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Low expression of CircRNA HIPK3 promotes osteoarthritis chondrocyte apoptosis by serving as a sponge of miR-124 to regulate SOX8

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Abstract. – OBJECTIVE: CircRNA, a type of circular RNA, has recently been shown to be a potential target for osteoarthritis (OA). Circular RNA HIPK3 (CircHIPK3) is reported to be abnormally expressed in various disease tissues and affects the occurrence and development of the disease. However, the role and underlying mechanism of CircRNA HIPK3 in osteoarthritis are still unclear. The purpose of this study is to explore the effect of CircRNA HIPK3 on osteoarthritis and analyze its regulatory mechanism.

PATIENTS AND METHODS: We took human OA tissues, normal knee cartilage, human OA chondrocytes and normal chondrocytes as the research objects. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to detect CircHIPK3 expression level, its target gene miRNA 124 (miR-124) and downstream target molecule SOX8. Flow cytometry analysis was applied to discover the apoptosis of CircHIPK3 and miR-124 on OA cartilage in different transfection situations. Moreover, Western blot and RT-qPCR were used to detect the expression of caspase-3 in OA chondrocytes. The binding site of CircRNA HIPK3 and miR-124, miR-124, and SOX8 were verified by using Dual-Luciferase assay.

RESULTS: High expressed CircHIPK3 and low expressed miR-124 were found in OA tissues and OA chondrocytes. In addition, the Dual-Luciferase report showed CircHIPK3 acted as a sponge of miR-124 in OA chondrocytes. CircHIPK3 and miR-124 expression in OA tissue were confirmed to be negatively correlated. To our surprise, knocking down CircHIPK3 and transfected miR-124 mimics both inhibited the apoptosis of OA chondrocytes. Further experiments verified that the downstream target molecule of miR-124 was SOX8 in OA chondrocytes. Besides, miR-124 inhibitors reversed the knockdown of CircHIPK3 while si-SOX8 reversed the miR-124 inhibitors effect of apoptosis on OA chondrocytes.

CONCLUSIONS: Our results demonstrated that CircHIPK3 was significantly upregulated in OA cartilage tissue and cells. Low expression of CircHIPK3 promoted the apoptosis of OA chondrocytes by promoting miR-124 to suppress SOX8 expression. The molecular mechanism of CircHIPK3 in present study is expected to provide new ideas for the treatment of osteoarthritis.

Key Words:
Circular RNA, CircHIPK3, Osteoarthritis, MiR-124, Apoptosis, SOX8.

Introduction

Osteoarthritis is the most common type of degenerative joint disease. The progression of osteoarthritis is age-related and it has a serious impact on old age life[1]. Osteoarthritis is the result of long-term joint actions, such as genetic susceptibility factors, imbalances in immune regulation and local physical factors[2]. The apoptosis of articular chondrocytes is a hallmark event and pathological feature of osteoarthritis. The apoptosis of OA chondrocytes played a dominant role in the destruction and disintegration of cartilage[3]. However, the pathogenesis of osteoarthritis is still not very clear, and its essence cannot be fully elucidated.

Circular RNA is a class of endogenous non-coding RNA molecules commonly found in eukaryotic cells[4-6]. Circular RNA has high stability, species conservation, and cell and tissue specificity[7]; it has been demonstrated to be involved in each process of the occurrence and development of various diseases, such as the proliferation, differentiation, invasion and apoptosis of cancel cells[7-10]. Circular RNAs served as ceRNAs[1,4-6,11] to regulate gene expression at the transcriptional or post-transcriptional level by interacting with microRNAs and other molecules. Li et al[12] revealed that the circRNAs/miRNAs/mRNAs axis played a significant role in regulating the process of OA cells. CircRNA hsa_circ_0005105

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was proved to be a sponge of miR-26a to increase the expression levels of NAMPT[13]. CircSERPINE2 could directly target miR-1271 to regulate the ETS-related gene expression, thus preventing against osteoarthritis[14]. Evidence[15] showed that hsa-circ-0005105 significantly increased the expression of NAMPT by targeting miR-26a.

Circular RNA HIPK3 (CircHIPK3) has recently been reported to be related to the occurrence and development of various diseases[16-18]. CircHIPK3 was highly expressed in various human tissues, especially enriched in liver and brain. Zheng et al[19] showed that silencing CircHIPK3 significantly inhibited human cell proliferation. CircHIPK3 acted as a sponge for various miRNAs in human cells including miR-152, miR-193a, miR-29a, miR-124, miR-338 etc. Since then, numerous studies[16-18] have focused on the effects of CircHIPK3 on liver cancer, cataracts and diabetes. However, the role of CircHIPK3 in OA is still unknown.

The present study aimed to investigate the influence of CircHIPK3 on OA, suggesting that CircHIPK3 significantly suppressed the apoptosis of OA chondrocytes by the miR-124/SOX8 pathway. CircHIPK3miR-124/ SOX8 axis had a great effect on the apoptosis of OA chondrocytes. The research in our study may provide an idea for the treatment of osteoarthritis.

Patients and Methods

Tissue Samples

A total of 36 OA patients and meniscal injury patients admitted to our hospital from April 2018 to January 2019 were selected. The OA diagnostic criteria were based on the American College of Rheumatology. 20 males and 16 females were included. The average age of the patients was 41.5 ± 11.3 years. The average age of meniscus injury patients was 44.8 ± 9.2 years, including 22 males and 14 females. None of the patients suffered from gouty arthritis, rheumatoid arthritis, or secondary osteoarthritis. The cartilage tissue was removed during the procedure. All tissue specimens were divided into the same size and shape and stored in liquid nitrogen. All patients signed the informed consent before surgery. The study was approved by the Medical Device Clinical Trial Ethics Committee of our hospital.

Cell Cultures

Human Primary chondrocytes (Biotechnology, Shanghai, China) and OA chondrocytes were placed in DMEM/F12 cell culture medium (Fuheng Biotechnology, Shanghai, China) containing 10% fetal bovine serum (FBS) and cultured in an incubator at 37°C and 5% CO_2 equilibrium humidity. Logarithmic growth phase chondrocytes were selected for experiment. All transfections were designed by Shanghai Xinfan Biotechnology Company (Shanghai, China).

RNA Extraction and RT-qPCR

TRIzol RNA reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from OA tissues and cells according to the manufacturer's instructions. Reverse transcription kit (TaKaRa, Otsu, Shiga, Japan) was used to synthesize cDNA for circRNA and mRNA. Total RNAs for miRNA was reversed using RiboBio reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The quantification of CircRNA mRNA and miRNA were measured using SYBR Green PCR Kit (TaKaRa, Otsu, Shiga, Japan). All primer sequences were planed and synthesized by Genery (Guangzhou, China). The GAPDH and U6 were used to normalize the levels of mRNA and circRNA expression. The $2^{-\Delta\Delta Ct}$ methods were used to measure the results. All primers are expressed as follows: CircHIPK3 Forward (F) 5'-TATGTTGGTGGATCCTGTTCGGCA-3', Reverse (R) 5'-TGGTGGGTAGACCAAGACTTGTGA-3'; miR-124 F 5'-CGCGCGCGUAAGGCACGCGGUGAA-3', R 5'-ATCCAGTGCAGGGTCCGAGG-3'; SOX8 F 5'-CGAGAGAAGACGCCTGCT-3', R 5'-CGTGTTGGAGAATGAGGG-3'; GAPDH F 5'-ATGTCGTGGAGTCTACTGGC-3', R 5'-TGACCTTGCCCACAGCCTTG-3'; U6 F 5'-CGCTTCGGCAGCACATATAC-3', R 5'-AAATATGGAACGCTTCACGA-3'.

Luciferase Reporter Assay

The wild type (WT) or mutant (MUT) CircHIPK3 were cloned into the pmirGLO plasmid receptor (Realgene, Nanjing, China), and miR-124 mimics or miR-NC were introduced into OA chondrocytes. After co-culture for 48 hours, the Dual-Luciferase activity was measured by Dual-Luciferase receptors system (Promega, Madison, WI, USA). SOX8-WT and SOX8-MUT were introduced into OA chondrocytes. The Dual-Luciferase activity was measured by Dual-Luciferase receptors system after co-culture for 48 hours.

Western Blot Analysis

After 24 h transfection of OA chondrocytes, the total protein was extracted and the solubil-

ity of the protein was measured using the BCA kit (Pierce, Rockford, IL, USA). Under 110 V electrophoresis and 250 mA electroporation to polyvinylidene difluoride (PVDF) membrane, 40 μg of the protein were added to be tested to each well of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Millipore, Billerica, MA, USA). The membrane was washed with TBST for 30 minutes, the secondary antibody was incubated at 37°C for 1 h, and the membrane was washed with TBST for 3 × 30 min. The gray value of protein bands was analyzed with Quantity one software, and β-actin (Abcam, Cambridge, UK) was used as an internal reference to calculate the relative expression.

Cell Apoptosis Assay

PE Annexin V apoptosis detection kits (BD Pharmingen, Franklin Lakes, NJ, USA) were used to analysis the apoptosis of OA chondrocytes according to the manufacturer's instruction. The data was analyzed by CellQuest analysis software (BectonDickinson, Brea, CA, USA). Besides, all experiments were performed in triplicate.

Statistical Analysis

All experimental data post-processing was expressed as mean ± standard deviation. Statistical software SPSS 21.0 (SPSS Inc., Armonk, NY, USA) and GraphPad 5.0 (Graph-Pad Software, Inc., La Jolla, CA, USA) were used to statistically analyze the data. Spearman's correlation analysis was used to evaluate the correlation of circHIPK3, miR-124 and SOX8 expression levels. Kaplan-Meier method and Student's *t*-test were applied to compare the difference between the two groups. Besides, $p<0.05$ was considered statistically significant.

Results

CircHIPK3 Mediated MiR-124 and Negatively Suppressed MiR-124 Expression in OA Tissues and OA Chondrocytes

The expression levels of CircHIPK3 in 36 OA patients and normal knee cartilage were detected *via* RT-qPCR. The results suggested that CircHIPK3 was frequently upregulated in OA tissues as compared to normal knee cartilage (Figure 1A). However, miR-124 was significantly downregulated in OA tissue, compared to normal knee cartilage (Figure 1B). Besides, low expression of CircHIPK3 effectively increased the expression level of miR-124 (Figure 1C). Interestingly, the expression levels of CircHIPK3 and miR-124 showed similar results in OA chondrocytes. Highly expressed CircHIPK3 and lowly expressed miR-124 were found in OA chondrocytes (Figure 1D and 1E). Transfection of si-CircHIPK3 enhanced the CircHIPK3 expression while LV-CircHIPK3 inhibited the CircHIPK3 expression in OA chondrocytes (Figure 1F). The Dual-Luciferase report confirmed the binding site of CircHIPK3 and miR-124 (Figure 1G). When the CircHIPK3-WT group was transfected with miR-124 mimics, the Dual-Luciferase activity was significantly lower than that of the miR-NC group. However, after CircHIPK3-MUT was transfected with miR-124 mimics and miR-NC, there was no significant difference in Dual-Luciferase activity (Figure 1H).

The Decrease of CircHIPK3 Enhanced OA Chondrocyte Apoptosis

To further explore the role of CircHIPK3 in OA, we performed flow cytometry to examine the effect of knocking down CircHIPK3 on OA chondrocyte apoptosis. Firstly, transfection of si-CircHIPK3 significantly decreased the expression of CircHIPK3 in OA chondrocyte (Figure 2A). Flow cytometry result showed that cell apoptosis rate was significantly increased compared to si-NC after transfection with si-CircHIPK3 (Figure 2B and 2C). Western blot and RT-qPCR showed that transfection of si-CircHIPK3 significantly increased the level of caspase-3 protein and mRNA in OA chondrocytes (Figure 2D and 2F).

Overexpression of MiR-124 Induced OA Chondrocyte Apoptosis

Considering that CircHIPK3 can target and regulate the expression of miR-124 in OA chondrocytes in our study, it is not clear whether miR-124 is involved in the process of OA chondrocyte apoptosis. We used transfected miR-124 mimics to promote the expression of miR-124 in OA chondrocytes (Figure 3A). Cell Apoptosis assay was applied to detect the apoptosis rate of OA chondrocytes after transfection with miR-NC and miR-124 mimics, respectively. As a result, miR-124 mimics was proved to induced OA chondrocytes apoptosis, compared to miR-NC group (Figure 3B and 3C). The mRNA and protein levels of apoptosis-related molecule caspase-3 were also further verified. Transfection of miR-124 mimics not only increased the level of caspase-3

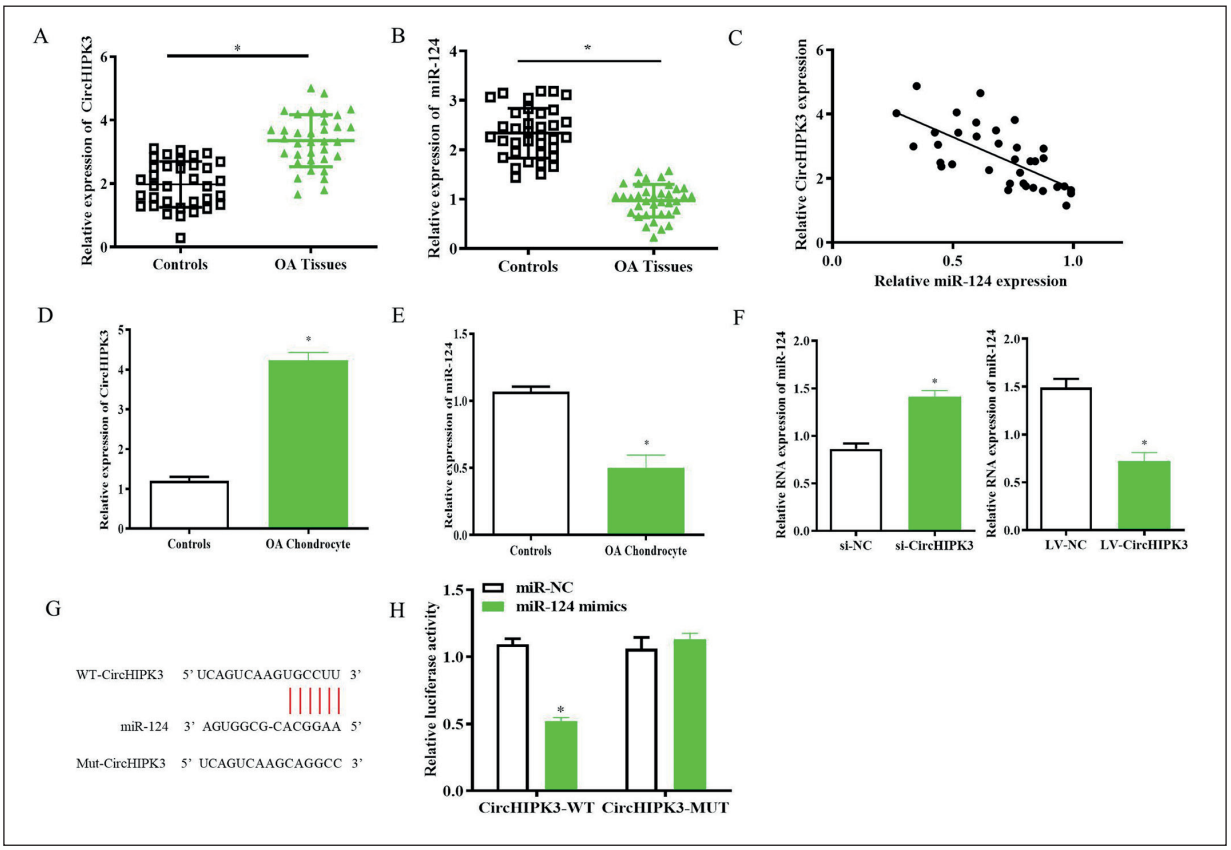

Figure 1. CircHIPK3 mediated miR-124 and negatively suppressed miR-124 expression in OA tissues and OA chondrocytes. **A,** CircHIPK3 relative expression was significantly up-regulated in OA tissues (OA Tissues) compared with normal knee cartilage (Controls). **B,** MiR-124 relative expression was significantly down-regulated in OA Tissues in comparison with Controls. **C,** The expression of the correlation between CircHIPK3 and miR-124 showed negative correlation in OA Tissues. **D-E,** CircHIPK3 and miR-124 expression were detected by RT-qPCR in normal cells and OA chondrocytes. **F,** MiR-124 relative expression was inhibited by transfection of LV-CircHIPK3 while promoted by transfection of si-CircHIPK3 on OA chondrocytes. **G,** The predicted miR-124 binging sites in CircHIPK3 mRNA 3'-UTR were measured by Dual-Luciferase assay. **H,** The Luciferase activity of CircHIPK3-WT after transfection of miR-124 mimics was lower than transfection of miR-NC group. However, there is no significant difference in Luciferase activity after CircHIPK3-MUT transfection with miR-NC or miR-124 mimics. Data are presented as the mean ± standard error of the mean. *p<0.05.

protein expression but also promoted the level of mRNA (Figure 3D and 3F).

MiR-124 Directly Regulated SOX8 in OA Chondrocytes

Xie et al[20] indicated that miR-124 served as a sponge of SOX8 in non-small cell lung cancer; however, we doubt whether miR-124 can also target SOX8 in OA chondrocytes. QRT-PCR results revealed that SOX8 was upregulated in OA tissues compared to normal tissues (Figure 4A). Spearman's correlation analysis showed that miR-124 was negatively correlated with the expression level of SOX8 and CircHIPK3 was positively correlated with the expression level of SOX8 (Figure 4B and 4C). We further studied the relative relationship between SOX8 and miR-124 in OA chondrocytes. The binding sites of miR-124 and SOX8 were shown by the Dual-Luciferase report (Figure 4D). After SOX-WT and SOW-MUT were transfected into OA chondrocytes with miR-124 mimics, the Dual-Luciferase activity of the SOX-WT group was significantly lower than that of the SOX-MUT group (Figure 4E). Moreover, transfection of miR-124 mimics significantly inhibited SOX8 expression in OA chondrocytes (Figure 4F).

CircHIPK3 Promoted Apoptosis of OA Chondrocytes by Acting as a Sponge MiR-124 Via SOX8

To further verify the effect of the CircHIPK3/miR-124/SOX8 signal axis on OA chondrocyte

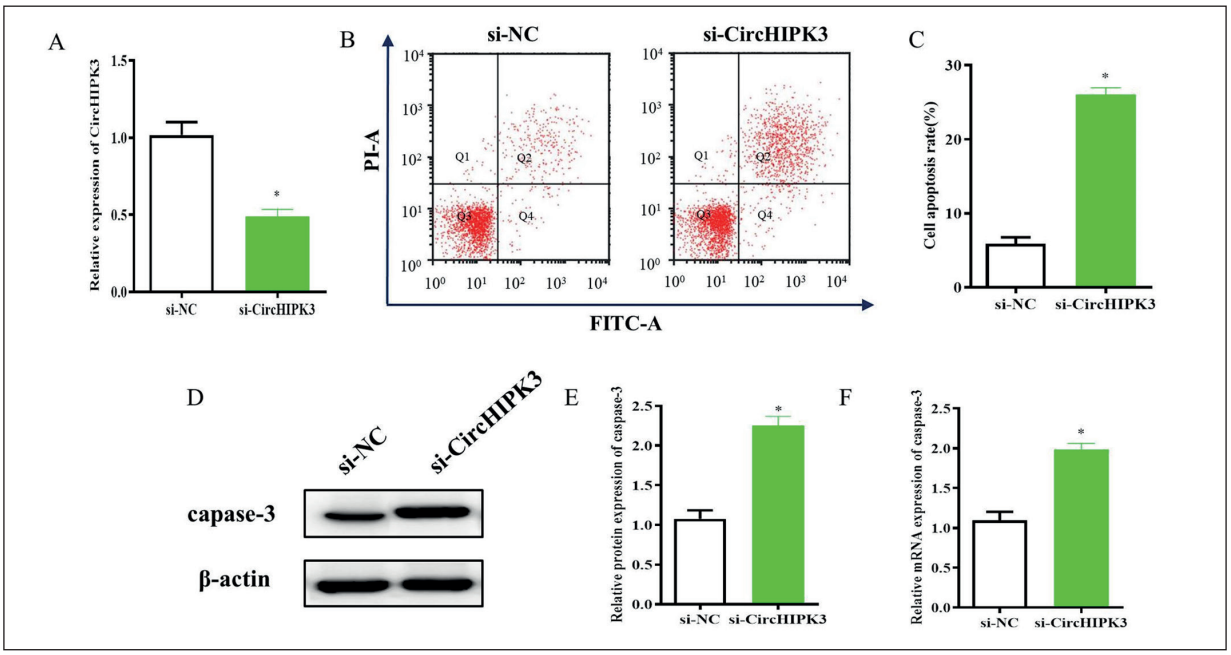

Figure 2. The decrease of CircHIPK3 enhanced OA chondrocyte apoptosis. **A,** Transfection of si-CircHIPK3 inhibited the expression of CircHIPK3 on OA chondrocyte. **B-C,** Cell apoptosis assay showed that knocking down CircHIPK3 significantly promoted apoptosis of OA chondrocyte. **D,** Western blot suggested that silencing CircHIPK3 increased caspase-3 protein expression. **E-F,** The relative expression of caspase-3 mRNA and protein was enhanced by si-CircHIPK3 transfection. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

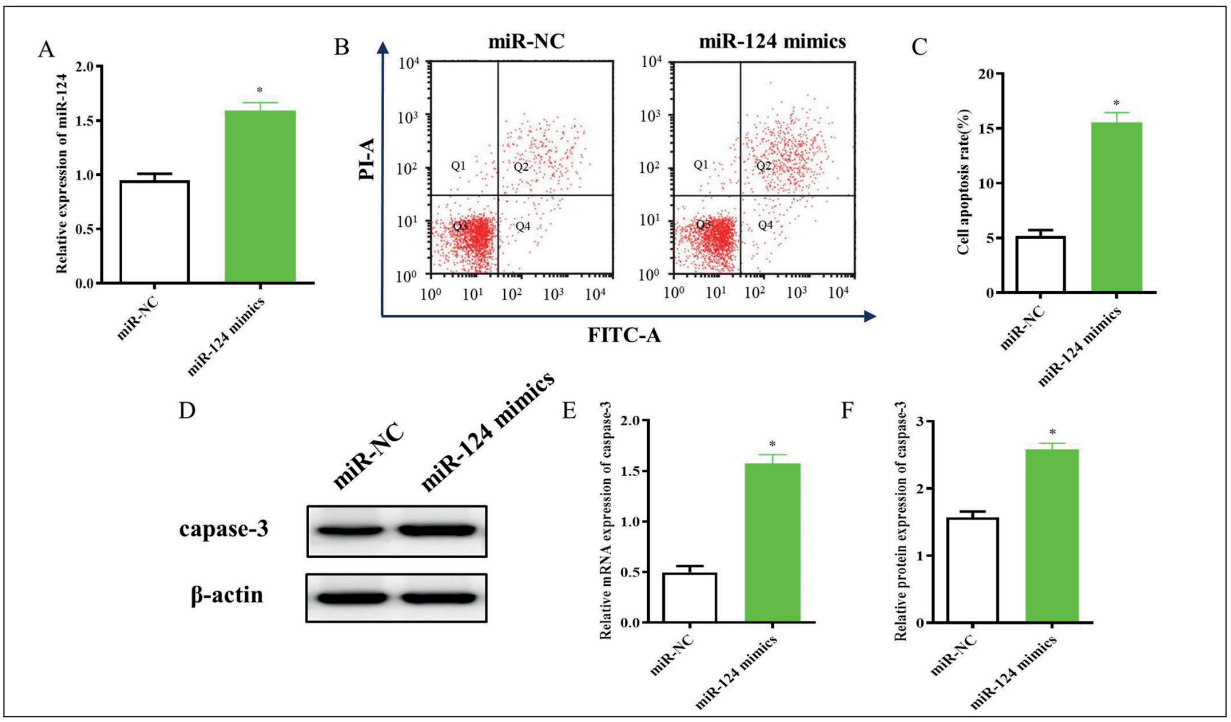

Figure 3. Over expression of miR-124 induced OA chondrocyte apoptosis. **A,** Transfection of miR-124 mimics obviously increased miR-124 expression on OA chondrocyte. **B-C,** Cell apoptosis assay showed that over expression of miR-124 significantly promoted apoptosis of OA chondrocyte. **D,** Western blot indicated that over expression of miR-124 increased caspase-3 protein expression. **E-F,** The relative expression of caspase-3 mRNA and protein was enhanced by miR-124 mimics transfection. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

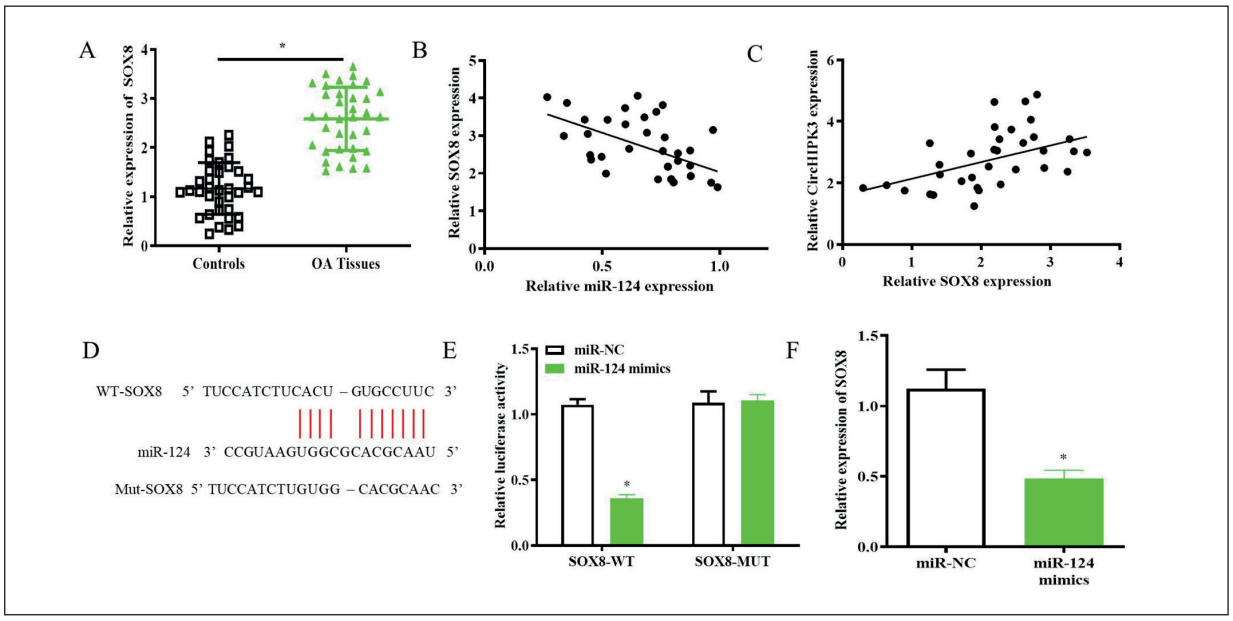

Figure 4. MiR-124 directly regulated SOX8 in OA chondrocytes. **A,** SOX8 expression in OA tissues was higher than normal knee cartilage. **B-C,** MiR-124 and SOX8 relative expression exhibited negative correlation while SOX8 and CircHIPK3 relative expression showed positive correlation in OA tissues. **D,** The predicted miR-124 binging sites in SOX8 mRNA 3'-UTR were measured by Dual-Luciferase report. **E,** The Luciferase activity of SOX8-WT after transfection of miR-124 mimics was lower than transfection of miR-NC group. However, there is no significant difference in Luciferase activity after SOX8-MUT transfection with miR-NC or miR-124 mimics. **F,** Over expression of miR-124 obviously increased SOX8 expression levels. Data are presented as the mean ± standard error of the mean. *p<0.05.

apoptosis, we explored the influence of miR-124 inhibitors on si-CircHIPK3 and LV-CircHIPK3. The results suggested that the expression levels of caspase-3 mRNA and protein levels simultaneous transfected with si-CircHIPM3 and miR-124 inhibitors were lower than transfection of si-CircHIPM3 in OA chondrocytes, so was apoptosis for different group. This means that miR-124 inhibitors reversed the effect of si-CircHIPM3 on OA cell apoptosis (Figure 5A). However, miR-124 inhibitors exhibited completely opposite effects on LV-CircHIPM3 (Figure 5B). The same idea has been applied to explore the effect of si-SOX8 on miR-124 mimics and miR-124 inhibitors. Our results showed that the expression levels of caspase-3 mRNA and protein levels simultaneous transfected with miR-124 inhibitors and si-SOX8 were higher than transfection of miR-124 inhibitors in OA chondrocytes, the apoptosis rate of different transfection groups also showed the same pattern (Figure 5C). However, si-SOX8 exhibited completely opposite effects on miR-124 mimics (Figure 5D). These results indicated that si-SOX8 reversed the effect of miR-124 inhibitors on OA cell apoptosis.

Discussion

Osteoarthritis is a common, frequently-occurring, inflammatory joint disease in orthopedics[21]. The main pathological manifestations are degeneration of articular cartilage, hyperplasia of bones and subchondral bone[22]. OA severely affects human health and lives. It may lead to joint deformation and joint dysfunction even lead to long-term disability of patients[23,24]. However, its pathogenesis has not been fully defined.

Circular RNA is a newly-recognized class of non-coding RNA molecules. This type of molecule was discovered by scientists as early as decades ago. CircRNA could serve as ceRNAs in regulating gene expression[25-27]. CircRNACDR1 could be used as a "molecular sponge". The expression of the target gene could be affected by adsorption. CircRNA was related to the occurrence of various diseases such as Alzheimer's disease, atherosclerotic disease, pathological myocardial hypertrophy and heart failure, osteoarthritis[8,9,13,14,28]. CircSERPINE2 was proved to serve as a sponge to miR-1271, thus alleviating apoptosis and promote anabolism of ECM on

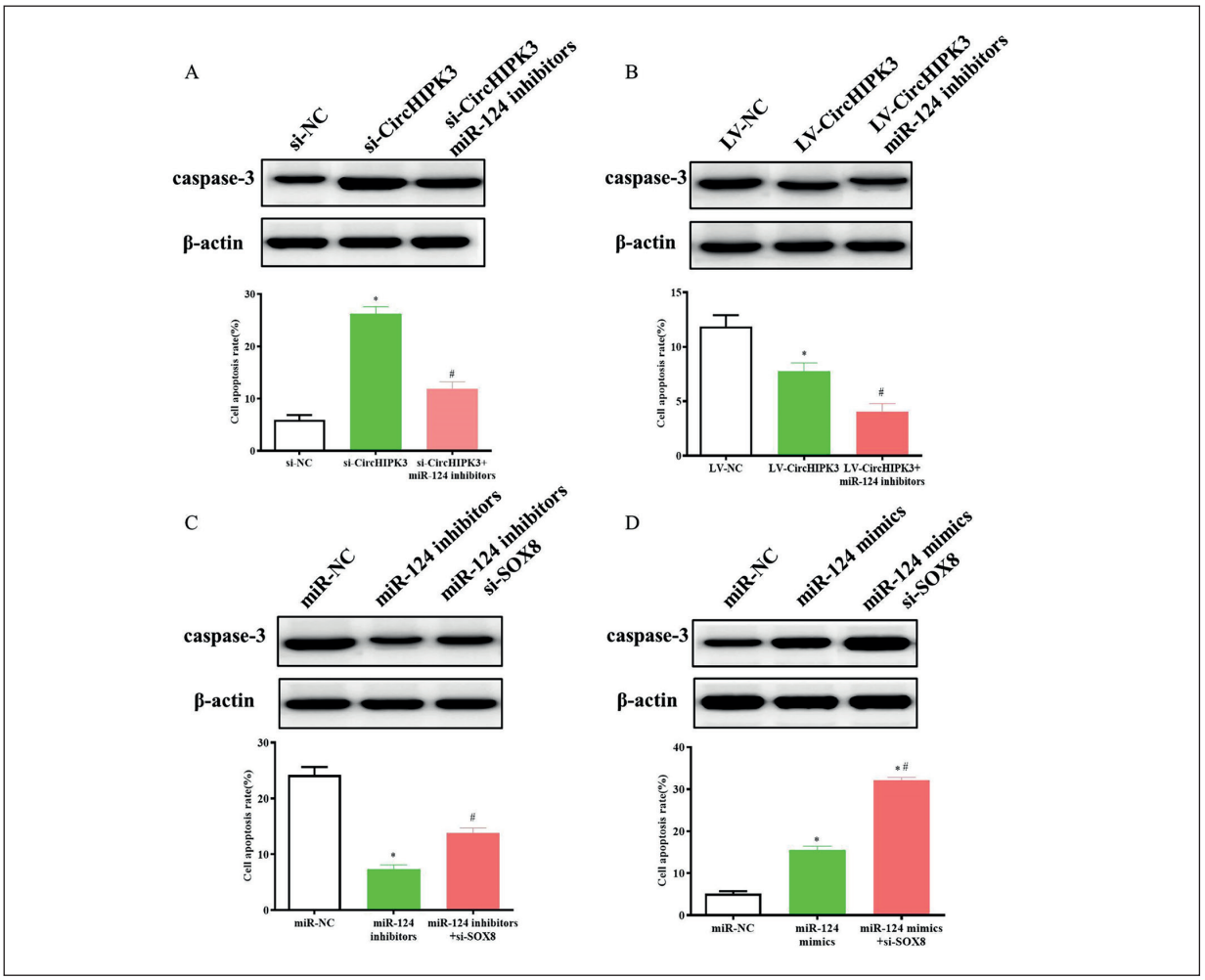

Figure 5. CircHIPK3 promoted apoptosis of OA chondrocytes by acting as a sponge miR-124 *via* SOX8. **A,** Transfection of miR-124 inhibitors significantly reversed the effect of the knockdown of CircHIPK3 on OA apoptosis. **B,** Transfection of miR-124 inhibitors further promoted the inhibitory effect of overexpression of CircHIPK3 on OA apoptosis. **C,** Knocking down SOX8 significantly reversed the low expression of miR-124 on OA chondrocyte apoptosis. **D,** Low expression of SOX8 further enhanced the promotion of overexpression of miR-124 on OA apoptosis. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

OA progression[11]. Hsa_circ_0005105 was demonstrated to accelerate ECM degradation by binding to miR-26a. In our study, we discovered that the expression level of CircHIPK3 was significantly up-regulated in OA tissue. Further exploring the effect of CircHIPK3 on OA chondrocytes, the results showed that low expression of CircHIPK3 significantly promoted the apoptosis of OA chondrocytes. This suggested that CircHIPK3 was involved in the apoptosis process of OA chondrocytes, but the specific mechanism remains to be studied.

MiRNA is a single-stranded non-coding RNA containing 19 to 25 nucleotide molecules. MiRNA is highly conserved in the evolution process. It can directly target the mRNA of the target gene by means of complementary base pairing and directly degrade. MiRNAs were actively involved in the process of the occurrence and development of OA chondrocytes[14,29]. MiR-146a showed strong therapeutic potential for OA treatment because it could promote the proliferation and inhibit apoptosis of OA chondrocytes by targeting TRAF6[29]. MiR-136 has been shown to participate in ECM degradation of OA chondrocytes by regulating the expression level of MMP13[30].

In our study, we found that CircHIPK3 was able to target miR-124. Low expression was found in OA chondrocytes and it significantly promoted the apoptosis of OA chondrocytes.

Statistical results showed that the expression level of CircHIPK3 and miR-124 showed a negative correlation. Low expression of CircHIPK3 significantly increased the expression level of miR-124. Conversely, the overexpression of CircHIPK3 significantly reduced the expression level of miR-124. To further study the mechanism of action of miR-124 in OA chondrocytes, the results showed that miR-124 could target to bind to SOX8. Statistics showed that miR-124 had a negative correlation with the expression level of SOX. Low expression of miR-124 significantly increased the expression level of SOX8 while high expression of miR-124 significantly reduced the expression of SOX8 Level. CircHIPK3 has a positive correlation with the expression level of SOX. Further experiment showed that low expression of miR-124 reversed the effect of si-CircHIPK3 on the apoptosis of OA chondrocytes while low expression of miR-124 enhanced the effect of LV-CircHIPK3 on the apoptosis of OA chondrocytes. Similarly, low expression SOX8 reversed the effect of miR-124 inhibitors on the apoptosis of OA chondrocytes while low expression of SOX8 enhanced the effect of miR-124 mimics on the apoptosis of OA chondrocytes.

Conclusions

Taken together, for the first time, we explored the role of CircHIPK3 in OA and verified its underlying mechanism. As a result, CircHIPK3 was proved to be significantly overexpressed in OA tissues and OA chondrocytes. Moreover, CircHIPK3 inhibited the apoptosis of OA chondrocytes by acting as sponge of miR-124 *via* SOX8. Therefore, our study will provide a reference for further understanding of OA cell apoptosis mechanism and OA treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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