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ORIGINAL RESEARCH

RETRACTED ARTICLE: Knockdown of CircCRIM1 Inhibits HDAC4 to Impede Osteosarcoma Proliferation, Migration, and Invasion and Facilitate Autophagy by Targeting miR-432-5p

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Background: Circular RNAs (circRNAs) serve as a genre of considerable modulatory molecules that have been largely researched in human cancers. However, the contribution of circRNA cysteine-rich transmembrane bone morphogenetic protein regulator 1 (circCRIM1) to osteosarcoma (OS) is completely unclear.

Methods: All the RNA levels were examined via quantitative real-time polymerase chain reaction (qRT-PCR). Cellular proliferation and migration/invasion were, respectively, analyzed using 3-(4, 5-dimethylthiazol-2-yl), 5-diphenyl tetrazolium bromide (MTT) assay and transwell assay. The determination of all protein expression was administrated by Western blot. Dual-luciferase reporter assay was used for proving the target combination. The exploration of circCRIM1 in vivo was performed by xenograft assay.

Results: In OS tissues and cells, circCRIM1 was differentially up-regulated. Functionally, cell proliferation, migration and invasion were suppressed while autophagy was promoted after circCRIM1 was down-regulated in OS cells. Mechanistically, mircoRNA-432-5p (miR-432-5p) was a miRNA target of circCRIM1 and the inhibitory effect of circCRIM1 knockdown on OS progression was achieved by targeting miR-432-5p. Moreover, histone deacetylase 4 (HDAC4) was a downstream gene of miR-432-5p and circCRIM1 targeted miR-432-5p to up-regulate HDAC4 level. MiR-432-5p inhibited proliferation, migration, and invasion but enhanced autophagy of OS cells through down-regulating HDAC4. In vivo, knockdown of circCRIM1 decreased OS growth via acting on the miR-432-5p/HDAC4 axis.

Conclusion: Our findings elucidated the oncogenic function of circCRIM1 in OS via the regulation of the miR-432-5p/HDAC4 axis, affording a novel view about how circRNA participated in OS development.

Keywords: circCRIM1, osteosarcoma, miR-432-5p, HDAC4

Introduction

Osteosarcoma (OS) is the most typical primary bone cancer, especially among children.[1] Currently, surgical resection conjoined with systemic chemotherapy has improved the overall survival but it remains poor for the metastatic OS patients.[2] To explore the molecular information of OS biology was considered rewarding for intensively understanding the pathological mechanisms underlying OS.[3] Thus, exploiting the novel molecular pathogenesis is exceedingly crucial towards developing the alternative therapies for OS patients.

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It is well known that circular RNAs (circRNAs) are classified into non-coding RNAs (ncRNAs) with closed-loop as the structural performance. Increasing studies have exhibited that circRNAs worked as essential cancer-regulatory factors as the sponges of microRNAs (miRNAs) to block the functions of miRNAs.[4,5] For example, circ_103809 was promoted proliferation and invasion of lung cancer cells by sponging miR-4302 to elevate ZNF121-mediated MYC expression;[6] circular RNA cTFRC served as a sponge of miR-107 to facilitate the carcinogenesis of bladder cancer.[7] Song et al proclaimed that circ_0001564 contributed to the tumorigenicity of OS as a miR-29c-3p sponge, and their circRNA microarray analysis also displayed the up-regulation of circRNA cysteine-rich transmembrane bone morphogenetic protein regulator 1 (circCRIM1, circ_0053958) in OS samples.[8] However, it has not been addressed regarding the function and the mechanism of circCRIM1 in OS.

MiRNAs, a class of regulatory ncRNAs with the characteristic of binding to 3′untranlsated regions (3′UTRs) of messenger RNAs (mRNAs), also have great implication in the clinical diagnosis and treatment of cancers, such as pancreatic cancer,[9] breast cancer,[10] endometrial cancer,[11] as well as OS.[12] Previous study has manifested that miR-432 was down-regulated in OS and functioned as a tumor repressor.[13] Here, the action of miR-432-5p in OS together with the relation to circCRIM1 were investigated.

As a subunit of class II histone deacetylases, histone deacetylase 4 (HDAC4) can be involved in the biological responses by controlling the expression of important genes in various cellular behaviors.[14] Kang et al purported that HDAC4 accelerated cell progression of gastric cancer via inhibiting p21[15] and Cui et al found the oncogenic role of HDAC4 in glioma.[16] Also, HDAC4 has been testified to enhance OS cell proliferation and invasion,[17] but its association with miR-432-5p and circCRIM1 is unknown.

Except for the function of circCRIM1 and miR-432-5p in OS, the correlation among these three molecules was another key point here. This study was designed to uncover a specific molecular pathogenesis concerning the progression of OS.

Materials and Methods

Tissue Samples and Cell Culture

The collected 35 pairs of OS tissues and peritumoral tissues from OS patients who have received the surgical resection at the Second Hospital of Jilin University were provisionally conserved in liquid nitrogen. Before surgery, we have informed all the patients about the purpose of our research and they signed the written informed consent. In addition, the Ethics Committee of the Second Hospital of Jilin University provided authorization for this study.

Two OS cell lines U2OS and MG63 were used in this research with human osteoblast hFOB1.19 as the normal control. The purchased cells from American Type Culture Collection (ATCC, Manassas, VA, USA) were sub-cultured in a 25mm^3 culture flask (Corning Inc., Corning, NY, USA) in the condition of 37°C, 5% CO_2 and 95% air, after the digestion of 0.25% trypsin (Gibco, Carlsbad, CA, USA). Among three cell lines, hFOB 1.19 and MG63 were cultivated using Dulbecco's modified eagle medium (DMEM; Gibco) while U2OS was maintained in McCoy's 5A medium (Gibco), both containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixed solution (Gibco).

Cell Transfection

Small interfering RNA (siRNA) targeting circCRIM1 (si-circCRIM1)/siRNA negative control (si-NC), miR-432-5p mimic (miR-432-5p)/miRNA mimic NC (miR-NC), miR-432-5p inhibitor (anti-miR-432-5p)/miRNA inhibitor NC (anti-miR-NC) were purchased from RIBIBIO (Guangzhou, China) and transfected into U2OS and MG63 cells through the riboFECT™ CP transfection Kit (RIBIBIO). CircCRIM1 and HDAC4 sequences were, respectively, inserted into the pCE-RB-Mam (RIBIBIO) and pcDNA (Invitrogen, Carlsbad, CA, USA) vectors to construct the overexpression vectors pCE-RB-Mam-circCRIM1 and pcDNA-HDAC4 with pCE-RB-Mam-NC and pcDNA-NC as the respective negative control. Vector transfection was performed employing Lipofectamine3000 (Invitrogen) following the manufacture's instruction book.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Using OS tissues and cells as the experimental specimens, RNA extraction was implemented using TRI Reagent (Sigma, St. Louis, MO, USA). RNA enrichment was measured by LuminoCt® SYBR® Green qPCR ReadyMix™ (Sigma) after the inverse transcription through the First-strand cDNA Synthesis Kit (Sigma) as per the manuals for users. The primers used in our study were listed as below: circCRIM1: Forward (F), 5′-CTGTGTGAGAC AGAGGTGTGC-3′ and Reverse (R),

5′TGCAGCCAGCAATAAGGTTTTCA-3′; miR-432-5p: F, 5′-AACGAGACGACGACAGAC-3′ and R, 5′-CTT GGAGTAGGTCATTGGGT-3′; HDAC4: F, 5′-AGA ATGGCTTTGCTGTGGTC-3′ and R, 5′-ATCT TGCTCACGCTCAACCT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): F, 5′-GGGCTGC TTTTAACTCTGGT-3′ and R, 5′-TGATTTTGGAG GGATCTCGC-3′; U6: F, 5′-GCTTCGGCAGCA CATATACTAAAAT-3′ and R, 5′-CGCTTCACGAATTTG CGTGTCAT-3′. The comparative cycle threshold ($2^{-\Delta\Delta Ct}$) approach was applied in this research to conduct the data analysis. For data normalization, GAPDH acted as the endogenous reference for circCRIM1 and HDAC4, like U6 for miR-432-5p.

Ribonuclease R (RNase R) Treatment

The stability of circCRIM1 was analyzed by the treatment of RNase R. In 37°C water bath, the mixture of 2 μg total RNA and 6 U RNase R (Epicentre Technologies, Madison, WI, USA) was incubated for 60 min. Then, qRT-PCR was exploited for assaying the expression levels of circCRIM1 and GAPDH.

3-(4, 5-Dimethylthiazol-2-Y1)-2, 5-Diphenyl Tetrazolium Bromide (MTT) Assay

The assessment of cell proliferation was administrated using Cell Proliferation Kit I (MTT) (Roche, Basle, Switzerland). In short, transfected cells in the 96-well plates were added with 10 μL/well MTT Labeling Reagent, which can transform the living cells into insoluble formazan. 4 h later, the formazan was solubilized by adding 100 μL Solubilization Solution into each well. Through the detection of a microplate reader (Sigma), cell proliferative ability could be indicated by the absorbance at 490 nm.

Transwell Assay

For the examination of cell migration, the upper chamber of the transwell 24-well chamber (Corning Inc.) was added with 2×10^4 cells resuspended in the serum-free medium, accompanied by the inoculation of 600 μL medium with 10% FBS into the lower chamber. At 24 h post-seeding, cells passed through the membrane were fixed via 4% paraformaldehyde (Sigma) and dyed using crystal violet (Sigma), followed by the quantification of migratory cells under a microscope. For invasion analysis, the upper chamber needed to be enveloped with matrigel (Corning Inc.) before cells were seeded and the other operating procedures were as same as the migration assay.

Western Blot

Proteins were extracted from OS tissues or cells using Tissue Extracts & Cell Lysates (Santa Cruz, San Diego, CA, USA), and 50 μg proteins were applied to carry out the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min. After proteins were transferred onto the UltraCruz Nitrocellulose Pure Transfer Membranes (Santa Cruz), TBST-Blotto A (Santa Cruz) was exploited as a blocking reagent to prevent the binding of nonspecific protein-binding signals. Then the membranes were incubated with primary antibodies in the diluted solution at 4°C overnight, followed by the combination of secondary antibody and primary antibodies at room temperature for 1 h to form the protein complex. The objective protein levels were analyzed by detecting the intensity of the immunoconjugated signals through Western Blotting Luminol Reagent (Santa Cruz) under the ImageLab software version 1 (Bio-Rad Hercules, CA, USA). All the antibodies were bought from Abcam (Cambridge, UK): anti-Beclin-1 (ab62557, 1:1000), anti-light chain 3B (anti-LC3B; ab51520, 1:1000), anti-P62 (ab109012, 1:1000), anti-HDAC4 (ab12172, 1:1000), internal control anti-GAPDH (ab9485, 1:3000) and secondary antibody goat anti-rabbit IgG/HRP (ab205718, 1:5000).

Dual-Luciferase Reporter Assay

After the site analysis, circCRIM1 and 3′UTR of HDAC4 containing the target binding sites for miR-432-5p were considered as wild-types (WTs) while mutant-types (MUTs) referred to those sequences mutated at the miR-432-5p binding sites. Then, the sequences of WTs and MUTs were, respectively, cloned into the pGL3 luciferase vector (Promega, Madison, WI, USA) to generate four new luciferase reporter plasmids circCRIM1-WT, circCRIM1-MUT, HDAC4 3′UTR-WT and HDAC4 3′UTR-MUT. Whereafter, transfection of circCRIM1-WT/circCRIM1-MUT+miR-NC/miR-432-5p or HDAC4 3′UTR-WT/HDAC4 3′UTR-MUT+miR-NC /miR-432-5p was performed in U2OS and MG63 cells for 48 h, and the relative luciferase activity (firefly/renilla) in cell lysate was determined according to the operation manual of the dual-luciferase assay system (Promega).

Xenograft Tumor Assay

To construct a xenograft model, short hairpin RNA (shRNA) against circCRIM1 (sh-circCRIM1) and shRNA NC (sh-NC) vectors (RIBIBIO) were separately transfected into MG63 cells and the transfected cells were subcutaneously injected into the BALB/c nude mice (6 weeks old) from Shanghai Animal Experimental Center (Shanghai, China), with 5 mice in each group. The measurement of tumor size was performed every 4 d, and tumor volume was calculated using the formula: length × width^2 × 0.5. After 20 d, all mice underwent euthanasia were dissected and tumor weight was recorded. Ultimately, the expression levels of circCRIM1, miR-432-5p and HDAC4 were examined by qRT-PCR or Western blot. Ethically, this animal assay was implemented after getting the favor of the Animal Ethics Committee of the Second Hospital of Jilin University, following the Management and Use Guidelines of Laboratory Animals of the National Institutes of Health (NIH).

Statistical Analysis

Statistical data processing was conducted via SPSS 22.0 and GraphPad Prism 7. Those data were expressed as the mean ± standard deviation (SD) after three repetitions. The analysis of linear connection was administrated by Spearman correlation coefficient. Student's *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's test were employed for comparing the difference between two groups or among multiple groups. Generally, $P < 0.05$ indicated a significant difference at the statistical level.

Results

CircCRIM1 Was Abundantly Expressed in OS Tissues and Cells

The differential expression of circCRIM1 was identified by qRT-PCR. In collected 35 OS tissues, the relative expression of circCRIM1 was found to be remarkedly upregulated by contrast to the corresponding 35 normal tissues (Figure 1A). Statistical analyses indicated that circCRIM1 was markedly associated with tumor size (P=0.020) and differentiation grade (P=0.028) (Table 1), suggesting that circCRIM1 was potentially related to OS progression. Also, an increased phenomenon of circCRIM1 level was observed in U2OS and MG63 cells using hFOB1.19 as the control (Figure 1B). After treatment of RNase R, there was almost no change of circCRIM1 expression while GAPDH level was conspicuously declined compared to the Mock group, exhibiting the higher stability of circCRIM1 than linear RNA (Figure 1C and D). CircCRIM1 might participate in the development of OS working as a crucial role.

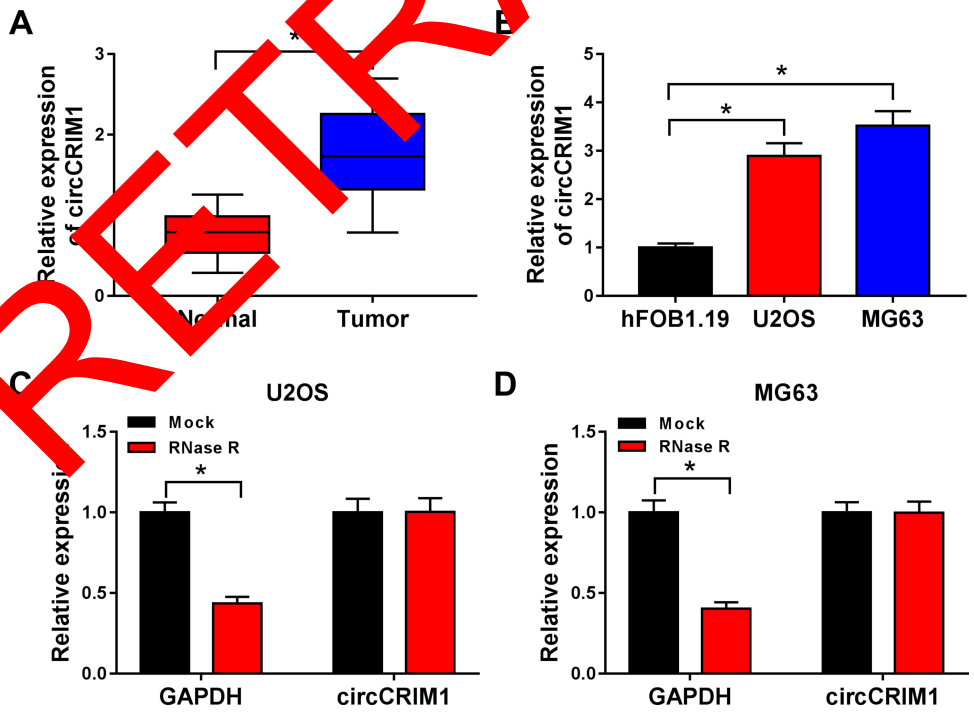

Figure 1 CircCRIM1 was abundantly expressed in OS tissues and cells. (**A** and **B**) The expression pattern of circCRIM1 in OS tissues (**A**) and cells (**B**) was analyzed via qRT-PCR. (**C** and **D**) The stability analysis of circCRIM1 was conducted using RNase R treatment and qRT-PCR. *P < 0.05.

Table 1 Association Between Clinical Features and circCRIM1 Expression of OS Patients (n=35)

Parameter	Case	circCRIM1 Expression[a]		*P* value
		High (n=18)	Low (n=17)	
Gender	20	12	8	0.241
Male	15	6	9	0.903
Female				
Age (years)				
≤40	23	12	11	
>40	12	6	6	
Tumor size				0.020*
≤6cm	22	8	14	
>6 cm	13	10	3	
WHO grade				0.601
I–II	16	9	7	
III	19	9	10	
Pulmonary metastasis				0.053
Yes	14	10	4	
No	21	8	13	
Differentiation grade				0.028 *
Well/moderately	18	6	12	
Poorly/ undifferentiated	17	12	5	

Notes: *$P<0.05$; [a]Using median expression level of circCRIM1 as cut-off.

CircCRIM1 Down-Regulation Restrained Cellular Proliferation, Migration and Invasion While Enhanced Autophagy of OS Cells

In view of the up-regulation of circCRIM1 in OS as above, the loss-function method was employed to research the potential role of circCRIM1 in OS by siRNA transfection. The qRT-PCR showed the successful interference of si-circCRIM1 on the level of circCRIM1 in U2OS and MG63 cells, compared with si-NC group (Figure 2A and B). After the administration of MTT and transwell assays, the knockdown of circCRIM1 was displayed to impede cell proliferation (Figure 2C and D), migration (Figure 2E) and invasion (Figure 2F), making a contrast with si-NC transfection. Beclin-1 and LC3B-II/I are the common indexes of autophagy occurrence, and P62 degradation in the autophagic process indicates that P62 serves as an autophagy inhibitor.[18] As the results of Western blot in Figure 2G and H, Beclin-1 and LC3B-II/I levels were evidently higher while P62 was decreased in U2OS and MG63 cells transfected with si-circCRIM1 contraposed to si-NC group, hinting that circCRIM1 knockdown caused the promotion of autophagy. Collectively, circCRIM1 down-regulation had inhibitory effects on OS cell proliferation, migration and invasion but a promoted effect on autophagy.

CircCRIM1 Targeted miR-432-5p in OS Cells

In Starbase3.0, it was obvious that circCRIM1 and miR-432-5p had the complemental nucleotide binding sites (Figure 3A). And visibly, the introduction of miR-432-5p repressed the luciferase activity in circCRIM1-WT reporter instead of circCRIM1-MUT, implicating the target combination of circCRIM1 and miR-432-5p (Figure 3B and C). In relation to the expression of miR-432-5p in OS, we performed qRT-PCR to analyze it and found the down-regulation of miR-432-5p not only in OS tissues (Figure 3D) but also in U2OS and MG63 cells (Figure 3E) compared to normal tissues and hFOB1.19 cells. After the analysis of Spearman correlation coefficient between the levels of circCRIM1 and miR-432-5p in OS tissues, a negative relationship ($R=-0.631$, $P<0.0001$) was discovered (Figure 3F). When circCRIM1 was overexpressed, the miR-432-5p level presented a striking decrease in U2OS and MG63 cells (Figure 3G). Conversely, circCRIM1 knockdown induced the up-regulation of miR-432-5p (Figure 3H). These data explained that circCRIM1 could target miR-432-5p and negatively regulated its expression in OS cells.

Knockdown of CircCRIM1 Retarded the Progression of OS via Targeting miR-432-5p

To further investigate the association between circCRIM1 and miR-432-5p in OS cellular behaviors, the anti-miR-432-5p was designed to rescue si-circCRIM1 in cell transfection. The addition of miR-432-5p inhibitor obviously assuaged the si-circCRIM1-induced miR-432-5p promotion, insinuating that the inhibitory efficiency of anti-miR-432-5p was relatively great (Figure 4A and B). Subsequent experiments proved that anti-miR-432-5p transfection could partly return the suppression of si-circCRIM1 on OS cell proliferation (Figure 4C and D), migration (Figure 4E) and invasion (Figure 4F). Simultaneously, circCRIM1 inhibition

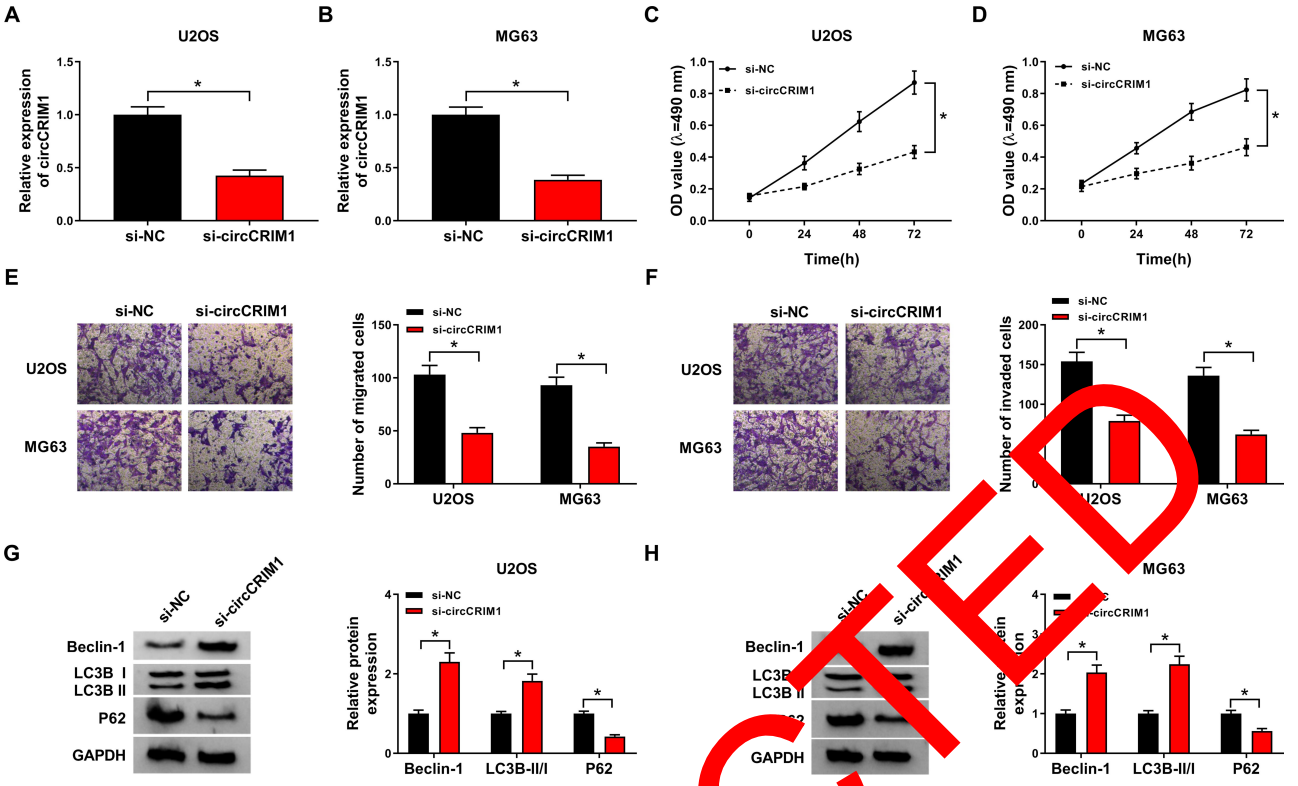

Figure 2 CircCRIM1 down-regulation restrained cellular proliferation, migration and invasion while enhanced autophagy of OS cells. In U2OS and MG63 cells, separate si-NC and si-circCRIM1 transfection was carried out. (**A** and **B**) The determination of circCRIM1 was performed by qRT-PCR. (**C** and **D**) MTT was exploited for the evaluation of cell proliferation. (**E** and **F**) Cellular migration and invasion were measured through the transwell assays. (**G** and **H**) The autophagy-related proteins were assayed via Western blot in the above transfected cells. *P < 0.05.

up-regulated miR-432-5p to trigger the enhancement of autophagy in U2OS and MG63 cells (Figure 4G and H). Altogether, the retardment of circCRIM1 knockdown on OS progression was achieved by targeting miR-432-5p.

CircCRIM1 Up-Regulated HDAC4 by Sequestering miR-432-5p in OS Cells

According to the bioinformatics analysis by Starbase3.0, the binding sites of miR-432-5p were noticed in the sequence of HDAC4 3′UTR (Figure 5A). Their combination was verified using the dual-luciferase reporter assay. As the illustration of Figure 5B and C, the relative luciferase activity of HDAC4 3′UTR-WT plasmid was notably lower after transfection of miR-432-5p in contrast to miR-NC group, while this decrease did not appear in HDAC4 3′UTR-MUT plasmid. By detecting the mRNA (Figure 5D) and protein (Figure 5E) levels, we found that HDAC4 was highly expressed in OS tissues relative to the normal tissues. Likewise, the overexpression of HDAC4 was identified in U2OS and MG63 cells by comparison with hFOB1.19 cells (Figure 5F and G). And miR-432-5p was negatively (R=−0.589, P<0.0001) associated with HDAC4 in OS tissues (Figure 5H) while the relation between circCRIM1 and HDAC4 was positive (R=0.589, P<0.0001) (Figure 5I). Interestingly, miR-432-5p mimic led to the suppressive effects on the mRNA and protein expression of HDAC4 while these effects were abolished after circCRIM1 overexpression (Figure 5J and K), showing that circCRIM1 could up-regulate HDAC4 through sponging miR-432-5p.

MiR-432-5p Targeted HDAC4 to Suppress Proliferation, Migration, Invasion and Expedite Autophagy of OS Cells

About the functional mechanism underlying miR-432-5p, the rescued assays were conducted by dividing into four transfection groups: miR-NC, miR-432-5p, miR-432-5p +pcDNA-NC, miR-432-5p+pcDNA-HDAC4. As Figure 6A and B depicted, the introduction of pcDNA-HDAC4 recovered the HDAC4 mRNA and protein expression reduction caused by miR-432-5p, demonstrating that HDAC4 was

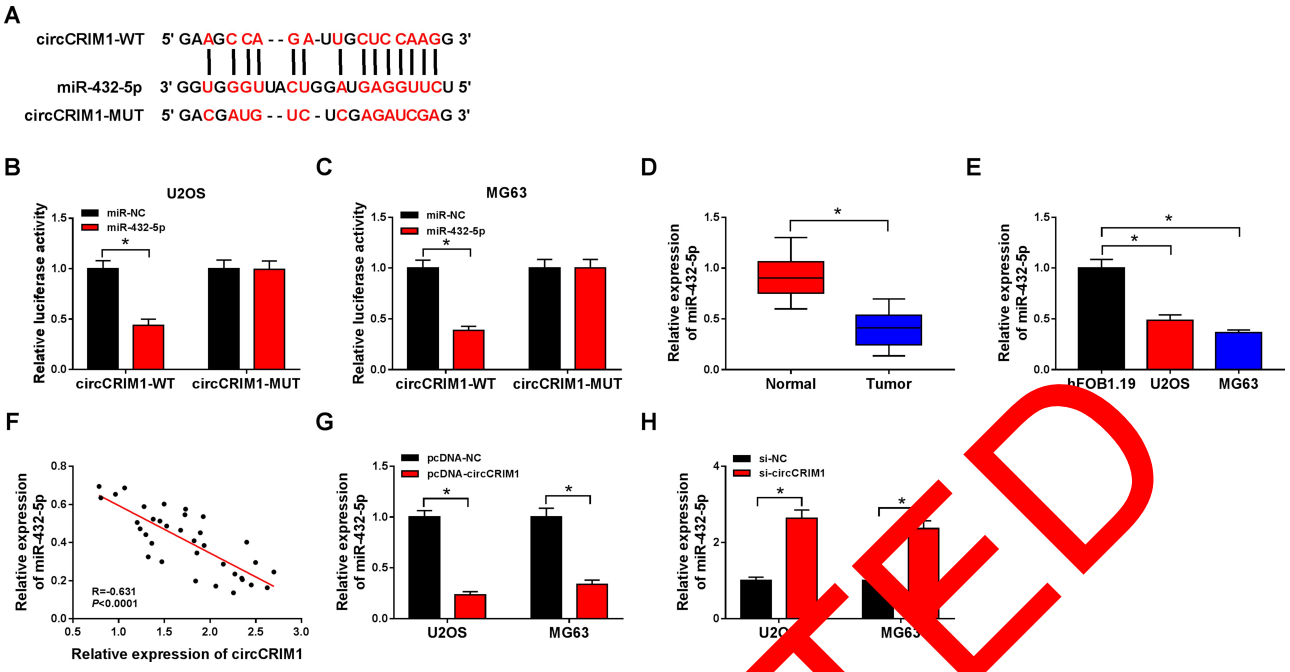

Figure 3 CircCRIM1 targeted miR-432-5p in OS cells. (**A**) The combinative region of circCRIM1 with miR-432-5p was analyzed using the online Starbase3.0. (**B** and **C**) The dual-luciferase reporter assay was implemented to affirm the actual combination between circCRIM1 and miR-432-5p in OS cells. (**D** and **E**) The qRT-PCR was used for the detection of miR-432-5p expression in OS tissues and cells. (**F**) The linear analysis between circCRIM1 and miR-432-5p in OS tissue samples was performed by Spearman correlation coefficient. (**G** and **H**) The influence of circCRIM1 overexpression (**G**) or down-regulation (**H**) on miR-432-5p level was assessed via qRT-PCR. *P < 0.05.

successfully overexpressed via the pcDNA-HDAC4 transfection. MTT and transwell assays indicated that cell proliferation (Figure 6C and D), migration (Figure 6E) and invasion (Figure 6F) were all repressed by miR-432-5p whereas overexpression of HDAC4 directly mitigated this inhibition on those cellular processes. Similarly, miR-432-5p mimic heightened the level of Beclin-1 and LC3B-II/I along with the decline of P62, but its stimulative effect on autophagy was lightened as a result of HDAC4 up-regulation (Figure 6G and H). These data together manifested that miR-432-5p was a tumor inhibitor in OS via targeting HDAC4.

Knockdown of CircCRIM1 Reduced OS Growth in vivo via the Regulation of miR-432-5p/HDAC4 Axis

After the establishment of xenograft model, we observed that the tumor volume (Figure 7A) and weight (Figure 7B) of sh-circCRIM1 group were considerably lower than that of sh-NC group. Following the analysis of qRT-PCR and Western blot, the sh-circCRIM1 group exhibited the decreased circCRIM1 level (Figure 7C), the promotive miR-432-5p expression (Figure 7D) and the downregulated HDAC4 protein expression (Figure 7E) by comparison to sh-NC group. All in all, knockdown of circCRIM1 up-regulated miR-432-5p to inhibit HDAC4 expression, hence causing the suppression of OS growth in vivo.

Discussion

NcRNAs have been found to act as oncogenes or tumor inhibitors in the initiation and development of OS.[19] In the present research, we announced that knockdown of circCRIM1 hindered the OS deterioration via inhibiting miR-432-5p-mediated HDAC4 expression. Innovatively, we provided a pathway manner about how circCRIM1 regulated OS evolution.

In OS, the involvement of several circRNAs has been recognized according to the issued documents. For instance, circ_0000285 heightened the TGFB2 level to increase cell proliferation and migration of OS cells through sponging miR-599.[20] Circ_0001658 boosted cellular proliferative and metastatic abilities in OS via the modulation of miR-382-5p/YB-1 axis.[21] And circMMP9 was validated to facilitate the oncogenesis of OS by restricting miR-1265 expression to up-regulate CHI3L1.[22] In this chapter, we not only discovered the abnormal overexpression of circCRIM1 in OS tissues and cells but also ascertained that circCRIM1 worked as a tumor driver of OS for the first time. In vitro experiments, knockdown of circCRIM1 restrained OS cell

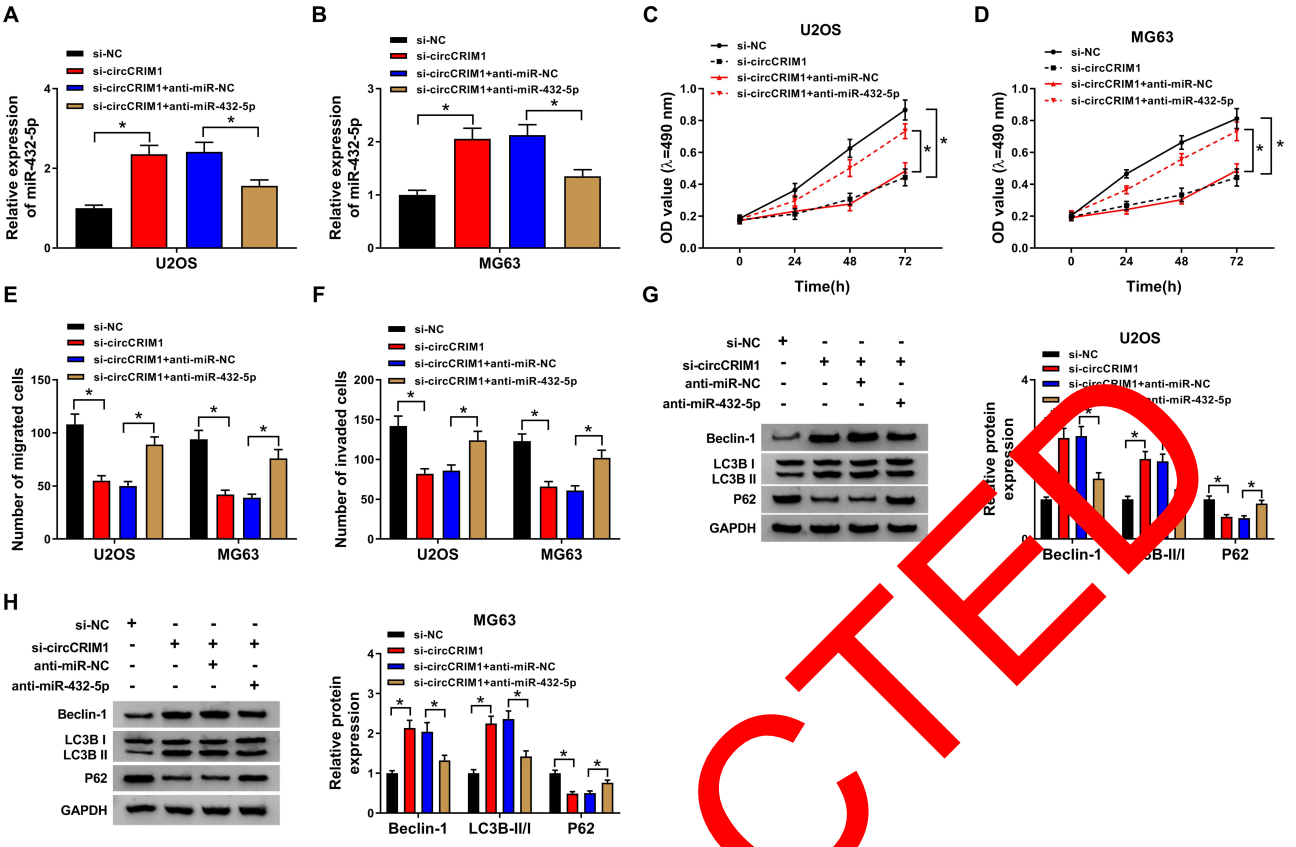

Figure 4 Knockdown of circCRIM1 retarded the progression of OS via targeting miR-432-5p. U2OS and MG63 cells were severally transfected with si-NC, si-circCRIM1, si-circCRIM1+anti-miR-NC, si-circCRIM1+anti-miR-432-5p. (**A** and **B**) The qRT-PCR was employed for measuring the level of miR-432-5p. (**C** and **D**) Cell proliferation was estimated using MTT assay. (**E** and **F**) The analysis of cell migration and invasion was implemented by transwell assay. (**G** and **H**) Autophagy was evaluated through the detection of related markers by Western blot. *P < 0.05.

proliferation, migration and invasion. Besides, autophagocytosis is widely regarded to be related to OS regulation. Zhang et al discovered that CCPS3 silencing could suppress the metastasis of OS cells via the inhibition of autophagy,[23] while Zhang et al asserted that TSSC3 promoted autophagy to obstruct the tumorigenesis and metastasis in OS cells.[24] Our experimental data indicated that circCRIM1 down-regulation reduced the autophagy generation in OS cells, possibly manifesting that circCRIM1 knockdown enhanced autophagy to hamper OS growth and metastasis in vitro.

CircRNAs are considered to sponge miRNAs in diverse cancers.[25] Circ_0000523 exerted the regulation in cell proliferation and apoptosis of colorectal cancer as a miR-31 sponge.[26] Circular RNA profiling identified circADAMTS13 as a sponge of miR-484 to repress the proliferation of hepatocellular carcinoma cells.[27] CircMMP9 played an oncogenic role in tumorigenesis of glioblastoma multiforme by sponging miR-124.[28] Herein, miR-432-5p was certified to be a target of circCRIM1 and circCRIM1 could directly sponge miR-432-5p in OS cells. Moreover, the role of circCRIM1 in OS oncogenesis was dependent on its negative regulation of miR-432-5p.

As regards the function of miR-432-5p in OS, our results revealed that miR-432-5p reduced proliferation, migration, invasion while promoted autophagy, which demonstrated the anti-tumor role of miR-432-5p in OS. A handful of downstream targets for miR-432-5p have been disclosed, such as E2F3 in breast cancer,[29] NFAT5 in glioblastoma,[30] p53 in neuroblastoma.[31] After our target analysis, we found that miR-432-5p interacted with the 3′UTR of HDAC4 and directly suppressed the level of HDAC4. Previous proofs have affirmed HDAC4 as an oncogene in OS to be regulated by miR-145-3p[32] and miR-140.[33] The rescued assays also suggested that HDAC4 inhibition was responsible for the antitumous effect of miR-432-5p on OS.

Furthermore, circCRIM1 was found to enhance the expression of HDAC4 in OS cells via sponging miR-432-5p in this research. The circRNA-miRNA-mRNA signaling

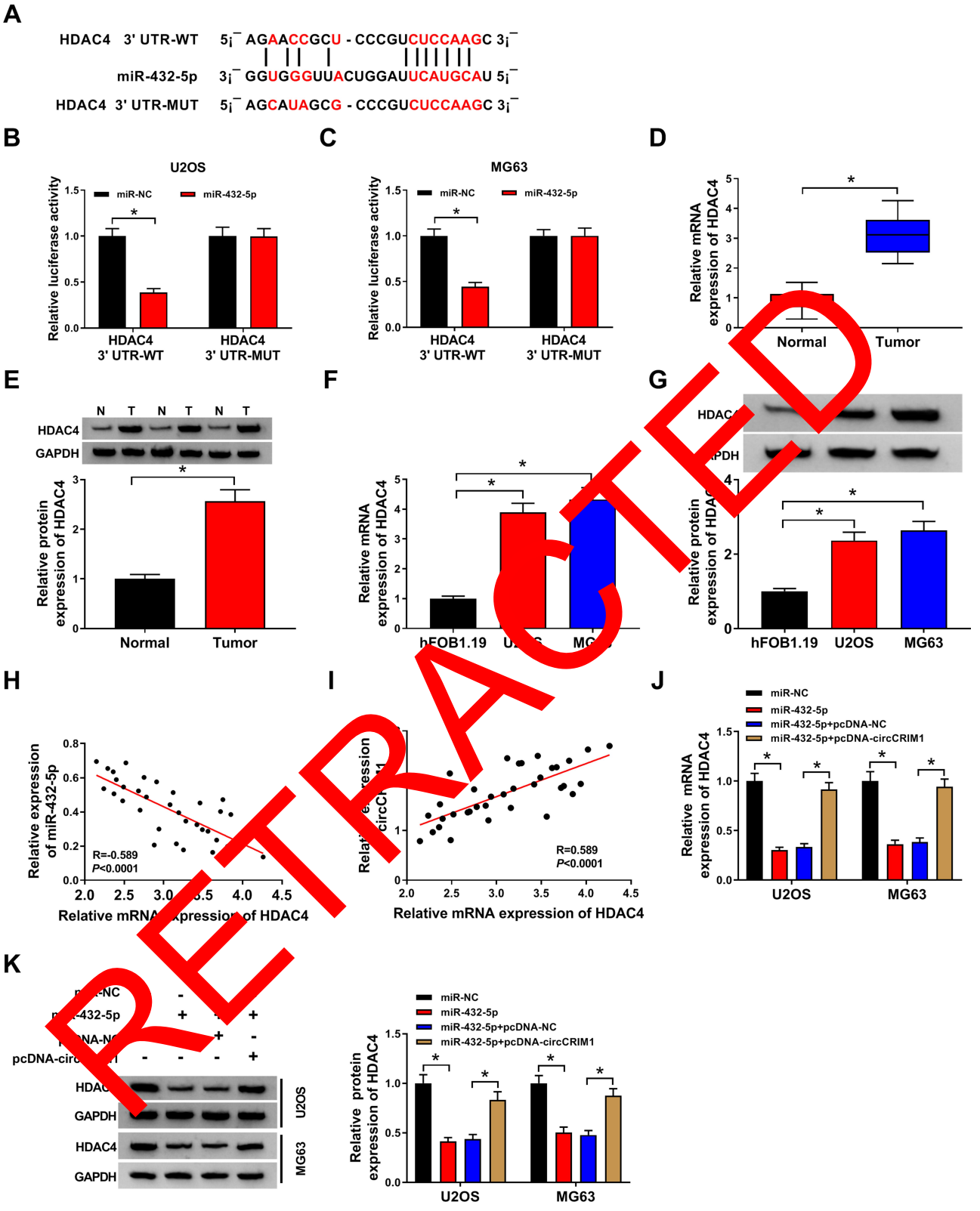

Figure 5 CircCRIM1 up-regulated HDAC4 by sequestering miR-432-5p in OS cells. (**A**) Starbase 3.0 was exploited to perform the site analysis of miR-432-5p and HDAC4 sequences. (**B** and **C**) The binding of HDAC4 3′UTR and miR-432-5p was assessed applying with the dual-luciferase reporter assay. (**D**–**G**) The qRT-PCR and Western blot were used for the determination of HDAC4 mRNA and protein expression in OS tissues (**D** and **E**) and cells (**F** and **G**). (**H** and **I**) Spearman correlation coefficient was applied to analyze the linear connection between miR-432-5p and HDAC4 (**H**), as well as circCRIM1 and HDAC4 (**I**). (**J** and **K**) HDAC4 expression was assayed using qRT-PCR and Western blot in U2OS and MG63 cells transfected with miR-432-5p, miR-432-5p+pCE-RB-Mam-circCRIM1 or their negative controls. *P < 0.05.

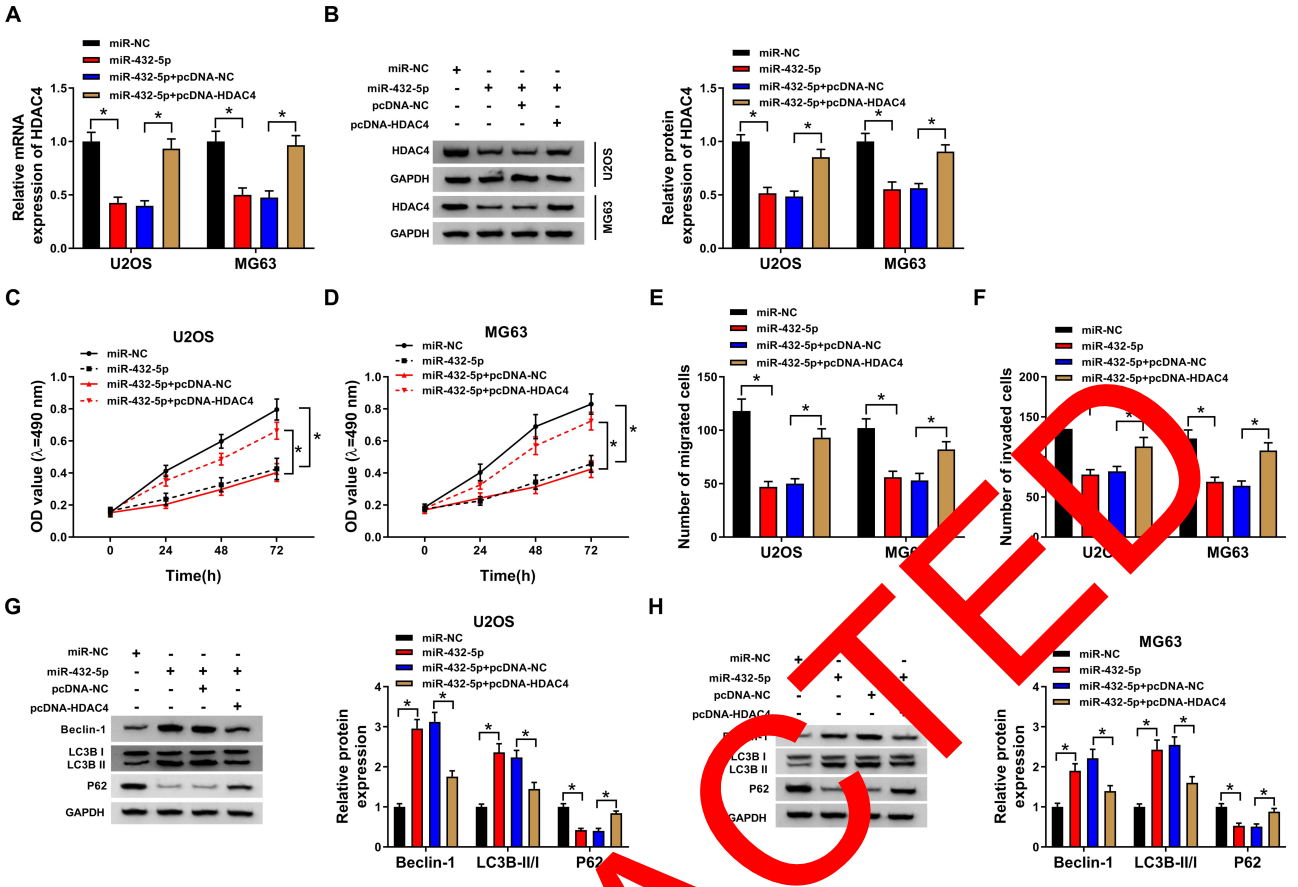

Figure 6 MiR-432-5p targeted HDAC4 to suppress proliferation, migration, invasion and autophagy of OS cells. (**A** and **B**) The mRNA and protein levels of HDAC4 were examined using qRT-PCR and Western blot after transfection of miR-432-5p, miR-432-5p+pcDNA-HDAC4 or the corresponding controls. (**C** and **D**) The measurement of cell proliferation was carried out by MTT assay. (**E** and **F**) Transwell assay was used to assess the migratory and invasive abilities of transfected OS cells. (**G** and **H**) Western blot was administrated for detecting the proteins associated with autophagy. *P < 0.05.

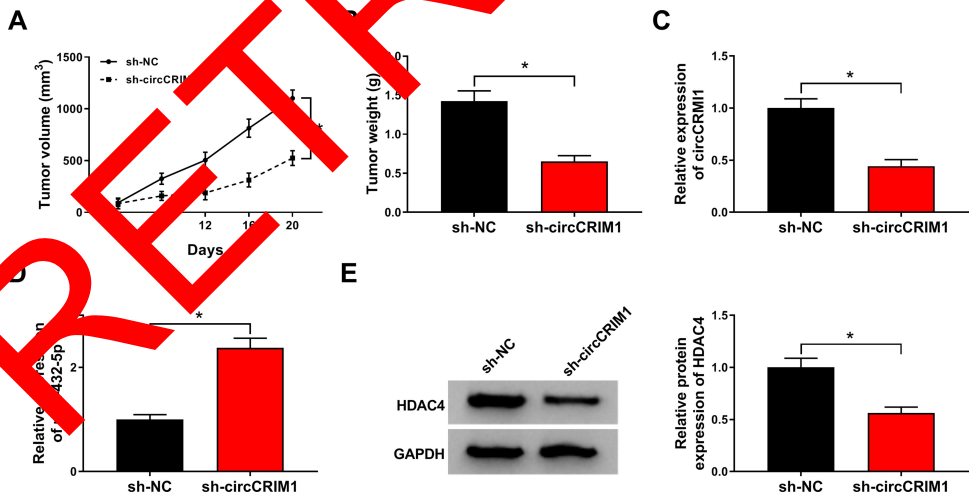

Figure 7 Knockdown of circCRIM1 reduced OS growth in vivo via the regulation of miR-432-5p/HDAC4 axis. (**A**) Tumor volume was measured every 4 d post-injection. (**B**) Tumor weight was recorded after mice were dissected. (**C** and **D**) The detection of circCRIM1 and miR-432-5p in tumor was performed using qRT-PCR. (**E**) The protein level of HDAC4 in tumor was determined through Western blot. *P < 0.05.

network has been in the illumination of tumor regulation.[34,35] Our data supported the emergence of circCRIM1/miR-432-5p/HDAC4 network in OS progression, and xenograft tumor assay also made clear that the inhibition of circCRIM1 decreased tumor growth of OS in vivo through regulating the miR-432-5p/HDAC4 axis.

To conclude, a novel molecular pathogenesis that circCRIM1/miR-432-5p/HDAC4 axis contributed to the carcinogenic action in OS was proposed in our study, contributing to intensify the comprehension of OS progression. In the diagnostic and therapeutic layers, circCRIM1 can be used as a target for OS diagnosis while its repression has the potential to relieve the exacerbation of OS in the treatment for patients.

Highlights

1. CircCRIM1 is overexpressed in osteosarcoma
2. Knockdown of circCRIM1 represses proliferation, migration and invasion while promotes autophagy in osteosarcoma cells
3. CircCRIM1 can up-regulate HDAC4 expression via targeting miR-432-5p
4. CircCRIM1 down-regulation refrains tumorigenesis of osteosarcoma in vivo by regulating miR-432-5p/HDAC4

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Disclosure of Interest

The authors declare that they have no financial or non-financial conflicts of interest

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