Lentivirus-Mediated Small Interfering RNA Targeting Piezo1 Inhibits Cartilage Matrix degeneration in Osteoarthritis Chondrocytes

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Research Article

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Abstract

Osteoarthritis (OA) is triggered by abnormal distraction stress, which eventually leads to cartilage degeneration. However, it remains unclear how the mechanical stress factor acts an effect on the destruction of the cartilage matrix. As a kind of mechanical sensitive ion channel protein, Piezo1 has the ability to sense and transmit mechanical signals to cells. Previous studies have found that the stress sensitivity of Piezo1 is closely related to the directionality of mechanical stress and the presence of extracellular matrix. This research aimed to explore the role of lentivirus-mediated Piezo1-siRNA transfection in extracellular matrix in chondrocytes of cultured osteoarthritis in vitro and to investigate the effect of Piezo1 on the progression of osteoarthritis. In this study, we demonstrated that the expression of Piezo1 mRNA in transfection group was significantly decreased after 24h mechanical stress was applied. However, the expression trend of collagen II and aggrecan was opposite to that of Piezo1, matrix metalloproteinase-13 (MMP-13), and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5). Our results indicate that Piezo1 could be used as a possible therapeutic target to attenuate the destruction of cartilage matrix induced by mechanical stress. Lentiviral-mediated Piezo1-siRNA can effectively interfere with the expression of Piezo1 and protect the expression of the extracellular cartilage matrix.

Impact Statement

Osteoarthritis (OA) is a degenerative disease characted cartilage abrasion and degeneration, with destruction of extracellular matrix. Abnormal mechanical stress is a key factor in OA pathology. Piezo1 is an ion channel that senses and transmits mechanical signals to cells. To investigate the expression and effect of Piezo1 in chondrocyte, we used Piezo1 inhibition mediated by lentivirus-mediated siRNA on chondrocytes, and chondrocytes underwent mechanical stress to simulate in vivo conditions. Our results showed that inhibition of Piezo1 on chondrocyte reduced MMP13, ADAMTS5 expression and promoted Aggrecan expression, which indicated that Piezo1 could be used as a possible therapeutic target to attenuate the destruction of cartilage matrix induced by mechanical stress. Lentiviral-mediated Piezo1-siRNA can effectively interfere with the expression of Piezo1 and protect extracellular cartilage matrix.

Introduction

Osteoarthritis (OA) is one of the crucial causes of chronic joint pain in the elderly. It can seriously interfere with the patient's daily activities, performance, and may eventually lead to lower extremity disorders and disabilities^{1,2}. Women and the elderly are susceptible to OA. The main symptoms include varying degrees of dysfunction and joint pain, and the quality of life of patients is severely affected³. OA is triggered by the macroscale stress related with joint kinematics, which is eventually converted into nanoscale stress at the individual extracellular matrix and cytoskeleton level, at which the mechanical failure ultimately result in joint pathology⁴⁻⁷. The primary pathology of OA are the decrease of chondrocytes, the degradation of extracellular matrix and the sclerosis of subchondral bone⁸. During the past few decades, OA has been identified as a kind of wear and tear disease, which can result in articular cartilage destruction and

disability. The prevalent conception is that the increased fragility of the cartilage, joint instability, and increased pressure on weight-bearing joints served as the major predisposing factors⁹. Chondrocytes and cartilage matrix can support force and regulate their own metabolic activities in response to mechanical loading^{10–12}. Under abnormal stress conditions, such as trauma and chronic strain, mechanical stress acts a crucial effect on the pathogenesis of osteoarthritis^{13, 14}.

Cartilage matrix is consists predominantly of collagen and proteoglycans, of which type II collagen (col-2) accounted for 90% of the total collagen, other types of collagen less; aggrecan is the highest proportion of proteoglycan in cartilage¹⁵. The most critical role of the cartilage matrix is to resist tensile stress and provide tissue tensile strength and stiffness¹⁶. The excessive collapse of col-2 and aggrecan caused by abnormal distraction stress is a critical factor in osteoarthritis cartilage degeneration¹⁷. However, it remains unclear whether the mechanical stress factor acts an effect on the destruction of the cartilage matrix. The pivotal mediators of cartilage matrix degradation can be classified into two categories, including the closely related a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and matrix metalloproteinases (MMPs), which is a group of zinc-dependent proteases degrading col-2^{18–21}. MMPs covers a variety of branches, such as MMP-1, -8, and -13, among which the aggrecan can be degraded by both ADAMTS and MMPs^{22, 23}. Unlike other MMPs, MMP-13 cleaves not only col-2 but also fibrinogen. In this, research, MMP-13 and ADAMTS5 were detected in order to explore the relationship between extracellular matrix degradation and Piezo1 protein.

Mechanosensitive channels can be used to mediate several coefficients, such as blood pressure regulation, proprioception, hearing, and touch²⁴. It was until the discovery of the evolutionarily-conserved Piezo family engaged in mechano-transduction that the molecular mechanism of the mechanical sensitive ion channels was discovered²⁵. Correctly, Piezo1 was regarded as molecules engaged in mechano-sensation channels, which were capable of sensing mechanical signals and keeping the cell volume homeostasis^{26, 27}. After being treated with the specific cationic blocker GsMTx4, it can act a beneficial effect on the chondrocytes of the stress model and reduce the rate of chondrocyte apoptosis induced by traumatic factors²⁸. Previous studies have found that the stress sensitivity of Piezo1 is closely related to the directionality of mechanical stress and the presence of extracellular matrix²⁹. Chondrocytes are mechano-sensation cells, so it is meaningful that Piezo1 could act an essential effect on the degradation of the OA cartilage matrix.

In this study, Piezo1 was used as the target gene to design specific siRNA targeting Piezo1 and transfected into human OA chondrocytes with a lentiviral vector. To study the role of Piezo1 in the extracellular cartilage matrix, it is intended to provide new ideas for gene therapy of OA.

Materials And Methods

Isolation and culture of OA chondrocytes

This project was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. All patients were informed and signed consent. Chondrocytes were harvested from articular cartilage derived

from the OA patients undergoing total knee arthroplasty. Cartilage tissue were briefly washed with phosphate-buffered saline (PBS) containing 400 U/ml penicillin and 0.4 mg/ml Streptomycin (Hyclone, USA) for 3 times. The samples were cut into 1 mm³ granules under aseptic conditions. Then trypsin and collagenase (Solaibio, China) sequential digestion for 4 h, the cells were collected after 1000 r/min centrifugation. OA chondrocytes were cultured in 25cm² flask density of $2 \times 10^4/\text{cm}^2$ which containing medium composed of DMEM (Hyclone, USA) provided with 15% fetal bovine serum (Hyclone, USA). The cells were cultured under the conditions of 5% CO₂ at 37°C, and the culture medium was replaced for a period of three days.

Preparation and transfection of small interfering RNA

The construction of the virus vector was completed by Gemma gene (Shanghai). All sequences were specific oligonucleotide chains (Table 1). Virus titer was determined by hole-by-hole dilution titer assay ($1.0 \times 10^8 \text{ TU/mL}$) after virus collection. The chondrocytes were divided into 5 groups blank control, Piezo1-siRNA1-3 and negative control. Totally 50 µl lentivirus vector, containing each interference sequences and negative control sequences was added to medium, respectively. The titer is $1 \times 10^8 \text{ TU/mL}$. The same amount of medium solution was added in blank control. After 48 h, the cells were observed and counted under fluorescence microscope.

Table 1
Interfering sequences and negative control sequences.

Oligo Name	Oligo Sequence
Piezo1-siRNA1	5'- GATCCGCGTCATCATCGTGTGTAAGATTCAAGAGATCTTACACACGATGATGACGCTTTTTTG- 3' 3'- AATTCAAAAAAGCGTCATCATCGTGTGTAAGATCTCTTGAATCTTACACACGATGATGACGCG- 5'
Piezo1-siRNA2	5'- GATCCGCGTCTTCCTTAGCCATTACTTTCAAGAGAAGTAATGGCTAAGGAAGACGCTTTTTTG- 3' 3'- AATTCAAAAAAGCGTCTTCCTTAGCCATTACTTCTCTTGAAAGTAATGGCTAAGGAAGACGCG- 5'
Piezo1-siRNA3	5'- GATCCGCCTCAAGTACTTCATCAACTTTCAAGAGAAGTTGATGAAGTACTTGAGGCTTTTTTG- 3' 3'- AATTCAAAAAAGCCTCAAGTACTTCATCAACTTCTCTTGAAAGTTGATGAAGTACTTGAGGCG- 5'
Negative control	5'- GATAGCGTCCATCATCGTGTGTAAGATTCAAGAGATCTTACACACGATGATGACGCTTTTTTG- 3' 3'- AATAGCGTCAAGCGTCATCATCGTGTGTAAGATCTCTTGAATCTTACACACGATGATGACGCG- 5'

Application of cyclic stress

The chondrocytes were inoculated into the Flexcell aseptic membranous 6-well plate, cultured in 2.5 ml DMEM containing 15% fetal bovine serum each well. When cells fusion reaches 40%, added the selected effective lentivirus and the empty vector virus 50 μ l per well. The virus titer is 1×10^8 TU/ml. The culture plates were placed in the multichannel cell stress loading system FX-4000T (Flexcell, USA) in 5%CO₂ incubator at 37°C in a humid atmosphere. Because of the different stress time applied to cells, each group was divided into five subgroups, and the cyclic stress of 0 h / 2 h / 12 h / 24 h / 48 h was loaded respectively, with the amplitude of 20% and the period of 10 times / min.

qRT-PCR

Each group added RNAiso (TaKaRa, Japan) to extract the total RNA. PrimeScript RT reagent Kit (TaKaRa, Japan) was used to reverse RNA to cDNA. Then used SYBR Premix ExTaq[®] kit (TaKaRa, Japan) for RT-PCR. The CT values of Piezo1, MMP-13, ADAMTS5, and Aggrecan genes were obtained by FTC-2000 system (Applied Biosystems, China) and the parameters were as follows: pre denatured: 95°C for 30 seconds, 1

cycle; PCR: 95°C for 5 seconds, 60°C for 30 seconds, 72°C for 30 seconds for 40 cycles. The relative expression of target gene was calculated by $2^{-\Delta\Delta C_t}$. GAPDH, Piezo1, MMP-13, ADAMTS5, and Aggrecan primers were produced by Shanghai Bioengineering Company (Table 2).

Table 2
The oligo sequences of the target genes.

Oligo Name	Oligo Sequence
Piezo-1	Forward PrimerCATCTTGGTGGTCTCCTCTGTCT
	Reverse Primer CTGGCATCCACATCCCTCTCATC
MMP-13	Forward PrimerGTGGTGTGGGAAGTATCATCA
	Reverse Primer CATCTGGAGTAACCGTATTG
ADAMTS5	Forward PrimerATGACCATGAGGAGCACTACGA
	Reverse Primer GGAGAACATATGGTCCCAACGT
Aggrecan	Forward PrimerAGCCTGCGCTCCAATGACT
	Reverse Primer GGAACACGATGCCTTTCACC
hGAPDH	Forward PrimerGCACCGTCAAGGCTGAGAAC
	Reverse Primer TGGTGAAGACGCCAGTGGA

Western blot analysis

Cells were lysed with the precooled RIPA lysate (Solarbio, China). The supernatant was obtained after centrifugation at 4°C for 12000 r/min. After adding appropriate amount of sample buffer to the protein sample, the protein samples and standard protein marker were added to polyacrylamide gel and then electrophoretic. After electrophoresis, the protein was transferred to the polyvinylidene fluoride membrane. The membranes immersed in the collagen II antibody (dilution concentration 1:5000; Abcam, China) were incubated overnight in 4°C. The second antibody (dilution concentration 1:10000; Abcam, China) was added for 1h. Enhanced chemiluminescence reagent was used to detect the target proteins. β -Tubulin was used as internal control

Immunofluorescence microscopy

The cells were cultured in a 24-wells plate. When the cells were covered with coverslips, the chondrocytes were fixed in 4% PFA (Hyclone, USA) and added with a blocking solution containing 1% goat serum and 0.5% Triton X-100. The chondrocytes were stained with the primary antibodies of rabbit polyclonal Piezo1 (1:400, Novus, USA) overnight at 4°C. AlexaFour 488 Goat Anti-Rabbit IgG was used as secondary antibodies. Then, cell nuclei were stained with 0.1 mg/mL DAPI (Invitrogen). Images were captured by laser scanning confocal microscopy and analyzed with Image-Pro Plus6.0 software.

Statistical analysis

The data were processed by SPSS22.0 statistical software, the measurement data were expressed as mean $\pm$ standard deviation (SD), *t test* were used when the two independent groups of measurement data were compared. One-way ANOVA was used to compare the mean values of multiple samples. $P < 0.05$ was defined as statistically significant.

Results

Culture of the OA chondrocytes

The OA chondrocytes grew into a polygonal shape (Fig. 1A). Immunohistochemical staining showed that the aggrecan showed a strong positive reaction. The cytoplasm of the chondrocytes was stained with yellow granules, and the nuclear staining was not visible (Fig. 1B).

Transfection of Piezo1-siRNA

We evaluated the transfection by detecting the expression of green fluorescence protein (Fig. 2A). In addition to the blank control group, the transfection efficiency (fluorescence field of cells / general field of cells $\times$ 100%) of the experimental group was greater than 90% (Fig. 2B). Compared with experimental group, the transfection efficiency of negative control group had no significant difference. The growth curves of Piezo1-siRNA1-3, blank control, and negative control groups were quite similar (Fig. 2C).

RT-qPCR of Piezo1, MMP-13, ADAMTS5, and Aggrecan

The mRNA expression characteristics of Piezo1 showed the first increase and then decrease, and reached the maximum expression upon 24 h mechanical stress (Fig. 3A). Transfected cells were mechanically stress for 24h, qRT-PCR results demonstrated that Piezo1, MMP-13, and ADAMTS5 levels of Piezo1-siRNA2 were reduced compared with Piezo1-siRNA1 and Piezo1-siRNA3, whereas the expression of aggrecan mRNA was presented the opposite trend (Fig. 3B, 3C, 3D, 3E). Piezo1-siRNA2 efficiently inhibits the expression of Piezo1.

Western blotting of Collagen II

Chondrocytes were mechanically stress for 24h, and Western blots were used to determine collagen II expression in OA chondrocytes transfected by Piezo1 siRNAs. Compared with the control group, the expression of collagen II in the Piezo1 siRNAs group was significantly increased ($P < 0.05$). Relative collagen expression in siRNA1, siRNA2, and siRNA3 groups was increased approximately 143%, 327% and 212% ($p < 0.01$) compared to the control group. There was no significant difference in the expression of collagen II between blank control group and negative control group ($P > 0.05$) (Fig. 4).

Immunofluorescence of the Piezo1 in OA chondrocytes

Cell immunofluorescent analysis revealed that the expression of Piezo1 was detected in OA chondrocytes. Moreover, Piezo1 was primarily located in the nucleus and cell membrane of chondrocytes (Fig. 5).

Discussion

Piezo1 is a novel type of non-selective mechanically sensitive ion channel which is expressed extensively in mammalian³⁰. However, the effect of Piezo1 on cells has not been fully elucidated. The pathogenesis of OA is related to many risk factors, especially the abnormal mechanical stress has a crucial effect on the progression of OA^{31,32}. Abnormal mechanical stress can not only lead to joint damage in physics but also affect the progress of OA through the baroreceptor signals³³. Previous studies had reported that TRPV4-mediated cartilage mechanotransduction was involved in the pathogenesis of aging- and injury-induced OA³⁴⁻³⁶. It is also meaningful to explore a novel mechanosensitive ion channel associated with the pathogenesis of OA chondrocytes. Piezo1, a molecule that activates the mechanical signal transduction pathway, is potentially beneficial for the therapy of OA. The excessive destruction of the Extracellular matrix is closely related to the decrease of Chondrocyte³⁷. When the synthesis of the extracellular matrix decreases, the living environment of chondrocytes will be destroyed, and it will cause the excessive apoptosis of chondrocytes, which was the critical factor of osteoarthritis³⁸. In this research, we tried to investigate the role of Piezo1, a new mechanical sensitive ion channel, in OA chondrocytes. Our results show that Piezo1 acts an effect on the destruction of the extracellular cartilage matrix. The degradation of Collagen II and Aggrecan can be inhibited by siRNA against Piezo1.

The previous study had explored that the presence of extracellular matrix proteins is the prerequisite for Piezo1 to transmit mechanical signals, which discovered that stress patterns could mediate the apoptosis of the cells. However, till now whether Piezo1 acts an effect on the destruction of extracellular matrix is not clear³⁹. We speculated that the mechanical stress could activate the Piezo1 and further resulted in the degradation of the Extracellular matrix during the progress of osteoarthritis. In this research, the FAM38A gene encoding Piezo1 was silenced by small interfering RNA, and the chondrocytes stress model was constructed by using a Flexcell Strain Unit. We monitored the expressions of the Piezo1, aggrecan, MMP-13, ADAMTS5 with RT-qPCR, and screened the best inhibitory sequence. The expression of Collagen II was detected by Western blot. The expression of the Piezo1, MMP-13, ADAMTS5 were increasing under the mechanical force and had the character of time-dependence, while the col-2 and aggrecan were decreasing. After the FAM38A gene was silenced by Piezo1-siRNA, the expression of the Piezo1, MMP-13, ADAMTS5 were decreasing, while the Collagen II and aggrecan were increasing. The results indicate that Piezo1 had a crucial effect on the degradation of the cartilage matrix and may be served as a potential target for the therapy of OA, especially for those due to trauma.

Understanding the mechanism of the mechanical sensitive ion channel Piezo1 function in chondrocytes is a viable way to understand cartilage mechanical biology and related diseases, especially osteoarthritis. Previous studies have discovered that Piezo1 and TRPV4 are independently activated, while stress induced Ca^{2+} influx pathway is primarily induced by Piezo1 and TRPV4 channels⁴⁰. There is evidence that L-type

Ca^{2+} voltage gated channel can affect Ca^{2+} influx upon mechanical stress⁴¹. It is meaningful to speculate that Ca^{2+} plays an essential role in activating Piezo1.

Conclusions

In conclusion, the present study found that Piezo1 acts a role on the destruction of the extracellular cartilage matrix. The degradation of col-2 and aggrecan can be inhibited by siRNA against Piezo1. Piezo1, functionally located in OA chondrocytes, provide potential targets for attenuating cartilage matrix degradation and reducing injury-induced cartilage degeneration. In this respect, Piezo1-siRNA could be capable to regard as a cartilage protective agent to prevent and treat osteoarthritis.

Declarations

Ethical Approval and Consent to Participate

All methods were carried out in accordance with the Helsinki' s Declaration. All experimental protocols were approved by the medical ethics committee of the Affiliated Hospital of Qingdao University (QYFYKYLL-2017-06-12-05), and informed consents were obtained from all patients.

Consent for publication

All authors consent the publication of this manuscript. Informed consent for publication were obtained from patients involved in the study.

Availability of data and materials

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

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Not applicable.

Authors' Contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; Liu Y and Lu X conducted the experiments; Zhang Z and Li T contributed in data curation; Liu Y write the manuscripts; Zhang H: Conceptualization, Supervision, Validation, Writing - review & editing.

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Figures

Figure 1

The OA chondrocytes with Identification and transfection, scale bars, 50 μm. (A) Primary of OA chondrocytes. (B) OA chondrocytes was also identified by detecting col-2. (C) Toluidine blue staining for primary OA chondrocytes.

Figure 2

The OA chondrocytes with transfection. (A) Green fluorescence protein expression was observed under fluorescence microscope at the end of 48h of post-transfection incubation, scale bars, 50 μm . (B) Fluorescence in each instance was quantified using FACS. The corresponding histogram showed transfection efficiency for Negative random and siRNA1-3. n.s. means $P > 0.05$. (C) Compared with the blank control group, the growth curve of cells in each group was almost parallel. The growth period was from 1 to 2 days, after 3 to 4 days cells turned into the period of growth platform, and then decreased.

Figure 3

RT-qPCR results of Piezo1, MMP-13, ADAMTS5 and Aggreacan expression in OA chondrocytes. (A) The expression of the Piezo1 in the stress group was increasing under the mechanical stress and had the character of time-dependence. Results represent mean $\pm$ SE. n.s. means $P>0.05$ the mechanical stress group versus the blank group; * $P<0.05$ ** $P<0.01$ the mechanical stress group versus the blank group. (B, C, D) The expression of Piezo1, MMP-13 and ADAMTS5 in small interfering RNA group was decreasing under the

mechanical stress for 24h. The expression of Piezo1, MMP-13 and ADAMTS5 in siRNA2 group was significantly decreased compared with siRNA1 and siRNA3. (E) Meanwhile, the expression of Aggrecan mRNA was presented the opposite trend.

Figure 4

The expression of col-2 regulated by Piezo1-siRNA. The expression level of col-2 in OA chondrocytes was analyzed by western blotting. β-tubulin was used as internal control. The expression of col-2 was opposite to that of Piezo1. Under the mechanical stress for 24h, col-2 in siRNA group was significantly higher than that in control group. In addition, the expression level of col-2 in siRNA2 group was higher than that in siRNA1 and siRNA3 group. Column, mean of three independent experiments; bars, SD; n.s. $P > 0.05$ * $P < 0.05$ ** $P < 0.01$.

Figure 5

LSCM results of the Piezo1 protein-specific immunolabeling in the OA, scale bars, 5 μm.

Supplementary Files

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