

Excessive tensile strain induced the change in chondrocyte phenotype

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Purpose: Chondrocyte extracellular matrix type II collagen and proteoglycans ensure an important compression-bearing structure in synovial joint. However, much more type I collagen is generated in osteoarthritis, which implies the presence of abnormal tensile strain in cartilage. We hypothesize that tensile stress influences chondrocyte phenotype at the cellular level, leading to potential osteoarthritis. *Methods:* Chondrocytes were stimulated with cyclic excessive tensile (10%) or mild tensile or compressive strain (5%) at 0.5 Hz, 3 h per day for 3 days. Chondrocyte morphology and matrix proteoglycans level was separately determined by Rhodamine phalloidin and toluidine blue staining. The expression of cartilage marker molecules was measured using quantitative reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assays. *Results:* Chondrocytes demonstrated significant fibroblastic morphology, reduced proliferation and increased apoptosis following exposure to 10% tensile strain. The 10% tensile strain group induced the lowest matrix proteoglycans level. It observably reduced the expression of COL2A1, Acan and SOX9, and increased COL1A1 expression level. The 5% tensile (5% compression) group, maintained the chondrocyte phenotype. *Conclusions:* The findings identified the effects of different magnitudes of tensile stress on chondrocyte phenotype compared to compressive strain. Further studies on cartilage biomechanical micro-environment might benefit from this study.

Key words: osteoarthritis, phenotype, chondrocyte, tensile strain

1. Introduction

Articular cartilage functions in cushioning of compressive loads across the joint surface and provides frictionless movement of joints. This function of cartilage is mainly based on its own material basis of extracellular matrix (ECM), which predominantly comprises type II collagen and proteoglycans [21].

Type II collagen is composed of a triple helix of three identical α chains. These molecules associate to form a fibril through intermolecular crosslinks and then form a network combined with proteoglycans and other components [6]. This fibrillar meshwork ensures cartilage shock absorption and compression resistance [10]. However, in the late stage of osteoarthritis, much more type I collagen is generated in the

articular cartilage tissue instead of the original type II collagen [18]. Type I collagen is typically found in ligaments and tendons as type I collagen fibrils have enormous tensile strength needed in these structures [14], which suggest that there may be an abnormal tensile stress environment in cartilage tissue when osteoarthritis is formed. Articular cartilage contains only one cell type, the chondrocyte. According to the changes of cartilage structure and stress environment in osteoarthritis cartilage, we hypothesized that the chondrocyte phenotype would change under the abnormal tensile stress, and the main component of extracellular matrix would transform from type II collagen and proteoglycan to type I collagen.

The present study aimed to verify this hypothesis by detecting the chondrocyte phenotype change under

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tensile/compressive strain *in vitro*. The results will offer an essential information and criteria of biomechanics for tissue-engineered cartilage and clinical treatments of osteoarthritis (OA).

2. Materials and methods

Isolation and culture of primary chondrocytes

Primary chondrocytes were isolated from the knee joints of 4-month-old New Zealand white rabbits of a weight between 2.5 to 3.0 kg. Briefly, cartilage from the condyles of femurs and tibias were aseptically removed, chipped and then minced, followed by digestion in 0.2% type II collagenase (Sigma-Aldrich) for 3 hours at 37 °C. After filtration through a 70 µm nylon filter, chondrocytes were plated in culture medium [Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin]. Passage 2 chondrocytes were used in subsequent experiments.

Fabrication of silicone cell culture chamber and application of mechanical load

The silicone elastomer, Sylgard[®] 184 (Dow Corning GmbH, Wiesbaden, Germany) was cast into flexible cell culture chambers. The silicone elastomer consisted of components A and B: component A contained a platinum catalyst and component B contained a cross-linker. Each chamber had a culture surface measuring 3 × 6 cm. After sterilization, the chambers were coated with fibronectin on their bottom surface to allow adherent chondrocyte culture.

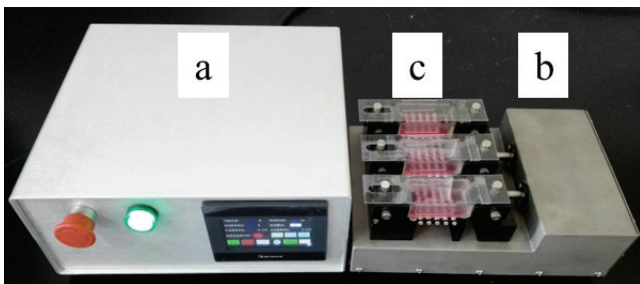


Fig. 1. Custom-designed mechanical loading system. The system consists of three parts: control unit (a), drive unit (b) and cell culture silicone dishes (c)

Tensile/compressive stress was applied using a custom-designed mechanical loading system (Fig. 1). The

system consisted of three parts: a control unit, a drive unit and silicone chambers for cell culture. One end of each chamber was stationary, the other attached to the drive apparatus and driven by the control unit. This arrangement enabled the entire silicon membrane area including the cultured cells to be stretched uniformly. To provide 5% compressive strain to the cells, the silicone dishes were stretched by 5% then fixed in place before seeding the cells. After 48h of culture, the chondrocytes were at 60% confluence. They were cycled at a tensile strain of 5% and 10% elongation or a compressive strain of 5% deformation (at 0.5 Hz) 3 h per day, for 3 days. Chondrocytes without mechanical stress were seeded onto the same chambers to be used as a control.

Chondrocyte cytoskeleton staining

Rhodamine phalloidin (MesGen, Shanghai, China) was utilized for staining the cellular cytoskeleton and observing cell morphology. 1 mL of the staining solution was added to each plate for 40 min at room temperature. Nuclei were counterstained with DAPI. Fluorescence images were captured with an Olympus IX51 microscope (Olympus Corporation, Tokyo, Japan).

Cell proliferation assay

A Cell Counting Kit-8 assay (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) was used to evaluate chondrocyte proliferation. 1mL of CCK-8 solution was added to each silicone plate and the cells were incubated at 37 °C for 1 h. Absorbance was determined at 450 nm using a microplate spectrophotometer (BioTek, Winooski, VT, USA).

Cell apoptosis analysis

After application of mechanical strain, the chondrocytes were tested with a FITC-labelled Annexin V Apoptosis Detection kit (BD Pharmingen[™], CA, USA). Briefly, cells were resuspended in binding buffer at a concentration of 10⁶ cells/mL. 5 µL FITC-conjugated Annexin V and 5 µL Propidium Iodide (PI) were then added to the cells and incubated at room temperature for 15 min in the dark. The samples were analyzed within 1 h post-staining by flow cytometry (Beckman-Coulter, Brea, CA, USA).

Toluidine blue staining

The chondrocytes were fixed in 4% paraformaldehyde and stained with toluidine blue for 1 h and then images were captured with an Olympus IX51 microscope (Olympus Corporation, Tokyo, Japan).

Table 1. Primer sequences of genes used for real-time PCR analysis

Gene name	Primer sequence (5'-3')	Primer sequence (3'-5')
COL2A1	AAGCTGGTGAGAAGGGACTG	GGAAACCTCGTTCACCCCTG
Acan	CTACACGCTACACCCTCGAC	ACGTCCTCACACCAGGAAAC
SOX9	AAGCTCTGGAGACTTCTGAACG	CGTTCCTCACCGACTTCCTCC
COL1A1	GGCAGATGACGCCAACG	CCAGTGTCATGTCCGAGA
GAPDH	CTATAAATTGAGCCCGCAGC	ACCAAATCCGTTGACTCCG

Analysis of gene expression using quantitative PCR

Total RNA was isolated from chondrocytes using TRIzol reagent (Invitrogen, USA). 1 µg of total RNA was then reverse-transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time quantitative polymerase chain reaction (qPCR) was performed with an iTaq™ Universal SYBR® Green Supermix kit (Bio-Rad, Hercules, CA, USA) on a CFX96™ Real-Time PCR System (Bio-Rad). Relative transcript levels of COL2A1 (type II collagen), Acan (aggrecan), SOX9 and COL1A1 (type I collagen) were measured against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) using the formula $2^{-\Delta\Delta CT}$. The primer sequences used for each gene are listed in Table 1.

Enzyme-linked immunosorbent assays (ELISA)

Measurement of type I and II collagen and aggrecan secretion from chondrocytes in the culture media was achieved using enzyme-linked immunosorbent assay (ELISA) kits (Yuyue Biological Technology, Shanghai, China). Protein concentrations were quantified from the absorbance at 450 nm measured using a microplate spectrophotometer (BioTek, Winooski, VT, USA).

Statistical analysis

Data from all experiments were presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Student's *t*-test were used to analyze the differences between groups. $P < 0.05$ was considered statistically significant. All tests were 2-sided and were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results

Effect of mechanical strain on chondrocyte morphology

In order to determine the effect of tensile or compressive strain on chondrocyte morphology, we viewed

staining of the cytoskeleton using Rhodamine phalloidin (Fig. 2a). After 3 days, chondrocytes treated with the largest elongation (10%) demonstrated significant fibroblastic morphology. Cells stretched with mild elongation (5%) exhibited a similar triangular morphology to the control group. However, cells that had undergone compression (5%) exhibited a smaller size but with original triangular morphology.

10% tensile strain decreased chondrocyte proliferation

The CCK-8 assay confirmed that 10% tensile strain reduced chondrocyte proliferation by 11.3% ($P < 0.001$), 18.9% ($P < 0.001$), 12.5% ($P = 0.001$), respectively, compared to the control, 5% tensile strain and 5% compressive strain group. 5% tensile strain accelerated cell proliferation that was 9.4% more than the static group ($P < 0.001$) and 7.7% more than the 5% compressive strain ($P = 0.006$) (Fig. 2b).

10% tensile strain promoted chondrocyte apoptosis

We further examined the effect of different forms of mechanical strain on chondrocyte apoptosis using an Annexin V/ PI staining assay (Fig. 3a). The percentage of apoptotic cells in the 5% tension group ($8.76 \pm 0.39\%$) was significantly lower than any of the other groups. Cells in the 10% tension group exhibited the highest rate of apoptosis ($12.57 \pm 0.35\%$), which was markedly more than 5% tensile strain group ($P = 0.002$), 5% compressive strain group ($10.37 \pm 0.60\%$, $P = 0.046$) and the control ($10.63 \pm 0.26\%$, $P = 0.011$).

10% tensile strain inhibited cartilage biomarker genes expression

The expression of proteoglycans was assessed by toluidine blue staining. 10% tension stimulated the lightest staining of all four groups. Meanwhile, the 5% compression exhibited the densest staining (Fig. 4).

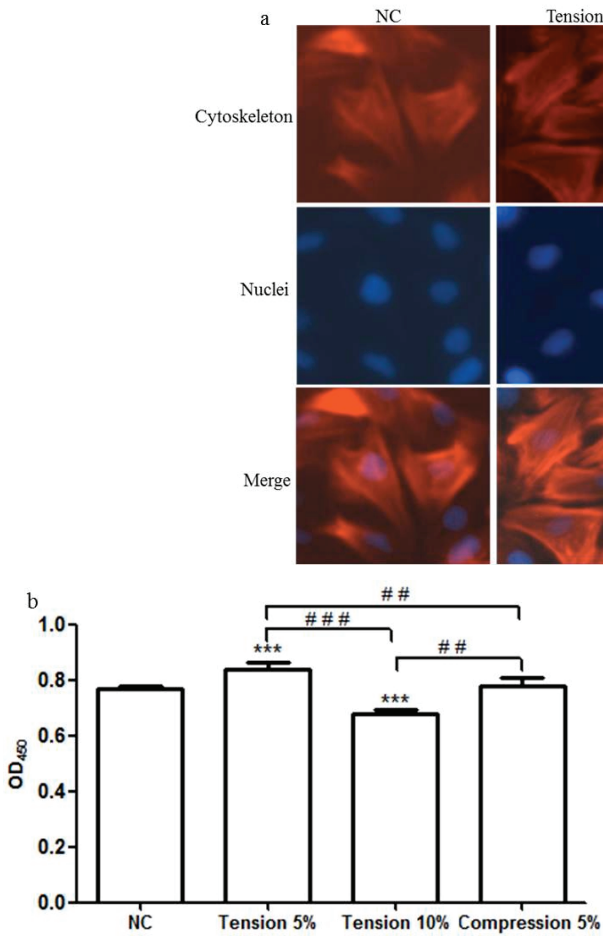


Fig. 2. Effects of mechanical strain on the cell morphology and proliferation of chondrocytes. (a) fluorescence images (200 \times). Scale bar = 50 μ m. (b) data of chondrocyte proliferation, mean \pm standard deviation of five independent experiments ($n = 5$). *** $P < 0.001$ compared with the control group at the same time point. ## $P < 0.01$, ### $P < 0.001$ in the indicated groups from an independent-sample t -test

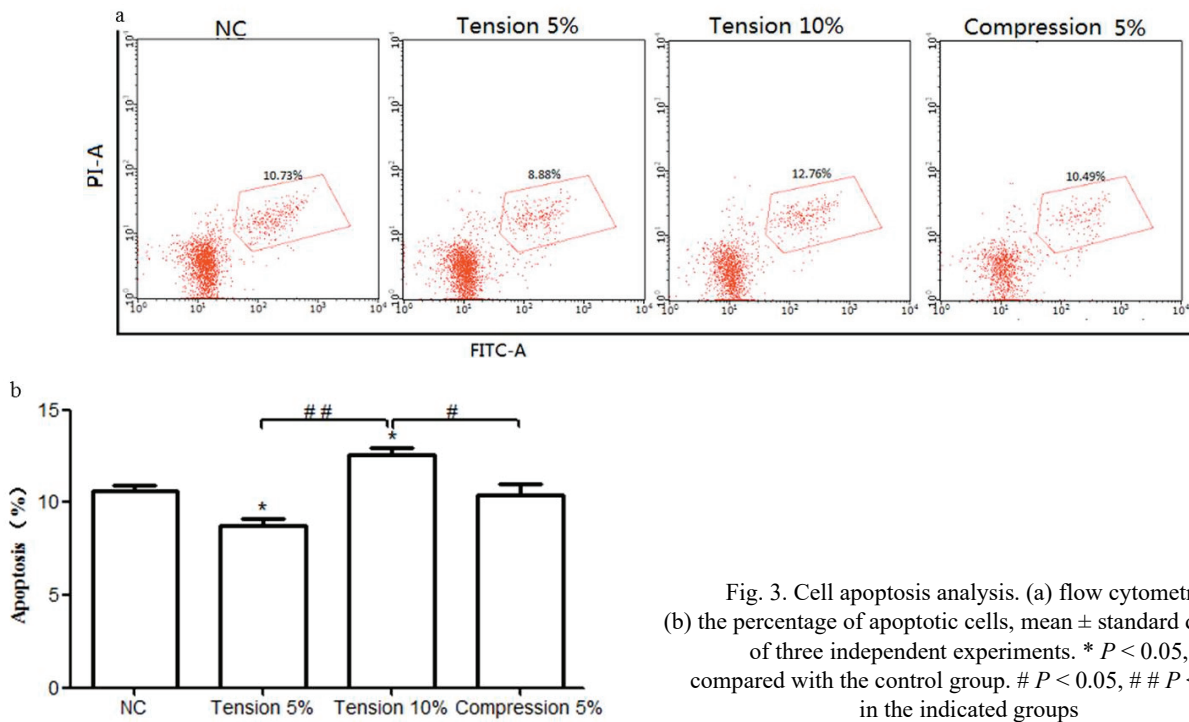


Fig. 3. Cell apoptosis analysis. (a) flow cytometry. (b) the percentage of apoptotic cells, mean \pm standard deviation of three independent experiments. * $P < 0.05$, compared with the control group. # $P < 0.05$, ## $P < 0.01$ in the indicated groups

To evaluate the extent to which the different forms of mechanical strain affected ECM expression, transcript levels of COL2A1, Acan, SOX9 and COL1A1

were examined. 10% tensile strain significantly decreased the expression of COL2A1 by 11.7%, 12.0% and 21.1%, separately compared with the static group

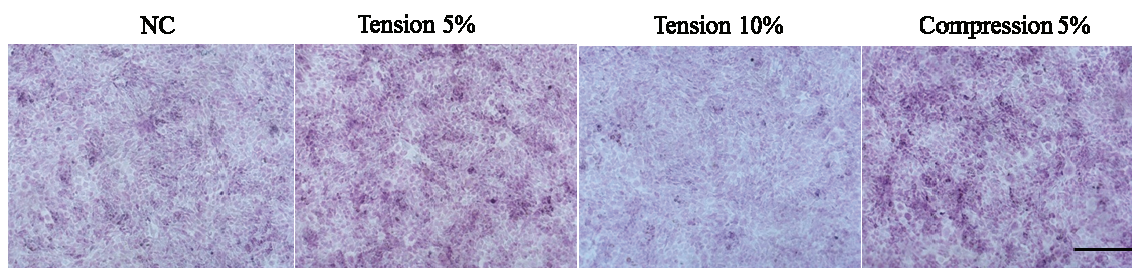


Fig. 4. Effect of mechanical strain on matrix proteoglycan expression. Scale bar = 200 μ m

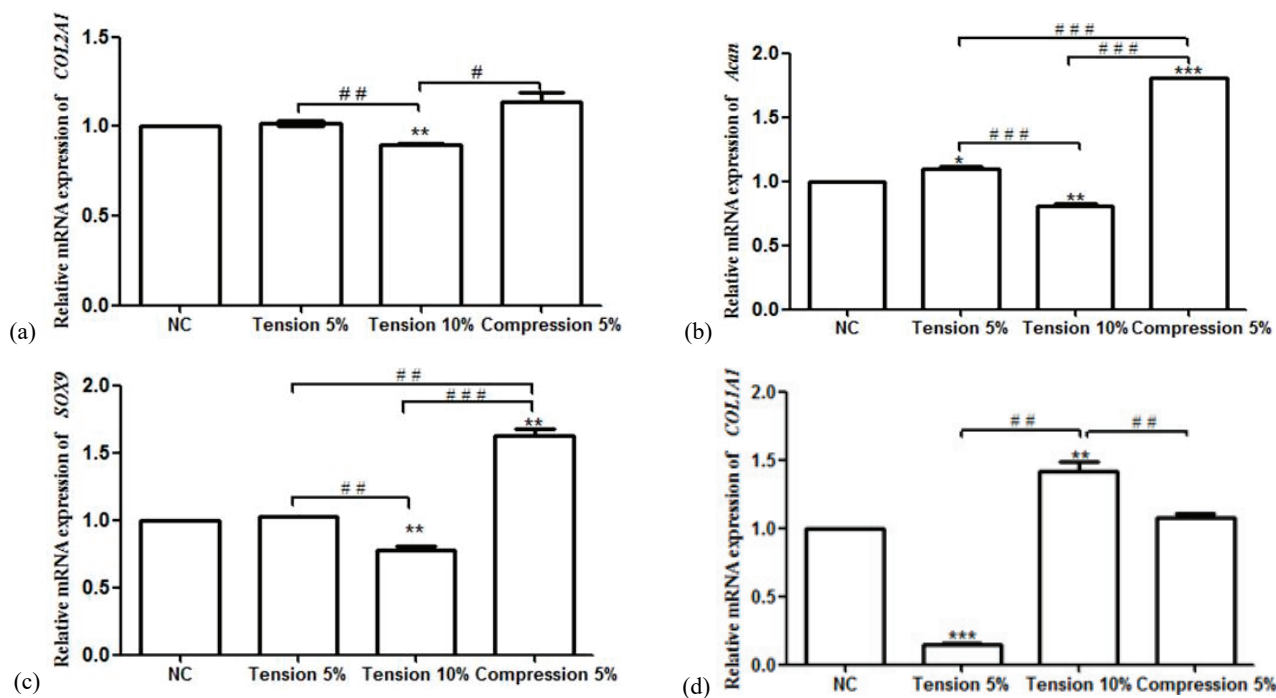


Fig. 5. Tensile/compressive strain modulates expression of mRNA encoding biomarkers genes.

Values are presented as mean \pm standard deviation of three independent experiments ($n = 3$).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the control group.

$P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ in the indicated groups

($P = 0.008$), 5% tensile strain group ($P = 0.001$), and 5% compressive strain group ($P = 0.046$) (Fig. 5a). Expression of *Acan* in the 5% compression group was elevated by 80.7% ($P < 0.001$) relative to the untreated cells, while 10% tensile strain decreased its expression level by 20.3% ($P = 0.009$), compared to the control (Fig. 5b). The transcription factor SOX9 plays a critical role in maintaining homeostasis of ECM. 5% compressive strain had a clear effect on its expression, increasing the level by 62.5% ($P = 0.003$), while 10% tensile strain downregulated its expression by 22.8% ($P = 0.007$), compared to the control group (Fig. 5c). Furthermore, 10% tensile strain also induced the mRNA level of *COL1A1* by 41.9%, 8.36-fold and 32.0%, respectively, compared to the control group ($P = 0.009$), 5% tensile strain ($P = 0.001$), and 5% compressive strain group ($P = 0.002$) (Fig. 5d).

We further examined the level of type II collagen, aggrecan and type I collagen in the culture medium of the chondrocytes using an ELISA assay. 5% tensile strain promoted the levels of type II collagen and aggrecan by 10.1% ($P = 0.023$) and 42.9% ($P < 0.001$), respectively, compared to the corresponding control group (Figs. 6a and b). Similarly, 5% compressive strain also elevated the two protein levels. In agreement with the results of the RT-qPCR and toluidine blue staining assays, 10% tensile strain clearly downregulated the levels by 18.4% for type II collagen, and 1.38 fold ($P < 0.001$) for aggrecan, compared to the control group (Figs. 6a and b). In relation to 5% tensile group, the decreased percentage induced by 10% tensile strain were 23.3% ($P = 0.028$) for type II collagen and 70.5% ($P < 0.001$) for aggrecan. 10% tensile strain promoted type I collagen expression level

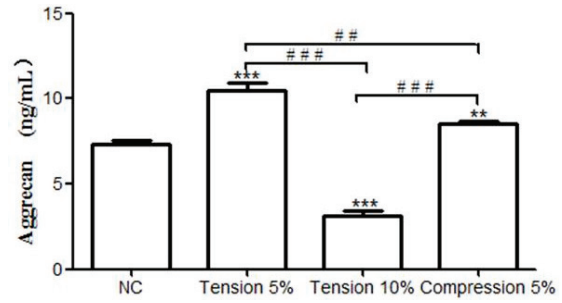
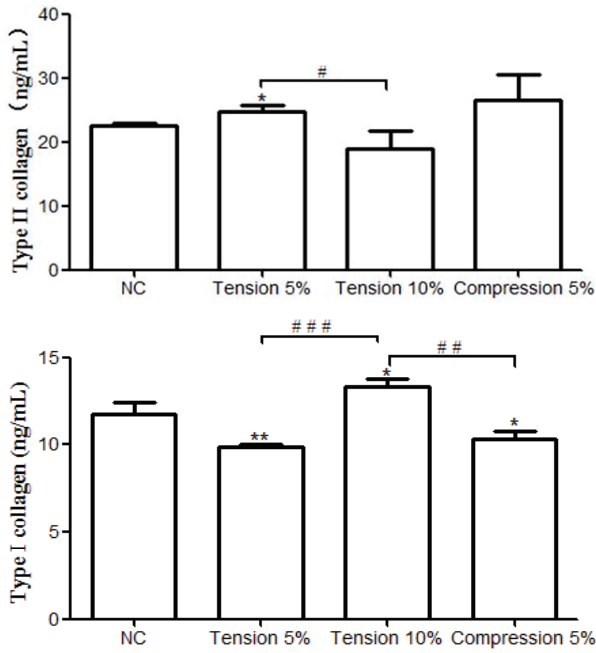


Fig. 6. Effects of mechanical strain on the secretion of: (a) type II collagen, (b) aggrecan and (c) type I collagen in the culture medium of chondrocytes. Values are presented as mean \pm standard deviation of three independent experiments ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ in the indicated groups

by 13.3% ($P = 0.027$), 34.8% ($P < 0.001$) and 29.3% ($P = 0.001$), separately in contrast with untreated cells, 5% tensile and compressive strain group (Fig. 6c).

4. Discussion

In the present study, we compared the effects of tensile and compressive stress on chondrocyte phenotype. Surprisingly, 5% tensile strain favored the maintenance of the chondrocyte phenotype, which was consistent with 5% compressive strain. Conversely, tensile strain with 10% elongation notably inhibited cell proliferation and promoted cell apoptosis. In addition, 10% tensile strain markedly decreased the expression level of type II collagen, aggrecan and SOX9, accompanying upregulated type I collagen. This suggested that only a sufficient magnitude of tensile stress could cause abnormal chondrocyte phenotype.

Mechanical signals acting on articular cartilage are critical regulators of tissue adaptation, structure, and function [19]. Chondrocyte is the only cell in the articular cartilage that can sense mechanical stimuli. It is of great value to explore the effect of different types and amplitudes of mechanical stimulation on chondrocyte phenotypes, which will be useful for the development of tissue-engineered cartilage and the treatment of osteoarthritis.

In recent years, there have been some studies on this subject [20], [25], [26], but there was no systematic and comprehensive comparison of the different

influences of tension and compression on chondrocyte phenotypes. First, we gave insight to the morphological changes of chondrocytes. Cells exposed to the two 5% strain group maintained an original polygonal shape and remained randomly orientated, but the 10% group exhibited a more elongated cell shape and consistent direction. The reason for this alignment might, among other things, be related to cytoskeletal remodeling which may represent a strategy to better withstand the loading. Secondly, we detected the proliferation and apoptosis of chondrocytes. Because of the lack of a blood supply in articular cartilage, which could provide a possible source of stem cells, chondrocyte viability is likely to be a key limiting factor in the repair response resulting in the development of osteoarthritis [1]. Chondrocyte death in cartilage has been identified as an important mechanism in the development of OA joint pathology [11]. It has been established that the death of chondrocytes in response to mechanical injury occurs due to apoptosis or necrosis [3], [15], [22]. As a result of chondrocyte apoptosis, cartilage matrix can degrade and the cells that are needed to repair and maintain extracellular matrix decrease [4], [23]. The 10% tensile group showed significantly decreased cell proliferation and increased apoptosis. This may be an important pathogenesis of OA formed after mechanical injury which often induced abnormal tensile strain in cartilage. Caspases are enzymes that play important roles in regulating and executing cell apoptosis [16]. Caspase inhibitors may be used to reduce chondrocyte apoptosis and restrain the development of OA. Thirdly, we explored the mRNA and protein level of chondrocyte biomarkers. During human physiological activi-

ties, such as walking and jogging, the articular cartilage constantly suffers repetitive compressive loading [2]. Appropriate joint load is a potent regulator for chondrocytes to sustain the cartilage ECM homeostasis. In the present study, 10% tensile strain that exceeded normal physiological amplitude downregulated both the mRNA and protein level of type II collagen and aggrecan. The degradative changes involving ECM may lead to cartilage degeneration and osteoarthritis [12]. SOX9, the first chondrogenic transcription factor, plays essential roles in chondrocyte differentiation and, thereby, in cartilage formation [13]. Reduced SOX9 expression would also affect the development of OA. Type I collagen, an extracellular molecule that predominates in tissues that resist stretch such as tendon or fibrocartilage, is also a marker of osteoarthritic chondrocytes [17]. Excessive tensile strain accelerated type I collagen expression, which would be another crucial reason for cartilage degeneration.

How excessive loading alters the chondrocyte phenotype? Thomas et al. [24] showed that mechanical loading influences downstream responses mediated by canonical Wnt signaling. Kawakita [9] and his colleagues demonstrated that chondrocyte ECM expression is regulated by p53R2 via Akt phosphorylation during chondrocyte mechanotransduction. Meanwhile, Gagarina et al. [7] found that SirT1 is a mediator of human chondrocyte survival via downregulation of PTP1B, a potent chondrocyte proapoptotic protein that is elevated in OA cartilage. SirT1 can also positively regulate the expression of type II collagen and aggrecan [5]. Huang et al. [8] suggested that SirT1 mediates the contractile differentiation of vascular smooth muscle cells under cyclic stretch. On the basis of these studies, we speculate that chondrocyte phenotype is most likely altered by excessive loading through regulation of SirT1. We will conduct further investigation on the exact mechanism.

Acknowledgements

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