#### **Cancer Management and Research**

#### ORIGINAL RESEARCH

# **RETRACTED ARTICLE: Knockdown of CircCRIMI** Inhibits HDAC4 to Impede Osteosarcoma Proliferation, Migration, and Invasion and Facilitate Autophagy by Targeting miR-432-5p

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Background: Circular RNAs (circRNA) considerable modulatory l in human However, the contribution of molecules that have been largely resear cer circRNA cysteine-rich transmembra. bone orphogenetic protein regulator 1 (circCRIM1) to osteosarcoma (OS) is completely unclear.

Methods: All the RNA leves were examined via quantitative real-time polymerase chain reaction (qRT-PCR). Cellur proliferation and migration/invasion were, respectively, analyzed using 3-(4, 5-dimethethiazol-2-y1) , 5-diphenyl tetrazolium bromide (MTT) assay and transwell assay. The demination of all protein expression was administrated by Western blot. Du land rese reporter assay was used for proving the target combination. The exploration of irccomprise wive was performed by xenograft assay.

Result QS tisst and cells, circCRIM1 was differentially up-regulated. Functionally, cell cration. nigration and invasion were suppressed while autophagy was promoted after pro CRIM<sup>1</sup> down-egulated in OS cells. Mechanistically, mircoRNA-432-5p (miR-432miRNA target of circCRIM1 and the inhibitory effect of circCRIM1 knockdown on 5p) ssion was achieved by targeting miR-432-5p. Moreover, histone deacetylase 4 OS pros (HDAC4) was a downstream gene of miR-432-5p and circCRIM1 targeted miR-432-5p to upulate HDAC4 level. MiR-432-5p inhibited proliferation, migration, and invasion but enh. ed 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.<sup>1</sup> Currently, surgical resection conjoined with systemic chemotherapy has improved the overall survival but it remains poor for the metastatic OS patients.<sup>2</sup> To explore the molecular information of OS biology was considered rewarding for intensively understanding the pathological mechanisms underlying OS.<sup>3</sup> 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 closedloop as the structural performance. Increasing studies have exhibited that circRNAs worked as essential cancerregulatory factors as the sponges of microRNAs (miRNAs) to block the functions of miRNAs.<sup>4,5</sup> For example, circ 103809 was promoted proliferation and invasion of lung cancer cells by sponging miR-4302 to elevate ZNF121-mediated MYC expression;<sup>6</sup> circular RNA cTFRC served as a sponge of miR-107 to facilitate the carcinogenesis of bladder cancer.<sup>7</sup> 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.<sup>8</sup> 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,<sup>9</sup> breast cancer,<sup>10</sup> endometrial cancer,<sup>11</sup> as well as OS.<sup>12</sup> Previous study has manifested that mine 432 was down-regulated in OS and functioned as a tumo repressor.<sup>13</sup> Here, the action of miR-432-5p in Ordersether with the relation to circCRIM1 were investigated.

As a subunit of class II histone deace clases the deacetylase 4 (HDAC4) can be involved in the tological responses by controlling the expression of important genes in various cellular behaviors <sup>14</sup> Kang et al purported that HDAC4 accelerated cell purgression of galaxic cancer via inhibiting p21<sup>15</sup> and Capet al found the oncogenic role of HDAC4 in glioma.<sup>16</sup> Alap, HOAC4 has been testified to enhance OS cellor diferation and iteration,<sup>17</sup> but its association with arR-432 p and capeRIM1 is unknown.

Except 1 othe formation SeircCRIM1 and miR-432-5p in OS, the contration 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 subcultured in a 25mm<sup>3</sup> culture flask (Corning Inc., Corning, NY, USA) in the condition of 37°C, 5% 602 and 95% air, after the digestion of 0.25% trypsin Gibco, Calsbad, CA, USA). Among three cell lines, h G63 were cultivated using Dulbeccer mode age medium (DMEM; Gibco) while U2OS was reantained in McCoy's 5A medium Sibco Joth containing 10% fetal bovine serum (2.5) a. 1% pre-cillin-streptomycin mixed solution ibco).

#### Cell mansfection

interfering RNA (siRNA) targeting circCRIM1 (si-Sm RIM1)/siRN negative control (si-NC), miR-432-5p circ mimic niR-43 5p)/miRNA mimic NC (miR-NC), miR-2-5p inhibitor (anti-miR-432-5p)/miRNA inhibitor NC from (? .a-m. NC) were purchased **RIBIBIO** Guangzhou, China) and transfected into U2OS and IG63 cells through the riboFECT<sup>™</sup> 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-MamcircCRIM1 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<sup>®</sup> SYBR<sup>®</sup> Green qPCR ReadyMix<sup>TM</sup> (Sigma) after the inverse transcription through the Firststrand cDNA Synthesis Kit (Sigma) as per the manuals for users. The primers used in our study were listed as below: circCRIM1: Forward (F), 5'-CTGTGTGAGACC 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' **5'-ATCT** and R, TGCTCACGCTCAACCT-3'; glyceraldehyde-3-phosphate (GAPDH): F, 5'-GGGCTGC dehydrogenase 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 (MT Assay

The assessment of cell proliferation was advanistrate, using Cell Proliferation Kit I (MTK) (Pache, Basie, Switzerland). In short, transferred cells is the 96-well plates were added with 00 µc/well MTK Labeling Reagent, which can transform the living cells into insoluble formazan. 4 h later, the formazan was solubilized by adding 100 µL sclubilization Solution into each well. Through the detection of a minipplate reader (Sigma), cell proliferative bility culdioe indicated by the absorbance w490 nm.

### Transwell Assay

For the examination of cell migration, the upper chamber of the transwell 24-well chamber (Corning Inc.) was added with  $2 \times 10^4$  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 Nitzena e Pure Transfer Membranes (Santa Cruz), TP Blotto A (Lonta Cruz) was exploited as a blocking reagent prevent the binding of nospecific protein-binding signals. The the embranes were incubated with printing antibudies in the diluted solution at 4°C overnight old the combination of secondary antibody apporting a bodie at room temperature for 1 h to form the potein comp. The objective protein levels were analyzed by letecting the intensity of the immuno-Jugated signals rough Western Blotting Luminol eagent (Same Cruz) under the ImageLab software version (Bio-Rad Hercules, CA, USA). All the antibodies were bootht from Abcam (Cambridge, UK): anti-Beclin-1 (ab62557, 1:1000), anti-light chain 3B (anti-LC3B; abs. 20, 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  $\times$  width<sup>2</sup>  $\times$  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  $\pm$  standard deviation (SD) after three repetitions. The analysis of linear connection was administrated b Spearman correlation coefficient. Student's *t*-test and oneway 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

# CircCRIMI Was Abundantly Expressed in OS Tissues and Cells

The differential expression of circCRIM1 was identified by qRT-PCR. In collected 35 OS tissues, the plotive expression of circCRIM1 was found to be repartedly up gulated by contrast to the corresponding 35 number of the corresponding 15 number of the corresponding 1 gure 1A). Statistical analyses indicated nat circe VIM1 w markedly Le (P=0.020) a associated with tumor Afferentiation grade (P=0.028) (Table surjesting that circCRIM1 was to OS gressic Also, an increased potentially relate was observed in U2OS phenomenon CRIM1 le and MG63 cells using FOB1.19 as the control (Figure 1B). After cannent of RNase, there was almost no change of RIM1 expression while GAPDH level was conspicucirc declined compared to the Mock group, exhibiting the ous higher bility circCRIM1 than linear RNA (Figure 1C 10). CircCRIM1 might participate in the development of won, ng as a crucial role.



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

Parameter	Case	circCRIMI Expression <sup>a</sup>		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	
ш	19	9	10	
Pulmonary				0.053
metastasis				
Yes	14	10	4	
No	21	8	13	
Differentiation grade				0.028 *
Well/moderately	18	6	12	
Poorly/	17	12	5	
undifferentiated				

 Table I Association Between Clinical Features and circCRIMI

 Expression of OS Patients (n=35)

Notes: \*P<0.05; <sup>a</sup>Using median expression level of circCR<sup>1</sup> as c

## CircCRIMI Down-Regulation Restrained Cellular Proliferation, Mignetion and Invasion While Enhanced Autophagy of OS Cells

-regue on of c<sup>i</sup> CRIM1 in OS as above, In view of the the loss-f employed to research the nethou Action potentia role of arcCRIM1 in OS by siRNA transfection. showed the successful interference of si-The qRTthe level of circCRIM1 in U2OS and circCRIM1 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.<sup>18</sup> 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.

## CircCRIMI Targeted miR-432-5p in OS Cells

In Starbase3.0, it was obvious that circC M1 and miR-432-5p had the complemental nucleotid binding sites (Figure 3A). And visit y, the introduction of miR-432-5p repressed the lucif use activity in characteristic RIM1-WT reporter instead of incertific MUT, implicating the target combination of circCk 11 an miR-432-5p (Figure 3B and C). I reason to the expression of miR-432-5p in OS, we performed qR PCR to analyze it and found the downalation of miR-4. 5p not only in OS tissues (Figure D) but also in U2OS and MG63 cells (Figure 3E) comared to not al tissues and hFOB1.19 cells. After the an sis of pearman correlation coefficient between the levels of circCRIM1 and miR-432-5p in OS tissues, a httive 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 CircCRIMI 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 CircCRIMI down-regulation restrained cellular proliferation, migration and invasion while enhanced as phagy of OS cells. In U2OS and MG63 cells, separate si-NC and si-circCRIMI transfection was carried out. (A and B) The determination of circCRIMI transfection was carried out. (A and B) The determination of circCRIMI transfection. (C and D) MTT was exploited for the evaluation of cell proliferation. (E and F) Cellular migration and invasion were measured to use the transfection of 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 enancement of autophagy in U2OS and MG63 cells (Figure 4Genue Altogether, the retardment of circCRP of knock own on OS progression was achieved by targeting miR-432-5p.

## CircCRIMI Up-Regulated HDA 4 by Sequestering min 432 op in OS Cells

According to the biginfor tics and sis by Starbase3.0, 5 were noticed in the the binding es of miR-4. sequence c HDAC ATTR (Figure 5A). Their combination was verice Lasing the dual-luciferase reporter assay. As the illustration of Figure 5B and C, the relative luciferase activity of HDAZ4 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- 32-5p 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 has analyzed using the online Starbase3.0. (B and C) The dual-luciferase reporter assay was implemented to affirm the actual combination between circles and miR-432-5p. 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 own-regulation (H) on miR-432-5p level was assessed via qRT-PCR. \*P < 0.05.

successfully overexpressed via the pcDNA-HDAC4 cosfection. MTT and transwell assays indicated that cell p oliferation (Figure 6C and D), migration (Figure 6E) or invasion (Figure 6F) were all repressed by m -432-. whereas overexpression of HDAC4 costly microated the inhibition on those cellular processes. Stabilary, miR-452-5p mimic heightened the level of Beclin-1 and LC3B-II/I along with the decline of PC, but this stimulative effect on autophagy was lightened is a result of N-AC4 up-regulation (Figure 6G and H). These datatogether manifested that miR-432-5p was a tumo, phibits r in OS via targeting HDAC4.

## Knockdown of CirceRIM1 Reduced OS Grown in vivo-na the Regulation of miR-432- p/HDAC4 Axis

After the estable ment 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-1, the miR-432-5p to inhibit HDAC4 expression, nee 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.<sup>19</sup> 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.<sup>20</sup> Circ 0001658 boosted cellular proliferative and metastatic abilities in OS via the modulation of miR-382-5p/YB-1 axis.<sup>21</sup> And circMMP9 was validated to facilitate the oncogenesis of OS by restricting miR-1265 expression to up-regulate CHI3L1.<sup>22</sup> 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



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Figure 4 Knockdown of circCRIM1 retarded the progression of OS via targeting circCRIMI+anti-miR-NC, si-circCRIMI+anti-miR-432-5p. (A and B) The qRT-PC estimated using MTT assay. (E and F) The analysis of cell migration and invasion detection of related markers by Western blot. \*P < 0.05.

proliferation, migration and invasion ophagocytosis is widely regarded to be r ed to OS I ulation. Zhang et al discovered that CCPS3 storing could suppress the metastasis of C cells via the inhibition of autophagy,<sup>23</sup> while Zhap et al a serted that TSSC3 promoted autophagy to object the tumorigenesis and metastasis in OS cells<sup>24</sup> Our coveriment data indicated that n-regulation by ed the autophagy gencircCRIM1 d oscibly manifesting that circCRIM1 eration in Cells knockdown e. ced autophagy to hamper OS growth and metastasis in ·tro.

CircRNAs are considered to sponge miRNAs in diverse cancers.<sup>25</sup> 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.<sup>27</sup> CircMMP9 played an oncogenic role in tumorigenesis of glioblastoma multiforme by sponging miR-124.<sup>28</sup> Herein, miR-432-5p was certified to be a target of circCRIM1 and

5p. U2OS : of cells were severally transfected with si-NC, si-circCRIMI, siwas en. for measuring the level of miR-432-5p. (C and D) Cell proliferation was rtanswell assay. (G and H) Autophagy was evaluated through the

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,<sup>29</sup> NFAT5 in glioblastoma,<sup>30</sup> p53 in neuroblastoma.<sup>31</sup> 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<sup>32</sup> 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 CircCRIMI 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 circCRIMI 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-circCRIMI or their negative controls. \*P < 0.05.



Figure 6 MiR-432-5p targeted HDAC4 to suppress proliferation, migration, invelop and provide 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 min 432, miR-43, 5p+pcDNA-HDAC4 or the corresponding controls. (C and D) The measurement of cell proliferation was carried out by MTT assay. (Figure 1) Transwere say was used to assess the migratory and invasive abilities of transfected OS cells. (G and H) Western blot was administrated for detecting the provenies associated with utophagy. \*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.<sup>34,35</sup> 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
- Knockdown of circCRIM1 represses proliferation, migration and invasion while promotes autophagy in osteosarcoma cells
- CircCRIM1 can up-regulate HDAC4 expression via targeting miR-432-5p
- CircCRIM1 down-regulation refrains tumorigenesis of osteosarcoma in vivo by regulating miR-432-5p/HDAC4

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