

## Effects of mechanosensitive ion channel Piezo1 on Osteoarthritic articular chondrocytes

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**Abstract:** Osteoarthritis (OA) is associated with abnormal mechanical stress and altering joint loads, such as obesity, trauma and joint instability, leading to joint degeneration. Abnormal mechanical stress can not only lead to joint damage in physics, but also affect the progress of OA through the baroreceptor signals. The novel stretch-activated ion channel (SACs), Piezo1, are expressed extensively in mammalian. Chondrocytes are mechano-sensation cells, so it is meaningful that Piezo1 may also exist in human OA chondrocytes and play an important role in osteoarthritis. In this research, we found that piezo1 protein was located in the cell membrane and nucleus of the OA chondrocytes. Piezo1, MMP-13, ADAMTS5 and the apoptosis-activated gene Bcl-associated X (Bax) and Bcl-2-associated death promoter (BAD) were significantly increased under mechanical stretch force and the expression was related to the degradation of Collagen II and Aggrecan. Meanwhile, the expression of the Collagen II, Aggrecan and B cell lymphoma/leukemia-2 (Bcl-2) were upregulated by GsMTx4, the specific inhibitor of Piezo1. So we got the conclusion that the Piezo1 plays an important role in human osteoarthritis, and it may be related to the degradation of extracellular matrix and the apoptosis of chondrocytes. The expression of Piezo1 protein can be inhibited by GsMTx4.

**Keywords:** Ion channel; Mechanical stress; Chondrocytes; Osteoarthritis; Apoptosis; Cartilage matrix

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### 1. Introduction

Osteoarthritis (OA) is one of the most common types of arthritis in the elderly. Clinically, OA is characterized by joint pain, crepitation, swelling, and stiffness, restricted range of motion, fatigue, and functional limitations[1,2]. Pathological changes can be seen with degradation of the Matrix and cell death resulting in the gradual loss of articular cartilage integrity. The widely held view was increased pressure on weight-bearing joints, Joint instability and increased fragility of cartilage tissue were the main predisposing factor[3]. Chondrocytes, the cells in the articular cartilage, can support loads and regulate their metabolic activities in response to mechanical loading[4-6]. However, it is still not clear about whether OA chondrocytes can sense and transduce mechanical signals by Piezo1.

Mechanosensitive channels mediate touch, hearing, proprioception, and blood pressure regulation[7]. The molecular mechanism of mechanical sensitive ion channels in mammals remained mysterious until the identification of the evolutionarily-conserved Piezo family of cation-permeable involved in mechano-transduction was discovered, including Piezo1 and Piezo2, by Patapoutian and colleagues in 2010[8-10]. It has been found that when epithelial cells become too crowded, they activate the stretch-activated channel Piezo1 to trigger extrusion of cells that later die[11]. The hPiezo protein has also been reported to be a key player in fluid shear stress response of the vascular endothelium and is critical

for development of the vascular system[12-14].

The pivotal mediators of cartilage degradation include the MMPs (a group of zinc-dependent proteases) and the closely related ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs)[15-20]. Collagen type II is degraded by MMPs, including MMP-1, -8, and -13, whereas aggrecan is degraded by both MMPs and ADAMTS[21,22]. Recent studies have identified cell death morphological and molecular characteristics in osteoarthritis (OA) cartilage, suggesting that chondrocyte apoptosis plays a key role in the pathogenesis of OA [23, 24]. B cell lymphoma/leukemia-2 (Bcl-2) is an anti-apoptosis signal factor, which promotes the cell proliferation and inhibits the apoptosis through many complex pathways. However, the Bcl-2-associated death promoter (BAD) can be activated by the Bcl-xl and lead to cell apoptosis by suppressing the Bcl-2 family, which acted as the function of Bax[25-27]. In this research, the expressions of the Bcl-2, Bax, and BAD were detected by RT-qPCR, in order to explore the connection between the mechanical stress-induced apoptosis and the Piezo1 protein.

### 2. Materials and methods

#### 2.1. Human chondrocyte isolation and culture

OA cartilage samples(n=20) were isolated from the knee of 20 patients suffered from osteoarthritis during the total knee arthroplasty from October, 2015 to December, 2016, without infections or blood diseases. The study was approved by the Medical

Ethics Review Committee of Affiliated Hospital of Qingdao University. Primary chondrocytes were isolated from knee cartilage tissues, as described previously. Briefly, cartilage specimens were washed with phosphate-buffered saline (PBS) solution (containing 0.4g/L Streptomycin and 0.4U/L Penicillin) under aseptic conditions and cut the cartilage into small pieces ( $1 \times 1 \times 1 \text{mm}^3$ ). Then the cartilage specimens were added into 0.25% pancreatic enzymes and 0.2% collagenase type II for 30 minutes and 4 hours respectively. Undigested tissue was separated from cells using a 40mm filter. Trypan blue staining was used to detect the activity of chondrocytes.

## 2.2. Application of cyclic stretch

For the application of cyclic stretch, the OA chondrocytes were seeded in growth medium (GM) at  $3 \times 10^6$  cells/well on six-well collagen-coated BioFlex plates containing a flexible silicone elastomer substratum and grown into 90% confluence under non-stretch conditions for 2-3 days. The cells were then cultured in serum-free  $\alpha$ -MEM for 24 h to be synchronized prior to mechanical stimulation. The medium was then replaced with fresh  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) containing 10% fetal bovine serum (FBS). The cells were then subjected to 20% surface elongation at a frequency of 6 cycles/min, each cycle consisting of 3s' stretch alternating with 3s' of relaxation with a computer-controlled vacuum stretch apparatus. The control cells were maintained under the same experimental conditions, but were not exposed to mechanical stretch.

## 2.3. Analysis of the dead cells

The lactate dehydrogenase (LDH) Activity Assay Kit (Roche Diagnostics, Indianapolis, USA) was used to determine the intracellular LDH activity of the OA chondrocytes after 0h, 2h, 12h and 48h. In brief, cells were plated at  $2.0 \times 10^4$  cells/well in flat-bottomed 96-well culture plates and precultured in DMEM supplemented with 5% FBS. The medium was discarded from each well, and then 50  $\mu$ l of 2% Triton X-100 solution was added to lyse the cells. Cells were incubated in the dark for 30 minutes at room temperature, and the lysate was detected by fluorescence (490nm) by a BIO-TEK spectrofluorometer plate reader with KC4 analysis software (BioTik, USA).

## 2.4. Total RNA extraction and quantitative real-time PCR analysis (qRT-PCR)

To quantify the levels of mRNA for Piezo1, MMP-13, ADAMTS5 and Aggrecan, as well as the apoptosis related genes, the B cell lymphoma/leukemia-2, Bcl-associated X protein and Bcl-2-associated death promoter, after 2h, 12h, 24h

and 48h of mechanical stretch force, respectively, reverse transcription PCR was applied using a PrimeScript RT Reagent Kit with cDNA Eraser (TaKaRa, Japan) and quantitative real-time PCR was performed using SYBR Premix Ex Taq (Perfect Real-Time, TaKaRa, Japan). GAPDH was used as an internal control. The PCR primers (synthesized by Sangon Biotech, Shanghai, China) were used to amplify the genes (Table 1). Gene expression was analyzed by the comparative  $2^{-\Delta\Delta CT}$  method.

## 2.5. Protein isolation and western blotting analysis

Equal amounts of protein were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (20mg per lane), and transferred onto a polyvinylidene fluoride membrane. After blocking in 10% non-fat dried milk in TBST for 2h, the membrane was then incubated with antibodies specific for Collagen II (1:200, ab34712; Abcam, Cambridge, United Kingdom), and  $\beta$ -actin at 4  $^{\circ}$ C overnight. The filters were then washed in Tris-Buffered Saline Tween-20 (TBST) and incubated for 1h in horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in TBST containing 2% BSA. After several washes with TBST, the immunoreactivity was detected using the ECL system according to the procedures recommended by the manufacturer.

## 2.6. Fluo-3 AM staining

Fluo3-AM was used to detect the intracellular  $\text{Ca}^{2+}$  concentration. Stock solutions of Fluo-3 AM were prepared by using a solution of 20% Pluronic F-127 in absolute dimethylsulphoxide. The aliquots were then diluted with Hanks balanced salt solution (HBSS) to give the final concentrations of 5  $\mu$ M. The cells were harvested from the 6-well plates after 0h, 2h, 12h, 24h and 48h. After being treated with GsMTx4, the specific inhibitor of the Piezo1, cells were then implanted into 24-wells plate containing the appropriate size of glass-made slices. The cells of each group were loaded with 5  $\mu$ M Fluo-3 AM at 37  $^{\circ}$ C for 60min, respectively. Then, they were washed in pure HBSS and left for a further 30 min at 37  $^{\circ}$ C. The calcium transients of OA chondrocytes under different mechanical tension were observed by laser scanning confocal microscopy. The expression of  $\text{Ca}^{2+}$  was assessed by the Image J2X (Rawak Software, Germany).

## 2.7. Immunofluorescence

Piezo1 expression in chondrocytes was examined by immunofluorescence staining. Briefly, chondrocytes were seeded on cover slips in 24-well plates at  $10^4$  cells/cm<sup>2</sup>. After 24h, the cells were fixed in 4% paraformaldehyde in PBS for 15min at room temperature. After washing with PBS, the cells were

treated with 0.5% Triton X-100 (MP Biomedicals, Senta Ana, CA, USA) in PBS for 15min at room temperature and incubated with bovine serum albumin for 60min at 37 °C. The samples were incubated in rabbit anti-Piezo1 antibody (1:400, NBP1-78537; Novus Biologicals, Littleton, CO, USA) at 4 °C overnight. Then, the cells were washed and incubated with secondary antibodies that were

conjugated to Fluorescein isothiocyanate isomer (FITC). Nuclei were stained with DAPI (Beyotime, China) for 3min at room temperature. The cover slips were then mounted with an anti fluorescence quenching reagent (Beyotime, China). Staining was observed using a fluorescence microscope (Zeiss, Jena, Germany) and photographed.

**Table1. The oligo sequences of the target genes**

Oligo Name	Oligo Sequence	5'-3'
Piezo-1	Forward Primer	CATCTGGTGGTCTCCTCTGTCT
	Reverse Primer	CTGGCATCCACATCCCTCTCATC
MMP-13	Forward Primer	GTGGTGTGGGAAGTATCATCA
	Reverse Primer	CATCTGGAGTAACCGTATTG
ADAMTS5	Forward Primer	ATGACCATGAGGAGCACTACGA
	Reverse Primer	GGAGAACATATGGTCCCAACGT
hBAD	Forward Primer	CCGGAGGATGAGTGACGAGT
	Reverse Primer	CCGATCCCACCAGGACTG
hBax	Forward Primer	CCTTTTGCTTCAGGGTTTCAT
	Reverse Primer	GAGACACTCGCTCAGCTTCTTG
hBcl-2	Forward Primer	TGGGATGCCTTTGTGGAAT
	Reverse Primer	GAGACAGCCAGGAGAAATCAAAC
Aggrecan	Forward Primer	AGCCTGCGCTCCAATGACT
	Reverse Primer	GGAACACGATGCCTTTCACC
hGAPDH	Forward Primer	GCACCGTCAAGGCTGAGAAC
	Reverse Primer	TGGTGAAGACGCCAGTGGA

## 2.8 Statistical analysis

All experiments were repeated at least thrice. Data were analyzed using GraphPad Prism Version 5.0. Quantitative results were expressed as mean ± standard deviation (SD). All the data groups for comparison were normally distributed. Data were analyzed by student's t-test or one-way ANOVA. Tukey's test was performed when the ANOVA test indicated a significant difference. Values were considered significantly different (P<0.05).

## 3. Results

### 3.1. Culture of the OA chondrocytes and Immunofluorescence of the Piezo1 in OA chondrocytes

The OA chondrocytes grew into polygonal shape (Figure 1A). Immunohistochemical staining showed that the aggrecan showed strong positive reaction (Figure 1B). The cytoplasm of the chondrocytes was stained with yellow granules, and the nuclear staining was not obvious. The immunofluorescence was used to test the expression and location of the MA ion channel Piezo1 protein. Cell immunofluorescent analysis revealed that the Piezo1 could be detected in the OA chondrocytes. We also found that piezo1 protein was located in the cell membrane and nucleus of the OA chondrocytes (Figure 1C-1E). The fluorescence intensity of Piezo1

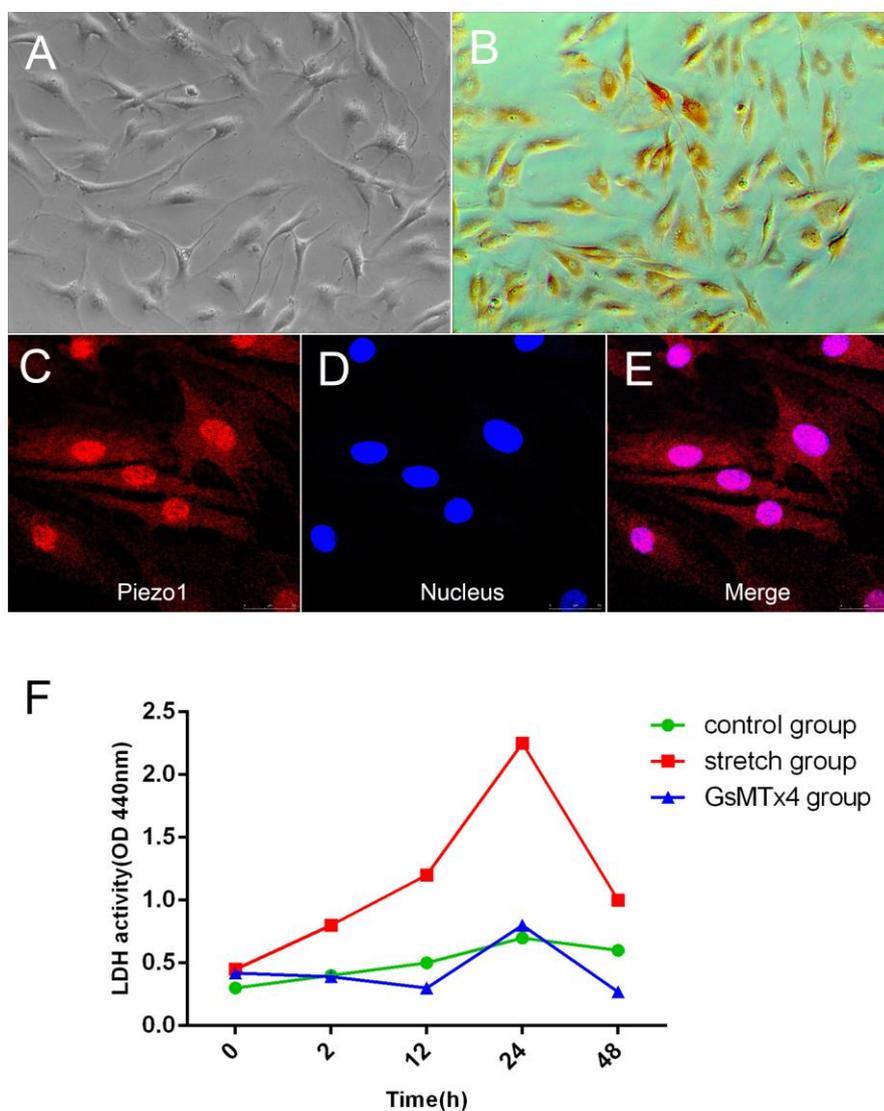
in the stretch group was increasing under the mechanical force and had the character of time-dependence (Figure 3A and B). The lactate dehydrogenase (LDH) in the cells of the control groups increased slowly without statistical significance (P>0.05), and in the mechanical stretch group, the LDH release was significantly higher than the control group (P<0.05) (Figure 1F). However, in the 48h stretch group, the LDH level was lower than the 24h stretch group (P<0.05). The LDH level was decreased by the GsMTx4.

### 3.2 Total RNA extraction and quantitative RT-qPCR analysis for Piezo1, MMP-13, ADAMTS5, hBcl-2, hBax, hBAD and Aggrecan

Piezo1, MMP-13, ADAMTS5, Aggrecan and the apoptotic associated genes like Bcl-2, Bax, and BAD were detected using the RT-qPCR (Figure 2). The expression of Piezo1 (encoded by FAM38A) was the lowest in 0h group. However, the expression of Piezo1 in 2h group and 12h group increased significantly compared with the control group (P<0.05). Under mechanical stretch for 24h, the expression of the FAM38A reached into the highest level, while the expression of FAM38A in 48h group decreased compared with 24h (P<0.05) (Figure 2A). This indicates that the Piezo1 expression in OA chondrocytes was time-dependent and was activated by mechanical stress. Meanwhile, the expression of

MMP-13, ADAMTS5 and apoptosis-activated gene Bax, BAD were presented the same trend (Figure 2B, C, E and F). However, the expression of the Aggrecan and Bcl-2 (a kind of anti-apoptosis gene, which could promote the cell proliferation) decreased

from 2h group ( $P < 0.05$ ), and reached to the lowest level at 24h compared with 0h group ( $P < 0.05$ ). In addition, the 48h group had higher expression than 24h group ( $P < 0.05$ ) (Figure 2D and G).



**Figure 1. Laser scanning confocal microscope (LSCM) results of the Piezo1 protein-specific immunolabeling in the OA chondrocytes and LDH cytotoxicity assay for the OA chondrocytes under mechanical stretch.** (A) Primary of OA chondrocytes ( $\times 100$ ). (B) OA chondrocytes was identified by detecting GAG ( $\times 100$ ). (C) Piezo1 protein was located in the cell membrane and nucleus of the OA chondrocytes. Piezo1 protein was located in the cell cytoplasm and nucleus of the OA chondrocytes. (D) The nucleus was stained by using DAPI (blue). (E) Merge of Piezo1 (red) and DAPI (blue). (F) LDH cytotoxicity assay for the OA chondrocytes under mechanical stretch. The cell death rate of the mechanical stretching group was significantly higher than that of the non-mechanical stretching group.

### 3.3 Western blot analysis for Collagen II

Collagen II was detected using the Western blot (Figure 2H and I). The expression of the Collagen II, a kind of protein, which associated with the osteoarthritis, decreased from 2h group ( $P < 0.05$ ), and reached to the lowest level at 24h compared with 0h group ( $P < 0.05$ ). In addition, the 48h group increased

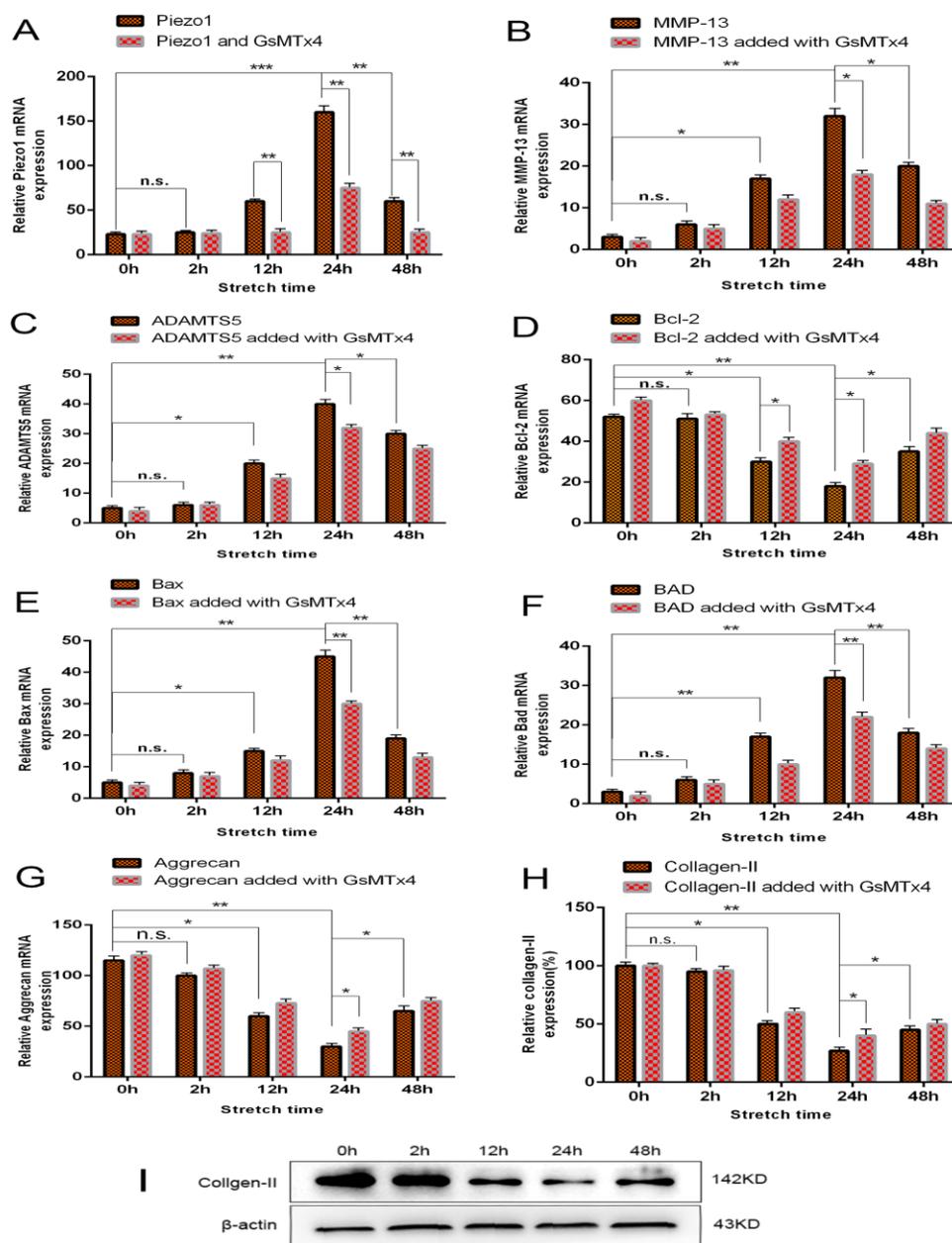
slightly compared with 24h group ( $P < 0.05$ ) (Figure 2H).

### 3.4 Analysis of calcium influx under tensile stress mechanical stress

The calcium in the chondrocytes increased from

2h to 24h as shown in the Fluo3-AM staining, as well as the expression of the Piezo1 (Figure 3C). During the mechanical stretch, the light intensity of  $Ca^{2+}$  fluorescent dyes showed a time-dependent trend. This indicates that mechanical stress-activated

Piezo1 increases  $Ca^{2+}$ . The increased of Piezo1 leads to the imbalance of synthesis and degradation of Cartilage matrix, as well as the apoptosis of the OA chondrocytes.



**Figure 2.** RT-qPCR results of the Piezo1, MMP-13, ADAMTS5, hBcl-2, hBax, hBAD and Aggrecan expression and western blotting results of Collagen II expression in OA chondrocytes. The expression of the Piezo1, MMP-13, ADAMTS5, hBax and hBAD in the stretch group was increasing under the mechanical force and had the character of time-dependence. However, the expression of Collagen II, Aggrecan and Bcl-2(a kind of anti-apoptosis gene) was decreasing. Column, mean of three independent experiments; bars, SD; n.s. P>0.05; \*P<0.05 \*\*P<0.01.

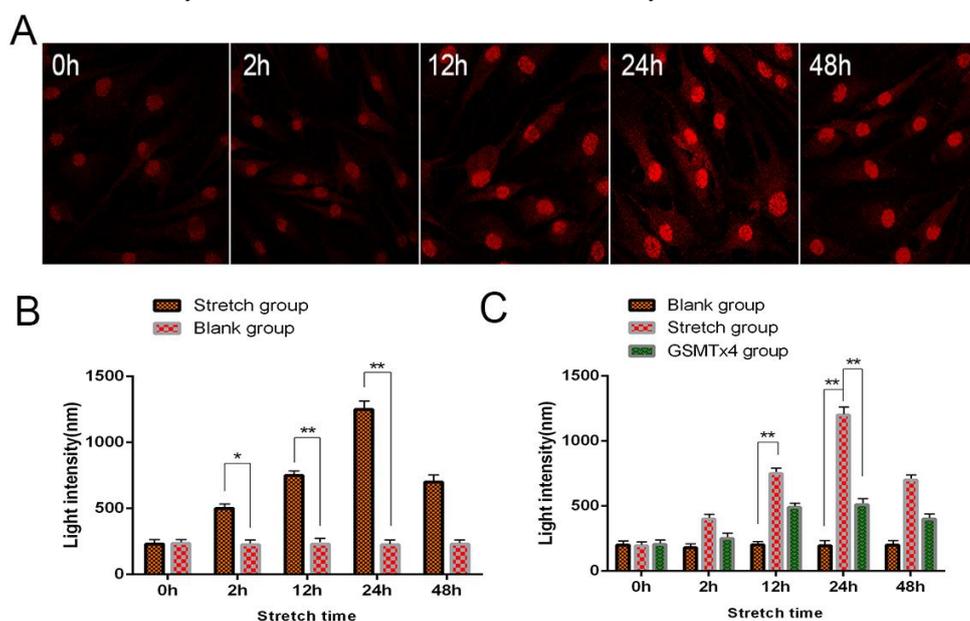
**4. Discussion**

Piezo1 represents a prototype of the mammalian mechanosensitive cation channel, but the function of Piezo1 is still not known completely[28-31]. In this

research, we tried to investigate the role of Piezo1, a novel mechanical sensitive ion channel, in OA chondrocytes. Our results show that the Piezo1 plays a role in the degradation of Cartilage matrix and is also associated with the apoptosis of OA

chondrocytes. The degradation of Collagen- II and Aggrecan can be inhibited by GsMTx4, the inhibitor

of the Piezo1, while the apoptosis rate of chondrocytes also decreased.



**Figure 3. The fluorescent light intensity of the Piezo1 and calcium in the OA chondrocytes cytoplasm.** (A, B) The fluorescent light intensity of the Piezo1 in the OA chondrocytes. The fluorescence intensity of Piezo1 in the stretch group was increasing under the mechanical force and had the character of time-dependence. (C) The fluorescent light intensity of the calcium in the OA chondrocytes cytoplasm. The fluorescence intensity of  $Ca^{2+}$  in the stretch group was increasing under the mechanical force and had the character of time-dependence. Column, mean of three independent experiments; bars, SD; \* $P < 0.05$  \*\* $P < 0.01$ .

Previous study had explored the connection between the degradation of Cartilage matrix with the apoptosis of the chondrocyte, which found that when the synthesis of Cartilage matrix decreases, the living environment of chondrocytes will be destroyed, and it will cause the excessive apoptosis of chondrocytes[32]. In this article, we hypothesized that the mechanical force could activate the Piezo1, and further resulted in the degradation of the Cartilage matrix and the apoptosis of chondrocyte during the progress of osteoarthritis.

In this research, we monitored the expressions of the Piezo1, MMP-13, ADAMTS5, Aggrecan and apoptosis-associate genes, including the Bcl-2, Bax, and BAD, with RT-qPCR after the mechanical-induced apoptosis of the human OA chondrocytes from OA patients. The expression of Collagen II was also detected by Western blot. We found that the expression of Collagen II and Aggrecan in 24h group were lower than that 0h group, as well as the Bcl-2, an anti-apoptosis and cell proliferation gene. However, the expression of Piezo1, MMP-13, ADAMTS5 and apoptosis-associate genes, including the Bax and BAD was higher in 24h group than 0h group, indicating that appropriate mechanical stretch increased the expression of MMP-13 and ADAMTS5 gene, which was a major degradation enzyme of Cartilage matrix. The results indicate that the Piezo1 played an important role in the degradation of Cartilage matrix, as well as mechanical-induced

apoptosis of the OA chondrocytes, and may be served as a possible target for the treatment of OA, especially for those due to traumatic arthritis.

Previous report found that Piezo1 and TRPV4 are activated independently and that the stretch-evoked  $Ca^{2+}$  influx pathway is mediated mainly through Piezo1 and TRPV4 channels [29]. It has evidence that  $Ca^{2+}$  influx can be influenced by L-type  $Ca^{2+}$  voltage-gated channels after mechanical staining [33]. It is meaningful to speculate that the  $Ca^{2+}$  plays an important role in activating Piezo1. Fluo-3 AM results in this experiment also demonstrate this view.

Cartilage matrix synthesis/ degradation imbalance has been known as a key mechanism leading to post-traumatic arthritis following traumatic joint injury [34-36]. Piezo1, functionally expressed in OA chondrocytes, provide novel molecular targets for reducing cartilage matrix degradation and mitigating injury-induced cartilage degeneration following joint trauma. In this respect, GsMTx4 may be able to serve as a cartilage protective agent to prevent and treat osteoarthritis and other mechanically-induced forms of the disease. A novel specific inhibitors for Piezo1 which are unharmed for the human being need to be studied in the future.

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## Author contributions

Xiaodong Li was responsible for all aspects of the research, including experimental design, data acquisition and analysis, and manuscript preparation. Qing Liu worked on primer design, technical and informatics' analyses of these genes. Haining Zhang was responsibility for the programs and all experiments, revised the manuscript and provided the final approval of the article.

## Conflict of interest

The authors declare that they have no conflicts of interest with content of the manuscript.

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