

HSA_Circ_0134111 Regulates Chondrocyte Degeneration in Osteoarthritis by miR-103a/Smad7 Axis

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Huang *et al.*: Mechanism of hsa_circ_0134111 Regulating miR-103a/Smad7 Axis in Osteoarthritis

To explore the mechanism of hsa_circ_0134111 regulating micro RNA-103a/Smad7 axis in chondrocyte degeneration in osteoarthritis. Human chondrocytes (HUM-CO-013) were cultured *in vitro* and divided into si-control group and si-circ_0134111 group. Chondrocytes were induced by monoiodoacetate to establish osteoarthritis injury model, the apoptosis rate was detected by flow cytometry, the expression of micro RNA-103a and Smad7 messenger RNA in osteoarthritis chondrocytes were detected by quantitative reverse transcription polymerase chain reaction; the targeting relationship between hsa_circ_0134111 and micro RNA-103a was detected by double luciferase report test. The contents of malondialdehyde, L-lactate dehydrogenase and glutathione peroxidase were detected by kit and the expressions of apoptosis-related proteins (B-cell lymphoma 2, B-cell lymphoma 2-associated protein x), collagen II, matrix metalloproteinase 13 and Smad7 in osteoarthritis chondrocytes were detected by Western Blot method. The apoptosis rate of osteoarthritis chondrocytes in si-circ_0134111 group was reduced than si-control group and the micro RNA-103a and Smad7 messenger RNA in osteoarthritis chondrocytes in si-circ_0134111 group were decreased than si-control group. Micro RNA-103a could reduce the activity of wild-type circ0134111 luciferase reporter gene, but had no effect on MUT-circ0134111. The contents of malondialdehyde and L-lactate dehydrogenase in osteoarthritis chondrocytes in si-circ_0134111 group decreased and the content of glutathione peroxidase increased, while the contents of B-cell lymphoma 2 and collagen II in osteoarthritis chondrocytes in si-circ_0134111 group increased, while the contents of B-cell lymphoma 2-associated protein x, matrix metalloproteinase 13 and Smad7 decreased compared with si-control group. Hsa_circ_0134111 can participate in osteoarthritis chondrocyte degeneration through micro RNA-103a/Smad7 axis and has_circ_0134111/micro RNA-103a/Smad7 axis may be a new regulatory mechanism and therapeutic target for osteoarthritis.

Key words: Osteoarthritis, has_circ_0134111, micro RNA-103a, Smad7, chondrocyte degeneration

Osteoarthritis (OA) is a common joint disease characterized by articular cartilage degeneration, osteophyte growth and arthritis^[1]. With the aging of the population, the incidence of OA is increasing, which brings a heavy burden to the society and families. Chondrocytes are the main cell types in articular cartilage and their functional impairment and cell death are important reasons for the progression of OA^[2]. Therefore, revealing the molecular mechanism of OA chondrocyte degeneration and finding new therapeutic targets are of great significance for the prevention and treatment of OA. It has been found that circular Ribonucleic Acid (circRNA) and micro RNA

(miRNA) play an important role in regulating the occurrence and development of OA chondrocytes and diseases^[3]. CircRNA has the characteristics of high stability, long half-life and remarkable regulation. It can regulate the expression of target genes by adsorbing miRNA, thus affecting the function of OA chondrocytes.

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It has been found that circ_013411 is differentially expressed in OA chondrocytes, but its specific mechanism is not clear. miRNA is a kind of non-coding RNA, which inhibits the expression of target genes or promotes the degradation of target genes by binding to the 3'-Untranslated Region (3'-UTR) of target genes and then regulates the biological processes such as cell proliferation, differentiation and apoptosis^[4]. Smad7 could regulate and inhibit Transforming Growth Factor-Beta (TGF- β) signal pathway, while TGF- β signal pathway plays critical role in OA chondrocyte degeneration. Some studies have found that miR-103a can inhibit Smad7 by binding to the mRNA of Smad7 and then participate in the regulation of TGF- β signal pathway^[5]. However, the specific molecular mechanism of OA chondrocyte degeneration is not completely clear. Therefore, the purpose of this study is to reveal the mechanism of hsa_circ_0134111 regulating miR-103a/Smad7 axis in the degeneration of OA chondrocytes and to provide a new theoretical basis and therapeutic strategy for the prevention and treatment of OA. To further clarify the mechanism of hsa_circ_0134111/miR-103a/Smad7 axis in OA chondrocyte degeneration, and provide a new potential target for the treatment of OA.

MATERIALS AND METHODS

Research object:

Human chondrocyte (HUM-CO-013) cells were purchased from a typical culture preservation centre in the United States of America. The cryopreservation tube containing HUM-CO-013 cells was melted in water at 37°. The cell suspension was transferred into the centrifuge tube and was centrifuged at 1000 r/min at room temperature for 5 min. The culture medium was added to the precipitated cells at 37° and cultured with 5 % CO₂. Discard the old culture medium, add 2 ml preheated Phosphate-Buffered Saline (PBS) washing cells and 0.5 ml trypsin digestion liquid, after complete digestion, add human bone marrow-derived stem cell medium containing 10 % Fetal Bovine Serum (FBS), stop digestion and subculture according to 1:3 proportion.

Cell grouping and treatment:

HUM-CO-013 cells were divided into si-control (si-con) group and si-circ_0134111 group. Lentivirus vector with circ_0134111 knockout was transfected into cells in si-circ_0134111 group,

while blank vector was transfected only in si-con group. OA cell model was prepared by culturing in 4 μ mol/l Monoiodoacetate (MIA) solution for 48 h in both groups.

Experimental materials, reagents and instruments:

HUM-CO-013 cell, FBS, Penicillin-Streptomycin double antibody (PS), MIA inducer (Sigma-Aldrich), aseptic PBS buffer, 2 % agarose gel, 10 % urea, Radioimmunoprecipitation Assay (RIPA) cleavage buffer, Bicinchoninic Acid (BCA) protein quantitative kit, polyacrylamide gel, Electrochemiluminescence (ECL) luminescent solution, B-cell lymphoma -2 (Bcl-2) antibody (ThermoFisherScientific), Bcl-2-associated x protein (Bax) antibody (ThermoFisherScientific), Malondialdehyde (MDA), Lactate Dehydrogenase (LDH), Glutathione Peroxidase (GSH-Px) detection kit, protein electrophoresis buffer (AgilentTechnologies), protein transfer membrane (AgilentTechnologies), protein extract, protein concentration kit, flow cytometry (BeckmanCoulter), USA), Westernblot (Roche, Switzerland), quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) kit (Bio-Rad).

Experimental method:

Flow cytometry: Each group of chondrocytes was washed with precooled PBS, the supernatant was discarded and the cells were resuscitated by adding 5 μ Annexin V- Fluorescein Isothiocyanate (FITC) to the cell precipitation μ buffer. After incubating with Propidium Iodide (PI) at room temperature for 10 min, the apoptosis rate was determined by FACSCalibur flow cytometry.

RT-PCR: HUM-CO-013 of human chondrocytes in each group was cultured *in vitro* for 15 d. Total RNA was extracted from chondrocytes of each group by Trizol method. The concentration and purity of RNA were measured by NanoDrop 2000° ultramicro spectrophotometer and stored in an ultra-low temperature refrigerator at 80°. The reverse transcription reaction system includes 5 \times g DNA buffer 2 μ l, 10 \times King RT buffer 2 μ l, fast King RT enzyme mixture 1 μ l, FQ-RT primer mixture 2 μ l and RNA (2 μ g). Using a deionized distilled H₂O (ddH₂O) supplement system without RNase, the total volume can be up to 20 μ l. The reaction conditions are 42° for 15 min and 95° for 3 min.

After the synthesized complementary DNA was diluted 20 times, qRT-PCR was carried out, and the operation reaction was described according to the kit. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the internal reference, and $2^{-\Delta Ct}$ was used to calculate the expression level of miR-103a and Smad7 mRNA (Table 1).

TABLE 1: PRIMER SEQUENCE

Primer	Sequence (5'-3')
miR-103a	F: TCCCCTTGAGTAGAGACCCG
	R: ACTGAAGGCTCAACAGCTCC
Smad7	F: GGGTCAGGTGCCTTAGTGAC
	R: TGTCGATGACACTGACGCAA
GAPDH	F: TGTCGATGACACTGACGCAA
	R: CCTGGAAGATGGTGATGGGAT

Double luciferase report experiment: Circular RNA Interactome prediction showed that there was a targeted relationship between hsa_circ_0134111 and miR-103a. The binding site and mutation site were cloned into pmirGLO vector to obtain wild type vector WT-circ0134111, mutant vector MUT-circ0134111 and amiR-103a co-transferred into chondrocytes with WT-circ0134111 and MUT-circ0134111, respectively. After 48 h of culture, cytopathic cell disease was collected and relative luciferase activity was detected.

Kit detection: The supernatant of cell culture in each group was collected, and the contents of MDA, LDH and GSH-Px were detected according to the instructions of the detection kit.

Western blot: The keratinocytes of psoriasis in each group were collected and lysed by adding RIPA lysate. The protein was quantified according to the working solution prepared by BCA kit. The supernatant was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to Poly Vinylidene Fluoride (PVDF) membrane. 10 ml 5 % skimmed milk powder was sealed at room temperature for 2 h or incubated

overnight at 4°. After the PVDF membrane was incubated in an anti-RhoA treatment at 4°, tris-buffered saline, 0.1 % Tween 20 (TBST) washed off the first antibody that binds to the membrane, and the corresponding second antibody was added to incubate at room temperature for 2 h. Coloration by enhanced chemiluminescence.

Statistical method:

Statistical Package for Social Sciences (SPSS) 22.0 software was used for statistical analysis. The measurement data were expressed by mean±standard deviation ($\bar{x} \pm s$). Pairwise comparison was conducted by least significant difference-t method.

RESULTS AND DISCUSSION

The apoptosis rate of OA chondrocytes in si-circ_0134111 group was reduced than si-con group (Table 2 and fig. 1). The miR-103a and Smad7 mRNA in OA chondrocytes in si-circ_0134111 group were reduced than si-con group (Table 3 and fig. 2). There was a binding site between hsa_circ_0134111 and miR-103a (fig. 3). Double luciferase reporter experiment showed that miR-103a could reduce the activity of wild-type circ0134111 luciferase reporter gene, but had no effect on MUT-circ0134111 (Table 4 and fig. 4). The contents of MDA and LDH in OA chondrocytes in si-circ_0134111 group were decreased while the content of GSH-Px was increased than si-con group (Table 5 and fig. 5).

The content of Bcl-2 in OA chondrocytes in si-circ_0134111 group was increased and the content of Bax was decreased than si-con group (Table 6 and fig. 6). The content of collagen II in OA chondrocytes in si-circ_0134111 group was increased, while the contents of Matrix Metalloproteinase 13 (MMP13) and Smad7 were decreased than si-con group (Table 7 and fig. 7).

TABLE 2: APOPTOSIS RATE OF OA CHONDROCYTES ($\bar{x} \pm s$, n=7)

Group	Cell apoptosis rate (%)
si-con	24.81±0.83
si-circ_0134111	14.23±0.81
t	24.136
p	<0.001

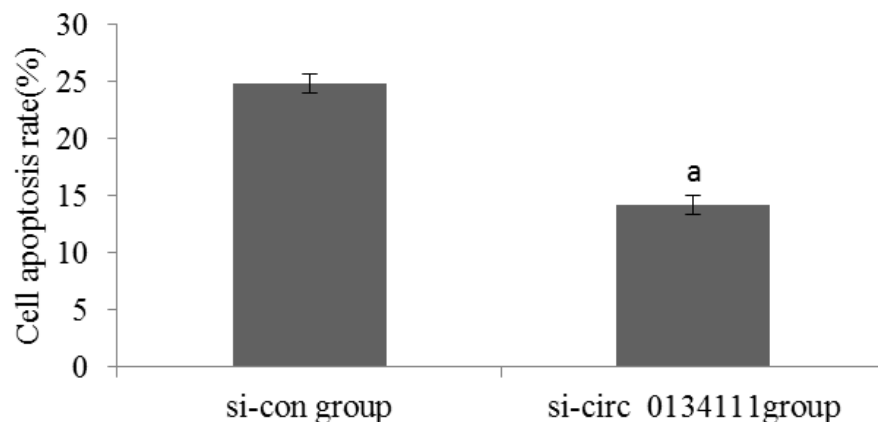


Fig. 1: Comparison of cell apoptosis rates in three groups of OA chondrocytes

Note: ^ap<0.05

TABLE 3: miR-103a AND Smad7 mRNA EXPRESSION IN OA CHONDROCYTES ($\bar{x} \pm s$, n=7)

Group	miR-103a	Smad7
si-con	1.12±0.08	1.14±0.05
si-circ_0134111	2.95±0.02	0.62±0.03
F	58.714	23.594
p	<0.001	<0.001

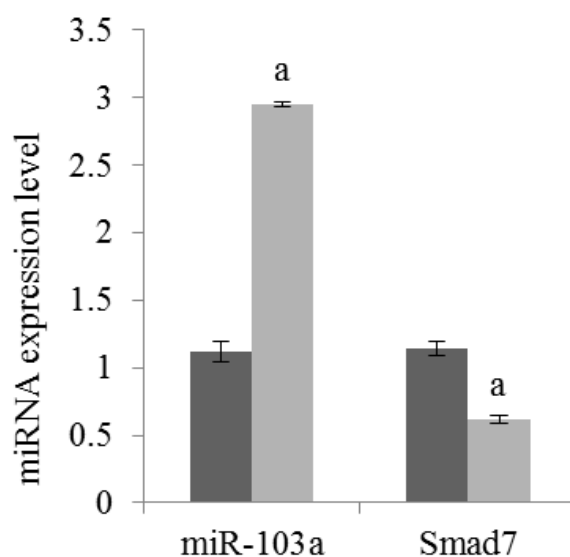


Fig. 2: Comparison of miR-103a and Smad7 mRNA expression levels in three groups of OA chondrocytes

Note: ^ap<0.05; (■): si-con group and (■): si-circ_0134111 group

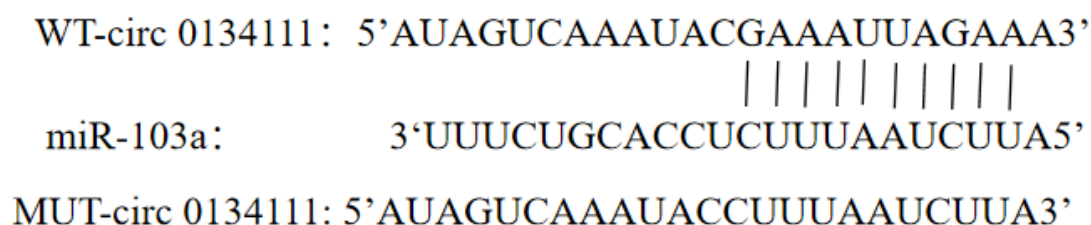
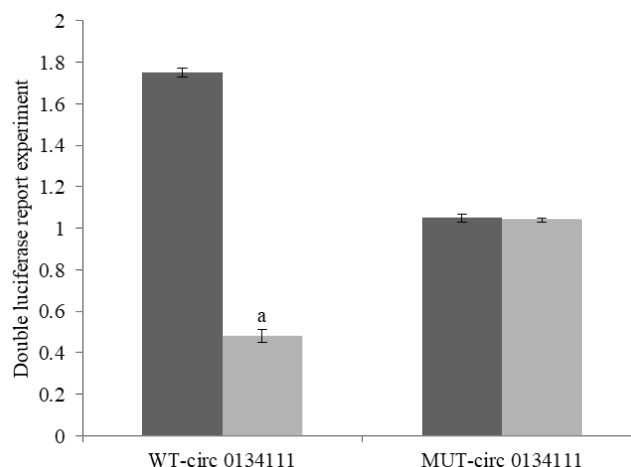


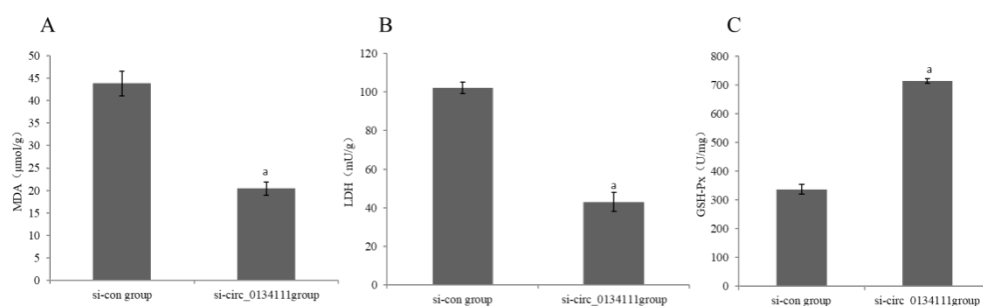
Fig. 3: Predicted results of circular RNA interactome

TABLE 4: DOUBLE LUCIFERASE REPORT EXPERIMENT ($\bar{x} \pm s$, n=7)

Group	WT-circ 0134111	MUT-circ 0134111
mimics	1.75 \pm 0.02	1.05 \pm 0.02
miR-103a	0.48 \pm 0.03	1.04 \pm 0.01
t	93.192	1.1832
p	<0.001	0.259

**Fig. 4: Double luciferase report experiment**Note: ^ap<0.05; (■): mimics and (■): miR-103a**TABLE 5: CONTENTS OF MDA, LDH AND GSH-Px IN OA CHONDROCYTES ($\bar{x} \pm s$, n=7)**

Group	MDA (μ mol/g)	LDH (mU/g)	GSH-Px (U/mg)
si-con	43.81 \pm 2.76	102.03 \pm 2.95	337.13 \pm 16.63
si-circ_0134111	20.43 \pm 1.46	43.06 \pm 4.86	713.29 \pm 7.87
t	19.811	27.442	54.093
p	<0.001	<0.001	<0.001

**Fig. 5: Comparison of MDA, LDH, and GSH-Px contents in three groups of OA chondrocytes**Note: ^ap<0.05**TABLE 6: EXPRESSION OF Bcl-2 AND Bax APOPTOTIC PROTEINS IN OA CHONDROCYTES ($\bar{x} \pm s$, n=7)**

Group	Bcl-2	Bax
si-con	0.23 \pm 0.02	0.57 \pm 0.04
si-circ_0134111	0.64 \pm 0.02	0.24 \pm 0.03
t	38.352	17.462
p	<0.001	<0.001

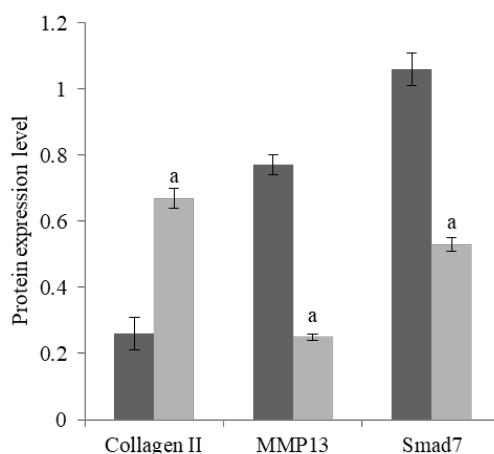


Fig. 6: Comparison of protein expression levels of collagen II, MMP13 and Smad7 between two groups

Note: ^ap<0.05; (■): si-con group and (■): si-circ_0134111 group

TABLE 7: PROTEIN EXPRESSION OF COLLAGEN II, MMP13 AND Smad7

Group	Collagen II	MMP13	Smad7
si-con	0.26±0.05	0.77±0.03	1.06±0.05
si-circ_0134111	0.67±0.03	0.25±0.01	0.53±0.02
t	18.603	43.506	26.039
p	<0.001	<0.001	<0.001

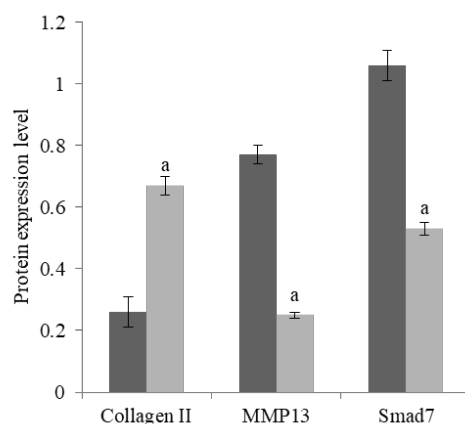


Fig. 7: Comparison of protein expression levels of collagen II, MMP13, and Smad7 between two groups

Note: ^ap<0.05; (■): si-con group and (■): si-circ_0134111 group

OA is the result of the imbalance between the synthesis and decomposition of articular chondrocytes, extracellular matrix and subchondral bone. The destruction of articular cartilage caused by the decrease of chondrocytes is one of the important reasons^[6]. Some studies have found that interleukin-1 inflammatory factors can induce apoptosis of chondrocytes. Chondrocyte degeneration is an important link in the pathogenesis of OA. Abnormal changes in the signaling pathways of TGF- β and Basic Metabolic Panel (BMP) play critical role in the pathogenesis

of OA, which leads to the loss of chondrocyte differentiation phenotype and the appearance of terminal hypertrophic chondrocytes during the development of growth plate^[7,8]. Some studies have found that circRNA can play an important regulatory role in chondrocyte injury in OA and may be used as a potential target for diagnosis and treatment to promote the degradation of extracellular matrix of chondrocytes^[9]. However, the mechanism of some circRNA on chondrocyte injury in OA has not been clarified. si-circ_0134111 is highly expressed in OA chondrocytes, but the

mechanism is not clear. Therefore, this study will explore the expression of circ013411 in OA chondrocytes and its mechanism.

The increased expression of miR-103a is related to the release lesion of bone. The relationship between the increased expression of miR-103a and bone degenerative diseases. The high expression of miR-103a was observed in bone marrow mesenchymal stem cells, osteoblasts, chondrocytes and synovial cells. By promoting osteoblast differentiation and inhibiting differentiation into adipocytes, it affects bone cell function^[10]. Among them, Smad7 can competitively bind to the receptor with R-Smads, prevent the phosphorylation of R-Smads, thus inhibit the signal transduction effect, promote the synthesis of collagen and matrix proteins in chondrocytes, and help to maintain the structure and function of cartilage tissue^[11]. Smad7 is a member of Smads. Smads molecule has two conserved Mad Homology 1 (MH1) domains at the N-terminal of Mad homology domain, which can bind to the CAZAC sequence of DNA^[12]. On the other hand, the C-terminal MH2 domain can interact with transcriptional helper repressors or coactivators to regulate the function of Smad. The short junction region between the two domains contains multiple phosphorylation sites, which is a negative regulatory region of Smad and can be inactivated by ERK phosphorylation. Some studies have found that^[13], Smad7 can reduce the damage of chondrocytes caused by inflammation by inhibiting the expression and activation of inflammatory factors, thus slowing down the progression of arthritis. The results showed that the expression of miR-103a and Smad7 mRNA in OA chondrocytes in si-circ_0134111 group were reduced than si-con group. It is suggested that the low expression of hsa_circ_0134111 can inhibit the degeneration of arthritic chondrocytes and protect articular cartilage through the miR-103a/Smad7 axis.

GSH-Px belongs to antioxidant enzymes, which can scavenge oxygen free radicals. MDA belongs to the product of lipid membrane oxidation. The activity of LDH increases significantly when chondrocytes are injured. The contents of MDA and LDH in OA chondrocytes in si-circ_0134111 group were reduced than si-con group, while the content of GSH-Px was raised in si-circ_0134111 group. It is suggested that the low expression of

hsa_circ_0134111 reduces intracellular oxidative stress and activates the activities of antioxidant enzymes.

Bax belongs to the pro-apoptotic protein family, whereas Bcl-2 belongs to the anti-apoptotic protein family. The increase of its level can activate the mitochondrial pathway and induce cell apoptosis. Collagen II is one of the important causes of extracellular matrix damage. Some studies have shown that oxidative stress injury can activate the expression of apoptotic protein Bax, and the increased expression of Bax can promote the release of CytC from mitochondria and then activate collagen II to induce apoptosis^[14]. MMP13 is one of the important enzymes in the matrix degradation of OA articular cartilage. Zhang *et al.*^[15], found that the expression of MMP13 in OA articular cartilage is more than 40 times that of normal chondrocytes. When cartilage is injured, the expression of MMP13 in chondrocytes increases, which breaks the balance between the production and degradation of Extracellular Matrix (ECM), leads to a large number of ECM degradation, destroys the collagen network and internal environment of chondrocytes, and then leads to the occurrence of OA. The contents of Bcl-2 and collagen II in OA chondrocytes in si-circ_0134111 group increased, while the contents of Bax and MMP13 decreased. It is suggested that the low expression of hsa_circ_0134111 can increase the levels of Bcl-2 and Bax, activate mitochondria, reduce apoptosis, and decrease the content of MMP13, thus inhibit the progression of OA.

To sum up, hsa_circ_0134111 regulates Smad7 through miR-103a to inhibit the degeneration of arthritis chondrocytes and reduce apoptosis. Has_circ_0134111/miR-103a/Smad7 provides a potential target for new OA treatment strategies.

Author's contributions:

Peiyan Huang and Qi Chen contributed equally to this work.

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Conflict of interests:

The authors declare no conflict of interest.

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