CircHYBID Regulates Hyaluronan Metabolism in Chondrocytes by Promoting the Expression of the hsa-miR-29b-3p Target Gene TGF- β 1

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Abstract

Background: Hyaluronan (HA) metabolism by chondrocytes is essential for cartilage development and homeostasis. However, the function of circular RNAs (circRNAs) in HA metabolism is limited. In this study, we profiled the role of the novel HA-related circRNA circHYBID in the progression of osteoarthritis.

Methods: The function of circHYBID in HA metabolism in chondrocytes was investigated using gain-of-function experiments. The mechanism of HA-binding protein involved in hyaluronan depolymerization (HYBID) was confirmed through bioinformatics analysis and luciferase assays. The expression correlation of the circHYBID–hsa-miR-29b-3p–transforming growth factor (TGF)- β 1 axis was examined by qRT-PCR and western blotting. CircHYBID, TGF- β 1, and HA levels in cartilage samples were evaluated using immunohistochemistry. ELISA assay was used to assess HA accumulation in chondrocyte supernatant.

Results: CircHYBID expression was significantly downregulated in damaged cartilage samples comparing with that in the corresponding intact cartilage samples. CircHYBID expression was inversely correlated with the Mankin score and positively correlated with HA expression. Interleukin-1 β stimulation in chondrocytes downregulated circHYBID expression and decreased HA accumulation. Gain-of-function experiments revealed that circHYBID overexpression in chondrocytes increased HA accumulation by regulating HA synthase 2 and HYBID expression. Further mechanism analysis illustrated that circHYBID upregulated TGF- β 1 expression by sponging hsa-miR-29b-3p.

Conclusions: Our results describe a novel HA-related circRNA that could promote the synthesis and accumulation of HA. The circHYBID–hsa-miR-29b-3p–TGF- β 1 axis may play a powerful regulatory role in HA metabolism and Osteoarthritis (OA) progression. Thus, these findings will provide new perspectives for studies on OA pathogenesis, and circHYBID may serve as a potential target for OA therapy.

Background

Osteoarthritis (OA) is the most common degenerative joint disease, and its socioeconomic impact is growing because of population aging and the rising prevalence of obesity (1). OA is also one of the major diseases leading to functional disability in the elderly (2). The chronic inflammatory response associated with OA is mediated by multiple factors, such as age, body weight, metalloproteinases (MMPs), cytokines, signaling pathways, metabolic disorders, and non-coding RNA (3, 4). However, the pathogenesis of OA is unclear. At present, early interventions for clinical OA mainly consist of anti-inflammatory analgesia and cartilage-protecting treatment, but there are no effective drugs to reverse the progression of OA (5).

Degradation of the cartilage extracellular matrix (ECM) and secondary osteogenesis are the most prominent characterizations of OA (6). The cartilage ECM is a highly specialized and dynamic three-dimensional scaffold in which chondrocytes reside in tissues. The ECM is composed of a variety of fibrillar components, such as collagens, fibronectin, and elastin, and non-fibrillar molecules, such as proteoglycans, hyaluronan (HA), and glycoproteins including matricellular proteins (7). Homeostasis of

ECM components including HA is tightly controlled by synthesis and degradation in cartilage tissue, and these processes are altered under pathological conditions. The initial pathological change of articular cartilage in patients with OA is the depletion of the HA-aggrecan(a major cartilage proteoglycan) network structure, which is followed by the degradation of collagen fibrils (8). HA is a non-sulfated glycosaminoglycan composed of disaccharide units containing N-acetylglucosamine and glucuronic acid (9). HA metabolism plays an important role in maintaining tissue homeostasis and regulating cell behavior (10, 11). However, the regulatory mechanism of HA metabolism in chondrocytes remains to be further explored. Elucidation of the mechanism regulating HA metabolism in chondrocytes is expected to provide a new theoretical basis for OA pathogenesis.

Circular RNAs (circRNAs) are endogenous non-coding RNAs that play central regulatory roles by functioning as miRNA sponges or competing endogenous RNAs (ceRNAs) that naturally sequester and competitively inhibit miRNA activity (12). Previous studies illustrated that multiple circRNAs that are differentially expressed in cartilage tissue play central regulatory roles in ECM degradation, inflammatory response, apoptosis, and intracellular signaling pathways (13-15). Another study has revealed that downregulation of circ_001653/miR-486-3p/CEMIP axis promoted proliferation of NPCs and ECM synthesis of nucleus pulposus cells in intervertebral disc degeneration (16). Cell migration-inducing hyaluronan binding protein (CEMIP), also known as HYBID, is closely associated with cellular HA metabolism. However, there is no clear evidence concerning whether circRNA can regulate HA metabolism in cartilage tissues.

Therefore, this study investigated the function of circRNAs in HA metabolism. The findings revealed a small number of up- and down-regulated circRNAs from different regions of cartilage tissue. Of interest, we specifically proposed a novel circRNA, named circHYBID (has_circ_00003893), which is related to HA metabolism in chondrocytes, and systematically explored its role in the pathogenesis of OA. We present the following article in accordance with the MDAR reporting checklist.

Methods

Patients and specimens

All cartilage with primary damage and the corresponding normal cartilage were isolated from patients who underwent total knee replacement. The articular cartilage of femoral heads was separated using a knife blade within 24 h of cartilage isolation. The chondrocytes were separated using a primitive enzyme digestion method for primary culture and subculture, and the remaining cartilage tissue was frozen in liquid nitrogen for subsequent histological examination. Informed consent was provided by all organizational donors. This study was approved by the Human Ethics Committee of Meizhou People's Hospital.

Histological examination

The isolated cartilage was fixed in 4% paraformaldehyde at 4°C for 2 days and then decalcified in 15% EDTA for 2 weeks. Cartilage specimens were dehydrated with ethanol and xylene, embedded in paraffin, and cut into 5-mm sections. The prepared sections were stained with toluidine blue, saffron O, and hematoxylin-eosin according to the routine procedures. Cartilage specimens were graded according to the Mankin score. Scores <4 and >6 indicated that the cartilage samples were intact and damaged, respectively (17).

Immunohistochemistry (IHC)

To evaluate HA and TGF-β1 expression, a semiquantitative scoring index reflecting both staining intensity and the percentage of positive cells percentage was used (18). The staining index (ranging from 0 to 12) was determined as the intensity of HA or TGF-β1 staining (0, negative; 1, weakly positive; 2, positive; 3, strongly positive) multiplied by the proportion of immunopositive cells (0, 0%; 1, <10%; 2, 10–49.9%; 3, 50–74.9%; 4, ≥75). All histological evaluations were performed in a double-blind manner by two expert pathologists (Liao and Zhang).

RNA sequencing and differential expression analysis

After RNA isolation, the quantity of total RNA in both sample types was measured using a Qubit RNA Assay Kit (Life Technologies, Waltham, MA, USA.), and the quality of RNA was assessed using the Agilent 2100 Bioanalyzer and RNA6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). The sequencing library was prepared as per the manufacturer's instructions for the NEBNext Ultra™ RNA Library prep kit for the Illumina platform. Next, we analyzed the functions of differentially expressed genes. OA and normal cartilage were selected using $\log_2$ ratio ≥ 1 and the false discovery rate of multiple hypothesis testing ($p < 0.05$).

Bioinformatics analysis

According to previously reported sequencing data, specific circRNAs were selected to construct the network. In particular, the potential target circRNAs were selected according to the circRNA microarray data, and a co-expression network was constructed according to the standardized signal intensity of circRNAs and mRNAs in the original microarray data. 6 circRNAs were selected to construct a circRNA-miRNA-mRNA network. In further research, the target circRNA, circHYBID, were selected to construct another circRNA-miRNA-mRNA network. The interactions of circRNAs and mRNAs with miRNAs were predicted with the Arraystar miRNA target prediction software based on TargetScan (http://www.targetscan.org/vert_72/) (17).

Primary culture of chondrocytes

Donor chondrocytes were isolated as previously described. Chondrocytes (5×10^5 /well) were obtained at passage 2 and grown in ProNectin F-coated Bioflex six-well culture plates (Flexcell International) to 80%

confluence (17). The chondrocytes were treated with the addition of 10 ng/ml of interleukin (IL)-1 β in the culture medium for 12 h. Untreated chondrocytes were used as controls.

Plasmid construction and transfection

CircHYBID and TGF- β 1 overexpression plasmid were purchased from RiboBio (Guangzhou, China) and transfected into cells to overexpress circHYBID and TGF- β 1. The wild-type (WT) or mutated type (MUT) plasmid of circHYBID and TGF- β 1, miR-29b-3p mimics were also purchased from RiboBio (Guangzhou, China) for subsequent experiments. All plasmids above were verified by direct sequencing. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was utilized for all transfections in accordance with the manufacturer's instructions. The cells with the highest transfection efficiency were obtained after 48 h for subsequent experiments.

Quantitative real-time polymer chain reaction (qRT-PCR)

Total RNA was isolated from cartilage tissues or primary chondrocytes using Trizol reagent. The gDNA was extracted from tissues or cultured cells according to the PureLink™ Genomic DNA Mini Kit protocol (Thermo Fisher Scientific). RNA was reverse-transcribed using HiScript II Q RT SuperMix for Q-PCR (+genomic DNA [gDNA] wiper) (Vazyme, Nanjing, China). AmpliTaq DNA Polymerase (Life Technologies) was used for PCR. The complementary DNA (cDNA) and gDNA PCR products were observed using 2% agarose gel electrophoresis. AceQqPCR SYBR Green Master Mix (Vazyme) was used for QPCR. GAPDH was used as internal controls. All primer sequences are listed in Table S1. The relative expression levels were determined using the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method.

Dual-luciferase assay

The sequence of circHYBID including the potential binding sites of miR-29b-3p was cloned into the dual-luciferase reporter vector pmirGLO (Promega, China). The WT/MUT plasmids of circHYBID or TGF- β 1 were co-transfected into cells with miR-29b-3p mimics respectively, together with the circHYBID overexpression vector or pcDNA3.1 empty vector, and changes in luciferase activity were analyzed using the Dual Glo Luciferase Assay System (Promega) following the manufacturer's instructions. Each experiment was performed at least three times independently.

Enzyme-linked immunosorbent assay (ELISA)

The HA concentration in the supernatant of chondrocytes cells in the presence or absence of interleukin-1 (IL)-1 β were determined using ELISA kits (R&D Systems, MN, USA) according to the manufacturer's instructions.

Western blotting (WB)

The cells were harvested and lysed using RIPA buffer (Beyotime, Shanghai, China). Proteins were quantified using a Bradford kit (Pierce, Rockford, IL, USA) and resolved on a 10% SDS-polyacrylamide

gradient gel. Proteins were then transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). The membranes were incubated in 5% bovine serum albumin for 1 h. To detect protein expression, membranes were incubated with antibodies including anti-HSA2 (Abcam, USA, diluted 1:2000), anti-KIAA1199 (Abcam, diluted 1:2000), and anti-TGF- β (CST, USA, diluted 1:1000) at 4°C overnight. Then, the membranes were incubated with secondary HRP-conjugated antibody (ab6721, Abcam, diluted 1:2000) at room temperature for 1 h. The HRP signal was detected using chemiluminescence reagent (Millipore, CA, USA). Protein expression was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). GAPDH (Abcam, diluted 1:5000) was used as the internal control.

Statistical analysis

Statistically significant differences between groups were calculated using ANOVA or the non-parametric Wilcoxon signed ranks test. The results from two groups were compared using a paired two-tailed Student's *t*-test. The strength of the association between continuous variables was tested with the Spearman correlation. The results are reported as the mean $\pm$ SEM. $p < 0.05$ was considered statistically significant. All experiments were performed and analyzed in triplicate. Data analysis was performed using SPSS 25.0 software (Abbott Laboratories, Chicago, IL, USA).

Results

HA accumulation was significantly decreased in damaged cartilage

Forty-eight pairs of cartilage samples from intact and damages areas were assessed histologically (Figure 1A) and graded using a modified Mankin score (Table S2). The mean score for intact cartilage was 4, compared with 9 for damaged cartilage ($p < 0.0001$, Figure 1B and Table S3). Regarding IHC, positive staining was observed primarily in the nuclei of chondrocytes, although positive staining was occasionally identified in the cytoplasm (Figure 1A). The staining index was used to access the level of HA accumulation. The median staining index of intact cartilage was 6.5, compared with 3.4 for damaged cartilage ($p < 0.0001$, Figure 1C and Table S3). Pearson's analyses performed using pathological and IHC data revealed a negative correlation between the Mankin score and HA expression in OA cartilage tissues (Figure 1D).

CircRNA expression profiles in intact and damaged cartilage

Based on the histologic examination, three pairs of cartilage samples were collected for further analysis (Figure 2A). Among these selected samples, the Mankin score of intact cartilage was ≤ 4 , versus ≥ 9 for damaged cartilage (Table S4). According to the results of qRT-PCR, IL-1 β and IL-6 expression was upregulated in damaged cartilage (Figure 2B).

Sequencing was performed to characterize the expression profiles of circRNAs in cartilage. We identified 450 differentially expressed circRNAs between paired intact and damaged cartilage (Table S5). Differentially expressed circRNAs between damaged and intact cartilages were subjected to hierarchical

clustering analysis (>1-fold difference in expression, $p < 0.05$). Among these differentially expressed circRNAs, 200 were upregulated in damaged tissue samples, whereas 250 were downregulated (Figure 2C). The column chart revealed the frequency distribution of circRNA length in the cartilage samples (Figure 2D). The volcano map was constructed according to fold changes and p-value, illustrating the varied circRNA expression between paired intact and damaged cartilage (Figure 2E).

CircHYBID was predicted to be related to HA metabolism

To further screen candidate circRNAs, we analyzed the host genes of the differentially expressed circRNAs. The result revealed that multiple circRNA-associated host genes were involved in the pathogenesis of OA, such as those involved in ECM degradation, collagen secretion, HA metabolism, and immune inflammatory response (Table S4).

Furthermore, six circRNAs (hsa_circ_0057390 (hsa-circ-COL3A1, chr2:189859772|189861222), hsa_circ_0003893 (circHYBID), hsa_circ_0003922(FBXW2), hsa_circ_0009125 (circHABP4), hsa_circ_0002882(circAXL), and hsa_circ_0006719(circVKORC1)) potentially related to OA pathogenesis were selected to construct a network of circRNAs, mRNAs, and their commonly bound miRNAs (Figure 3A). To validate the circRNA sequencing results, qRT-PCR results confirmed that these six circRNAs were differentially expressed between damage and intact cartilage, and the expression trends were consistent with the sequencing results (Figure 3B). The mRNA analysis revealed that multiple target genes, such as IL6, Bcl2, TGF- β 1, and MMP9, are involved in HA metabolism, inflammatory response, apoptosis, and ECM degradation.

Among these circRNAs, we noticed a special cartilage-associated circRNA (circHYBID, circRNA ID hsa_circ_0003893 in circBase; <http://circbase.org>), and its 398-bp gene was located at chr15:81229014–81230320. The symbol of the associated gene is KIAA1199, which encodes HA-binding protein involved in HA depolymerization (HYBID) through linear expression. Because HYBID was previously identified as an important enzyme involved in HA degradation, we assumed that circHYBID might also play an important role in HA metabolism and OA pathogenesis. Therefore, circHYBID was chosen for further investigation.

To further characterize circHYBID, divergent and convergent primers were designed to amplify the circular and linear transcripts, respectively, in both cDNA and gDNA. The PCR results revealed that the circular form was amplified using the divergent primers only from cDNA, whereas convergent primers amplified both cDNA and gDNA (Figure 3C). Reverse splicing of circHYBID was successfully confirmed using Sanger sequencing (Figure 3D).

CircHYBID expression and HA accumulation were downregulated by IL-1 β

To further validate the role of CircHYBID, primary chondrocytes were successfully isolated (NC group) and stimulated by IL-1 β to construct the in vitro OA chondrocyte model (IL-1 β group). QRT-PCR illustrated that TNF- α expression was downregulated after IL-1 β stimulation, confirming that the OA chondrocyte model

was successfully constructed (Figure 4A). CircHYBID expression of the chondrocytes was downregulated after IL-1 β stimulation, which was consistent with the sequencing results of cartilage tissue (Figure 4B).

The mRNA expression of HA synthase 2 (HAS2) was downregulated and HYBID was upregulated in chondrocytes treated with IL-1 β . However, there is no significant difference of the HAS1 expression under IL-1 β stimulation (Figure 4C). WB data revealed that the protein expression of HAS2 was downregulated and HYBID was upregulated in OA chondrocytes (Figure 4D). HA level in cell culture supernatant was increased after IL-1 β stimulation (Figure 4E).

CircHYBID increases HA accumulation by regulating the expression of HA-metabolizing enzymes

To investigate the relationship between circHYBID and HA metabolism, a circHYBID overexpression vector was constructed and transfected into chondrocytes. CircHYBID upregulation of circHYBID overexpression chondrocytes was verified by qRT-PCR (Figure 5A). CircHYBID-overexpressing chondrocytes (OV-circHYBID+ IL-1 β group) were further incubated with IL-1 β . TNF- α expression was upregulated in standard chondrocytes with IL-1 β stimulation. However, there is no significant TNF- α upregulation under IL-1 β stimulation in circHYBID-overexpressing chondrocytes (Figure 5B). Overexpression of circHYBID shows chondroprotective effect.

As mentioned previously, HAS2 expression was downregulated and HYBID expression was upregulated in IL-1 β group. Comparing to IL-1 β group, HAS2 downregulation was restored, and the upregulation of HYBID was offset in OV-circHYBID+ IL-1 β group (Figure 5C). The protein expression of HAS2 and HYBID was consisted with the mRNA results (Figure 5D). HA content in the cell culture supernatant decreased after IL-1 β stimulation, and HA production was recovered by circHYBID overexpression (Figure 5E). Base on the above results, circHYBID increases HA accumulation of the chondrocytes.

CircHYBID upregulates the expression of the hsa-miR-29b-3p target gene TGF- β 1

Given that many circRNAs commonly function as miRNA sponges that competitively sequester and suppress miRNAs, we assumed that circHYBID may also bind to miRNAs as a sponge and regulate targets via the ceRNA mechanism. Thus, the circRNA-miRNA-mRNA network of circHYBID was constructed to explore the molecular mechanisms of circHYBID (Figure 6). Among the potential downstream targets of circHYBID, hsa-miR-29b-3p has caught our attention because it has been demonstrated to participate in the progression of OA (19). The expression of hsa-miR-29b-3p was detected using qRT-PCR, and the result show that hsa-miR-29b-3p expression was upregulated in chondrocytes treated with IL-1 β . However, the expression of hsa-miR-29b-3p was inhibited by circHYBID overexpression (Figure 7A). Hsa-miR-29b-3p may be a potential binding target miRNA of circHYBID. For further confirmation, the sequence of circHYBID was separately cloned into dual-luciferase reporter vectors (circHYBID-WT), and then the potential binding sites of hsa-miR-29b-3p were mutated (circHYBID-MUT). The aforementioned plasmids were co-transfected into cells, and changes in luciferase activity were analyzed. Luciferase activity was significantly downregulated in cells co-transfected with circHYBID-WT and hsa-miR-29b-3p mimics but obviously restored in cells co-transfected with circHYBID-MUT and

hsa-miR-29b-3p mimics, indicating that the sequence is the potential binding site of circHYBID and hsa-miR-29b-3p (Figure 7B).

From the circRNA-miRNA-mRNA network, we found that TGF- β 1 was a potential downstream target of hsa-miR-29b-3p (Figure 6). TGF- β 1 has been reported to be closely related to HA metabolism and OA pathogenesis (20). Furthermore, a previous study has described the interaction between hsa-miR-29b-3p and TGF- β 1 (21). Thus, hsa-miR-29b-3p was selected as the target mRNA for further research. TGF- β 1 mRNA and protein expression was detected using qRT-PCR (Figure 7C) and WB (Figure 7D), respectively. The result illustrated that IL-1 β inhibits the expression of TGF- β 1 in chondrocytes, whereas circHYBID overexpression restored TGF- β 1 expression. TGF- β 1 expression was further verified in 48 pairs of cartilage samples using IHC. The median staining index of intact cartilage was 7.3, versus 4.1 in damaged cartilage. TGF- β 1 expression was downregulated in damaged cartilage samples (Figure 7E). Meanwhile, pearson's analyses indicated a positive correlation between relative circHYBID and TGF- β 1 expression in OA cartilage tissues (Figure 7F). Dual luciferase reporter assays as previous described was used to confirm the binding sites of hsa-miR-29b-3p and TGF- β 1. Similarly, the luciferase activity of cells cotransfected with TGF- β 1-WT and hsa-miR-29b-3p mimics was significantly downregulated but obviously restored in cells co-transfected with TGF- β 1-MUT and hsa-miR-29b-3p mimics, indicating that the sequence is the potential binding site of TGF- β 1 (Figure 7G). Altogether, these results reveal that circHYBID upregulates the expression of TGF- β 1, which is the potential target gene of hsa-miR-29b-3p. We initially constructed the circHYBID- hsa-miR-29b-3p- TGF- β 1 axis, which play regulatory function in HA metabolism of chondrocytes.

Discussion

OA is a common disabling joint disease. No existing clinical options exist for preventing early OA from progressing to severe disease. To improve this situation, the underlying mechanisms of OA must be further explored to identify strategies to promote the biological regeneration of cartilage tissue in the early stage of OA (22).

HA is a ubiquitous high-molecular-weight polymer of repeated disaccharides of glucuronic acid and N-acetylglucosamine, and it is majorly found in the cartilage ECM between chondrocytes (23). HA constitutes the ECM and stabilizes its integrity. HA participates in varied biological processes, such as cellular and tissue development, migration, and repair after injury or inflammation, by binding to secretory proteins and cell surface receptors (24).

The synthesis and degradation metabolism of HA are maintained by the dynamic regulation of key enzymes such as CD44, HAS, hyaluronidase (HYAL), and HYBID (25, 26). Dysregulated HA metabolism is an important factor in cartilage ECM degeneration and the development of OA. However, little is known about the functions of HA in the development and progression of OA. In the present study, IHC results using cartilage specimens from patients with OA revealed that HA levels were lower in damaged cartilage specimens. The staining index of HA was inversely correlated with the Mankin score, suggested that HA

levels in cartilage gradually decrease with OA progression. Thus, assessing the mechanism of HA metabolism in OA may provide a new perspective for clarifying the pathogenesis of OA.

The role of circRNAs in the pathogenesis of OA has drawn increasing attention (17, 27, 28). Previous studies revealed that circRNAs play regulatory roles in cartilage ECM degradation, inflammatory response in chondrocytes, and apoptosis (12, 29). This study investigated the pathogenesis of OA from the perspective of the regulation of HA metabolism by circRNAs. Among the identified differentially regulated circRNAs, we identified circHYBID as a key downregulated circRNA related to HA metabolism in OA cartilage for the first time.

CircHYBID is generated by back splicing of the KIAA1199 gene, which comprises the protein coding sequence of HYBID mRNA. KIAA1199 has a key role in the binding and depolymerization of HA, and the encoded protein is also named HYBID or cell migration-inducing protein (26). HYBID can catabolize HA via the clathrin-coated pit pathway and play an important role in ECM homeostasis. HYBID is essential for the degradation of HA in skin, arthritic synovial fibroblasts, nucleus pulposus cells and cartilage independently of HYAL1 and HYAL2/CD44. HYBID is highly expressed by chondrocytes in the HA-depleted area of OA cartilage (4). Our data indicated that circHYBID plays a protective role against OA progression.

Accumulating evidence supports the key regulatory role of the ceRNA mechanism in many diseases, including OA. The mechanistic ceRNA network of circHYBID was explored in this study. Our data indicated that TGF- β 1 was a potential target for circHYBID. TGF- β 1 was recently demonstrated to play an important role in HA metabolism. Specifically, TGF- β 1 has a stimulatory effect on equine chondrocytes, enhancing HA synthesis and promoting ECM generation (20). Shen et al reported that CircCDK14 promote Smad2 expression in TGF- β signaling pathway, which protect against OA by maintaining the ECM of chondrocytes and regulating chondrocyte apoptosis and proliferation (30).

Based on our study findings, we propose a mechanism by which circHYBID acts as an hsa-miR-29b-3p sponge to upregulate TGF- β 1 expression. This axis promotes HA anabolism and suppresses HA catabolism, thereby increasing HA levels in cartilage.

Conclusion

we identified the circHYBID–hsa-miR-29b-3p–TGF- β 1 axis as a novel mechanism for HA metabolism in OA. CircHYBID overexpression elevated HA accumulation, suggesting its potential use as a target for preventing and treating OA. Deciphering the precise molecular mechanisms of circHYBID in OA is critical for understanding the pathogenesis of OA and exploring new potential therapeutic targets.

Lists Of Abbreviation

circRNA: circular RNAs

CEMIP: cell migration-inducing hyaluronan binding protein

ECM: extracellular matrix

ELISA: Enzyme-linked immunosorbent assay

HA: Hyaluronan

HAS2: HA synthase 2

HYAL: hyaluronidase

HYBID: HA-binding protein involved in hyaluronan depolymerization

IHC: Immunohistochemistry

MMP: metalloproteinase

MUT: mutated type

OA: osteoarthritis

qRT-PCR: Quantitative real-time polymer chain reaction

TGF: transforming growth factor

WB: Western blotting

WT: wild type

Declarations

Ethics approval and consent to participate

The current experimental proposal was approved by This study was approved by the Human Ethics Committee of Meizhou People's Hospital and conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was provided by all organizational donors.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

1. L. designed and performed the experiments and prepared figures and/or tables. Z. Z. designed and performed the experiments and prepared figures and/or tables. H. C., Y. H. prepare the clinical samples, performed pathological experiments. Z. L. and J. H. performed partial verification experiments and analyzed the data. All authors read and approved the final manuscript.

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Figures

Figure 1

Hyaluronan (HA) accumulation was decreased in damaged cartilage. (A) Pathological examination and HA immunohistochemistry of cartilage from intact and damaged areas. The histological change was assessed using the Mankin score. Scale bar=50 μ m. (B) The Mankin score was significantly higher for damaged cartilage than for intact cartilage (Wilcoxon's test, $p < 0.0001$). (C) The HA staining index was significantly higher for damaged cartilage than for intact cartilage (Wilcoxon's test, $p < 0.0001$). (D) Correlation scatter plot of relative HA expression and the Mankin score (Pearson's correlation, $r = -0.6596$).

Figure 2

Differential expression of circular RNAs(circRNAs) in cartilage. (A)Screening flow of circRNAs. (B) Quantitative real-time PCR was used to assess interleukin (IL)-1 β and IL-6 expression in three selected pairs of intact and damaged cartilage samples. (C) Hierarchical clustering analysis of differentially expressed circRNAs between damaged and intact cartilage (>1-fold difference in expression; $p < 0.05$). (D) Frequency distribution of circRNA length (x-axis, range of circRNA length; y-axis: frequency of circRNAs of

A

Figure 3

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represents circRNA, and their relationship is depicted by one edge. (B) The mRNA expression trends of circHYBID, has-circ-COL3A1, hsa_circ_0003922, hsa_circ_0009125, hsa_circ_0002882 and hsa_circ_0006719 were confirmed by quantitative real-time PCR. (C) PCR analysis of circHYBID and its linear isoform HA-binding protein involved in hyaluronan depolymerization (HYBID) using complementary and genomic DNA. GAPDH was used as a linear RNA control. (D) The back-splice junction of circHYBID was identified by Sanger sequencing. * is used for the comparison between intact and damaged group. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Figure 4

CircHYBID expression and hyaluronan (HA) accumulation were decreased by interleukin (IL)-1 β . The mRNA expression of TNF- α (A), circHYBID (B), HA synthase 1 (HAS1), HA synthase 2 (HAS2), and HA-

binding protein involved in hyaluronan depolymerization (HYBID) (C) were analyzed by quantitative real-time PCR in chondrocytes in the presence or absence of IL-1 β . (D) The protein expression of HAS2 and HYBID was analyzed by Western blotting in chondrocytes in the presence or absence of IL-1 β . (E) HA levels in cell culture supernatant were measured using enzyme-linked immunosorbent assay. β -actin was used as internal control. * is used for the comparison between NC and IL-1 β group. *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$.

Figure 5

CircRNA-miRNA-mRNA network of circHYBID. The oval represents mRNA, the rhombus denotes miRNA, the inverted triangle represents circRNA, and their relationship is depicted by one edge.

Figure 7

CircHYBID upregulates the expression of the hsa-miR-29b-3p target gene transforming growth factor (TGF)-β1. (A) The expression of hsa-miR-29b-3p in chondrocytes with or without overexpressed circHYBID was analyzed using quantitative real-time PCR. (B) Dual-luciferase reporter assays confirmed the

potential binding site of circHYBID and hsa-miR-29b-3p. (C) The relative mRNA expression of TGF- β 1 in chondrocytes with or without overexpressed circHYBID was analyzed using quantitative real-time PCR. (D) The protein expression of TGF- β 1 in chondrocytes with or without overexpressed circHYBID was analyzed using Western blotting. (E) The expression of TGF- β 1 in paired samples of intact and damaged cartilage was analyzed using immunohistochemistry. Scale bar=50 μ m.(F) Correlation scatter plot of relative circHYBID and TGF- β 1 expression (Pearson's correlation, $r=0.5489$). (G) Dual-luciferase reporter assays confirmed the potential binding site of TGF- β 1 and hsa-miR-29b-3p.

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