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Piezo1-driven mechanotransduction as a key regulator of cartilage degradation in early osteoarthritis

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ABSTRACT

Osteoarthritis (OA) is a prevalent degenerative disease characterized by pain and cartilage damage in its later stages, while early OA is marked by the loss of cartilage's mechanical function. Recent studies suggest that Piezo1, a mechanotransducer, may contribute to cartilage degradation under abnormal physical stress. This study investigates the mechanism by which Piezo1 mediates the loss of cartilage's mechanical properties. Using rat chondrocytes cultured in a 3D in vitro model, we found that fluid flow-induced physical stress activates constitutively expressed Piezo1, leading to increased catabolic activity and apoptosis, which, in turn, disrupts the matrix structure. Ex vivo cartilage experiments further demonstrated that the mechanical stress-induced loss of cartilage's physical properties (approximately 10% reduction in relaxation modulus) is mediated by Piezo1 and depends on cell viability. Notably, Piezo1 agonists alone did not alter the mechanical behavior of cartilage tissue. In vivo, using an OA rat model induced by anterior cruciate ligament transection, we observed cartilage integrity degradation and loss of mechanical properties, which were partially mitigated by Piezo1 inhibition. RNA sequencing revealed significant modulation of the PI3K signaling and matrix regulation pathways. Collectively, this study demonstrates that Piezo1-mediated catabolic activity in chondrocytes is a key driver of the loss of cartilage's mechanical function during the relaxation phase.

Keywords: Osteoarthritis, cartilage degradation, Piezo1, mechanotransduction, catabolic activity.

INTRODUCTION

Osteoarthritis (OA) is a chronic degenerative disease affecting over 300 million people globally, with prevalence expected to rise due to aging and obesity(1). Due to the limited treatment options, there was no cure for OA until knee/hip replacement taken. Numerous promising therapeutic targets were investigated but mot fully understood. OA is characterized by cartilage destruction, leading to joint pain and dysfunction, typically evident in the later stages of the disease (2). However, loss of cartilage's mechanical function, as indicated by changes in modulus, is an early sign of OA pathology. These mechanical properties, including elastic modulus, permeability, and relaxation strength, are typically assessed during the creep and relaxation phases of cartilage(3). Early detection methods have shown promise (4, 5), but the molecular mechanisms underlying the loss of mechanical properties in early-stage OA remain poorly understood. Unraveling these mechanisms could reveal potential targets for early OA treatment.

OA etiology involves factors such as trauma, aging, obesity, and catabolic activity, with abnormal physical stress playing a key role in OA pathogenesis (2). Healthy cartilage disperses mechanical stress and reduces friction through a hydrated matrix of aggrecan and type II collagen. In contrast, OA cartilage exhibits decreased proteoglycan content and disrupted collagen structure, leading to matrix degradation(6). This degradation is particularly evident in cartilage from weight-bearing joints, where abnormal mechanical stimuli contribute to OA progression(7). Chondrocytes, the sole cell type in cartilage, regulate both anabolic and catabolic processes, and changes in mechanical properties, such as stiffness and permeability, reflect cartilage degradation.

Piezo channels, discovered in 2010(8), include Piezo1 and Piezo2, which function as mechanosensitive ion channels involved in various physiological processes(9). Piezo1, in particular, transduces mechanical signals into cellular responses, such as osteogenesis in bone marrow stem cells(10). In chondrocytes, Piezo1 activation by abnormal stress has been linked to senescence, apoptosis, and catabolic activity, suggesting its critical role in OA development(10-12). Abnormal mechanical stimulation such as compression and shear stress leads to decreased chondrocyte activity and matrix degradation, which is closely related to OA damage. In OA cartilage tissue, the expression of Piezo1 was up-regulated. Based on the above observations, several studies suggested that Piezo1 represented a promising therapeutic target to limit OA. However, whether Piezo1 mediates changes in the mechanical properties of

cartilage during early OA remains unclear.

By elucidating how Piezo1 influences cartilage's mechanical behavior, particularly during the early stages of OA, this research aims to uncover potential therapeutic targets for early diagnosis and treatment. The purpose is to establish the role of Piezo1 in regulating mechanical properties and catabolic activities in chondrocytes, thereby providing insights into the early detection and management of OA.

MATERIALS AND METHODS

Preparation of rat chondrocytes and cartilage explants

Healthy and clean 200 g SD rats (male and female not limited) were purchased from animal centre of Zhengzhou university (licence number: SCXK 2021-0009). All experiments involving animals were conducted according to the ethical policies and procedures approved by the ethic committee of the first affiliated hospital of Zhengzhou university.

For cartilage harvest, all the animals were euthanized by carbon dioxide asphyxiation. Then, the proximal ends of the tibia were harvested and the cartilage tissue were cut and transferred rapidly at room temperature, cultured and preserved at 37 °C. The cartilage tissue was placed into a 6-well plate containing 2 mL culture medium (10% fetal bovine serum + DMEM). The cartilage tissue were then pumped to make cartilage slices with same circle area, descripted as cartilage explants ready for experiments *ex vivo* and mechanical testing.

For chondrocyte isolation, the cartilage tissue was collected and then digested by several enzymes. The cartilage tissue was diced before transferring to a conical tube and incubated for 1 hour on the roller in the 37°C oven, in 7 units/mL pronase prepared in culture media. Thereafter, pronase was carefully aspirated and the tissue was incubated in 100 units/mL collagenase on the roller at 37°C for a further 16 hours. After discarding the supernatant, the cell pellet was re-suspended in the culture media with 10% fetal bovine serum. The pre-autoclaved agarose gel was placed in an oven at 80°C for 20 min and cool naturally. Subsequently, chondrocytes suspension was subsequently added to an equal volume of molten 6% (w/v) agarose making cells in 3% (w/v) agarose construct within certain moulds ready for further experiments. For staining, constructs were ready for use. While for RNA/protein detection and apoptosis detection, papainase solution were used to digest the crushed specimens.

Apoptosis assay

Apoptosis of cells induced was assessed using Annexin V-FITC labelling by flow cytometer. The ratio of apoptotic cells was calculated by dividing the number of positive cells by the total number of cells. The working solution of Annexin V-FITC was prepared immediately prior to use. Treated cells were washed 3 times with PBS and resuspended in binding buffer. Flow cytometric analysis began immediately following incubation with 100 ng/mL Annexin V-FITC in the dark for 10 min. 5 mg/mL for 5 min and then washed 3 times with PBS prior to examination.

Animals and experimental design

SD rats were OA-induced via the way of anterior cruciate ligament (ACL) transection. This method has been widely applied (1) that the rat knee joint was exposed after inhalational anesthetization followed by the resection of ACL. The contralateral capsule was cut as a sham control. Rats were randomly grouped into Sham group, OA model group and OA model + intra-articular injection of GsMTx4 group. For Shame group, operation only included the cut of skin to expose the cartilage and 100μL of saline was injected into the articular space every week. An amount of 100 μL of 40 μM Gsmtx4 was injected intra-articularly every week for the OA model + intra-articular injection of GsMTx4 group (14). At six weeks after surgery, right knee joints of rats were collected. After fixing with 4% paraformaldehyde and decalcifying with 0.5 M EDTA, tissue was then sliced for staining. Meanwhile, total RNA was collected using Lysate and then subjected to RNA sequencing.

Mechanical loading of shear stress by fluid flow

The way to generate the shear stress by fluid flow to cartilage explants/cells has been reported previously (15). The cells/agarose constructs and cartilage tissue were 0.25 mm in radius, and cut into 1mm in thickness. The fluid flow stress device was made by 3D printer to generate a mould producing several small chambers. After sterilization, the agarose in 2% weight/volume PBS was placed until totally gelled. Then, the mould was removed while the constructs placed into the small chambers. The device fulfilled with culture media was placed on the orbital shaker in the incubator. The exact flow fluid stress was calculated in the ANSYS software. The rotator was set at 100PRM inducing the fluid flow to produce a shear stress for 48h.

Detection of physical properties of cartilage tissue

The mechanical properties or behaviour of cartilage tissue have been widely explored, basing on various devices for instance the strain detector which compressing the cartilage explants. Considering the tiny size of rat cartilage, we used the way of

microindentation test which has been previously reported (16). Briefly, the proximal part of rat tibia were removed and embedded in the fixing ring by adhesive gel. On the top of cartilage tissue, one drop of culture media was used to maintain the water content of the samples. A set of testing programs and appropriate probes from the indentation test system were prepared. During the indentation, the indenter displacement was documented with a cross read speed necessary to generate the regulated strain and strain rate. Each explant was pre-loaded to 0.01N ensuring the direct contact with the displacement recorded. Then, cartilage tissue were subjected to a 20% compressive strain applied at a strain rate of 20%/min (creep phase). For example, a cartilage slice with a thickness of 200μm was compressed by 40μm within one minute. A recovery period of four minutes was induced by maintaining the compressor in the same location. The strain values were recorded every second to depict a scatted line for further analysis.

Live/dead staining of cartilage tissue

The viability of chondrocytes was assessed by the kit of live/dead staining consisting Calcein AM (Sigma) and Ethidium homodimer-1 (EthD-1, Fisher Scientific). Tissue was merged in media which contained 5μM Calcein AM and 5μM EthD-1 for 30 minutes at 37°C. After washing with PBS for three times, tissue were positioned on coverslips and immediately imaged on a microscope with a x10 objective. Calcein AM labelled live chondrocytes green (excitation 495nm, emission 515nm) while EthD-1 labelled dead cells red (excitation 528nm, emission 617nm).

RNA -sequencing analysis of cartilage tissue

The cartilage tissue were harvested. Then, total mRNA was isolated and collected using Lysate of TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocol. The enriched mRNAs were reverse transcribed into cDNA with random primers. Second-strand cDNA were synthesized by DNA polymerase I, RNase H, dNTP and buffer. Next, the cDNA fragments were purified with QiaQuick PCR extraction kit, end repaired and ligated to Illumina sequencing adapters. The digested products were size selected by agarose gel electrophoresis, PCR amplified, and sequenced. Six cDNA libraries were constructed using the Illumina NovaSeq 6000 system by Gene Denovo Biotechnology Co. (Guangzhou, China). Results from Gene Ontology (GO) analysis were analysed and used.

Histological staining

Serial sagittal sections of rat samples were sectioned in 6 μm and stained with H&E, Alcian blue and Safranin-O. Based on the images, Osteoarthritis Research Society

International (OARSI) scoring was used to degrade the severity of OA. For immunohistochemistry experiments, sections were incubated overnight at 4 °C with anti-Piezo1 (1:1000). Sections were visualized using an HRP detection system and the rate of positive cells was quantified by two blinded pathologists.

Quantitative polymerase chain reaction (q-PCR)

To validate the osteogenic gene expression, the total RNA was isolated from chondrocytes or cartilage tissue using isolation column from Qiagen company (74004). Then, cDNA was synthesised with TRIzol and Oligo-dT (15596026, SO132 from Invitrogen). In some cases, the concentration and quality of RNA were quite differed between samples. The PCR was done with SYBR Green assay kit (A25779, also from Invitrogen), according to the amplification process of 95°C for 3 min followed by 40 cycles alternating 95 °C for 15 s and 60 °C for 30s. The primes were designed based on the sequences in GenBank (Primers sequences were listed below. MMP-13: F-CTTGATGCCATTACCAGTC, R- GGTTGGGAAGTTCTGGCCA. ADAMTs5: F-TATGACAAGTGCGGAGTATG, R- TTCAGGGCTAAATAGGCAGT; Collagen: F-ACGTCCAGATGACCTTCCTG, R- GGATGAGCAGAGCCTTCTTG. Agg: F-GAGTTTGTCAACAACAATGCC, R- TGGTAATTACATGGGACATCG; GAPDH: F-GACAAAATGGTGAAGGTCGG, R- TCCACGACATACTCAGCACC. 100-300 bp in length). The fold changed in gene expressions of were analysed by $2^{-\Delta\Delta T}$ method and normalised to the expression of GAPDH. The reported data represent the mean expression from 3 experiments.

Western blot analysis

We used western blotting to determine the protein expressions of Piezo1, Matrix metalloproteinase-13 (MMP-13), ThromboSpondin motifs5 (ADAMTs5), Aggrecan and Collagen II. The cells or tissue were extracted and dissolved in 150 μl of lysis buffer supplemented with 1% (v/v) cocktail buffer (78440 from Invitrogen). Proteins were separated using SDS–PAGE and transferred to membranes (1704156 and 1620176 from Bio-Rad). After blocking (37565 from Invitrogen), the membranes were stained with primary antibodies against tubulin, Piezo1, MMP-13, ADAMTs5, Aggrecan and Collagen II purchased from Santa Cruz Biotechnology. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (31460 and 31430 from Invitrogen) overnight and visualized by chemiluminescence. Image J was used to quantify the intensities of the target protein bands in each blot.

Ethics approval

This study was reviewed and approved by the Ethic Committee of the first affiliated hospital of Zhengzhou University. No patient was involved in this study.

Statistical analysis

GraphPad Prism was used to conduct statistical analyses. The gene/protein expression data and mechanical test data are presented as the means ± SD from over three repeats and were analysed using Student's t tests for two separate groups. For over three groups, one-way ANOVA was taken post Tukey's tests. For frequency data, eact Fisher's test was used. The significance level was set to $p < 0.05$.

RESULTS

Shear stress stimulated catabolic response and apoptosis via Piezo1 mediating calcium influx in 3D cultured chondrocytes *in vitro*

Experiments *in vitro* identified the harmful mechanical stress by fluid flow to chondrocytes metabolism and apoptosis. Fig. 1a showed the protocol of experiments that rat chondrocytes were harvested by enzyme digestion on Day 1 followed by a 5 day cell culture in monolayer. Then, Cells were reseeded in the 3D construct with 3% v/w agarose gel. They were placed in the static control or loaded with shear stress by fluid flow for 48h. The device mechanism of shear stress conduction was depicted in Fig. 1b imitating the stimulation of shear strain of from a 100 RPM orbital shaker. This was further applied to the cartilage explants. In the unloaded or loaded cells, Piezo1 was stable expressed with no changes in mount detected by the western blot (Fig. 1c) and located in the nuclear area tracked by immunofluorescence staining and DAPI staining (Fig. 1d). In response to shear stress, the Ca^{2+} influx was monitored by Fura-2 AM indicator. Fig. 1e showed the calcium influx initiated by mechanical loading while Piezo1 inhibitor GsMTx4 blocked this effect indicating the requirement of Piezo1 in mechanically calcium signalling.

The apoptotic cells were tested then by a flow cytometry that injury mechanical loading did induce the apoptosis which was blocked by Piezo1 blockage (Fig. 1f). Interestingly, the pathological stress injury activating Piezo1 to elevate the catabolic activities by gene transcription and protein expressions. Mechanically-regulated catabolic genes as MMP-13 and ADAMTs5 were promoted in expressions but reduced after Piezo1 inhibition (Fig. 1g). The 4.9±1.1 and 3.1±0.4-fold changes were observed in terms of MMP-13 and ADAMTs5 after physical loading, but reduced to 1.9±0.3 and 1.7±0.7-fold changes with Piezo1 inhibition. The alterations in the proteins of MMP-13 and

ADAMTs5 were similar to gene expressions indicating Piezo1 mediated mechanically catabolic activity in chondrocytes (Fig. 1h). The 2.2±0.2-fold change was observed regarding the protein expressions in ADAMTs5 in loaded cells, Piezo1 antagonist only significantly reduced the ADAMTs5 expression to the control level (Fig. 1i).

Piezo1 mediated the loss of mechanical properties during relaxation phase in cartilage explants *ex vivo* via regulating live chondrocytes

We collected the cartilage tissue from rat knee and cut them into explants for mechanical test *ex vivo*. The device for mechanical testing was shown in Fig. 2a. The compression strain value over time was conducted into the creep and relaxation phases. Fig. 2b illustrated the creep behaviour from σ0 to σ1 (peak of strain) and the relaxation phase from σ1 to σ2 (final stable condition). Based on the equations (Fig. 2c), the elastic modulus and relaxation percentage of cartilage tissue were measured. Prior to mechanical testing, the Piezo1 expressions within tissue were detected that Piezo1 (+) cells hardly seen in the control while the Piezo1 (+) cells was frequently observed in the strained tissue (blue arrow, Fig. 2d). This was statistically significant as shown in Fig. 2e.

The live/dead staining was used (Fig. 2f) showing the cell viability not changed by treatment, indicating the cell apoptosis differently affected in the 3D culture and cartilage tissue. The representative lines of strain over time in explants from three groups were shown in the scattered plot (Fig. 2g) that the elastic modulus and relaxation modulus maintained, but the relaxation percentage significantly differed. In unloaded, loaded, loaded with GsMTx4-treated cartilage explants, the elastic modulus was approximately 2MPa with no significant difference between groups (Fig. 2h). Notably, the percentage of relaxation was increased from 70.9±8.0% in the control to the 84.5±6.7% in the loaded tissue, which was restored to 77.1±4.4% by additional treatment with GsMTx4 (Fig. 2i). This alteration in the mechanical property was reflective to the matrix integrity of cartilage.

To test the role of chondrocytes, we used cartilage tissue to do the mechanical test with no live cells (showing in Fig. 2j). Obviously, mechanical properties from both the creep and relax behaviour were unchanged (Fig. 2k and i) demonstrating the requirement of live chondrocytes in injury mechanically-regulated loss in physical properties. It was proposed that the metabolism activity of live cells was of great value to Piezo1 mechanism. By contrast, Piezo1 activation with Yoda1 alone did not alter the mechanical behaviour on the elastic modulus (Fig. 2m) and relaxation percentage (Fig.

2n), suggesting the regulating role but not the initial role of Piezo1 in the loss of mechanical properties.

Piezo1 mediated the physical properties loss of cartilage in rat osteoarthritis model *in vivo*

We further investigated the effects of Piezo1 antagonist on OA damages in rats. The OA model was created on Day 1 by resection of ACL in rat knee and continued for the next 6 weeks (Fig. 3a). Rats were divided into three groups: shame control, OA model, OA model with intra-articular injection of GsMTx4. To this end, the cartilage tissue was fixed for staining or collected for RNA sequence detection. The results from H&E stain, Alcian blue stain and Safranin o stain (Fig. 3b) showed that OA model markedly exacerbated the OA lesions in the knee joints while GsMTx4 injection exhibited a protective effect. In details, the structure of collagen-proteglycan structure was disrupted (blue arrows in the OA model group, Fig. 3b) and decreased in the collagen mount. Additional treatment with GsMTx4 prevented the OA lesion but small tiny injury to cartilage were still founded (blue arrows, Fig. 3b). The OARSI score from three kinds of staining all reflected this trend that the score in control rats was lower than OA model group and OA model + GsMTx4 group (Fig. 3c). A significant difference was found between the latter two groups indicating Piezo1 modulation partially protected the cartilage in the OA model. In terms of the mechanical tests, representative lines of strain over time in cartilage tissue from three groups were shown in the scattered plot (Fig. 3d). The elastic modulus of cartilage was significantly decreased in the OA model which was not attenuated by Piezo1 inhibition (Fig. 3e). The relaxation behaviour of cartilage was interestingly different between groups that relaxation percentage (81.7±1.8% in control) was up-regulated to 89.1±1.6% in the OA model which was reduced to 85.7±2.6% after GsMTx4 treatment (Fig. 3f).

RNA sequence data provided more information suggesting the possible pathways such as the PI3K signalling, PPAR signalling and hippo signalling pathways in the KEGG analysis (Fig. 3g). These pathway signalling may participate into the Piezo1 mediated loss of cartilage mechanical properties during relaxation stage due to the regulating of "Piezo1 - matrix degradation - cartilage mechanical behaviour". The differently expressed genes regulating bone metabolism of MMPs and ADAMTs were involved (Fig. 3h and Fig. 3i) with GO analysis showing different genes enriched in the matrix regulation.

DISCUSSION

This study highlights the crucial role of the mechanosensitive Piezo1 channel in the loss of cartilage physical properties. Our findings from *in vitro*, *ex vivo*, and *in vivo* experiments demonstrate that Piezo1 activation by shear stress enhances the catabolic activity of chondrocytes, thereby affecting the relaxation behavior of cartilage during mechanical testing.

At the molecular level, shear stress induces apoptosis and upregulates catabolic genes such as MMP-13 and ADAMTS5 in chondrocytes through Piezo1-mediated Ca^{2+} influx. Ex vivo, shear stress increases the proportion of Piezo1(+) cells but maintains cell viability. While shear stress impairs the relaxation modulus, this effect is counteracted by Piezo1 inhibition with GsMTx4, although no significant impact on the elastic modulus was observed. In vivo, GsMTx4 injection prevents OA progression but only restores the relaxation behavior of cartilage. RNA sequencing reveals that PI3K signaling and matrix regulation pathways involving MMPs and ADAMTSs modulate inflammatory responses and matrix stability. Thus, Piezo1-mediated loss of mechanical relaxation properties in cartilage is evident.

Mechanical loading through shear stress can have both beneficial and detrimental effects on cartilage homeostasis. While low-frequency shear stress promotes proteoglycan and collagen synthesis, excessive shear stress is harmful (17). For instance, shear stress exceeding 0.21 Pa has been shown to damage cartilage (15). In this study, we focused on injury-level shear stress to explore the Piezo1-mediated catabolic effects on cartilage. The shear stress was approximately 1 to 2 Pa which was estimated by the ANSYS software. We designed and applied a loading machine to simulate abnormal mechanical loading, which is characteristic of osteoarthritis (OA) pathology (18). Although other forms of mechanical loading, such as tensile strain and compression, could be applied, shear stress was consistently used to load 3D chondrocyte constructs and explants throughout the study due to practical constraints.

Piezo1 is widely recognized for its mechanical responsiveness and its role in regulating OA lesions and osteophyte formation (12-14). Previous studies have shown that knocking out the Piezo1 gene or administering GsMTx4 can prevent OA progression (14), which aligns with our findings. However, our study delved further into the mechanical behavior of cartilage tissue. Cartilage's viscoelastic properties, characterized by creep and relaxation phases, are early indicators of OA pathology (4, 5). Although we did not find evidence that Piezo1 regulates creep behaviour, this might

be due to the compressive modulus formed by negatively charged proteoglycans, which Piezo1 minimally affects. This phenomenon was in accordance with to the loss of supportive force of OA cartilage during joint movement. Previous reviews have reported the elastic modulus of cartilage varies depending on loading conditions, with rat cartilage showing an elastic modulus of approximately 2 MPa, consistent with studies using similar methodologies (16). In contrast, relaxation responses, which are tied to matrix integrity—specifically the collagen-proteoglycan matrix—are crucial for preventing crack nucleation in cartilage (19). Previous study demonstrated the inhibition of Piezo1 by siRNA reduced the expressions of MMP-13 and ADAMTS5, two of which were key regulators of cartilage degrading factors. We demonstrated that Piezo1 governs the relaxation response through factors like MMP-13 and ADAMTS5, while RNA sequencing data suggested additional pathways such as PI3K and Col6.

Piezo channels are well-characterized in terms of their structure and function (8, 9), participating in various biological and pathological processes, including vascular mechanobiology (20), erythrocyte volume regulation, and sensing pressure changes in the genitourinary tract (21). Recent studies have highlighted the role of Piezo1 in bone homeostasis, showing its necessity for bone formation by mediating osteogenesis in osteoblasts (22)and facilitating osteoblast-osteoclast crosstalk (23). However, unlike its anabolic role in bone, Piezo1 seems to mediate OA progression in chondrocytes, likely due to differences in cell types. Mechanical loading triggers intracellular calcium influx via Piezo1, activating the calcineurin/NFAT signalling pathway (24), which mediates OA lesions. Other ion channels may also be involved; for example, strain-induced calcium influx through transient receptor potential vanilloid 4 (TRPV4) is another major contributor(25, 26). Additionally, harmful mechanical strain predominantly induces calcium influx through the Piezo2 channel. YAP, a known mechanotransducer, may act as a downstream factor(27). Previous studies have linked Piezo1-mediated mechanical responses to the Wnt/β-catenin signaling pathway, with elevated Wnt/Ctnnb1 expressions identified through KEGG pathway mapping(24). Our RNA sequencing results are consistent with these observations.

The findings of this study also have significant implications for cell-based therapies in the treatment of osteoarthritis (OA). Given that Piezo1 activation contributes to the degradation of cartilage by transducing mechanical injury signals and compromising the extracellular matrix, targeting Piezo1 could enhance the efficacy of cell-based approaches. For instance, mesenchymal stem cells (MSCs) or chondrocyte-based

therapies could potentially be optimized by either modulating Piezo1 activity or engineering cells with reduced Piezo1 expression. This could protect the newly formed cartilage from mechanical stress-induced degradation, thereby improving tissue integration and longevity.

This study has several limits. First, more molecular regulating pathways were needed to be illustrated in Piezo1-regulated events. Among them, some factors may directly affect the mechanical behaviour of cartilage. Second, we took shear stress to monitor the cell responses to mechanical loading. Other specific forms of mechanical loading such as compression, tensile strain, hypostatic pressure, both dynamic and static, may be regime-varying. Last, the implications of Piezo1-mechanical behavior to clinical treatment in OA patients was potential to be explored on the future.

CONCLUSION

In conclusion, this study highlights the role of Piezo1 activation in the deterioration of cartilage mechanical properties during OA pathogenesis. Piezo1 transduces mechanical injury signals that lead to extracellular matrix degradation and a reduction in the relaxation capacity of cartilage. These findings enhance our understanding of the early stages of OA and have important implications for the development of drugs and treatments aimed at modulating OA progression.

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TABLES AND FIGURES

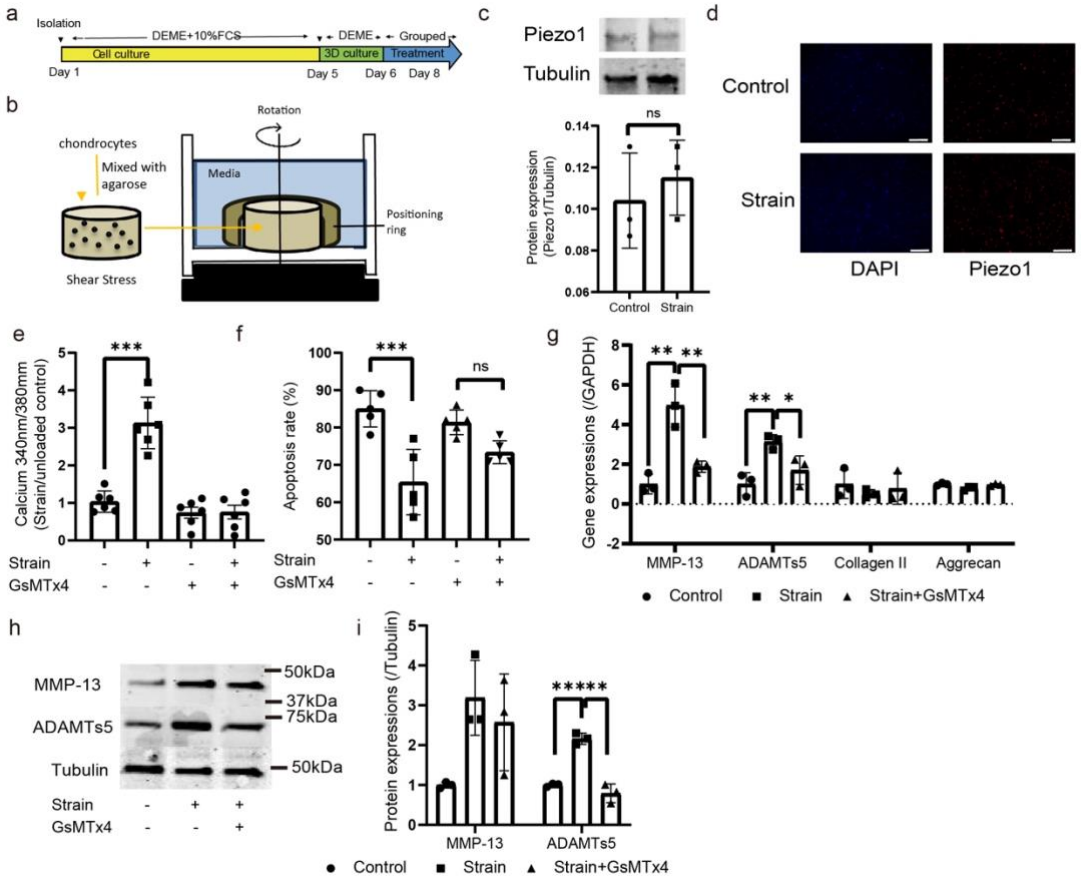

Figure 1. Mechanical strain activated Piezo1-Ca^{2+} influx to induce apoptosis and catabolic activity in chondrocytes cultured in 3D construct *in vitro*. Isolated chondrocytes cultured in 3D construct were unloaded, loaded, or loaded with GxMTx4 after culture and serum deprivation, which was illustrated (a). The mechanical loading pattern with special device was shown in (b). The Piezo1 protein expressions were shown in unloaded and loaded cases (c). Meanwhile, the immunofluorescence labelling of Piezo1 protein in cells constructs were present in unloaded and loaded cells (d). (e) The Ca^{2+} influx in response to strain in cells without and with GxMTx4. The apoptosis frequency of cells in unloaded or loaded condition, without and with GxMTx4 were shown in (f). Then, the mRNA isolated from cells was analysed by using qRT–PCR (g) showing the gene expression levels of MMP-13, ADAMTs5, Aggrecan, Collagen II normalized to the unloaded. The bands and protein expressions of MMP-13, ADAMTs5/Tubulin are also shown (h and j). Scattered dots were together with bars representing the means ± SD (N=3 in c, N=6 in e, N=5 in g, N=3 in h and i). Statistically significant differences based on Student's t-tests (c). One-way ANOVA was used for (e, f, g, j). *p<0.05, **p< 0.01, ***p< 0.001 mean the difference with statistical

significance. The symbol of "ns" means no statistical difference.

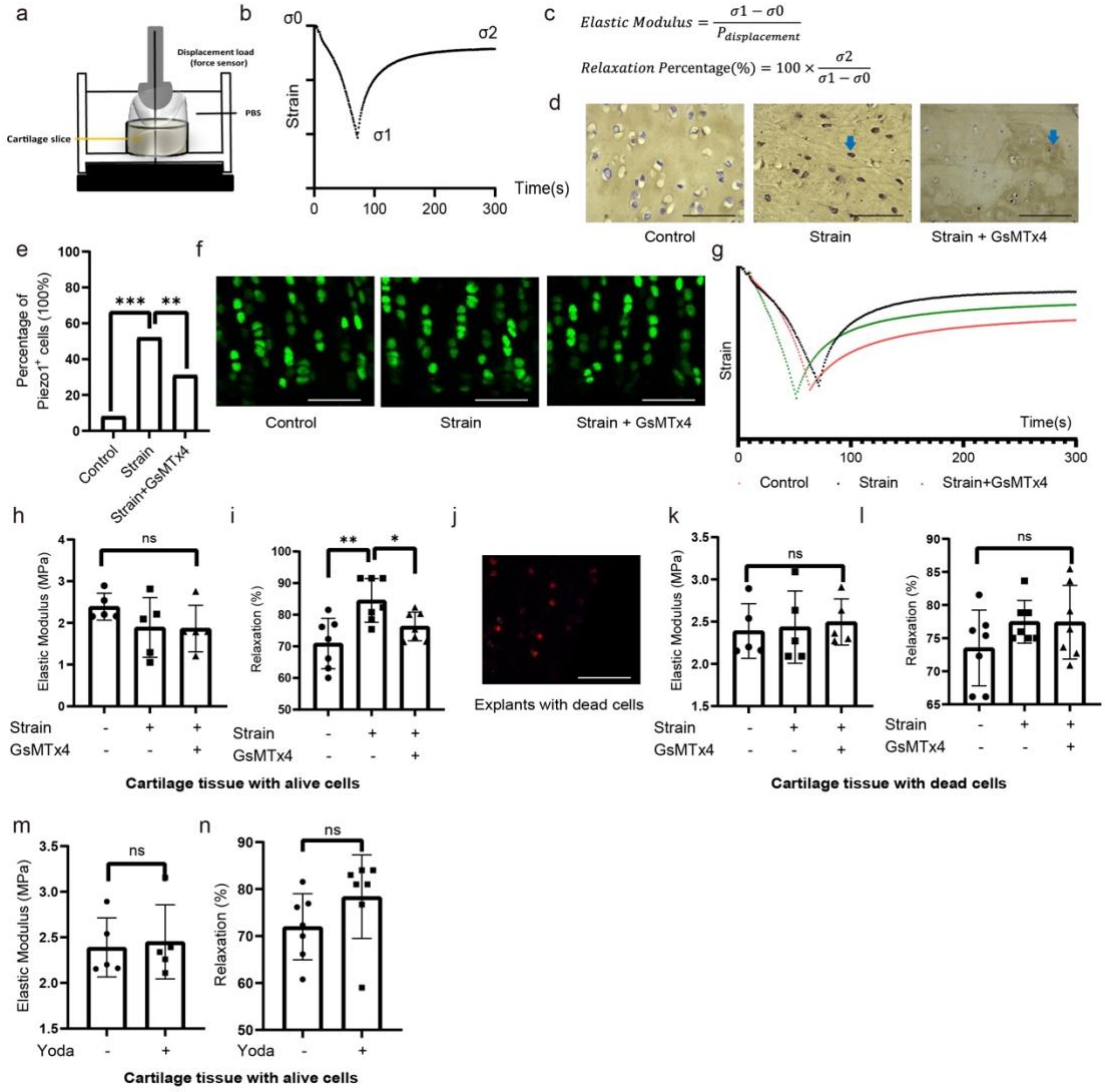

Figure 2. The loss of mechanical properties during relaxation phase by mechanical loading in cartilage explants *ex vivo* was mediated by Piezo1-regulating live chondrocytes. The mechanical testing method was shown in (a) that the compression strain value over time was conducted into the creep and relaxation phases (b). Based on the equations (c), the modulus both in elastic and relax phases were calculated, as well as the percentage of relaxing. Cartilage explants were unloaded, loaded with shear stress or strain-loaded with additional GsMTx4. Piezo1 expressions within tissue was first detected (d) with the frequency results of positive cells (e). Meanwhile, live cells within tissue were identified (f). The representative curve during mechanical testing was showing in (g). For explants with alive cells, the elastic modulus (h) and percentage of relax (i) were calculated showing the significant difference. By using cartilage explants with all dead cells with the confirmation of dead cartilage tissue (j) by

live/dead staning, the mechanical behaviour on elastic modulus (k) and percentage of relax (l) were shown, along. In explants without and with Yoda1 treatment, the mechanical properties were unchanged on modulus (m) and relaxing parentage (n). Scattered dots were together with bars representing the means ± SD (N=5 in h, k, m, N=7 in i, l, n). Statistically significant differences based on Mann-Whitney tests (h, k, m, i, l, n) and exact Fisher's test (e). *p<0.05, **p< 0.01, ***p< 0.001 mean the difference. The symbol of "ns" means no statistical difference.

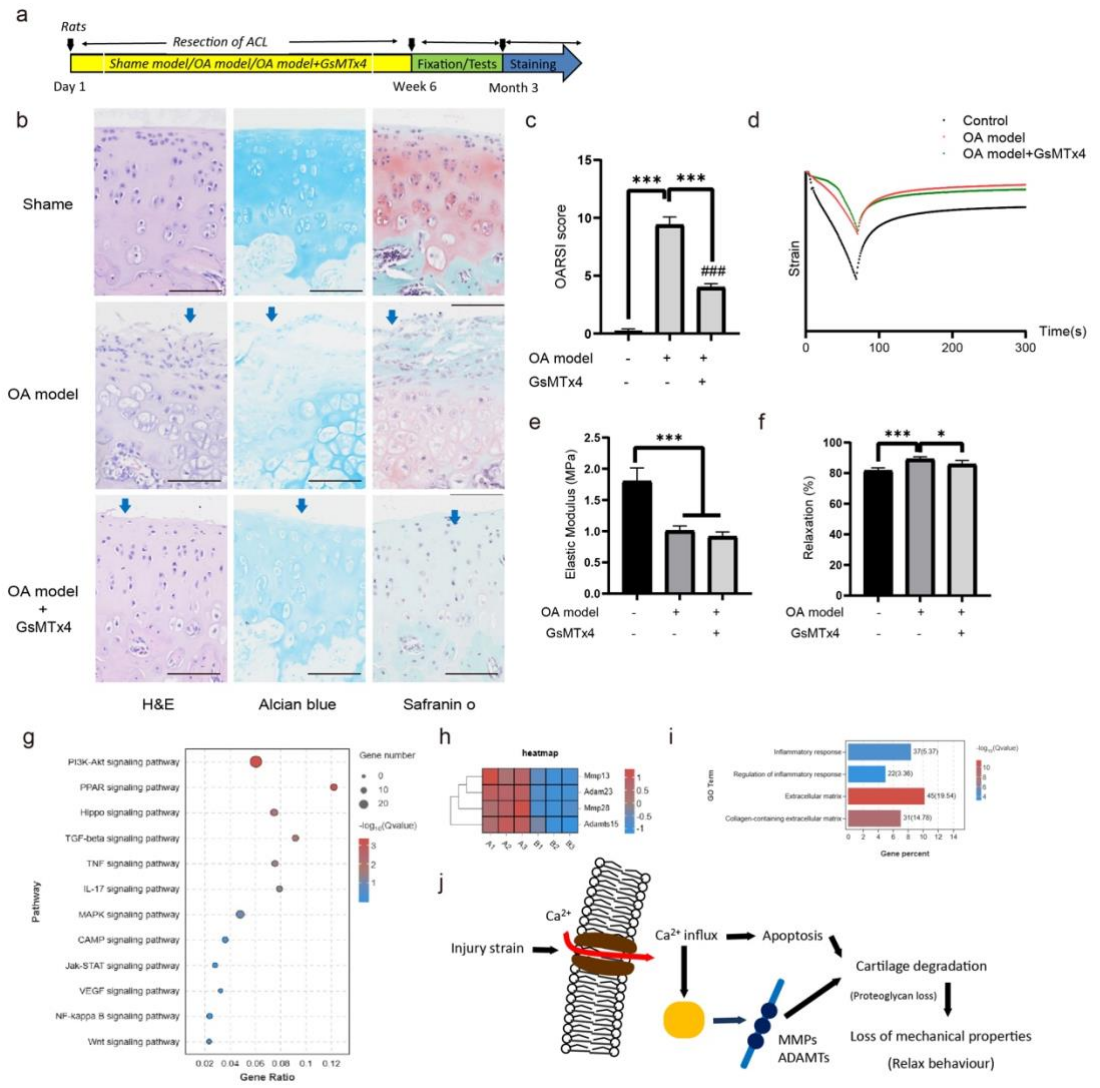

Figure 3. Piezo1 mediated the physical properties loss of cartilage in rat OA model *in vivo*. The OA model was created and divided into three groups: sham control, OA model, OA model with intra-articular injection of GsMTx4 (a). The results from H&E stain, Alcian blue stain and Safranin o stain (b) demonstrated the OA lesions in three groups. The OARSI score from each staining also reflected this trend (c). As shown in the representative strain curve (d), the elastic modulus (e) and relax percentage (f)

indicated the protective role of Piezo1 antagonist on mechanical loss of cartilage. RNA sequence data indicated several possible pathways (g), differently expressed genes (h) and part of GO analysis results on matrix regulating (i). The final mechanism of Piezo1 on mechanical properties was shown in schematic drawing (j). Bars represent the means ± SD (N=3 in c, N=6 in f and g). Statistically significant differences based on One-way ANOVA (c, f, g). *p<0.05, ***p< 0.001 mean the difference. The symbol of "ns" means no statistical difference.