhibited osteogenic differentiation of osteoblasts.

Articular cartilage and the underlying subchondral bone have been considered as a functional unit (41). Yao *et al* (7)

reported that mechanical stimulation altered the cartilage metabolism by directly affecting the subchondral bone during exercise and mechanical loading. Chondrocytes in the cartilage and osteoblasts in the subchondral bone are the primary cells involved in communication under mechanical conditions (42). Previous studies have performed numerous co-culture experiments to investigate the interactions between chondrocytes and osteoblasts (43,44). Co-culture can be divided into direct co-culture and indirect co-culture. Direct co-culture models permit cell-cell contact and paracrine interactions between osteoblast and chondrocytes in a 3-dimensional culture. The direct co-culture model has previously been used to determine the effects of co-culture on the phenotypic maintenance of osteoblasts and chondrocytes (43). On the other hand, indirect co-culture models allow cells to share medium without direct contact, which has also been used to investigate the interactions between osteoblasts and chondrocytes in previous studies (7,39). In the present study, an indirect chondrocyte-osteoblast co-culture model was established to investigate the effect of osteoblasts on excessive stretched chondrocytes. Conditioned medium from Scm and Ucm osteoblasts was collected to stimulate articular chondrocytes. Consistent with previous studies (39,45), the Scm group displayed significantly decreased mRNA expression levels

of Col 2a and SOX 9 compared with the control group. Furthermore, the mRNA expression levels of MMP 13, OCN and Col X were significantly upregulated in the Scm group compared with the control group. OCN and Col X are factors of the hypertrophic chondrocyte phenotype (35). These results indicated that excessive stretched osteoblasts inhibited chondrocyte matrix synthesis and promoted a hypertrophic change in chondrocytes. Furthermore, the results of the present study demonstrated that the apoptosis rate was significantly increased in the Scm group compared with the control group. Collectively, the results suggested that subchondral osteoblasts adversely affected chondrocytes under excessive mechanical loading.

There are molecular communications between articular cartilage and subchondral bone, which may be altered during the development of OA (34,35,44). Previous studies have reported that excessive mechanical stimulation increases the production of cytokines and MMPs by chondrocytes (11,46). In addition, a previous report indicated that excessive stimulation increases the expression of MMP 13, leading to bone remodeling (47). High magnitude stretch applied to osteoblasts increases PG E2 production, which is critical for bone remodeling (48). Consistent with previous studies, the results of the present study demonstrated that, compared with the un-stretched group, excessive mechanical stretch significantly promoted osteoblast production of MMP 13, IL-6 and PG E2, which have been reported as factors for OA (49). These findings suggested that excessive mechanical stretch may induce the catabolism and apoptosis of chondrocytes via an osteoblast-associated mechanism.

The Wnt/ β -catenin signaling pathway serves an important role in the development and maintenance of cartilage, but its detailed function remains controversial (22,23). During Wnt signaling, GSK-3 β causes the degradation of newly synthesized β -catenin. Inactivation of GSK-3 β leads to the accumulation of β -catenin, thus mediating proliferation and apoptosis (25,27,50). The results of the present study revealed that the protein expression levels of β -catenin were significantly upregulated in the Scm group compared with the control group, and the effect of stretched osteoblasts on chondrocytes was partly alleviated by Wnt inhibitor XAV-939. Compared with the Scm group, chondrocytes in the Scm + XAV-939 group displayed significantly decreased mRNA expression levels of MMP 13, ADAMTS 5, Col X and OCN. Meanwhile, the mRNA expression levels of Col 2a and SOX 9 were significantly upregulated. These findings suggested that blocking the Wnt/ β -catenin signaling pathway can promote the anabolism of chondrocytes and inhibit chondrocyte hypertrophy. These results demonstrated that excessive mechanically stretched osteoblasts affected the characteristics of chondrocytes via the Wnt/ β -catenin signaling pathway.

The present study had a number of limitations. First, osteoblasts were isolated from rat calvaria rather than the subchondral bone; however, a previous study demonstrated that osteoblasts from calvaria or long bones respond similarly to mechanical strain (41), ensuring the validity of the model in the present study. Secondly, the results of the present study demonstrated that high mechanical stretch increased MMP 13 and ADAMTS 5 expression levels by

direct mechanical stretching of chondrocytes and indirect co-culture with stretched osteoblast conditioned culture medium, but it is unknown which happened first. Third, a previous study demonstrated that chondrocytes can alter the characteristics of osteoblasts in a co-culture model, suggesting that stretched chondrocytes may affect osteoblasts (51). Both chondrocytes and osteoblasts are subjected to mechanical loading in the human body. Similar to a number of previous studies (7,39,45), the present study primarily focused on the effect of mechanically stretched osteoblasts on chondrocytes. Further investigations are required to understand the effect of mechanically stretched osteoblasts on mechanically stretched chondrocytes, as well as their interactions. Forth, Dickkopf1 (DKK1) is a canonical Wnt/ β -catenin inhibitor, but it has also been reported to serve as an inducer of AKT (52). Our preliminary experiment suggested that DKK1 may partly alleviate the effect of stretched osteoblasts on chondrocytes (data not shown); however, further investigation is required to understand whether or not this effect is due to the activation of AKT signaling.

In conclusion, the findings of this study indicated that excessive mechanical stretch promoted chondrocyte catabolism and inhibited osteoblast osteogenesis. In addition, alterations in chondrocyte can be induced by stressed osteoblasts, providing a possible explanation for the onset and progression of OA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GXN designed the study. CXS, SYL and SYX performed the experiments and interpreted the data. CXS and SYL collected the data. CXS, SYL and WTZ analyzed the data. GXN, CXS, SYL, WTZ and SYX wrote the manuscript. All authors read and approved the final version of the manuscript. GXN and CXS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of Nanfang Hospital, Southern Medical University (approval no. NFYY-2016-128; Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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