

CircHIPK3 knockdown suppresses E2F3 expression to inhibit the viability and promote the apoptosis of lens epithelial cells in age-related cataract by sponging miR-499a-5p

Type

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Keywords

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Abstract

Introduction

Circular RNA (circRNA) is considered to be a vital regulator of disease progression, including age-related cataract (ARC). However, the molecular mechanism of circHIPK3 in ARC progression has not been fully elucidated.

Material and methods

The expression levels of circHIPK3, miR-499a-5p and E2F transcription factor 3 (E2F3) were measured by quantitative real-time PCR. Cell counting kit 8 assay and flow cytometry were performed to detect cell viability and apoptosis, respectively. Western blot analysis was employed to test the protein levels of apoptosis-related markers and E2F3. Biotin-labeled RNA pull-down assay was used to select miRNAs that could be targeted by circHIPK3, and dual-luciferase reporter assay was performed to confirm the interaction between miR-499a-5p and circHIPK3 or E2F3.

Results

CircHIPK3 is a stable circRNA that is significantly under-expressed in the anterior lens capsule tissues of ARC patients. Knockdown of circHIPK3 suppressed SRA01/04 cell viability and accelerated apoptosis. Moreover, miR-499a-5p could be targeted by circHIPK3, and its inhibitor reversed the effect of circHIPK3 silencing on cell viability and apoptosis. Furthermore, E2F3 was a target of miR-499a-5p, and its overexpression reversed the effect of miR-499a-5p on the viability and apoptosis of SRA01/04 cells. In addition, circHIPK3 positively regulated E2F3 expression by sponging miR-499a-5p.

Conclusions

CircHIPK3 knockdown inhibited viability and enhanced apoptosis of lens epithelial cells to promote ARC progression by regulating miR-499a-5p/E2F3 axis.

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Conclusion: CircHIPK3 knockdown inhibited viability and enhanced apoptosis of lens epithelial cells to promote ARC progression by regulating miR-499a-5p/E2F3 axis.

Keywords: Age-related cataract, apoptosis, circHIPK3, miR-499a-5p, E2F3

Introduction

Age-related cataract (ARC) is the most common type of cataract, which is more common in middle-aged and older people over 50 years of age [1, 2]. The etiology of ARC is more complicated, which may be due to physiological aging, malnutrition and genetic factors [3, 4]. In addition, at the molecular level, abnormal apoptosis of lens epithelial cells (LECs) caused by various factors is considered to be the direct cause of ARC [5, 6]. At present, surgical treatment is still the most basic and effective means of ARC treatment [7]. However, the occurrence of postoperative complications has also seriously affected the quality of life of patients [8]. Therefore, there is still necessary to find the potential mechanisms that affect the occurrence of ARC to provide new evidence for slowing the progression of ARC.

Circular RNAs (circRNAs) are non-coding RNA molecules that do not have 5' end caps and 3' end poly(A) tails and form circular structures by covalent bonds in living organisms [9, 10]. With the deepening of research, circRNA has been found to be an important player in cell biology process and disease development [11, 12]. CircRNA has been shown to be a promising biomarker for ocular diseases [13]. For example, circKMT2E has been reported to be up-regulated in diabetic cataract and may be related to the pathogenesis of DC [14]. CircHIPK3 has been found to be differentially expressed in many diseases and is associated with disease progression in recent years [15, 16]. Some studies have confirmed that circHIPK3 is significantly up-regulated in diabetes mellitus and can promote retinal vascular dysfunction [14]. In ARC, circHIPK3 was demonstrated to be significantly down-regulated and was associated with the proliferation and apoptosis of LECs [17]. Therefore, its role in

ARC development is worth further exploration.

CircRNA contains a large number of microRNA (miRNA) binding sites and can function as “miRNA sponge”, which indirectly regulates the expression of downstream targets of miRNA [18, 19]. Therefore, the circRNA-miRNA-mRNA axis is an important way to elucidate the molecular mechanism of circRNA. The purpose of this study is to explore the role of circHIPK3 in ARC progression and to explore its potential molecular mechanism through the circRNA-miRNA-mRNA axis, thus providing a more reliable theoretical basis for mitigating ARC progression.

Materials and methods

Clinical tissues

ARC patients (9 men/11 women, age range 56-72 years) and donors (12 men/8 women, age range 55-71 years) in this study were recruited from Zaozhuang Municipal Hospital Shandong Province. Inclusion criteria: lens was opaque, visual acuity was ≤ 0.6 , and double lacrimal passage was unobstructed and free of other ocular diseases. Exclusion criteria: other ocular diseases, high myopia, ocular trauma, diabetes, ocular inflammation and women during pregnancy or lactation. 20 eyes of normal anterior lens capsule tissues (Control) were collected within 10 hours of the donor's death, while 20 eyes of anterior lens capsule tissues were obtained during routine surgery in ARC patients. Informed consent was obtained from each patient and donor. All procedures were conducted in accordance with the principles of the Helsinki declaration and were approved by the Ethics Committee of Zaozhuang Municipal Hospital Shandong Province.

Cell culture and transfection

Human LECs (SRA01/04) were obtained from Biovector (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in an incubator with 5% CO₂.

Small interference RNA against circHIPK3 (si-circHIPK3), miR-499a-5p mimic and inhibitor (miR-499a-5p and anti-miR-499a-5p), pcDNA3.1 circHIPK3 and E2F transcription factor 3 (E2F3) overexpression vector (circHIPK3 and E2F3), and negative controls (si-NC, miR-NC, anti-miR-NC and vector) were obtained from General Biosystems (Anhui, China). The above oligonucleotides (50 nM) or vectors (4.0 µg) were transfected into SRA01/04 cells using Lipofectamine 3000 (Invitrogen) when cells reached 70% confluences (did not undergo serum deprivation). After transfection for 48 h, the cells were harvested for function analysis.

Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Invitrogen) was used to isolate total RNA. After reverse-transcribed to cDNA using cDNA Synthesis Kit (Takara, Dalian, China), SYBR Premix Dimer Eraser kit (Takara) was used to perform qRT-PCR in PCR system (Applied Biosystems, Foster, CA, USA). GAPDH and U6 were used as the internal controls. The primers were shown as below:

circHIPK3, F 5'-TTCAACATATCTACAATCTCGGT-3', R
5'-ACCATTACATAGGTCCGT-3'; HIPK3, F 5'-TGGAGACTGGGGGAAGATGA-3', R
5'-CACACTAACTGGCTGAGGGG-3'; E2F3, F 5'-CACTTCCACCACCTCCTGTT-3', R
5'-TGACCGCTTTCTCCTAGCTC-3'; GAPDH, F 5'-GGGAGCCAAAAGGGTCAT-3', R
5'-GAGTCCTTCCACGATACCAA-3'; miR-499a-5p, F
5'-GCCGAGTTAAGACTTGCAGTG-3', R 5'-CAGTGCGTGTCTGGAGT-3'; U6, F

5'-CTCGCTTCGGCAGCACACA-3', R 5'-AACGCTTCACGAATTTGCGT-3'. The $2^{-\Delta\Delta Ct}$ method was used to quantify relative expression.

Actinomycin D (ActD) assay

ActD was obtained from AAT Bioquest (Sunnyvale, CA, USA). In brief, SRA01/04 cells were incubated with ActD for 0, 4, 8, 12 and 24 h, respectively. Then, circHIPK3 and HIPK3 expression levels were detected using qRT-PCR. Linear HIPK3 was used as negative control. For detecting the effect of circHIPK3 on the degradation of miR-499a-5p, SRA01/04 cells were transfected with circHIPK3 overexpression vector or vector and then incubated with ActD for 0, 12 and 24 h, respectively. The expression of miR-499a-5p was measured by qRT-PCR.

Ribonuclease R (RNase R) assay

After extracted RNA using TRIzol reagent, the RNA (2.0 μg) was treated with RNase R (Geneseed, Guangzhou, China) for 15 min. The RNA without RNase R treatment was used as negative control (Mock). After that, circHIPK3 and liner HIPK3 expression was detected by qRT-PCR.

Subcellular fractionation assay

The cytoplasm and nuclear RNA of SRA01/04 cells were extracted using Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek Corp, Ontario, Canada). U6 was used as nuclear control and GAPDH was used as cytoplasm control. QRT-PCR was performed to test the expression levels of circHIPK3, U6 and GAPDH in the cytoplasm and nuclear of SRA01/04 cells.

Cell counting kit 8 (CCK8) assay

After transfection, SRA01/04 cells were harvested and re-inoculated in 96-well plates (1×10^3 cells/well). After 48 h, CCK8 solution (Dojindo, Tokyo, Japan) was added into each well and incubated for 2 h. The absorbance was detected at 450 nm using a microplate reader to evaluate cell viability.

Flow cytometry

SRA01/04 cells were digested with trypsin after transfection, and the cell suspension was collected (1×10^5 cells). Then, Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Kit (Beyotime, Shanghai, China) was used to stain the cell suspension. Cell apoptosis rate was detected using a flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot (WB) analysis

Total proteins were extracted using RIPA Lysis and Extraction Buffer (Thermo Scientific, Waltham, MA, USA). Extracted proteins (20 μ g) were separated on 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Subsequent to blocking in 5% skim milk for 1 h, the membranes were incubated with primary antibodies targeting B-cell lymphoma-2 (Bcl-2; 1:1,000, Boster, Wuhan, China), Bcl-2 associated x (Bax; 1:1,000, Boster), Cleaved-caspase3 (C-caspase3; 1:1,000, Boster), E2F3 (1:2,000, Boster) or GAPDH (1:4,000, Boster) overnight at 4°C, and then interacted with HRP-conjugated secondary antibodies (1:10,000, Boster) for 2 h at room temperature. Finally, protein signals were visualized using Enhanced Chemiluminescent Kit (Boster).

Biotin-labeled RNA pull-down assay

Biotin-labeled circHIPK3 probe and oligonucleotide (oligo) probe were obtained by Invitrogen. The probes were incubated with streptavidin magnetic beads (Thermo Scientific)

to generate probe-coated beads. SRA01/04 cells were lysed and cell lysates were collected. Then, the probe-coated beads were incubated with cell lysates at 4°C overnight, followed by eluted and performed qRT-PCR. The primers of candidate miRNA were synthesized by Invitrogen.

Dual-luciferase reporter assay

The predicted sequences and mutant sequences of miR-499a-5p on circHIPK3 or E2F3 3'UTR were sub-cloned into the psiCHECK-2 reporter vector (Promega, Madison, WI, USA) to generate the wild-type (WT) and mutant-type (MUT) circHIPK3 or E2F3 3'UTR reporter vectors (circHIPK3 WT/MUT or E2F3 3'UTR WT/MUT), respectively. Lipofectamine 3000 was used to transfect with the reporter vectors and miR-NC or miR-499a-5p into SRA01/04 cells. After 48 h, the luciferase activities were analyzed by Dual-Luciferase Assay Kit (Promega).

Statistical analysis

All experiments were repeated 3 times, and each independent experiment was set 3 times to take the average value. All data were presented as mean \pm standard deviation. Student's *t*-test and one-way analysis of variance were used for comparisons, and Pearson correlation analysis was used for analyzing the correlation. Statistical analyses were executed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). *p* value of less than 0.05 was considered to be statistically significant.

Results

CircHIPK3 was downregulated in the anterior lens capsule tissues of ARC patients

By analyzing the genomic locus of circHIPK3 using the circPrimer software

(<http://www.bioinf.com.cn/>), it was found that circHIPK3 was derived from the Exon 2 of HIPK3 gene, and its splicing maturity sequence length was 1099 bp (Fig. 1A). In the anterior lens capsule tissues of ARC patients, circHIPK3 expression was significantly lower than in normal anterior lens capsule tissues ($p = 0.003$, Fig. 1B). To determine the authenticity of the circHIPK3, ActD assay and RNase R assay were performed. As shown in Fig. 1C-D, compared to linear HIPK3, circHIPK3 was more stable and could resist the digestion of RNase R ($p < 0.001$). Using the subcellular fractionation assay, we detected the expression of circHIPK3 in the nuclear and cytoplasm and confirmed that circHIPK3 was mainly distributed in the cytoplasm of SRA01/04 cells ($p < 0.001$, Fig. 1E).

CircHIPK3 silencing inhibited the viability and promoted the apoptosis of SRA01/04 cells

To confirm the function of circHIPK3, we transfected with si-circHIPK3 into SRA01/04 cells. QRT-PCR results showed that si-circHIPK3 could markedly suppress the expression of circHIPK3 in SRA01/04 cells, confirming the transfection was successful ($p < 0.001$, Fig. 2A). CCK8 assay results indicated that circHIPK3 silencing obviously repressed the viability of SRA01/04 cells ($p < 0.001$, Fig. 2B). Besides, the results of flow cytometry assay suggested that the apoptosis rate of SRA01/04 cells could be promoted by circHIPK3 knockdown ($p < 0.001$, Fig. 2C). Additionally, the protein levels of apoptosis markers were determined by WB analysis and the results presented that silenced circHIPK3 significantly reduced the Bcl-2 level and remarkably enhanced the Bax and C-caspase3 protein levels ($p < 0.001$, Fig. 2D). All data revealed that circHIPK3 knockdown could promote the progression of ARC.

CircHIPK3 could sponge miR-499a-5p

In order to find the targeted miRNAs of circHIPK3, we constructed a circHIPK3 probe and proved that it could significantly recruit circHIPK3 ($p < 0.001$, Fig. 3A). Subsequently, we used Starbase and Circbank tools to jointly predict the targeted miRNAs of circHIPK3, and identified 26 targeted miRNAs through Venn Diagram software analysis (Fig. 3B). Biotin-labeled RNA pull-down assay results showed that of the 26 predicted miRNAs, 22 miRNAs could be pulled down by the circHIPK3 probe, and we selected the miR-499a-5p with the highest expression for our study ($p < 0.001$, Fig. 3C). The Starbase tool predicted the targeting binding sites between circHIPK3 and miR-499a-5p were shown in Fig. 3D. Then, dual-luciferase reporter assay indicated that miR-499a-5p mimic significantly decreased the luciferase activity of the circHIPK3 WT vector without affecting that of the circHIPK3 MUT vector ($p < 0.001$, Fig. 3E). Meanwhile, our data also indicated that circHIPK3 silencing could markedly enhance miR-499a-5p expression in SRA01/04 cells ($p < 0.001$, Fig. 3F). The ActD assay results suggested that circHIPK3 could accelerate the degradation of miR-499a-5p ($p < 0.001$, Fig. 3G). In addition, we found that miR-499a-5p expression was markedly increased in the anterior lens capsule tissues of ARC patients ($p < 0.001$, Fig. 3H), and its expression was negatively correlated with circHIPK3 expression ($p < 0.001$, Fig. 3I).

Inhibition of miR-499a-5p partially reversed the effect of circHIPK3 silencing on the viability and apoptosis of SRA01/04 cells

Then, we co-transfected with si-circHIPK3 and anti-miR-499a-5p into SRA01/04 cells to explore whether circHIPK3 regulated ARC progression through sponging miR-499a-5p. The detection results of miR-499a-5p expression revealed that anti-miR-499a-5p could reverse the promotion effect of si-circHIPK3 on miR-499a-5p expression, indicating that the transfection

efficiency of both was good ($p < 0.001$, Fig. 4A). By measuring the viability of cells using CCK8 assay, we discovered that miR-499a-5p inhibitor could partially reverse the suppressive effect of circHIPK3 knockdown on the viability of SRA01/04 cells ($p < 0.001$, Fig. 4B). Moreover, flow cytometry results showed that the increasing effect of circHIPK3 silencing on the apoptosis of SRA01/04 cells also could be inverted by miR-499a-5p inhibitor ($p < 0.001$, Fig. 4C). The results of WB analysis revealed that miR-499a-5p inhibitor also reversed the inhibition effect of circHIPK3 knockdown on Bcl-2 protein level and the promoting effect on Bax and C-caspase3 protein levels in SRA01/04 cells ($p < 0.001$, Fig. 4D). Therefore, all data suggested that circHIPK3 could sponge miR-499a-5p to regulate ARC progression.

E2F3 was a target of miR-499a-5p

For searching the targets of miR-499a-5p, we used the Starbase and Targetscan tools and found that E2F3 3'UTR had binding sites with miR-499a-5p (Fig. 5A). The results of dual-luciferase reporter assay showed that the luciferase activity of E2F3 3'UTR WT vector could be inhibited by miR-499a-5p overexpression compared to miR-NC, while the luciferase activity of E2F3 3'UTR MUT vector had not any changed ($p < 0.001$, Fig. 5B). Besides, we uncovered that miR-499a-5p overexpression could markedly repress the mRNA and protein levels of E2F3 in SRA01/04 cells ($p < 0.001$, Fig. 5C-D). Through detecting the expression of E2F3 expression in ARC tissues, we discovered that E2F3 expression was decreased in the anterior lens capsule tissues of ARC patients compared to normal anterior lens capsule tissues in the mRNA and protein levels ($p < 0.001$, Fig. 5E-F). Also, the results of Pearson correlation analysis revealed that there was a negative relationship between E2F3 expression and

miR-499a-5p expression in **the anterior lens capsule tissues of ARC patients** ($p < 0.001$, Fig. 5G).

E2F3 overexpression partially reversed the effect of miR-499a-5p on the viability and apoptosis of SRA01/04 cells

To verify whether E2F3 participated in the regulation of miR-499a-5p on ARC progression, miR-499a-5p mimic and E2F3 overexpression vector were co-transfected into SRA01/04 cells. Compared with the miR-499a-5p + vector group, the increased E2F3 mRNA and protein expression levels in the miR-499a-5p + E2F3 group confirmed the effectiveness of miR-499a-5p mimic and E2F3 overexpression plasmid transfection ($p < 0.001$, Fig. 6A-B). By detecting the viability and apoptosis of SRA01/04 cells **using CCK8 assay and flow cytometry**, we found that miR-499a-5p overexpression could suppress the viability and increase the apoptosis of SRA01/04 cells, while this effect could be partially reversed by E2F3 overexpression ($p < 0.001$, Fig. 6C-D). In addition, miR-499a-5p also could decrease the protein level of Bcl-2 and improve the protein levels of Bax and C-caspase-3 in SRA01/04 cells. However, the effects of miR-499a-5p mimic on the protein levels of apoptosis markers also could be reversed by E2F3 overexpression ($p < 0.001$, Fig. 6E). Hence, these results confirmed that miR-499a-5p regulated ARC progression by targeting E2F3.

CircHIPK3 regulated E2F3 by sponging miR-499a-5p

Correlation analysis indicated that E2F3 expression was positively correlated with circHIPK3 expression in **the anterior lens capsule tissues of ARC patients** ($p < 0.001$, Fig. 7A). To further confirm the regulation of circHIPK3 on E2F3, we detected E2F3 expression in SRA01/04 cells transfected with si-circHIPK3 and anti-miR-499a-5p. As presented in Fig. 7B-C, we

uncovered that circHIPK3 knockdown could suppress the mRNA and protein levels of E2F3, while this effect could be reversed by miR-499a-5p inhibitor ($p < 0.001$). Our data suggested that circHIPK3 sponged miR-499a-5p to positively regulate E2F3 expression.

Discussion

Lens turbidity and cloudiness are considered to be the characteristic manifestations of ARC [1, 2]. Although it is well known that the abnormal apoptosis of LECs is closely related to the occurrence of ARC, the specific mechanism is still unclear. Increasing evidence suggests that circRNA is a key regulator of cell biological processes, including cell viability and apoptosis [11, 12]. However, there are few related studies on circRNA in ARC. In our study, we discovered that circHIPK3 was lowly expressed in ARC tissues, which was consistent with the previous study [17]. In addition, we found that circHIPK3 knockdown could restrain the viability and enhance the apoptosis of SRA01/04 cells. Hence, we speculated that the low expression of circHIPK3 in ARC might contribute to the development of ARC.

There are many mechanisms of circRNA, among which the most studied is act as competitive endogenous RNA of miRNA [18, 19]. After the prediction of bioinformatics software and preliminary experiments, we determined that miR-499a-5p could be sponged by circHIPK3. MiR-499a-5p has been reported as a differentially expressed miRNA in a variety of diseases and has been shown to be associated with cell proliferation, differentiation and apoptosis [20-22]. Zong *et al.* reported that miR-499a-5p overexpression could inhibit the proliferation and promote the apoptosis of glioma [23], and Yang *et al.* showed that circ_0079593 knockdown could promote miR-499a-5p expression to suppress glioma proliferation and enhance apoptosis [24]. Therefore, miR-499a-5p may be an important

miRNA that regulates cell biology process. In this study, we found that miR-499a-5p was highly expressed in ARC, and its inhibitor could reverse the suppressive effect of circHIPK3 knockdown on the viability and the promotion effect on the apoptosis of SRA01/04 cells. In addition, we also uncovered that miR-499a-5p overexpression could repress the viability and accelerate the apoptosis of SRA01/04 cells. Therefore, we speculated that miR-499a-5p might play a positive role in the progression of ARC, mainly by promoting LECs apoptosis.

E2F3 is a member of the transcription factor E2F family and is involved in the regulation of cell cycle processes [25]. A large number of studies have confirmed that E2F3 is highly expressed in many cancers, mainly promoting cell proliferation and inhibiting cell apoptosis [26]. The results of Xiang *et al.* indicated that E2F3 silencing could result in the apoptosis of SRA01/04 cells [27], and Gong *et al.* showed that E2F3 was underexpressed in ARC and its overexpression could enhance the viability and hinder the apoptosis of SRA01/04 cells [28]. Consistent with the previous studies, we discovered that E2F3 expression was decreased in ARC. Further experiments confirmed that miR-499a-5p contributed to the viability inhibition and apoptosis of SRA01/04 cells by inhibiting E2F3 expression. In addition, we also determined that circHIPK3 could regulate E2F3 by sponging miR-499a-5p. Our data suggested that circHIPK3 and E2F3 had a decrease expression, while miR-499a-5p had an elevated expression in the in the anterior lens capsule tissues of ARC patients compared with that in control. Also, correlation analysis suggested that circHIPK3 expression was positively correlated with E2F3 expression, while miR-499a-3p expression was negatively correlated with circHIPK3 or E2F3 expression. These data further revealed that circHIPK3 sponged miR-499a-5p to positively regulate E2F3. This was a new mechanism to reveal how

circHIPK3 regulated ARC progression.

Similarly, in a recent study, circHIPK3 was shown to promote the proliferation and inhibit the apoptosis of LECs, thereby protecting LECs from dysfunction [29], which was consistent with our findings. However, Cui *et al.* proposed that circHIPK3 regulated the proliferation and apoptosis of LECs mainly through the regulation of the miR-221-3p/PI3K/AKT axis [29], indicating that circHIPK3 could mediate the proliferation and apoptosis of LECs by regulating the multiple pathways. Therefore, further research is necessary.

In general, our study showed that silencing of circHIPK3 inhibited the viability and promoted the apoptosis of LECs by mediating the miR-499a-5p/E2F3 axis, which provided new insights into the pathogenesis of ARC. In addition, our study pointed out that circHIPK3 might be a new target for the treatment of ARC, which will help promote the development of targeted therapy for ARC in clinical.

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Disclosure of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1 The expression and identification of circHIPK3 in ARC. (A) The information of circHIPK3 was shown. (B) The expression of circHIPK3 in ARC tissues (ARC) and normal anterior lens capsule tissues (Control) was detected by qRT-PCR. (C) ActD assay was used to determine the stability of circHIPK3 and linear HIPK3 in SRA01/04 cells. (D) RNase R assay was employed to confirm the circular characteristic of circHIPK3 in SRA01/04 cells. (E) The expression levels of circHIPK3, U6 and GAPDH in the nuclear and cytoplasm of SRA01/04 cells were measured by qRT-PCR.

Fig. 2 Effects of circHIPK3 silencing on the viability and apoptosis of SRA01/04 cells. SRA01/04 cells were transfected with si-NC or si-circHIPK3. (A) The expression of circHIPK3 was detected by qRT-PCR to evaluate transfection efficiency. (B) CCK8 assay was used to measure the viability of cells. (C) The apoptosis of cells was determined using flow cytometry. (D) WB analysis was used to assess the protein levels of Bcl-2, Bax and C-caspase-3.

Fig. 3 CircHIPK3 could sponge miR-499a-5p. (A) The expression of circHIPK3 was measured by qRT-PCR to confirm the effectiveness of the circHIPK3 probe. (B) The Venn Diagram software analyzed the number of targeted miRNAs identified by Starbase and Circbank tools. (C) Biotin-labeled RNA pull-down assay was used to confirm the candidate miRNAs that could be pulled down by the circHIPK3 probe. (D) **According to the prediction of Starbase tool, the binding sites and mutate sites between circHIPK3 and miR-499a-5p were shown.** (E) Dual-luciferase reporter assay was used to verify the interaction between circHIPK3 and miR-499a-5p. (F) The expression of miR-499a-5p was detected by qRT-PCR

in SRA01/04 cells transfected with si-NC or si-circHIPK3. (G) ActD assay was employed to explore the effect of circHIPK3 on the degradation of miR-499a-5p. (H) QRT-PCR was used to determine the expression of miR-499a-5p in ARC tissues (ARC) and normal anterior lens capsule tissues (Control). (I) The correlation between circHIPK3 and miR-499a-5p was analyzed using Pearson correlation analysis.

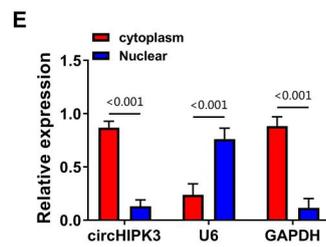
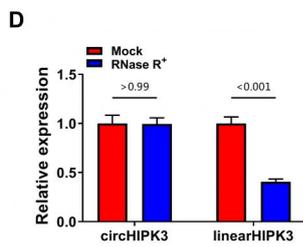
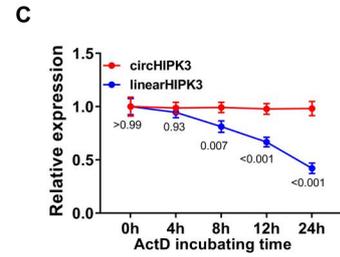
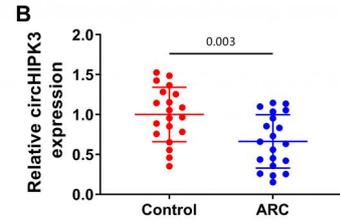
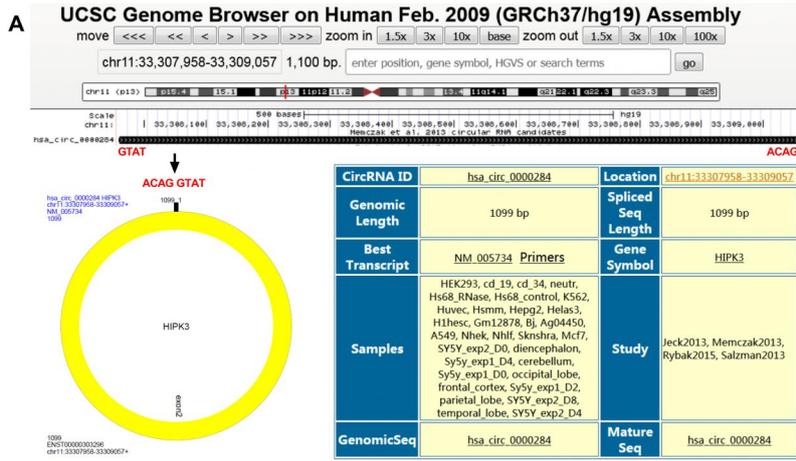
Fig. 4 Effects of circHIPK3 silencing and miR-499a-5p inhibitor on the viability and apoptosis of SRA01/04 cells. SRA01/04 cells were transfected with si-NC, si-circHIPK3, si-circHIPK3 + anti-miR-NC or si-circHIPK3 + anti-miR-499a-5p. (A) QRT-PCR was used to determine the expression of miR-499a-5p to evaluate transfection efficiency. (B) The viability of cells was detected by CCK8 assay. (C) The apoptosis of cells was measured by flow cytometry. (D) The protein levels of Bcl-2, Bax and C-caspase-3 were detected by WB analysis.

Fig. 5 E2F3 was a target of miR-499a-5p. (A) According to the prediction of Starbase tool, the binding sites and mutate sites between miR-499a-5p on E2F3 3'UTR were presented. (B) The interaction between E2F3 and miR-499a-5p was confirmed using dual-luciferase reporter assay. The mRNA and protein levels of E2F3 were measured by qRT-PCR (C) and WB analysis (D) in SRA01/04 cells transfected with miR-NC or miR-499a-5p mimic. QRT-PCR (E) and WB analysis (F) were used to detect the mRNA and protein levels of E2F3 in ARC tissues (ARC) and normal anterior lens capsule tissues (Control). (G) The correlation between E2F3 and miR-499a-5p was assessed by Pearson correlation analysis.

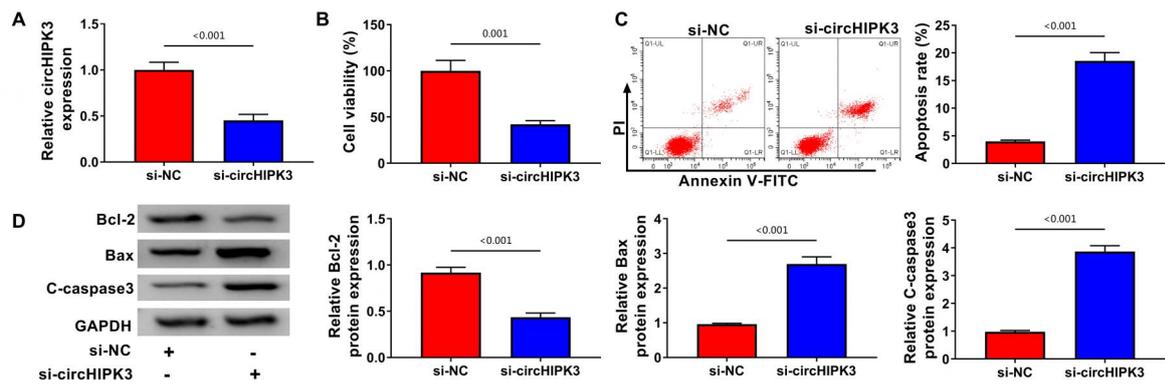
Fig. 6 Effects of miR-499a-5p and E2F3 overexpression on the viability and apoptosis of SRA01/04 cells. SRA01/04 cells were transfected with miR-NC, miR-499a-5p, miR-499a-5p

+ vector or miR-499a-5p + E2F3. The mRNA and protein levels of E2F3 were determined using qRT-PCR (A) and WB analysis (B) to assess transfection efficiency. (C) The viability of cells was measured by CCK8 assay. (D) The apoptosis of cells was determined by flow cytometry. (E) WB analysis was employed to measure the protein levels of Bcl-2, Bax and C-caspase-3.

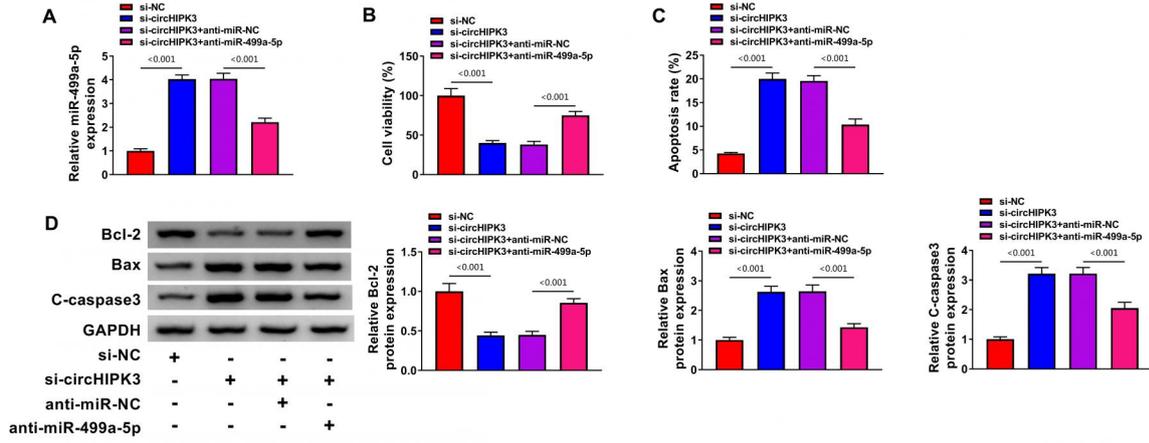
Fig. 7 CircHIPK3 regulated E2F3 by sponging miR-499a-5p. (A) Pearson correlation analysis was performed to evaluate the correlation between E2F3 and circHIPK3. SRA01/04 cells were transfected with si-NC, si-circHIPK3, si-circHIPK3 + anti-miR-NC or si-circHIPK3 + anti-miR-499a-5p. QRT-PCR (B) and WB analysis (C) were used to measure the mRNA and protein levels of E2F3.



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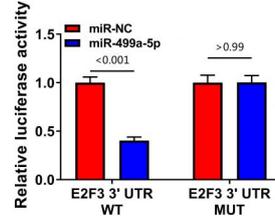
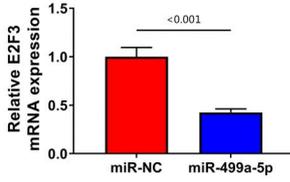
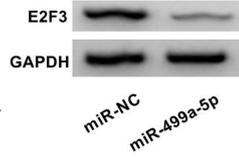
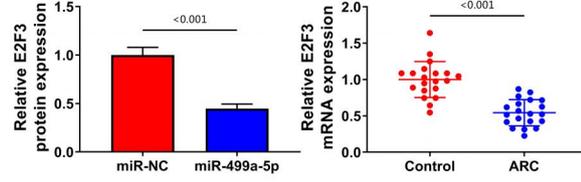
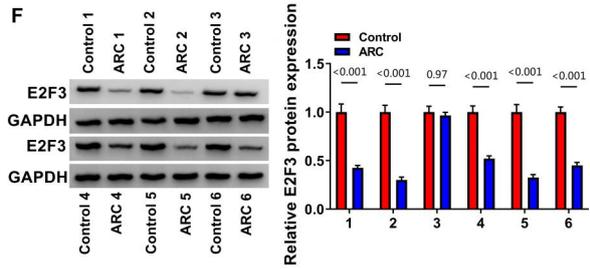
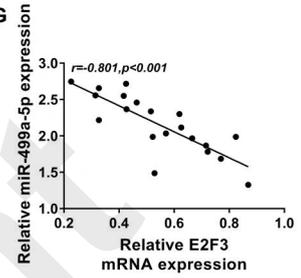


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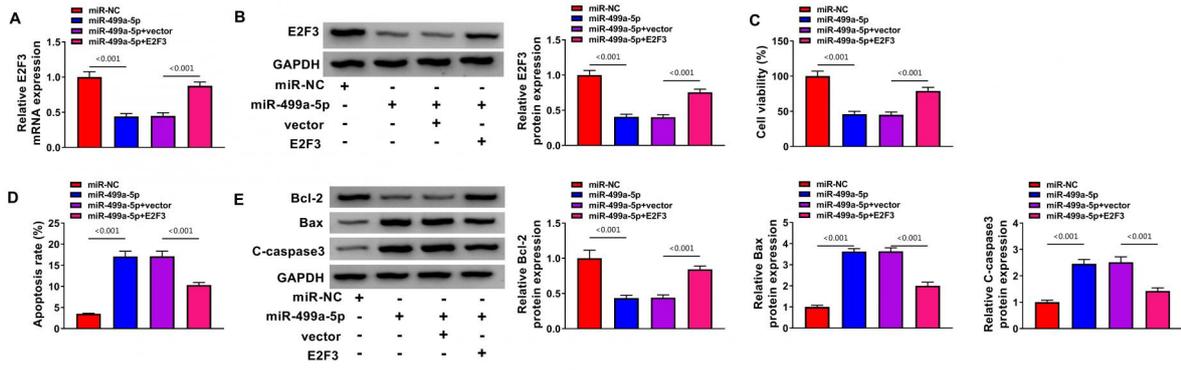
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Binding Site of miR-499a-5p on E2F3 chr6:20492947-20492954[+]

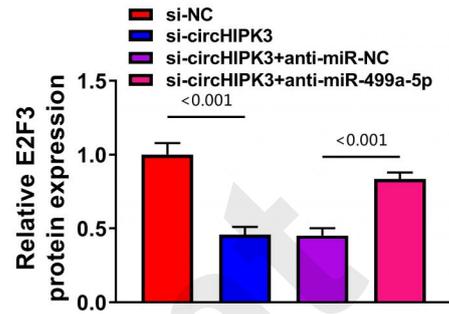
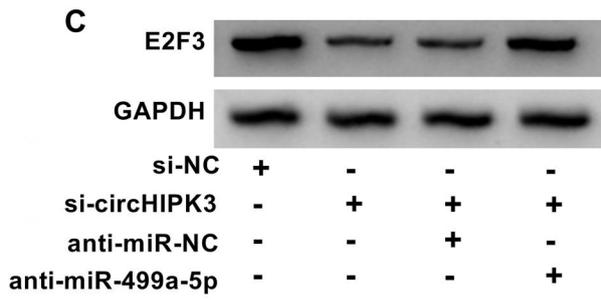
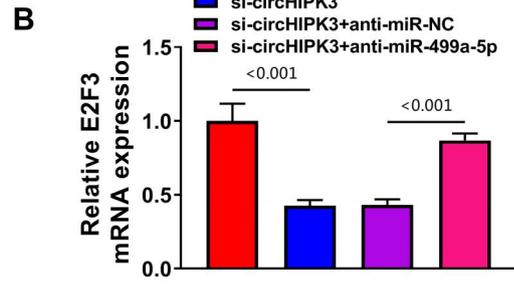
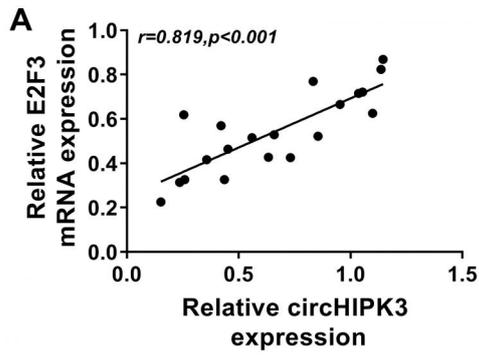
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 E2F3 3' UTR MUT 5' aaggGAUGAGAAUGGACUGCGa 3'

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