

# TIMP3/TGF- $\beta$ 1 axis regulates mechanical loading-induced chondrocyte degeneration and angiogenesis

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**Abstract.** Chondrocytes in injured cartilage tissue are susceptible to mechanical loading; mechanical overloading can induce cartilage degeneration. The aim of the present study was to investigate whether mechanical loading can regulate chondrocyte degeneration and angiogenesis via the tissue inhibitor of matrix metalloproteinase-3 (TIMP3)/transforming growth factor (TGF)- $\beta$ 1 axis. Primary human chondrocytes were obtained from knee articular cartilage of a healthy donor. Then, normal chondrocytes or TIMP3 lentivirus-transfected (LV-TIMP3) chondrocytes were subjected to mechanical loading (10 MPa compression). Then, chondrocytes were stimulated with 1  $\mu$ g/ml lipopolysaccharide (LPS) or treated with LDN-193189 (inhibitor of TGF- $\beta$ 1 signaling pathway). In addition, human umbilical vein endothelial cells (HUVECs) were co-cultured with chondrocytes or LV-TIMP3 chondrocytes. The expression levels of collagen-I, proteoglycan, TIMP3, TGF- $\beta$ 1, Smad2 and Smad3 were detected by reverse transcription-quantitative PCR and western blotting. Moreover, cell apoptosis and viability were determined using flow cytometry and MTT analysis, while cell migration was observed by Transwell assays. In addition, the vascular endothelial growth factor (VEGF)/VEGF receptor (R)2 binding rate in HUVECs was detected by a solid-phase binding assay. It was demonstrated that mechanical loading significantly inhibited the expression levels of collagen-I and proteoglycan in chondrocytes, as well as reducing cell proliferation and promoting cell apoptosis. In addition, the expression levels of TIMP3, TGF- $\beta$ 1, phosphorylated (p)-Smad2 and p-Smad3 were significantly decreased in degenerated chondrocytes that were induced by LPS, as well as in chondrocytes treated with LDN-193189. Furthermore, TIMP3 overexpression suppressed cell

migration and reduced the VEGF/VEGFR2 binding rate in HUVECs. Mechanical loading significantly inhibited the expression levels of TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3 in chondrocytes, and also increased cell migration of HUVECs; TGF- $\beta$ 1 treatment or TIMP3 overexpression reversed these effects. Thus, the TIMP3/TGF- $\beta$ 1 axis may be a vital signaling pathway in mechanical loading-induced chondrocyte degeneration and angiogenesis.

## Introduction

Osteoarthritis (OA), as a degenerative joint inflammatory disease, influences the structure and function of synovial joints, which is characterized by articular cartilage degeneration, resulting in the narrowing of joint space, subchondral sclerosis and osteophyte formation (1,2). OA is one of the leading causes of global disability, and affects ~40% of the elderly population (3). Furthermore, OA causes a significant socioeconomic burden, including direct cost of health care and indirect cost associated with productivity loss (4). Thus, it is important to investigate the mechanisms involved in OA progression.

Cartilage degeneration as the pivotal cause in OA has attracted increased attention (5). It is well-known that chondrocytes in cartilage tissues exhibit cell hypertrophy and extracellular matrix (ECM) degradation, followed by the vascular invasion to the cartilage, thus exacerbating the injury of affected joints (6). Chondrocytes also play an important role in maintaining the balance of cartilage degeneration and repair, and catabolic and abnormal differentiation of cytokines and growth factors in chondrocytes can induce ECM degradation (5,7). A previous study has reported that transforming growth factor (TGF)- $\beta$ 1 signaling is a pivotal mediator in cartilage injury and OA development (8). Moreover, TGF- $\beta$ 1 may induce ECM mineralization, cell hypertrophy and angiogenesis, thus accelerating cartilage degeneration (9). Smad2/3, as essential downstream molecules of the TGF- $\beta$ 1 pathway, can induce tissue inhibitor of matrix metalloproteinase-3 (TIMP3) expression in human chondrocytes (10,11). Therefore, the TIMP3/TGF- $\beta$ 1 pathway has the potential to participate in OA development; however, this requires further investigation.

As mechanosensitive cells, chondrocytes in the injured cartilage tissues are susceptible to mechanical loading, and mechanical overloading can induce cartilage

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degeneration (12). In addition, mechanical overloading can lead to the activation of TGF- $\beta$ 1 signaling in chondrocytes (13). Thus, it was investigated whether mechanical loading can regulate chondrocyte degeneration and angiogenesis via the TIMP3/TGF- $\beta$ 1 axis. In the current study, primary human chondrocytes were isolated, and then the effects of mechanical loading on chondrocyte metabolism were examined. Furthermore, a TIMP3 overexpression lentivirus (LV-TIMP3) was used to investigate the role of the TIMP3/TGF- $\beta$ 1 axis in mechanical loading induced-chondrocyte degeneration and angiogenesis.

## Materials and methods

**Ethical consideration.** This study was approved by the Ethics Committee of First Affiliated Hospital, Heilongjiang University of Chinese Medicine (approval no. 2017HZYLL-021), and written patient consent was obtained.

**Isolation and culture of primary human chondrocytes.** Primary human chondrocytes were isolated from healthy knee articular cartilage of a 70-year old male donor who underwent lower extremity traumatic trauma surgery on 10 December 2018 at First Affiliated Hospital, Heilongjiang University of Chinese Medicine (Harbin, China). First, knee articular cartilage was processed into pieces, and then treated by enzymatic digestion (25% trypsin and 0.2% type II collagenase) as previously described (14). The isolated chondrocytes were cultured in complete DMEM (Sigma-Aldrich; Merck KGaA) containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) under standard incubation conditions (5% CO<sub>2</sub> and 37°C).

Primary human chondrocytes were identified using 1% toluidine blue (Sigma-Aldrich; Merck KGaA) staining for 2 h at room temperature. In addition, the expression levels of chondrocyte markers (collagen-I and collagen-II) were detected by immunocytochemistry. In brief, the cells were subjected to 4% paraformaldehyde for 15 min at room temperature for cell fixation and goat serum for 30 min for cell blockage at room temperature. Then, the cells were reacted with collagen-I (1:50; cat. no. ab34710; Abcam) or collagen-II antibody (1:50; cat. no. ab34712; Abcam) overnight at 4°C, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:500; cat. no. ab150077; Abcam) for 1 h at room temperature. Following washing with PBS, cells were observed by light microscope (MX 5200H; Meiji Techno Co., Ltd.) (magnification, x100 and x400). Primary human chondrocytes at the third passage were used for the following experiments.

**Lentivirus infection.** The LV-TIMP3 overexpression lentivirus (titer with  $2 \times 10^8$  TU/ml) was purchased from Shanghai GeneChem Co., Ltd. Chondrocytes were infected with LV-TIMP3 (MOI=20). The stable LV-TIMP3-infected cell lines (named as LV-TIMP3 chondrocytes) were established using culture medium containing puromycin (10  $\mu$ l/ml) for 5 days before the expression level of LV-TIMP3 was assessed using western blotting.

**Mechanical loading to chondrocytes.** To evaluate the effect of mechanical overloading on cartilage metabolism, chondrocytes

were treated with TGF- $\beta$ 1 (5 ng/ml; Sigma-Aldrich; Merck KGaA) or LV-TIMP3 with or without mechanical loading for 24 h at 37°C. Chondrocytes with mechanical loading were subjected to a mechanical pressure unit (15) with 10 MPa compression for 72 h. Chondrocytes without loading were set as the control group. After 72 h, the culture medium of chondrocytes in these groups was collected for the Transwell experiments.

**Cell treatment with lipopolysaccharide (LPS) or LDN-193189.** To establish the chondrocyte degeneration model, chondrocytes were stimulated with 1  $\mu$ g/ml LPS (Sigma-Aldrich; Merck KGaA) for 24 h (LPS group) at 37°C. Then, in order to examine the relationship of the TIMP3/TGF- $\beta$ 1 signaling pathway and cartilage degeneration, chondrocytes treated with LDN-193189 (0.5  $\mu$ M; Sigma-Aldrich; Merck KGaA), an inhibitor of the TGF- $\beta$ 1 signaling pathway, for 24 h (LDN-193189 group) at 37°C. The chondrocytes without treatment were used as the control group.

**Reverse transcription-quantitative (RT-q)PCR.** Chondrocytes after the various treatments were collected and total RNA from these cells was obtained by TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Then cDNA was obtained by reverse transcription of RNA using a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The RT-qPCR was performed using SYBR Premix Ex Taq TM II (Takara Biotechnology Co., Ltd.). The PCR primers for collagen-I, proteoglycan, TIMP3, TGF- $\beta$ 1, Smad2, Smad3 and GAPDH are presented in Table I. The PCR parameters were set as follows: 95°C for 10 min, 40 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 15 sec. GAPDH was used as the reference gene. The relative expression levels of these genes were calculated using comparative threshold (Ct) cycle method ( $2^{-\Delta\Delta Ct}$ ) (16).

**Western blotting.** Chondrocytes after various treatments were collected, and then lysed in RIPA lysis buffer (Gibco; Thermo Fisher Scientific, Inc.) supplemented with PMSF (1 mM; Sigma-Aldrich; Merck KGaA). Protein was extracted by centrifugation at 12,000 x g for 20 min at 4°C and detected using a bicinchoninic acid kit (Sangon Biotech Co., Ltd.). Protein samples (40  $\mu$ g) were separated via 10% SDS-PAGE, and transferred to PVDF membranes, followed by the blocking with 5% non-fat milk for 1 h at room temperature. Then, the membrane was incubated with anti-human collagen-I (1:200; cat. no. sc-59772; Santa Cruz Biotechnology, Inc.), proteoglycan (1:200; cat. no. MAB1582; Sigma-Aldrich; Merck KGaA), TIMP3 (1:200; cat. no. sc-373839; Santa Cruz Biotechnology, Inc.), TGF- $\beta$ 1 (1:200; cat. no. sc-130348; Santa Cruz Biotechnology, Inc.), phosphorylated (p)-Smad2 (1:200; cat. no. sc-59772; Santa Cruz Biotechnology, Inc.), Smad2 (1:200; cat. no. sc-393312; Santa Cruz Biotechnology, Inc.), p-Smad3 (1:200; cat. no. ab-53100; Abcam), Smad3 (1:200; cat. no. sc-101154; Santa Cruz Biotechnology, Inc.) or  $\beta$ -actin (1:200; cat. no. sc-8432; Santa Cruz Biotechnology, Inc.) antibodies overnight at 4°C. Membranes were washed with PBS, followed by incubation with peroxidase-labeled second antibody (1:5,000; cat. no. 115-035-003; Jackson ImmunoResearch Laboratories, Inc.) for 2 h at room temperature. Then, enhanced chemiluminescence (EMD Millipore) was used to detect the

Table I. Primer sequences for PCR.

Gene	Primer sequence
Collagen-I	F: 5'-GACATCCCTGAAGTCAGCTGC-3'
Collagen-I	R: 5'-TCCCTTGGGTCCCTCGAC-3'
Proteoglycan	F: 5'-ATGGAGTTCAGATTCTTCATC-3'
Proteoglycan	R: 5'-TCAGTAGGCTTCACCGACCT-3'
TIMP3	F: 5'-CTCGAGCAAGGAGGAAGTGGGTG-3'
TIMP3	R: 5'-GCGGCCGCAATACAGAAGTGTCT-3'
TGF- $\beta$ 1	F: 5'-GACCGCAACAACGCAATCTA-3'
TGF- $\beta$ 1	R: 5'-AGGTGTTGAGCCCTTTCCA-3'
Smad2	F: 5'-GTTCTGCTTTGCTGAGAC-3'
Smad2	R: 5'-TCTCTTTGCCAGGAATGCTT-3'
Smad3	F: 5'-AGCACACAATAACTTGGACC-3'
Smad3	R: 5'-TAAGACACACTGGAACAGCGGATG-3'
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3'
GAPDH	R: 5'-TGGTGAAGACGCCAGTGGA-3'

Forward, forward; r, reverse; TIMP3, tissue inhibitor of matrix metalloproteinase-3; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

protein expression levels. Band quantification was performed using ImageJ software (version 1.54; National Institutes of Health).

**MTT assay.** Chondrocytes ( $5 \times 10^4$ /well) were cultured in a 96-well plate. The next day, chondrocytes were treated with or without mechanical loading as aforementioned and then the cells were incubated for 72 h (saturation humidity, 37°C, 5% CO<sub>2</sub>). MTT (10  $\mu$ l; 5 mg/ml; Sigma-Aldrich; Merck KGaA) was added into wells at 24, 48 or 72 h, and incubated for 4 h. Then, 100  $\mu$ l DMSO (Sigma-Aldrich; Merck KGaA) was added. The zero hole (medium, MTT, DMSO) and blank hole were set up. The absorbance values at 570 nm were measured by microplate reader (Molecular Devices, LLC).

**Flow cytometry analysis of cell apoptosis.** An Annexin V-FITC Apoptosis Detection kit (cat. no. ab14085; Abcam) was used to evaluate the cell apoptosis. Chondrocytes after various treatments were digested with 0.25% trypsin. After washing with PBS, chondrocytes were resuspended with 1X Binding Buffer, followed by incubation with 5 propidium iodide (PI) and 5  $\mu$ l FITC-Annexin V for 15 min at 25°C in the dark, with a mixture of 1X Binding Buffer. Cells were detected using a flow cytometer (FACSCalibur; BD Biosciences) and analyzed using CellQuest software (v. 3.0; Becton, Dickinson and Company). The apoptotic rate was calculated as the percentage of early + late apoptotic cells.

**Transwell assay for migration of human umbilical vein endothelial cells (HUVECs).** HUVECs were provided by Obio Technology (Shanghai) Corp. and maintained in RPMI-1640 medium with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) under 37°C and 5% CO<sub>2</sub>. The migration of HUVECs was analyzed using Transwell assays (pore size, 0.4  $\mu$ M; EMD Millipore). HUVECs ( $5 \times 10^5$  cells/ml in 300  $\mu$ l serum-free medium) were seeded into the upper chamber. Then, 300  $\mu$ l cell serum-free medium from chondrocytes in

the control, mechanical loading and LV-TIMP3 + mechanical loading groups was placed in the lower chamber for 24 h. Subsequently, the cells were stained with crystal violet for 10 min at 37°C. Migration of HUVECs was observed using a light microscope in five randomly selected fields (Olympus Corporation) (magnification, x200).

**Co-culture of HUVECs and chondrocytes.** Co-culture of HUVECs with the chondrocytes was constructed using Transwell inserts (EMD Millipore). HUVECs ( $5 \times 10^5$  cells/ml in 300  $\mu$ l medium) were seeded into the upper chamber. Chondrocytes (control group) or LV-TIMP3 chondrocytes were cultured in the lower chamber and treated with 1  $\mu$ g/ml LPS for 24 h at 37°C. Migration of HUVECs was observed under a light microscope (Olympus Corporation) (magnification, x200) and HMVECs were collected for the following solid-phase binding assay.

**Solid-phase binding assay.** The binding of vascular endothelial growth factor (VEGF) to VEGF receptor (R)2 was detected by solid-phase binding assays. Chondrocytes were collected, and then lysed in RIPA lysis buffer supplemented with 1 mM PMSF. Recombinant VEGFR2 protein (50 ng/ml) in PBS was coated in a 96-well plate at 4°C overnight, and then each well was washed with PBS. After blocking with 4% bull serum albumin (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, supernatant obtained from chondrocytes by centrifugation at 12,000 x g for 15 min at 4°C was added into wells at 4°C overnight. After washing with PBS, the bound proteins were detected by biotinylated-anti-VEGF antibody (1:200; cat. no. BAF293; R&D Systems China Co., Ltd.) at 37°C for 1 h, followed by the incubation of avidin-horseradish peroxidase at 37°C for 30 min. Then, plates were developed with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and measured at 450 nm.

**ELISA.** The culture supernatant from the aforementioned treated cells (centrifugation at 2,500 x g for 5 min at room temperature) was measured by commercial ELISA kits (Cusabio Technology LLC) for the concentrations of proteoglycan (cat. no. CSB-E14124h), interleukin (IL)-1 $\beta$  (cat. no. CSB-E08053h), IL-6 (cat. no. CSB-E04638h), IL-4 (cat. no. CSB-E04633h), IL-13 (cat. no. CSB-E04601h) and TGF- $\beta$ 1 (cat. no. CSB-E04725h), according to the manufacturer's instructions. Supernatant samples (100  $\mu$ l) were incubated in a 96-well plate that was coated with antigen in each well at 37°C for 1.5 h, followed by incubation with biotinylated antibody (100  $\mu$ l) at 37°C for 1 h. Then, avidin peroxidase (100  $\mu$ l) was added to each well and incubated at 37°C for 30 min, followed by substrate solution (90  $\mu$ l) in the dark at 37°C for 15 min and stopping solution (50  $\mu$ l). Absorbance at 450 nm was detected by a microplate reader (Thermo Fisher Scientific, Inc.) to calculate the level of each cytokine.

**Statistical analysis.** SPSS 12.0 statistical analysis software (SPSS, Inc.) was used for statistical analysis. Data are presented as the mean  $\pm$  SD of three experimental repeats. Data were analyzed by one-way ANOVA followed by post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

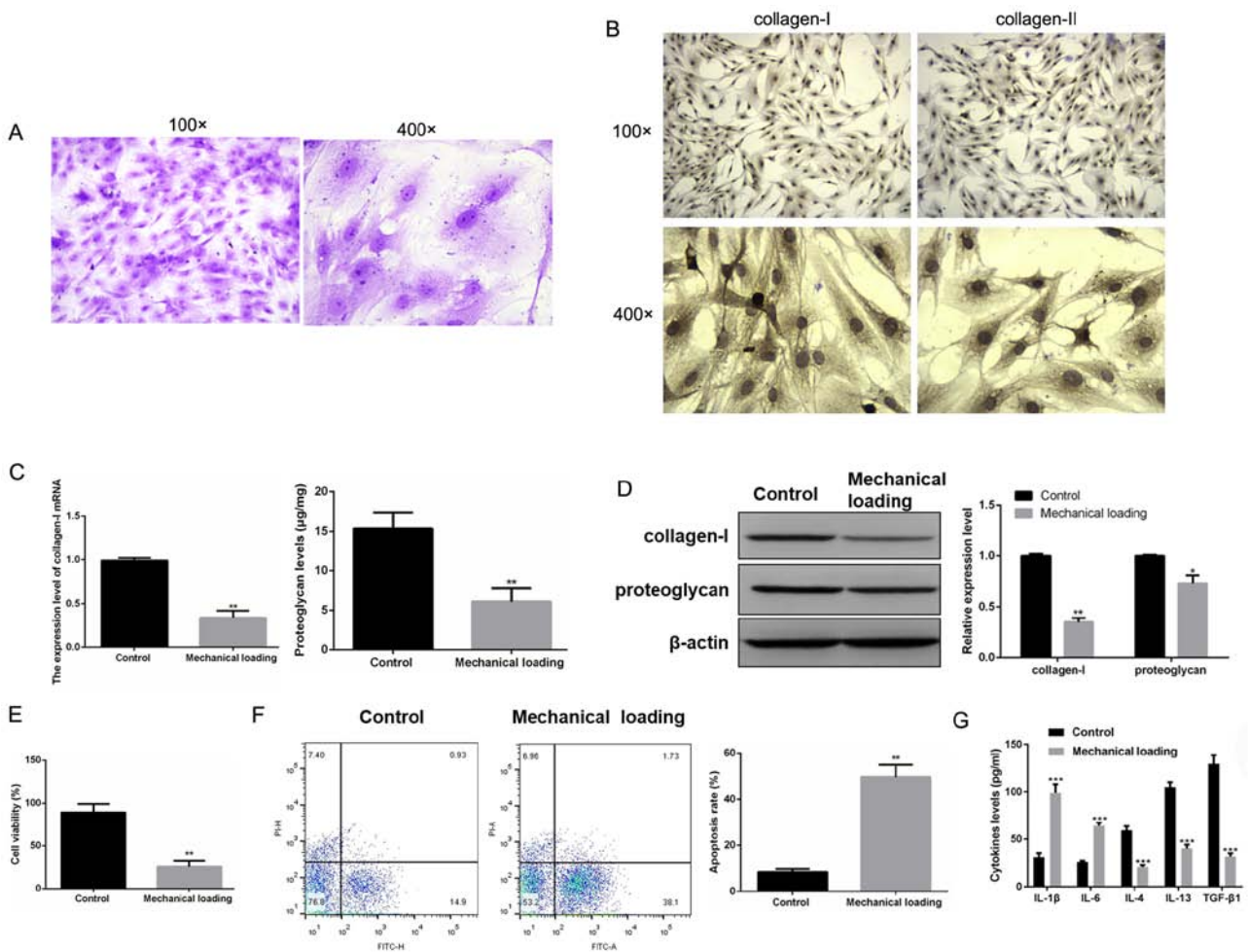


Figure 1. Effects of mechanical loading on chondrocyte metabolism. (A) Toluidine blue staining of primary human chondrocytes. (B) Expression of chondrocyte markers, collagen-I and collagen-II, in primary human chondrocytes determined via immunocytochemistry. (C) mRNA expression of collagen-I and the level of proteoglycan in chondrocytes from control and mechanical loading groups measured by reverse transcription-quantitative PCR and ELISA. (D) Protein expression levels of collagen-I and proteoglycan in chondrocytes from control and mechanical loading groups assessed by western blotting. (E) Cell viability of chondrocytes in the control group and mechanical loading group measured by MTT assay. (F) Cell apoptotic rate of chondrocytes in the control group and mechanical loading group determined by flow cytometry analysis. (G) Levels of inflammatory factors in the control group and mechanical loading group measured by ELISA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control group. IL, interleukin; TGF- $\beta 1$ , transforming growth factor  $\beta 1$ .

## Results

**Mechanical loading influences chondrocyte metabolism.** To determine the effects of mechanical loading on chondrocyte metabolism, primary human chondrocytes were isolated and subjected to mechanical loading. Toluidine blue staining identified that primary human chondrocytes were stained as blue-violet, which suggested the positive expression of proteoglycan (Fig. 1A). Moreover, immunocytochemistry results demonstrated the positive expression levels of collagen-I and collagen-II (Fig. 1B). Thus, it was indicated that primary human chondrocytes were successfully isolated.

The mRNA expression and protein levels of collagen-I and proteoglycan were both significantly lower in chondrocytes subjected to mechanical loading compared with normal chondrocytes ( $P < 0.05$ ; Fig. 1C and D). MTT assay results also suggested that mechanical loading inhibited chondrocyte proliferation ( $P < 0.01$ ; Fig. 1E). Furthermore, flow cytometry analysis also found that the cell apoptotic rate was significantly increased in chondrocytes subjected to mechanical loading compared with normal chondrocytes ( $P < 0.01$ ; Fig. 1F).

Furthermore, ELISAs demonstrated that the levels of IL-1 $\beta$  and IL-6 were significantly increased, while the levels of IL-4, IL-13 and TGF- $\beta 1$  were significantly decreased in chondrocytes subjected to mechanical loading compared with normal chondrocytes ( $P < 0.001$ ; Fig. 1G).

**Relationship between the TIMP3/TGF- $\beta 1$  axis, chondrocyte degeneration and angiogenesis.** To investigate the effects of the TIMP3/TGF- $\beta 1$  signaling pathway on chondrocyte degeneration, degeneration was induced by LPS. It was found that LPS treatment significantly inhibited the protein expression levels of collagen-I and proteoglycan compared with in normal chondrocytes ( $P < 0.05$  and  $P < 0.01$ , respectively; Fig. 2A), which indicated that chondrocyte degeneration was successfully induced by LPS.

In addition, the levels of IL-1 $\beta$  and IL-6 were significantly increased, and the levels of IL-4, IL-13 and TGF- $\beta 1$  were significantly decreased in chondrocytes treated with LPS compared with normal chondrocytes ( $P < 0.001$ ; Fig. 2B). Western blotting results identified that the protein expression levels of TIMP3, TGF- $\beta 1$ , p-Smad2 and p-Smad3 were



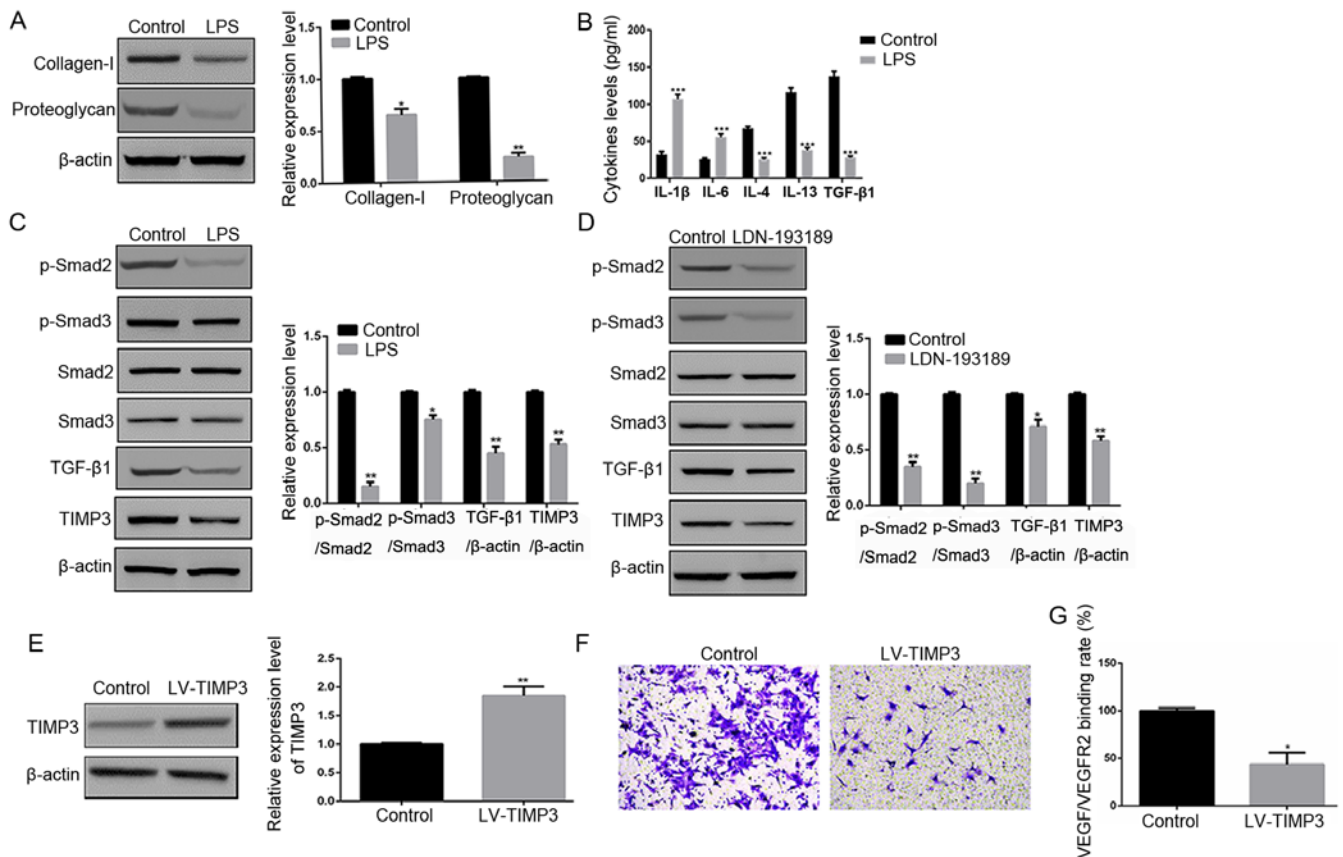


Figure 2. Effects of the TIMP3/TGF- $\beta$ 1 axis on chondrocyte degeneration and angiogenesis. (A) Protein expression levels of collagen-I and proteoglycan in chondrocytes from the control group and LPS group as determined via western blotting. (B) Levels of inflammatory factors in chondrocytes from the control group and LPS group as determined via ELISA. (C) Protein expression levels of TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3 in chondrocytes from the control group and LPS group assessed via western blotting. (D) Protein expression levels of TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3 in chondrocytes from the control group and LDN-193189 group determined via western blotting. (E) Protein expression of TIMP3 in chondrocytes from the control group and LV-TIMP3 group assessed via western blotting. (F) Cell migration of HUVECs when co-cultured with chondrocytes or LV-TIMP3 chondrocytes determined via Transwell assays. (G) VEGF/VEGFR2 binding rate in HUVECs when co-cultured with chondrocytes or LV-TIMP3 chondrocytes as determined via solid-phase binding assays. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control group. LPS, lipopolysaccharide; p-, phosphorylated; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; TIMP3, tissue inhibitor of matrix metalloproteinase-3; HUVECs, human umbilical vein endothelial cells.

significantly decreased in chondrocytes treated with LPS, compared with normal chondrocytes ( $P < 0.05$ ; Fig. 2C).

It was also demonstrated that LDN-193189 (an inhibitor of the TGF- $\beta$ 1 signaling pathway) significantly inhibited the protein expression levels of TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3 ( $P < 0.05$ ; Fig. 2D). Furthermore, LV-TIMP3 chondrocytes were constructed, and western blotting results indicated that TIMP3 was overexpressed in LV-TIMP3 chondrocytes ( $P < 0.01$ ; Fig. 2E).

Co-culture of chondrocytes and HUVECs showed that LV-TIMP3 chondrocytes suppressed cell migration of HUVECs compared with normal chondrocytes (Fig. 2F). Moreover, the VEGF/VEGFR2 binding rate in HUVECs in the LV-TIMP3 group was significantly lower compared with the control group ( $P < 0.05$ ; Fig. 2G).

**Mechanical loading influences chondrocyte degeneration and angiogenesis via the TIMP3/TGF- $\beta$ 1 axis.** It was found that, compared with control chondrocytes, mechanical loading significantly inhibited the protein expression levels of collagen-I, proteoglycan, TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3, while TGF- $\beta$ 1 treatment or TIMP3 overexpression significantly increased their expression levels compared

with control chondrocytes. Furthermore, the combination of mechanical loading and TGF- $\beta$ 1 treatment or TIMP3 overexpression significantly reduced the protein expression levels of collagen-I, proteoglycan, TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3 compared with TGF- $\beta$ 1 treatment or TIMP3 overexpression alone, and significantly increased their expression compared with chondrocytes subjected to mechanical loading alone ( $P < 0.05$ ; Fig. 3A).

Compared with control chondrocytes, TGF- $\beta$ 1 treatment or TIMP3 overexpression alone significantly decreased the levels of IL-1 $\beta$  and IL-6, but increased the levels of IL-4, IL-13 and TGF- $\beta$ 1; however, the combination of mechanical loading and TGF- $\beta$ 1 treatment or TIMP3 overexpression partly reversed their levels compared with TGF- $\beta$ 1 treatment or TIMP3 overexpression alone ( $P < 0.05$ ; Fig. 3B).

Furthermore, cell migration of HUVECs was increased in the mechanical loading group, while it was decreased in the TGF- $\beta$ 1 and LV-TIMP3 groups, compared with the control group. It was also indicated that the combination of mechanical loading and TGF- $\beta$ 1 treatment or TIMP3 overexpression promoted cell migration of HUVECs compared with cells subjected to TGF- $\beta$ 1 treatment or TIMP3 overexpression alone (Fig. 3C).

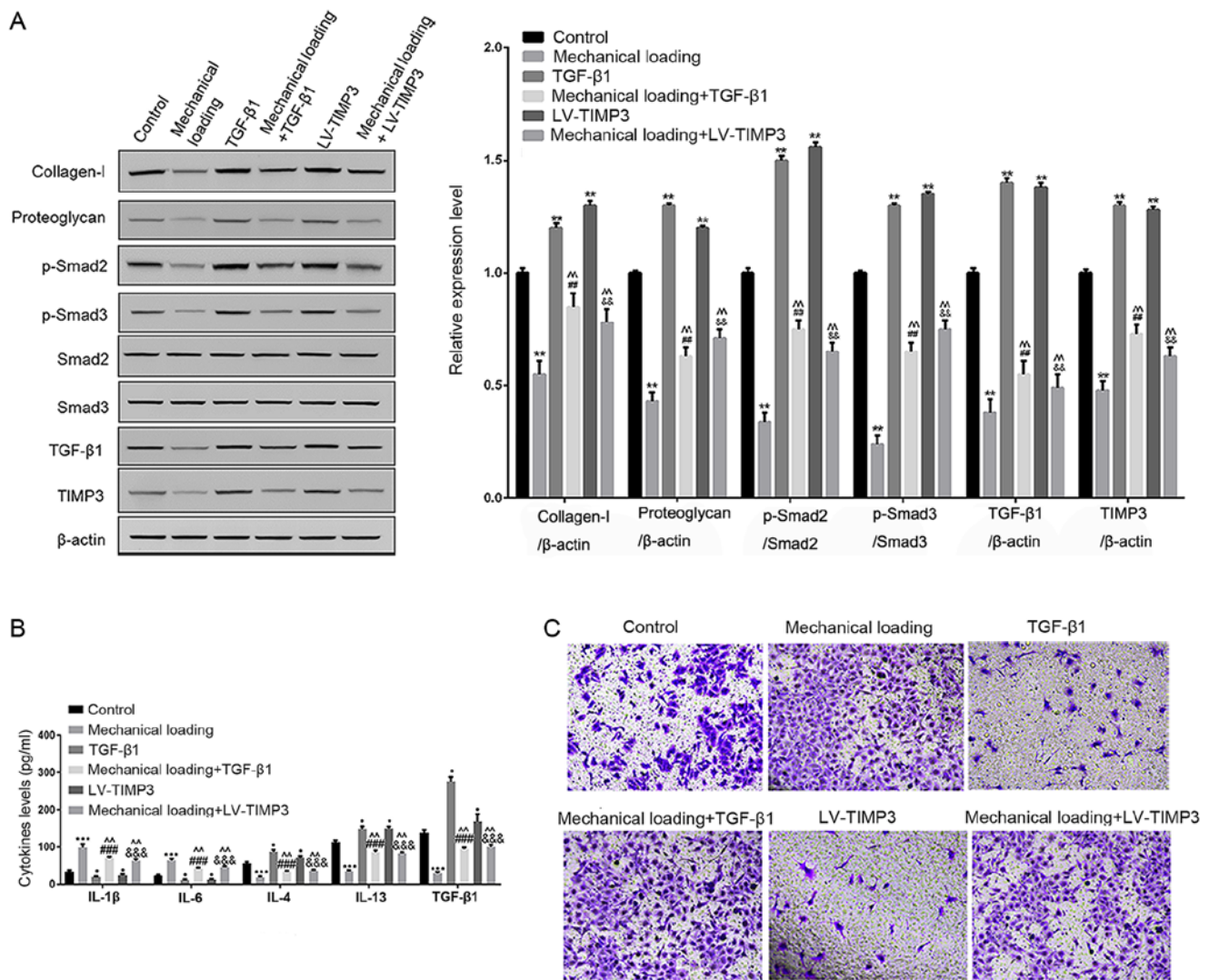


Figure 3. Effects of mechanical loading on the TIMP3/TGF- $\beta$ 1 axis and angiogenesis. (A) Protein expression levels of collagen-I, proteoglycan, TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3 in chondrocytes in the control group, mechanical loading group, TGF- $\beta$ 1 group, mechanical loading + TGF- $\beta$ 1 group, LV-TIMP3 group and mechanical loading + LV-TIMP3 group assessed via western blotting. (B) Levels of inflammatory factors in chondrocytes in the control group, mechanical loading group, TGF- $\beta$ 1 group, mechanical loading + TGF- $\beta$ 1 group, LV-TIMP3 group and mechanical loading + LV-TIMP3 group determined via ELISA. (C) Cell migration of HUVECs in the control group, mechanical loading group, TGF- $\beta$ 1 group, mechanical loading + TGF- $\beta$ 1 group, LV-TIMP3 group and mechanical loading + LV-TIMP3 group determined using Transwell assays. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. control group. ^^ $P$ <0.01, ^^& $P$ <0.001 vs. TGF- $\beta$ 1 group. && $P$ <0.01, &&& $P$ <0.001 vs. LV-TIMP3 group. ^^ $P$ <0.01 vs. mechanical loading group. p-, phosphorylated; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; TIMP3, tissue inhibitor of matrix metalloproteinase-3; HUVECs, human umbilical vein endothelial cells.

## Discussion

In the present study, primary human chondrocytes were successfully isolated. It was demonstrated that mechanical loading significantly inhibited the expression levels of collagen-I and proteoglycan in chondrocytes, as well as reducing cell viability and promoting cell apoptosis, thus suggesting that mechanical loading leads to chondrocyte degeneration. In addition, the expression levels of TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3 were significantly decreased in degenerated chondrocytes induced by LPS, as well as in chondrocytes treated with LDN-193189. Furthermore, TIMP3 overexpression suppressed cell migration and reduced the VEGF/VEGFR2 binding rate in HUVECs. It was also identified that mechanical loading significantly inhibited the expression levels of collagen-I, proteoglycan, TIMP3, TGF- $\beta$ 1, p-Smad2 and p-Smad3 in chondrocytes, increased the contents

of IL-1 $\beta$  and IL-6, decreased the levels of IL-4, IL-13 and TGF- $\beta$ 1, and promoted the migration of HUVECs; TGF- $\beta$ 1 treatment or TIMP3 overexpression reversed these results.

It is well-known that articular chondrocytes are responsible for the maintenance of articular cartilage homeostasis, and the dysregulation of this is related to the pathological processes of cartilage degeneration in OA (17). TGF- $\beta$ 1 has been researched in chondrocytes and OA for several years. A previous study reported that TGF- $\beta$ 1 is an essential growth factor that maintains cartilage integrity and ECM, and that the deactivation of TGF- $\beta$ 1 signaling can induce cartilage degeneration (18). However, it has also been shown that TGF- $\beta$ 1 is upregulated in subchondral bone of OA, and cartilage degradation is attenuated by inhibiting the activity of TGF- $\beta$ 1, indicating that TGF- $\beta$ 1 overexpression may be a trigger of OA (19,20). Moreover, the present results suggested that the expression levels of TGF- $\beta$ 1 and p-Smad2/3 were reduced in degenerated chondrocytes induced by LPS.

Furthermore, it was demonstrated that TIMP3 expression was downregulated in degenerated chondrocytes. TIMPs are endogenous inhibitors of matrix metalloproteinases (MMPs), and MMPs are involved in ECM degradation in chondrocytes of OA (21). It has been previously revealed that TGF- $\beta$ -induced TIMP3 can inhibit MMP activity in cartilage (10,21). In addition, Smad2/3, as classical intracellular mediators of the TGF- $\beta$  signaling pathway, can upregulate TIMP3 in human chondrocytes; TIMP3 is considered as a target gene of Smad2/3 in the TGF- $\beta$  signaling pathway (10,22). In line with these studies, the present results suggested that TIMP3 expression was reduced when the TGF- $\beta$ 1/Smad2/3 pathway was inhibited. Furthermore, it was identified that TIMP3 overexpression suppressed cell migration and reduced the VEGF/VEGFR2 binding rate in HUVECs. A previous study has also reported that the TGF- $\beta$  signaling pathway can promote angiogenesis in endothelial progenitor cells by upregulating VEGF expression (23). Moreover, it has been shown that TGF- $\beta$ 1 induces angiogenesis in cartilage of OA (9,19). Collectively, it was speculated that the TIMP3/TGF- $\beta$ 1 axis may be involved in chondrocyte degeneration and angiogenesis.

Mechanical stress is considered as a risk factor for OA development (24). It has been revealed that excessive loading in joints results in cartilage degeneration (25,26). The present results indicated that mechanical loading significantly inhibited the expression levels of collagen-I and proteoglycan in chondrocytes. Collagen-I and proteoglycan, as anabolic genes, are involved in ECM metabolism of chondrocytes, and the abnormal expression of these genes may lead to chondrocyte degeneration (27). In addition, a previous study reported an increased cell apoptosis rate in the chondrocytes of OA cartilage compared with healthy cartilage, thus suggesting that chondrocyte apoptosis is closely associated with OA progression (28). Moreover, it has been shown that mechanical injury can induce chondrocyte apoptosis (29,30). Consistent with these findings, the present results demonstrated that mechanical loading reduced cell proliferation and promoted cell apoptosis. Therefore, it was speculated that mechanical loading led to chondrocyte degeneration. Furthermore, a previous study revealed that mechanical overloading promotes the activation of TGF- $\beta$ 1 signaling in chondrocytes, and upregulation of TGF- $\beta$ 1 is responsible for mechanical stress-induced chondrocyte degradation (13). In line with this, the present results suggested that mechanical loading inhibited the expression of the TIMP3/TGF- $\beta$ 1 pathway in chondrocytes, and also increased cell migration of HUVECs. Moreover, TIMP3 overexpression could reverse the role of mechanical loading in chondrocytes. Collectively, it was indicated that the TIMP3/TGF- $\beta$ 1 axis may be involved in mechanical loading-induced chondrocyte degeneration and angiogenesis.

In conclusion, it was demonstrated that mechanical loading led to chondrocytes degeneration. In addition, the TIMP3/TGF- $\beta$ 1 axis may be a vital signaling pathway in mechanical loading induced-chondrocytes degeneration and angiogenesis in OA development.

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#### Availability of data and materials

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

#### Authors' contributions

SHD designed the experiments. HTL provided the patient sample and designed the experiments. DLZ and SHD carried out the experiments. DLZ prepared the manuscript. All authors read and approved the manuscript.

#### Ethics approval and consent to participate

This study was approved by the Ethics Committee of First Affiliated Hospital, Heilongjiang University of Chinese Medicine (approval no. 2017HZYLL-021). Written patient consent was obtained from the donor.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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