Contents lists available at ScienceDirect

Journal of Bone Oncology

Down-regulation of circular RNA hsa_circ_0007534 suppresses cell growth by regulating miR-219a-5p/SOX5 axis in osteosarcoma

Peng Zhang, Jun Li *

Department of Orthopedics, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiangyang, China

ARTICLE INFO

ABSTRACT

Introduction: Circular RNA circ_0007534 and microRNA-219a (miR-219a-5p) were reported to be involved in osteosarcoma (OS) development. Osteosarcoma (OS) is one of the most common malignant bone tumors, which was more prone to occur in the metaphysis of long bones, including distal femur and proximal tibia. However, the detailed mechanisms were not fully clear. The purpose of this research was to reveal the functional mechanisms of circ_0007534 and miR-219a-5p in OS.

Methods: The levels of genes were determined by quantitative real-time polymerase chain reaction (qRT-PCR) or western blot assay. Cell proliferation ability was detected by cell counting kit-8 (CCK-8) and colony formation assay. Cell migration and invasion abilities were measured using the transwell assay. Furthermore, the interaction between miR-219a-5p and circ_0007534 or SRY (sex-determining region Y)-box 5 (SOX5) was predicted by starbaseV3.0, and confirmed by the dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assay. Besides, tumor xenograft experiment was performed to analyze the effect of circ_0007534 depletion on tumor growth *in vivo*.

Results: The levels of circ_0007534 and SOX5 were increased, while the miR-219a-5p level was decreased in OS tissues and cells. Circ_0007534 knockdown repressed the proliferation, colony formation, migration, and invasion in OS cells. Circ_0007534 targeted miR-219a-5p, and miR-219a-5p interacted with SOX5. Furthermore, circ_0007534 regulated the growth of OS cells through modulating the levels of miR-219a-5p and SOX5.

Conclusion: Our finding demonstrated that circ_0007534 knockdown suppressed the growth of OS cells via regulating miR-219a-5p/SOX5 axis, providing a potential target for OS treatment and diagnosis.

1. Introduction

Osteosarcoma (OS) is a common malignant tumor that mainly affects children and adolescents worldwide, which inclines to occur in the metaphysis of long bones, including the distal femur and proximal tibia [1]. OS is the second leading cause of tumor-related death in children and adolescents [2,3]. With the development of science and technology, the 5-year survival rate of OS patients after surgery and chemotherapy has been greatly improved, but the mortality rate of patients with recurrent or metastatic OS is still very high [4–6]. Thus, it is imperative to investigate the molecular mechanism of OS development for the therapy of OS patients.

An increasing number of studies suggested circRNAs exerted a pivotal function in OS development [7,8]. For instance, Li et al. revealed that circRNA circ_ORC2 accelerated OS cell proliferation and mobility through regulating miR-19a [9]. Yan et al. indicated that circRNA circSAMD4A promoted cell growth via regulating miR-1244/murine double minute2 (MDM2) pathway in OS [10]. Liu et al. demonstrated that circRNA circFAT1 positively regulated cell development via binding to miR-375 in OS [11]. These data confirmed that circRNAs acted as a class of important regulators in OS development. Therefore, the studies of circRNAs functional mechanism are essential for the therapy and diagnose of OS patients.

It is reported that approximately 90% of human transcripts are non-coding RNAs, including long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and circular RNA (circRNAs) [12]. LncRNAs and miRNAs were well reported and closely related to the development of human cancers [13,14]. Unlike the linear RNAs, circRNAs are mainly characterized by covalently closed loop structures that lack 3′ and 5′ polyadenylated tails [15]. On account of its special structure, circRNAs are resistant to nuclease and more stable than linear RNA, which exhibited an obvious advantage in the development and application of new clinical diagnostic markers [16]. Li et al. indicated that the expression level of circ-KLHDC10 could successfully distinguish the colorectal cancer patients and normal individuals [17]. Increasing evidence has suggested that circRNAs could function as tumor activators or suppressors in various cancers, including OS [18,19]. A previous report suggested that circRNA circ_0007534 mediated the growth of OS cells through modulating protein kinase B (AKT) activity, and was considered as a marker for the prediction of OS occurrence [20]. However, the functional mechanism of circ_0007534 is not fully understood.

MiRNAs, with about 22 nucleotides, regulated the levels of downstream genes via targeting 3′untranslated region (UTR) of the messenger RNA (mRNA) in human diseases [21]. Amounting evidence demonstrated that miRNAs were abnormally expressed in a variety of cancers, such as breast cancer [22], hepatocellular carcinoma [23], lung cancer [24], and liver cancer [25]. MiR-219a-5p, lowly expressed in OS tissues and cells, was reported to suppress the mobility of OS cells [26], revealing that miR-219a-5p exerted pivotal function in OS development. However, the study of miR-219a-5p function in OS has rarely been reported.

SRY (sex-determining region Y)-box 5 (SOX5) was identified as a transcription factor and played a crucial role in embryonic development and cell growth [27]. Increasing evidence indicated that SOX5 was involved in tumor growth. For example, You et al. confirmed that SOX5 level was upregulated in gastric cancer (GC) tissues, and SOX5 induced the invasion as well as epithelial-mesenchymal transition (EMT) of GC cells [28]. Sun et al. suggested that SOX5 positively regulated the growth of breast cancer cells through modulating the recombinant enhancer of zeste homolog 2 (EZH2) expression. In OS, SOX5 was reported to act as an oncogene and was highly expressed in OS tissues and cells [29]. However, the molecular mechanism of SOX5 function in OS development is poorly explored.

Here, we detected the levels of circ_0007534, miR-219a-5p, and SOX5 in OS tissues and cells. Furthermore, the functions of circ_0007534, miR-219a-5p and SOX5 in OS cell progression were also investigated. Besides, we also explored the effect of circ_0007534 on tumor growth *in vivo*.

2. Materials and methods

2.1. Tissues and cell culture

OS tissues and paratumor tissues were obtained from the patients with OS at the hospital of Xiangyang Central Hospital, and then stored at −80 °C for further experiment. The clinical information of the OS patients included age, gender, location, tumor size, enneking stage, and lymph node metastasis was summarized in Table 1. This research was approved by the Ethics Review Committees of Xiangyang Central Hospital. All patients in this research signed informed content.

Human osteoblast cell line (hFOB1.19: ATCC® CRL-11372), which is often used as a normal human osteoblast cell model, and four OS cell lines as the research subjects (143B: ATCC® CRL-8303, mixed fibroblast and epithelial-like cell; MG63: ATCC® CRL-1427, fibroblast-like cell; HOS: ATCC® CRL-1543, mixed

Table 1
The clinicopathological factors in osteosarcoma.

Clinicopathological features	Number of cases
Age	
>20 years	16
≤20 years	26
Gender	
Male	24
Female	18
Location	
Femur/Tibia	30
Elsewhere	12
Tumor size (cm)	
>8	15
≤8	27
Enneking stage	
I–II	19
III–IV	23
lymph node metastasis	
Negative	17
Positive	25

fibroblast and epithelial-like cell; and U2OS: ATCC® HTB-96, epithelioid-like cell) were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO_2.

2.2. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA in OS tissues or cells was isolated using TRIzol (Invitrogen). Then, the PrimeScript RT reagent kit (TaKaRa Bio, Shiga, Japan) was used to generate complementary DNA (cDNA) and the SYBR Green Mix (Beyotime Biotechnology, Shanghai, China) was applied to conduct the qRT-PCR assay. The reaction program was as follows: Initial denaturation at 95 °C for 3 min; followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, and a final extension of 72 °C for 5 min. Relative expression levels were determined by the comparative cycle threshold (Ct) method, using the $2^{-\Delta\Delta Ct}$ method [30], and normalized by the level of U6 (for miR-219a-5p, Accession: MIMAT0000276) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH for circ_0007534, Accession: NM_007372, and SOX5, Accession: NM_006940). The primer sequences of RT-qPCR were shown in Table 2.

2.3. Cell transfection

Small hairpin RNA against circ_0007534 (sh-circ_0007534), miR-219a-5p mimic (miR-219a-5p), miR-219a-5p inhibitor (anti-miR-219a-5p: 3′-ACTAACAGGTTTGCGTTAAGA-5′), and their negative control (sh-NC, miR-NC, and anti-NC) were obtained from Genepharma (Shanghai, China). For overexpression of circ_0007534 or SOX5, The sequences of circ_0007534 or SOX5 were cloned into the pcDNA3.1 plasmid (Genepharma) to construct plasmids over-expressing circ_0007534 or SOX5. Lipofectamine 2000 was employed to perform cell transfection assay according to the manufacturer's instruction.

2.4. Cell proliferation assay

Cell counting kit-8 (CCK-8) was employed to measure cell proliferation according to the manufacturer's instruction. Briefly,

Table 2
Primer sequences for RT-qPCR used in this study.

Gene	Names	Sequences (5′–3′)
circ_0007534	RT (Random primer)	pd(N)6
	circ_0007534-F	CTGGTGTGGTTCAGGAGGAA
	circ_0007534-R	ATGGAATTGCTGGCGAGTTG
miR-219a-5p	RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGG CAATTGCACTGGATACGACACTGGA
	miR-219a-5p-F	TCTACAGTGCACGTGTCTCCAGT
	miR-219a-5p-R	CTCTCATTTGCTATATTCA
SOX5	SOX5-F	CTTCATGCCTGACTGATAT
	SOX5-R	GACGTCTCAGGACTTACG
U6	U6-F	TGCGGGTGCTCGCTTCGGCAGC
	U6-R	CCAGTGCAGGGTCCGAGGT
GAPDH	GAPDH-F	ATCACTGCCACCCAGAAGAC
	GAPDH-R	TTTCTAGACGGCAGGTCAGG

U2OS and MG63 cells upon transfection were cultured for 24 h, 48 h, or 72 h. Subsequently, cells were incubated with CCK-8 solution for 2 h. Finally, the absorbance was examined by a microplate reader (Bio-Rad, Richmond, CA, USA) at 450 nm.

2.5. Colony formation assay

In brief, transfected U2OS or MG63 cells were plated into 6-well-plate, and then cultured at 37 °C for 10 days. Subsequently, 0.5% crystal violet was applied to stain the colonies for 20 min. Finally, the colonies were imaged, and then counted with ImageJ software (NIH, Bethesda, MD, USA).

2.6. Cell migration and invasion assay

Cell migration was examined using the transwell chamber (Millipore, Bedford, MA, USA). BD Matrigel™ Basement Membrane Matirx (BD Biosciences, San Jose, CA, USA) was pre-coated to the chamber membrane in the invasion assay, whereas BD Matrigel™ Basement Membrane Matirx (BD Biosciences) was not coated in the migration assay. In brief, transfected U2OS or MG63 cells were harvested and then re-suspended with a serum-free medium. Subsequently, 100 μL re-suspended cells were introduced into the upper chamber, and a relative 500 μL medium with 10% FBS was seeded into the lower chamber. After 24 h of incubation, migratory or invasive cells were analyzed under a microscope (Olympus, Tokyo, Japan).

2.7. Western blot assay

Total proteins from OS tissues or cells were extracted with lysis buffer (Beyotime Biotechnology). Next, the proteins were subjected to 10% sodium dodecyl sulfonate (SDS)-polyacrylamide gel electrophoresis (PAGE), and then electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). Subsequently, the membranes were blocked by 5% non-fat milk, incubated with the primary antibodies against SOX5 (1:1000: Abcam, Cambridge, MA, USA), Vimentin (1:1000: Abcam), N-cadherin (N-cad) (1:1000: Abcam), E-cadherin (E-cad) (1:1000: Abcam), or GAPDH (1:1000: Abcam), and then incubated with corresponding secondary antibodies (1:2000; Abcam). Finally, the protein signals were determined by the ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Dual-luciferase reporter assay

According to the prediction of bioinformatics tool starbaseV3.0, miR-219a-5p interacted with circ_0007534 or SOX5 3′UTR. Circ_0007534 or SOX5 3′UTR sequences contained wide-type or mutant-type miR-219a-5p binding sites (WT/MUT-circ_0007534 or WT/MUT-SOX5 3′UTR) were cloned into pGL3 luciferase reporter vector (Promega). Then, luciferase reporter plasmids and miR-219a-5p or miR-NC were co-transfected into U2OS and MG63 cells. The luciferase activities were examined using the dual-luciferase reporter kit (Beyotime Biotechnology).

2.9. RNA immunoprecipitation (RIP) assay

The Magna RIP Kit (Millipore) was chosen to carry out RIP assay based on the user's manual. Briefly, U2OS and MG63 cells at 80% confluency were harvested, lysed by RIP lysis buffer, and incubated with RIP buffer containing magnetic beads conjugated with anti-argonaute RISC catalytic component 2 (Ago2) or anti-IgG for 2 h at 4 °C. Subsequently, samples were digested with proteinase K, and coprecipitated RNA was isolated. Then, qRT-PCR assay was employed to analyze RNA enrichment.

2.10. Mouse xenografts

4-week-old male BALB/c-nude mice were used to conduct xenograft assay. Briefly, the mice were injected subcutaneously with MG63 cells stably transfected with sh-circ_0007534 or sh-NC. Tumor volume (length $\times$ width^2 $\times$ 0.5) was calculated every 5 d. Mice were sacrificed after 32 days of injection, and the tumors were collected for weight analysis and further experiments. The animal experiment was conducted in line with the National Animal Care and Ethics Institution's guidance and was authorized by the Animal Research Committee of Xiangyang Central Hospital.

2.11. Statistical analysis

Each assay was repeated independently at least three times. All data were expressed as the mean ± standard deviation (SD), and were analyzed using paired or unpaired Student's *t*-test. The relationship between the levels of two genes was investigated through the analysis of spearman's correlation coefficient. *P* values <0.05 were considered statistically significant.
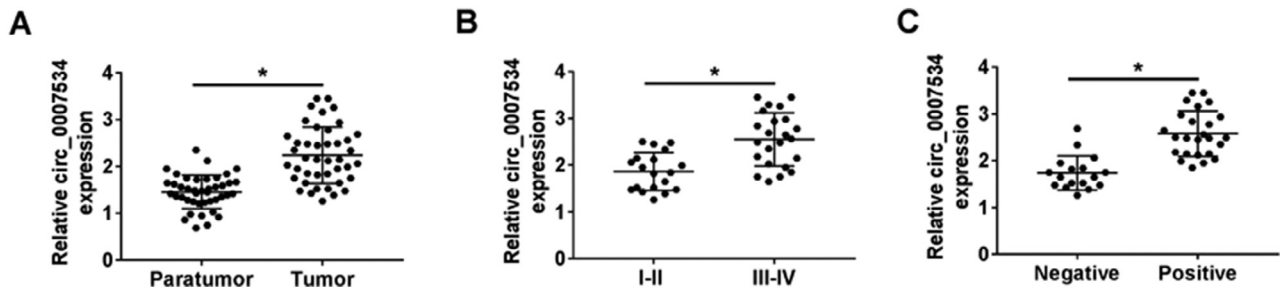
Fig. 1. The level of circ_0007534 in OS tissues. (A–C) The expression level of circ_0007534 was detected by qRT-PCR assay in OS tissues and paratumor tissues (A), OS tissues from patients at different clinical stage (B), and OS tissues from lymph node metastasis patients (C). ***$P < 0.001$, n = 3.

3. Results

3.1. Circ_0007534 expression was upregulated in OS tissues

To investigate the role of circ_0007534 in OS, the expression of circ_0007534 was detected by qRT-PCR assay in OS tissues and

paratumor tissues. The results suggested that circ_0007534 expression was significantly upregulated in OS tissues (Fig. 1A). We then investigated the relationship between circ_0007534 level and clinical-pathological parameters. As demonstrated in Fig. 1B, the OS patients at tumor stage III–IV exhibited higher circ_0007534 level compared with that in OS patients at tumor
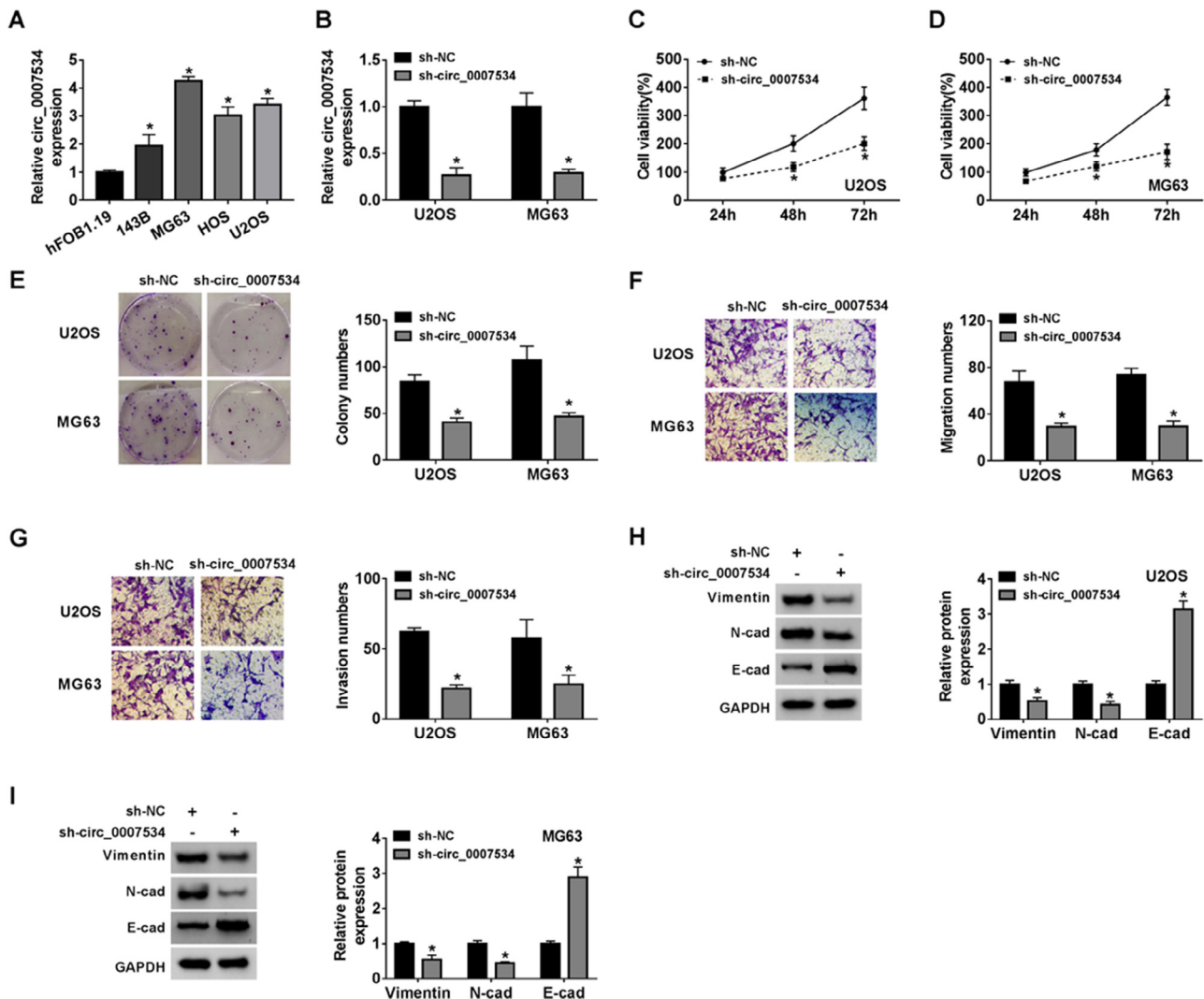
Fig. 2. The effect of circ_0007534 on OS cell progression. (A) Circ_0007534 expression was determined in normal cells (hFOB1.19) and OS cells (143B, MG63, HOS, and U2OS). (B) Circ_0007534 expression was examined in U2OS and MG63 cells transfected with sh-NC or sh-circ_0007534. (C and D) CCK-8 was used to assess cell proliferation ability in U2OS (48 h, $P = 0.004$; 72 h, $P < 0.001$) and MG63 cells (48 h, $P = 0.004$; 72 h, $P < 0.001$). (E) Colony formation assay was performed to measure cell clone formation ability. (F and G) Cell migration and invasive abilities were determined using transwell assay. (H and I) Western blot assay was employed to investigate the levels of three EMT markers. *$P < 0.05$, n = 3.

stage I–II. Moreover, we found that circ_0007534 expression was remarkably increased in patients with lymph node metastasis relative to negative lymph node metastasis (Fig. 1C). These data indicated that circ_0007534 was related to OS development.

## 3.2. Circ_0007534 knockdown repressed the proliferation, migration, and invasion in OS cells

Next, we determined the expression of circ_0007534 in OS cells. As shown in Fig. 2A, circ_0007534 expression was higher in OS cells (143B, MG63, HOS, and U2OS) than in normal cells (hFOB1.19). To further explore the function of circ_0007534 in OS cells, U2OS and MG63 cells were transfected with sh-circ_0007534 to downregulate its level. Then, knockdown efficiency was confirmed by qRT-PCR assay (Fig. 2B). Subsequently, CCK-8 was employed to detect cell proliferation ability. As shown in Fig. 2C and D, cell viability was significantly reduced in U2OS cells by 45%, and up to 53% in MG63 cells due to the circ_0007534 knockdown. Furthermore, colony formation assay indicated that clone formation ability was remarkably inhibited by the knockdown of circ_0007534 (Fig. 2E). On the other hand, we carried out transwell assay to investigate cell mobility, and found that cell migration and invasion abilities were dramatically repressed by circ_0007534 depletion in U2OS and MG63 cells (Fig. 2F and G). Besides, the levels of three EMT markers, Vimentin, N-cad, and E-cad, were detected by western blot assay. The results showed that the levels of Vimentin and N-cad were downregulated, and E-cad level was increased in circ_0007534-depleted U2OS and MG63 cells (Fig. 2H and I), which suggested that circ_0007534 downregulation might be related to the EMT. Therefore, circ_0007534 depletion inhibited the growth of OS cells.

3.3. Circ_0007534 targeted miR-219a-5p and inhibited miR-219a-5p expression

Using the bioinformatics tool starbaseV3.0, we recognized that miR-219a-5p was a target miRNA of circ_0007534 (Fig. 3A). Then, dual-luciferase reporter and RIP assay were employed to confirm this interaction in U2OS and MG63 cells. As shown in Fig. 3B and C, cells transfected with WT-circ_0007534 and miR-219a-5p displayed lower luciferase activity than the cells transfected with MUT-circ_0007534 and miR-219a-5p, revealing the interaction between circ_0007534 and miR-219a-5p. And it was confirmed by the enrichment of circ_0007534 or miR-219a-5p in Anti-Ago2 group (Fig. 3D and E). Next, we investigated the effect of circ_0007534 on miR-219a-5p expression via transfecting sh-circ_0007534 or circ_0007534 into OS cells. QRT-PCR assay showed that the transfection with circ_0007534 indeed increased the level of circ_0007534, and miR-219a-5p expression was significantly upregulated by circ_0007534 knockdown and downregulated by circ_0007534 overexpression (Fig. 3F and G). Furthermore, decreased miR-219a-5p was observed in OS tissues and cells (Fig. 3H and I). Besides, the miR-219a-5p level was negatively correlated with circ_0007534 level in OS tissues (Fig. 3J). These data indicated that circ_0007534 interacted with miR-219a-5p and negatively regulated the expression of miR-219a-5p.

3.4. Circ_0007534 regulated the growth of OS cells by suppressing miR-219a-5p expression

To explore whether miR-219a-5p was functionally related to circ_0007534-regulated OS cell progression, U2OS and MG63 cells were transfected with sh-NC, sh-circ_0007534, sh-circ_0007534 +
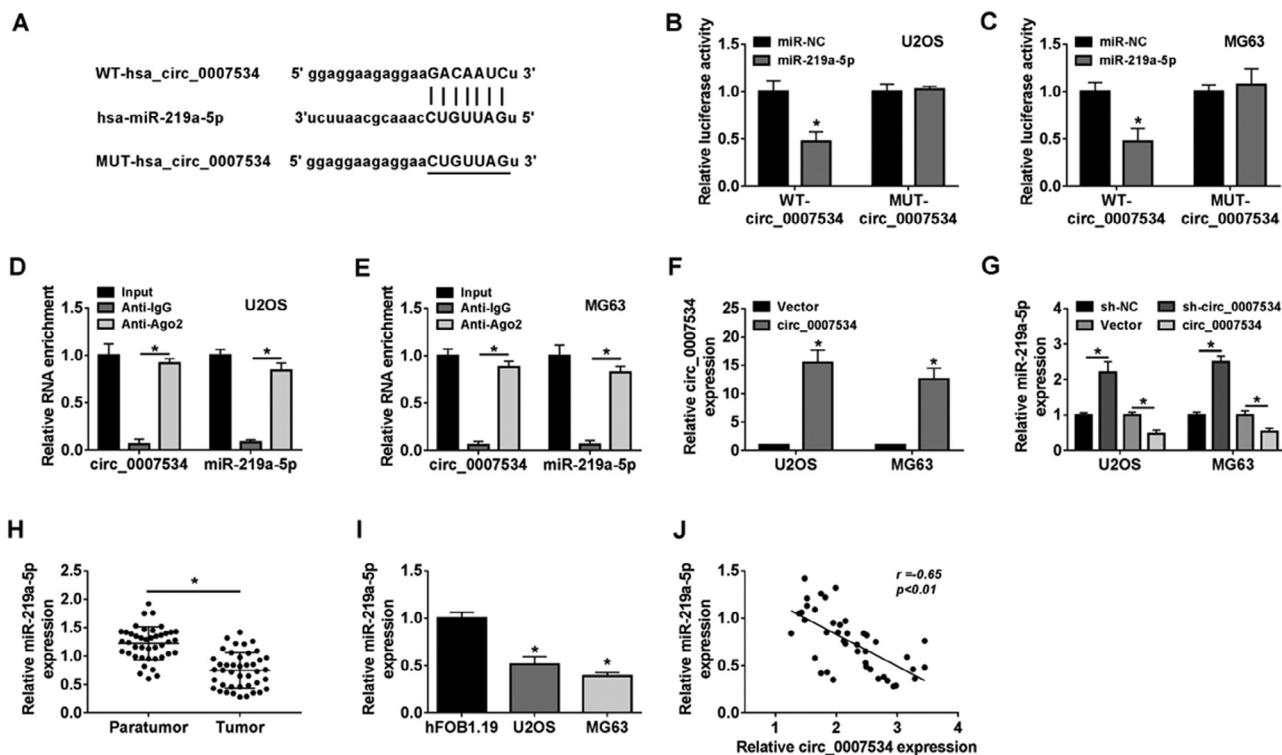
Fig. 3. The relationship between circ_0007534 and miR-219a-5p. (A) The interaction between circ_0007534 and miR-219a-5p was predicted by bioinformatics tool starbaseV3.0. (B and C) The luciferase activity of U2OS and MG63 cells transfected with WT-circ_0007534 or MUT-circ_0007534 and miR-219a-5p or miR-NC was determined. (D and E) RIP assay was employed to confirm the interaction between circ_0007534 and miR-219a-5p. (F) The level of circ_0007534 was analyzed in U2OS and MG63 cells transfected with Vector or circ_0007534. (G) MiR-219a-5p level was measured in U2OS and MG63 cells transfected with sh-NC, sh-circ_0007534, Vector, or circ_0007534, respectively. (H and I) MiR-219a-5p level was examined in OS tissues and paratumor tissues (H) as well as OS cells and normal cells (I). (J) QRT-PCR assay was carried out to explore the association between circ_0007534 and miR-219a-5p. *$P < 0.05$, n = 3.
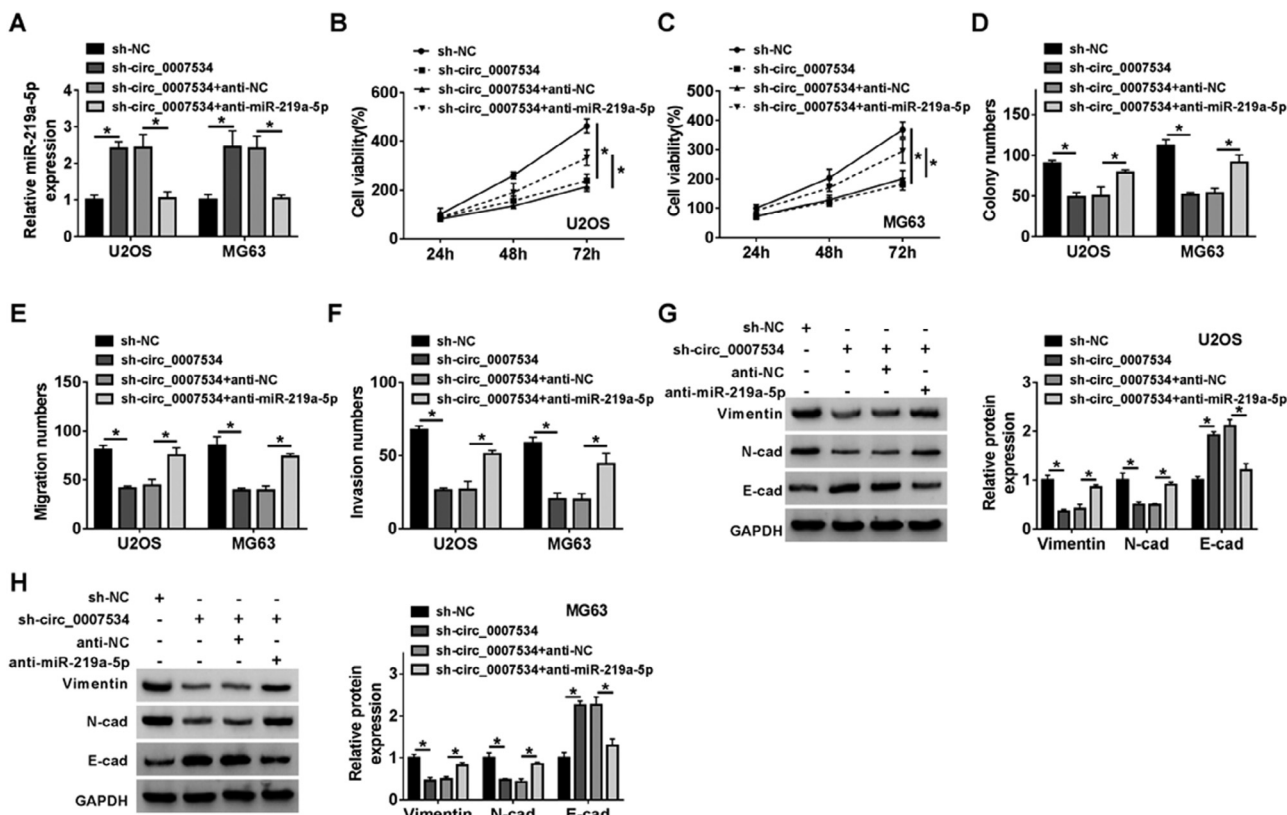
Fig. 4. The function of miR-219a-5p in circ_0007534-regulated OS cell progression. (A) MiR-219a-5p expression was detected in U2OS and MG63 cells transfected with sh-NC, sh-circ_0007534, sh-circ_0007534 + anti-NC, or sh-circ_0007534 + anti-miR-219a-5p, respectively. (B and C) Cell proliferation ability was measured using CKK-8. (D) Clone formation ability was examined by colony formation assay. (E and F) Transwell assay was employed to determine cell migration and invasive abilities. (G and H) The levels of EMT markers were investigated by western blot assay. *P < 0.05, n = 3.

anti-NC, or sh-circ_0007534 + anti-miR-219a-5p, respectively. QRT-PCR assay indicated that the level of miR-219a-5p was upregulated by circ_0007534 knockdown, and was downregulated by the transfection with anti-miR-219a-5p (Fig. 4A). Besides, cell proliferation was reduced by 49% and 51% in U2OS and MG63 cells with si-circ_0007534 transfection, and this suppression effect was reversed by miR-219a-5p inhibitor in U2OS (increased to 155%) and MG63 cells (increased to 147%) (Fig. 4B and C). Moreover, colony formation assay was performed, and results indicated that colony numbers reduced by circ_0007534 knockdown were increased by miR-219a-5p depletion in U2OS and MG63 cells (Fig. 4D). Next, transwell assay was conducted to examine cell migration and invasion abilities. As demonstrated in Fig. 4E and F, cell migration was inhibited by circ_0007534 knockdown, and it was partly rescued by miR-219a-5p depletion. Besides, this phenomenon that miR-219a-5p depletion reversed the effect of circ_0007534 knockdown on the levels of EMT markers was discovered in U2OS and MG63 cells (Fig. 4G and H). Taken together, circ_0007534 could modulate OS cell progression by targeting miR-219a-5p expression.

### 3.5. SOX5 was a target gene of miR-219a-5p

Through the bioinformatics tool starbase, we found that SOX5 was a potential target gene of miR-219a-5p (Fig. 5A). To verify this prediction, WT-SOX5 3′UTR or MUT-SOX5 3′UTR and miR-219a-5p or miR-NC were co-transfected into U2OS and MG63 cells to perform the dual-luciferase reporter assay. As shown in Fig. 5B and C, miR-219a-5p reduced the luciferase activity of WT-SOX5 3′UTR group, whereas it had little effect on the luciferase activity of MUT-

SOX5 3′UTR group, indicating that miR-219a-5p interacted with SOX5. Then, miR-NC, miR-219a-5p, miR-219a-5p + Vector, or miR-219a-5p + circ_0007534 were transfected into U2OS and MG63 cells to investigate the effects of miR-219a-5p and circ_0007534 on SOX5 expression. QRT-PCR assay suggested that miR-219a-5p expression was increased in cells transfected with miR-219a-5p, while it was decreased by circ_0007534 overexpression (Fig. 5D). Besides, SOX5 expression was reduced by miR-219a-5p overexpression, and then rescued by circ_0007534 upregulation (Fig. 5E and F). These data indicated that circ_0007534 increased the level of SOX5 via inhibiting miR-219a-5p expression.

Next, the expression of SOX5 in OS was determined. As demonstrated in Fig. 5G–J, SOX5 expression was significantly increased in OS tissues and cells. Finally, we explored the relationship between SOX5 and circ_0007534 or miR-219a-5p. As expected, the SOX5 level was negatively correlated with miR-219a-5p level and positively correlated with circ_0007534 level in OS tissues (Fig. 5K and L). Therefore, SOX5 might act as an oncogene in OS.

3.6. Circ_0007534 depletion inhibited the growth of OS cells via downregulating SOX5 expression

To analyze the function of SOX5 in circ_0007534-regulated OS cell progression, U2OS and MG63 cells were transfected with sh-NC, sh-circ_0007534, sh-circ_0007534 + Vector, or sh-circ_00075 34 + SOX5, respectively. Firstly, qRT-PCR assay and western blot assay were performed to determine the level of SOX5. The results showed that SOX5 level was significantly downregulated by circ_0007534 depletion, and rescued by the upregulation of SOX5 (Fig. 6A and B). Then, Cell proliferation ability was assessed by
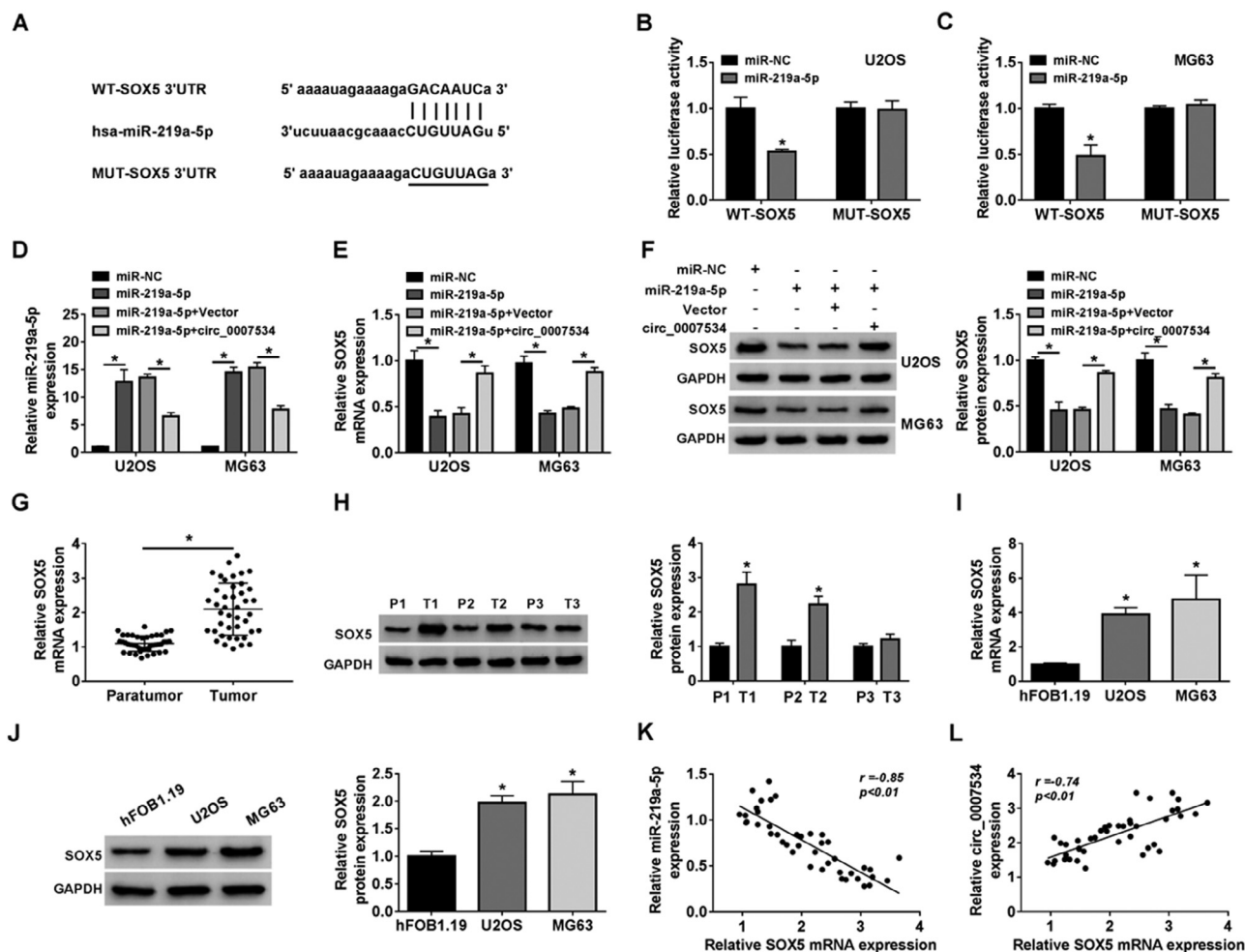
Fig. 5. The relationship between miR-219a-5p and SOX5. (A) The interaction between miR-219a-5p and SOX5 was predicted by bioinformatics tool starbaseV3.0. (B and C) The luciferase activity was determined in U2OS and MG63 cells transfected with WT-SOX5 or MUT-SOX5 and miR-219a-5p or miR-NC. (D–F) The levels of miR-219a-5p (D) and SOX5 (E and F) were detected in U2OS and MG63 cells transfected with miR-NC, miR-219a-5p, miR-219a-5p + Vector, or miR-219a-5p + circ_0007534, respectively. (G–J) The mRNA level and protein level of SOX5 were examined in OS tissues and paratumor tissues (G and H) as well as OS cells and normal cells (I and J). (K) The relationship between SOX5 and miR-219a-5p was analyzed. (L) The relationship between SOX5 and circ_0007534 was investigated. *$P < 0.05$, n = 3.

CCK-8. As shown in Fig. 6C and D, cell viability was decreased in U2OS cells by 42%, and 50% in MG63 cells due to the silencing of circ_0007534, whereas it was reversed by SOX5 upregulation in U2OS (increased to 135%) and MG63 cells (increased to 191%). In addition, circ_0007534 depletion induced suppression on clone formation ability was promoted by SOX5 upregulation (Fig. 6E). Next, transwell assay was conducted to investigate cell migration and invasion, and we found that SOX5 upregulation weakened the effect of circ_0007534 knockdown on cell migration and invasion (Fig. 6F and G). Besides, the effect of circ_0007534 knockdown on the levels of EMT markers was attenuated by SOX5 overexpression (Fig. 6H and I). These results suggested that circ_0007534 regulated the growth of OS cells by modulating SOX5 expression.

3.7. Circ_0007534 depletion impeded tumor growth in vivo

To investigate whether circ_0007534 affecting tumor growth *in vivo*, MG63 cells stably transfected with sh-circ_0007534 was injected into male BALB/c-nude mice. Then, tumor volume was calculated every 5 days. The data showed that tumor volume was decreased by 46% in the sh-circ_0007534 group (Fig. 7A). Similarly, circ_0007534 knockdown significantly reduced tumor weight (Fig. 7B). Besides, circ_0007534 depletion significantly reduced the growth of tumors, as assessed by Ki67 expression (Fig. 7C). Fur-

thermore, the levels of circ_0007534, miR-219a-5p, SOX5, and EMT markers were detected in sh-circ_0007534 group and sh-NC group. As expected, the levels of circ_0007534, SOX5, Vimentin, and N-cad were decreased, and the levels of miR-219a-5p and E-cad were increased in sh-circ_0007534 group (Fig. 7C–F). Therefore, circ_0007534 depletion repressed tumor growth *in vivo*.

4. Discussion

Circ_0007534, identified as an oncogene, was highly expressed in a lot of cancers, including pancreatic ductal adenocarcinoma [31], glioma [32], colorectal cancer [33], and breast cancer [34]. Moreover, circ_0007534 promoted cell proliferation and migration, and induced apoptosis in these cancers by regulating the downstream genes' levels. Here, we found that circ_0007534 level was remarkably increased in OS tissues and cells. Furthermore, we suggested that circ_0007534 knockdown counteracted OS cell proliferation, mobility, and EMT. Besides, the depletion of circ_0007534 dramatically repressed the growth of OS tumors *in vivo*. These results were consistent with the previous results [20]. Our results further indicated that circ_0007534 acted as a tumor activator in OS, which might be a potential detection marker and novel therapeutic target for OS.
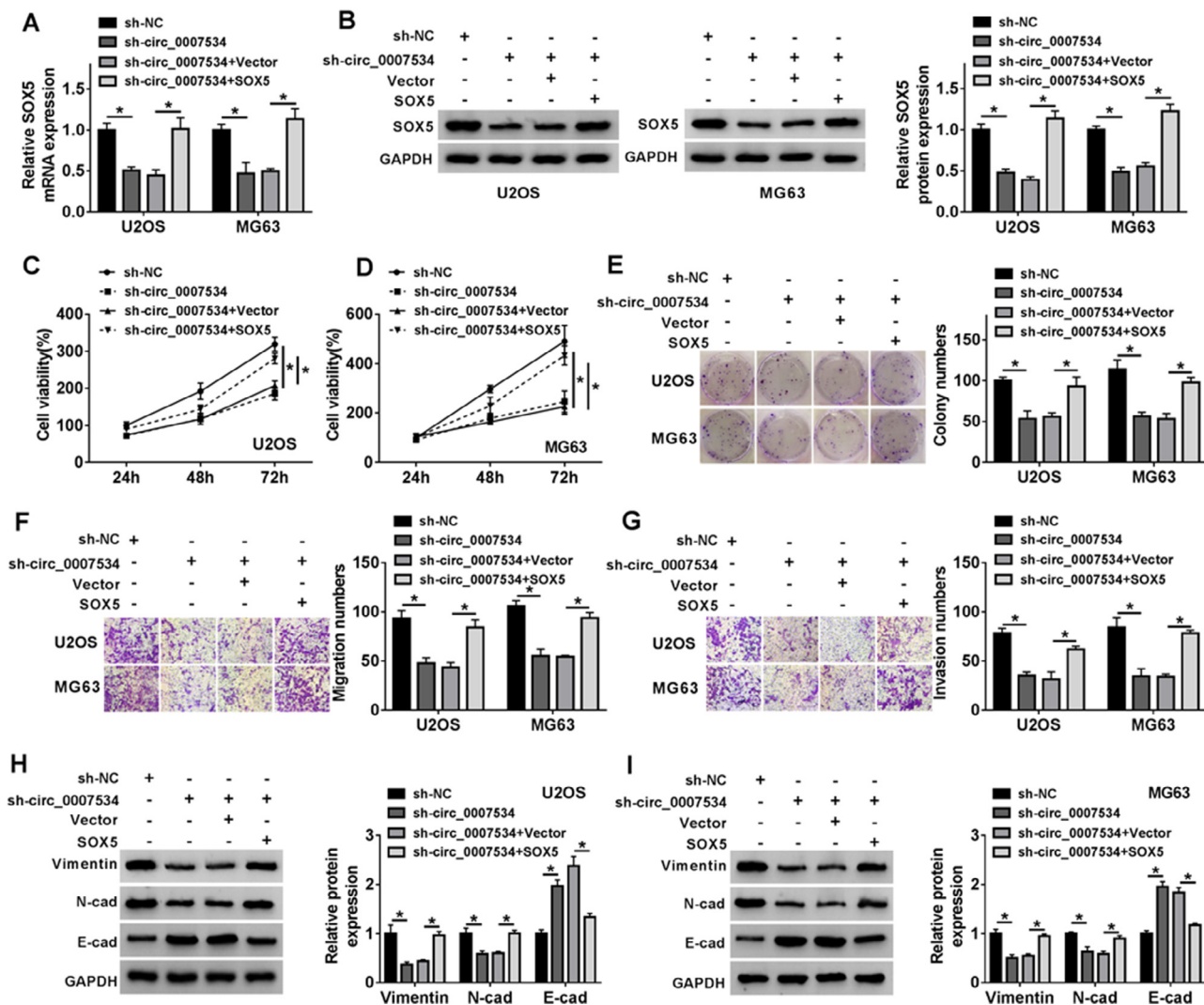
Fig. 6. The function of SOX5 in circ_0007534-regulated OS cell progression. (A and B) The mRNA level and protein level of SOX5 were detected in U2OS and MG63 cells transfected with sh-NC, sh-circ_0007534, sh-circ_0007534 + Vector, or sh-circ_0007534 + SOX5, respectively. (C and D) CCK-8 was employed to determine cell proliferation ability ($P < 0.001$). (E) Colony formation assay was used to assess the clone formation ability. (F and G) Transwell assay was performed to measure cell migratory and invasive abilities. (H and I) The levels of EMT markers were examined by western blot assay. *$P < 0.05$, n = 3.

It is widely accepted that circRNAs serve as a type of sponges for miRNAs in the tumorigenesis of cancers [35]. For example, circ_0035483 targeted miR-335 and suppressed miR-335 expression in renal cancer [36]. Bioinformatics tool starbaseV3.0 predicted the potential target genes of circ_0007534 and found that miR-219a-5p could bound to circ_0007534. And it was further confirmed by the dual-luciferase reporter assay and RIP assay. Moreover, we discovered that circ_0007534 downregulated the level of miR-219a-5p. MiR-219a-5p, identified as a tumor suppressor, inhibited tumorigenesis in many cancers. For instance, miR-219a-5p repressed cell growth and EMT through targeting the estimated glomerular filtration rate (ERFR) in ovarian cancer [37]. Zhuang et al. confirmed that miR-219a-5p attenuated the mobility and EMT of breast cancer cells by modulating the expression of myocardin-related transcription factor A (MRTF-A) [38]. In OS, miR-219a-5p was reported to inhibit proliferation and invasion, and the miR-219a-5p level was significantly reduced in OS tissues and cells [26,39]. These data were in agreement with our results, which suggested that miR-219a-5p was upregulated and suppressed cell growth in OS. Taken together, we hypothesized that circ_0007534 regulated the growth of OS cells through modulating miR-219a-5p expression.

MiRNAs have been reported as a family of sponges for mRNAs to affect cancer development by regulating the expression of downstream genes [40]. MiR-219a-5p has been reported participates in the process of OS progression. In OS cells, miR-219a-5p represses cell migration and invasion via targeting EYA [26]. Besides, miR-219a-5p was negatively regulated by TUG1 and target to PI3K3A to mediate osteosarcoma cell tumorigenesis and metastasis [39]. Then, the bioinformatics tool starbase was employed to forecast the potential target genes of miR-219a-5p. The result showed that SOX5 possessed a complementary sequence with miR-219a-5p. Subsequently, the interaction between miR-219a-5p and SOX5 was verified by the dual-luciferase reporter assay. Furthermore, our findings demonstrated that miR-219a-5p negatively affected SOX5 expression. In this study, we also showed that the SOX5 level was increased in OS tissues and cells. These data were accordant with the previous results [29,41]. CircRNAs/miRNAs/SOX5 pathways played important roles in the OS cell progression, including proliferation, metastasis, and EMT [29,42]. Our results suggested that circ_0007534 impeded miR-219a-5p expression to promote the expression of SOX5. Taken together, circ_0007534 could modulate miR-219a-5p/SOX5 axis to regulate OS cell progression.
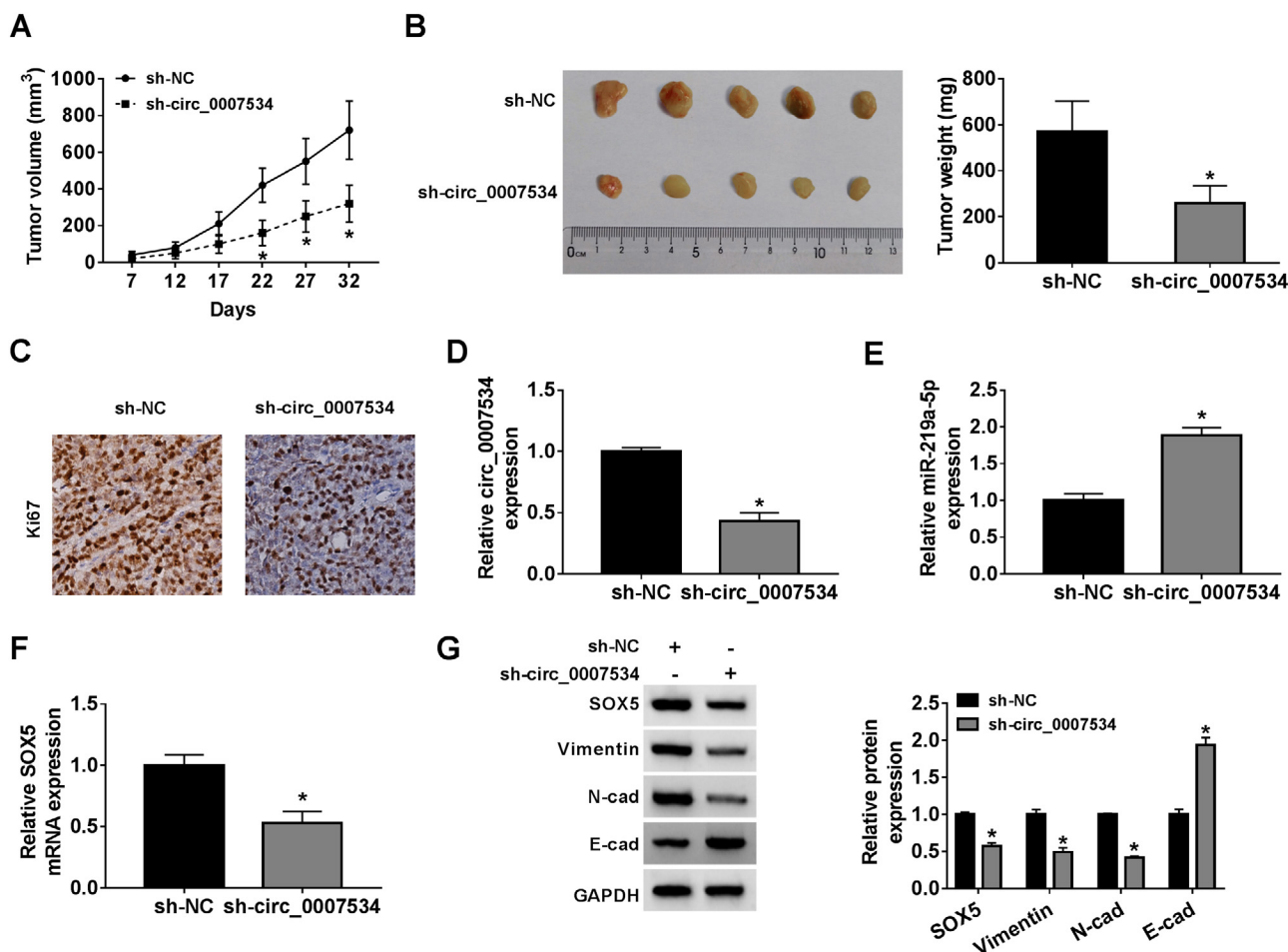
Fig. 7. The effect of circ_0007534 depletion on tumor growth *in vivo*. (A) Tumor volume was calculated in circ_0007534-depleted mice every 5 d. (B) 32 days upon injection, the mice were euthanized, and tumor tissues were photographed and weighted. (C) The proliferation ability of transplanted tumor tissues in nude mice was determined by immunohistochemical analysis of Ki-67. (D–F) The mRNA levels of circ_0007534, miR-219a-5p, and SOX5 were detected by qRT-PCR assay. (G) The protein levels of SOX5 and EMT markers were determined using western blot assay. *$P < 0.05$, n = 3.

Clinical trials supported the validity of miRNA inhibition in clinical applications, such as antagomiRs or miRNA mimics [43]. Artificial circRNAs that contained an array of microRNA response elements could sponge sequesters miRNAs in a manner similar to antagomiR, which provide novel future miRNA-based therapy in OS [44]. In our research, we confirmed that circ_0007534 regulated OS cell progression by miR-219a-5p/SOX5 axis, which indicating that circ_0007534/miR-219a-5p could be used as therapeutics for OS. However, there are still a few concerns that need to be addressed before clinical application. Circ_0007534 expression and function should be confirmed using larger sample sizes, and the mechanism by which circ_0007534 is dysregulated in OS still needs further explored.

In summary, our research indicated that circ_0007534 serves as a novel oncogenic circRNA by sponging miR-219a-5p to upregulate SOX5 expression, thereby promote OS cell proliferation, migration and invasion (Fig. 8). Our findings suggested that targeting circ_0007534/miR-219a-5p axis was a potential treatment strategy for OS treatment and diagnose.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
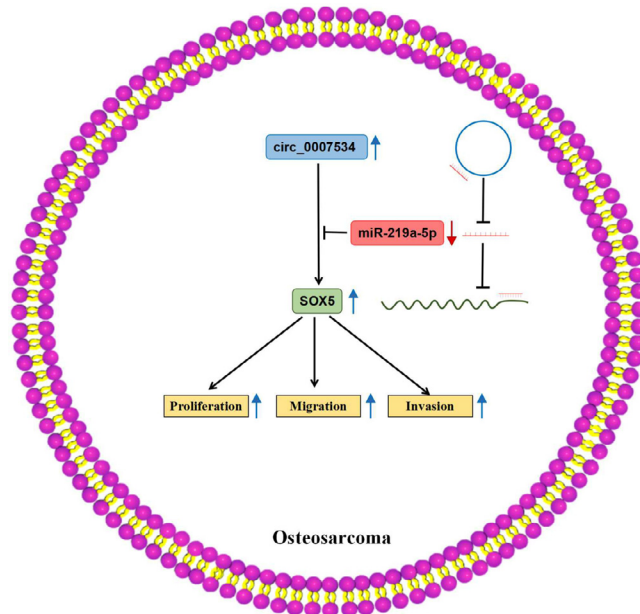
Fig. 8. Circ_0007534 promoted proliferation, migration, invasion by regulating the miR-219a-5p/ SOX5 in OS cells.

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The present study was approved by the ethical review committee of Jining No.1 People's Hospital.

Patient consent for publication

Not applicable.

References

[1] D.D. Moore, H.H. Luu, Osteosarcoma, Cancer Treat. Res. 162 (2014) 65–92.

[2] B. Fuchs, D.J. Pritchard, Etiology of osteosarcoma, Clin. Orthop. Rel. Res. (397) (2002) 40–52.

[3] V.A. Siclari, L. Qin, Targeting the osteosarcoma cancer stem cell, J. Orthop. Surg. Res. 5 (2010) 78.

[4] P.A. Meyers, Muramyl tripeptide (mifamurtide) for the treatment of osteosarcoma, Expert Rev. Anticancer Ther. 9 (8) (2009) 1035–1049.

[5] A. Lamora, J. Talbot, G. Bougras, J. Amiaud, M. Leduc, J. Chesneau, J. Taurelle, V. Stresing, M.C. Le Deley, M.F. Heymann, D. Heymann, F. Redini, F. Verrecchia, Overexpression of smad7 blocks primary tumor growth and lung metastasis development in osteosarcoma, Clin. Cancer Res. 20 (19) (2014) 5097–5112.

[6] P. Picci, M. Mercuri, S. Ferrari, M. Alberghini, A. Briccoli, C. Ferrari, E. Pignotti, G. Bacci, Survival in high-grade osteosarcoma: improvement over 21 years at a single institution, Ann. Oncol. 21 (6) (2010) 1366–1373.

[7] C. Wang, M. Ren, X. Zhao, A. Wang, J. Wang, Emerging roles of circular RNAs in osteosarcoma, Med. Sci. Monit. 24 (2018) 7043–7050.

[8] Y. Zhang, J. Li, Y. Wang, J. Jing, J. Li, The roles of circular RNAs in osteosarcoma, Med. Sci. Monit. 25 (2019) 6378–6382.

[9] X. Li, X.H. Sun, H.Y. Xu, H.S. Pan, Y. Liu, L. He, Circ_ORC2 enhances the regulatory effect of miR-19a on its target gene PTEN to affect osteosarcoma cell growth, Biochem. Biophys. Res. Commun. 514 (4) (2019) 1172–1178.

[10] Z. Yanbin, Z. Jing, CircSAMD4A accelerates cell proliferation of osteosarcoma by sponging miR-1244 and regulating MDM2 mRNA expression, Biochem. Biophys. Res. Commun. 516 (1) (2019) 102–111.

[11] G. Liu, K. Huang, Z. Jie, Y. Wu, J. Chen, Z. Chen, X. Fang, S. Shen, CircFAT1 sponges miR-375 to promote the expression of Yes-associated protein 1 in osteosarcoma cells, Mol. Cancer 17 (1) (2018) 170.

[12] E. Anastasiadou, L.S. Jacob, F.J. Slack, Non-coding RNA networks in cancer, Nat. Rev. Cancer 18 (1) (2018) 5–18.

[13] A. Bhan, M. Soleimani, S.S. Mandal, Long noncoding RNA and cancer: a new paradigm, Cancer Res. 77 (15) (2017) 3965–3981.

[14] Y. Tutar, miRNA and cancer; computational and experimental approaches, Curr. Pharm. Biotechnol. 15 (5) (2014) 429.

[15] J. Li, J. Yang, P. Zhou, Y. Le, C. Zhou, S. Wang, D. Xu, H.K. Lin, Z. Gong, Circular RNAs in cancer: novel insights into origins, properties, functions and implications, Am. J. Cancer Res. 5 (2) (2015) 472–480.

[16] Y. Xi, M. Fowdur, Y. Liu, H. Wu, M. He, J. Zhao, Differential expression and bioinformatics analysis of circRNA in osteosarcoma, Biosci. Rep. 39 (5) (2019).

[17] Y. Li, Q. Zheng, C. Bao, S. Li, W. Guo, J. Zhao, D. Chen, J. Gu, X. He, S. Huang, Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis, Cell Res. 25 (8) (2015) 981–984.

[18] S. Haque, L.W. Harries, Circular RNAs (circRNAs) in health and disease, Genes 8 (12) (2017).

[19] W. Liu, J. Zhang, C. Zou, X. Xie, Y. Wang, B. Wang, Z. Zhao, J. Tu, X. Wang, H. Li, J. Shen, J. Yin, Microarray expression profile and functional analysis of circular RNAs in osteosarcoma, Cell. Physiol. Biochem. 43 (3) (2017) 969–985.

[20] B. Li, X. Li, Overexpression of hsa_circ_0007534 predicts unfavorable prognosis for osteosarcoma and regulates cell growth and apoptosis by affecting AKT/GSK-3β signaling pathway, Biomed. Pharmacother. 107 (2018) 860–866.

[21] B. Liu, J. Li, M.J. Cairns, Identifying miRNAs, targets and functions, Briefings Bioinf. 15 (1) (2014) 1–19.

[22] M.V. Iorio, M. Ferracin, C.G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Ménard, J.P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Nenci, G.A. Calin, P. Querzoli, M. Negrini, C.M. Croce, MicroRNA gene expression deregulation in human breast cancer, Cancer Res. 65 (16) (2005) 7065–7070.

[23] X. Zhao, G.Q. Chen, G.M. Cao, Abnormal expression and mechanism of miR-330-3p/BTG1 axis in hepatocellular carcinoma, Eur. Rev. Med. Pharmacol. Sci. 23 (16) (2019) 6888–6898.

[24] Z. Wang, X. Wu, X. Hou, W. Zhao, C. Yang, W. Wan, L. Chen, miR-548b-3p functions as a tumor suppressor in lung cancer, Lasers Med. Sci. 35 (4) (2020) 833–839.

[25] Y. Ye, L. Zhang, Y. Song, J. Zhuang, G. Wang, J. Ni, S. Zhang, W. Xia, MicroRNA-373 exerts anti-tumor functions in human liver cancer by targeting Rab22a, Mol. Med. Rep. 20 (4) (2019) 3874–3882.

[26] X. Zhu, L. Chen, J. Lin, miR-219a-5p represses migration and invasion of osteosarcoma cells via targeting EYA2, Artif. Cells Nanomed. Biotechnol. 46 (sup3) (2018) S1004–S1010.

[27] R. Ueda, K. Yoshida, T. Kawase, Y. Kawakami, M. Toda, Preferential expression and frequent IgG responses of a tumor antigen, SOX5, in glioma patients, Int. J. Cancer 120 (8) (2007) 1704–1711.

[28] J. You, Q. Zhao, X. Fan, J. Wang, SOX5 promotes cell invasion and metastasis via activation of Twist-mediated epithelial-mesenchymal transition in gastric cancer, OncoTargets Ther. 12 (2019) 2465–2476.

[29] X.H. Hu, J. Dai, H.L. Shang, Z.X. Zhao, Y.D. Hao, SP1-mediated upregulation of lncRNA ILF3-AS1 functions a ceRNA for miR-212 to contribute to osteosarcoma progression via modulation of SOX5, Biochem. Biophys. Res. Commun. 511 (3) (2019) 510–517.

[30] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method, Methods (San Diego, California) 25 (4) (2001) 402–408.

[31] L. Hao, W. Rong, L. Bai, H. Cui, S. Zhang, Y. Li, D. Chen, X. Meng, Upregulated circular RNA circ_0007534 indicates an unfavorable prognosis in pancreatic ductal adenocarcinoma and regulates cell proliferation, apoptosis, and invasion by sponging miR-625 and miR-892b, J. Cell. Biochem. 120 (3) (2019) 3780–3789.

[32] G.F. Li, L. Li, Z.Q. Yao, S.J. Zhuang, Hsa_circ_0007534/miR-761/ZIC5 regulatory loop modulates the proliferation and migration of glioma cells, Biochem. Biophys. Res. Commun. 499 (4) (2018) 765–771.

[33] R. Zhang, J. Xu, J. Zhao, X. Wang, Silencing of hsa_circ_0007534 suppresses proliferation and induces apoptosis in colorectal cancer cells, Eur. Rev. Med. Pharmacol. Sci. 22 (1) (2018) 118–126.

[34] L. Song, Y. Xiao, Downregulation of hsa_circ_0007534 suppresses breast cancer cell proliferation and invasion by targeting miR-593/MUC19 signal pathway, Biochem. Biophys. Res. Commun. 503 (4) (2018) 2603–2610.

[35] M. Dori, S. Bicciato, Integration of bioinformatic predictions and experimental data to identify circRNA-miRNA associations, Genes 10 (9) (2019).

[36] L. Yan, G. Liu, H. Cao, H. Zhang, F. Shao, Hsa_circ_0035483 sponges hsa-miR-335 to promote the gemcitabine-resistance of human renal cancer cells by autophagy regulation, Biochem. Biophys. Res. Commun. 519 (1) (2019) 172–178.

[37] L. Wang, M. Yu, S. Zhao, lncRNA MEG3 modified epithelial-mesenchymal transition of ovarian cancer cells by sponging miR-219a-5p and regulating EGFR, J. Cell. Biochem. 120 (10) (2019) 17709–17722.

[38] C. Zhuang, Y. Yuan, T. Song, H. Wang, L. Huang, X. Luo, H. He, L. Huo, H. Zhou, N. Wang, T. Zhang, miR-219a-5p inhibits breast cancer cell migration and epithelial-mesenchymal transition by targeting myocardin-related transcription factor A, Acta Biochim. Biophys. Sin. 49 (12) (2017) 1112–1121.

[39] Y. Li, T. Zhang, Y. Zhang, X. Zhao, W. Wang, Targeting the FOXM1-regulated long noncoding RNA TUG1 in osteosarcoma, Cancer Sci. 109 (10) (2018) 3093–3104.

[40] Y. Fei, P. Guo, F. Wang, H. Li, Y. Lei, W. Li, X. Xun, F. Lu, Identification of miRNA-mRNA crosstalk in laryngeal squamous cell carcinoma, Mol. Med. Rep. 16 (4) (2017) 4179–4186.

[41] D. Zhang, S. Liu, SOX5 promotes epithelial-mesenchymal transition in osteosarcoma via regulation of Snail, J. B.U.ON. 22 (1) (2017) 258–264.

[42] X. Liu, J. Zheng, Y. Xue, C. Qu, J. Chen, Z. Wang, Z. Li, L. Zhang, Y. Liu, Inhibition of TDP43-mediated SNHG12-miR-195-SOX5 feedback loop impeded malignant biological behaviors of glioma cells, Molecular therapy, Nucleic Acids 10 (2018) 142–158.

[43] N. van Zandwijk, N. Pavlakis, S.C. Kao, A. Linton, M.J. Boyer, S. Clarke, Y. Huynh, A. Chrzanowska, M.J. Fulham, D.L. Bailey, W.A. Cooper, L. Kritharides, L. Ridley, S.T. Pattison, J. MacDiarmid, H. Brahmbhatt, G. Reid, Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study, Lancet Oncol. 18 (10) (2017) 1386–1396.

[44] B. Wan, H. Hu, R. Wang, W. Liu, D. Chen, Therapeutic potential of circular RNAs in osteosarcoma, Front. Oncol. 10 (2020) 370.