Circular RNA circ_001422 Promotes Progression and Metastasis of Osteosarcoma via the miR-195-5p/FGF2/PI3K/Akt Axis

Bingsheng Yang

Southern Medical University

Lutao Li

Southern Medical University

Ge Tong

Sun Yat-Sen University

Zhirui Zeng

Guizhou Medical University

Jianye Tan

Southern Medical University

Zexin Su

Sun Yat-Sen University

Zhengwei Liu

Southern Medical University

Jiezhao Lin

Southern Medical University

Wenwen Gao

Southern Medical University

Jianping Chen

Southern Medical University

Sisi Zeng

Southern Medical University

Guofeng Wu

Southern Medical University

Lin Li

Southern Medical University

Shuang Zhu

Southern Medical University

Qiuzhen Liu

Southern Medical University

Lijun Lin (✉ gost1@smu.edu.cn)

Research

Keywords: Osteosarcoma, Circ_001422, miR-195-5p, FGF2, PI3K/Akt pathway

Posted Date: February 4th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-168231/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Journal of Experimental & Clinical Cancer Research on July 16th, 2021. See the published version at <https://doi.org/10.1186/s13046-021-02027-0>.

Abstract

Background

Circular RNAs (circRNAs) are involved in diverse processes that drive cancer development. Nevertheless, the expression landscape and mechanistic function of circRNAs in osteosarcoma (OS) remain to be studied.

Methods

Bioinformatics analysis and high-throughput RNA sequencing tools were employed to determine differentially expressed circRNAs between OS and adjacent healthy tissues. The expression levels of circ_001422 in clinical specimens and cell lines were measured using qRT-PCR. A total of 55 OS patients were recruited to analyze the association of circ_001422 expression with clinicopathologic features. Loss- and gain-of-function tests were conducted to explore the role of circ_001422 in OS cells. RNA immunoprecipitation, fluorescent in situ hybridization, bioinformatics databases, RNA pull-down assay, dual-luciferase reporter assay, mRNA sequencing, and rescue experiments were conducted to decipher the competitive endogenous RNAs regulatory network dominated by circ_001422.

Results

We characterized a novel and abundant circRNA, circ_001422, which promoted the progression of OS. Circ_001422 expression was dramatically higher in OS cell lines and tissues relative to normal samples. Increased circ_001422 correlated with more advanced clinical stage, larger tumor size, more distant metastases and poorer overall survival of OS patients. Knockdown of circ_001422 markedly repressed proliferation and metastasis and promoted apoptosis of OS cells in vivo and in vitro, whereas overexpression of circ_001422 exerted an opposite effect. Mechanistically, competitive interactions between circ_001422 and miR-195-5p elevated the expression of FGF2 while also initiating the PI3K/Akt signaling pathway. These events enhanced the malignant characteristics of OS cells.

Conclusions

Circ_001422 accelerates OS tumorigenesis and metastasis through modulating the miR-195-5p/FGF2/PI3K/Akt axis, implying that circ_001422 could be therapeutically targeted to treat OS patients.

Background

Osteosarcoma (OS) is the most prevalent primary malignant bone neoplasm causing substantial morbidity in adolescents and children [1]. It is derived from mesenchymal cells and characterized by rapid infiltrating growth, early lung metastasis and high recurrence rate [2]. Studies show that the overall 5-year survival rate of patients with localized OS ranges between 65 and 75%, and is only 20% for those with recurrent and metastatic tumors [3]. Despite advances in OS treatment approaches such as adjuvant chemotherapy and surgical resection, the survival rates have plateaued in the last 3 decades, below

satisfactory rates [4]. Indeed, there are no specific biomarkers for the diagnostic and prognostic prediction of OS. Consequently, molecular studies aiming to identify promising targets for the treatment of OS are urgently needed.

Circular RNAs (circRNAs) are known to regulate various functions of eukaryotic cells [5]. Based on the order of splicing events and different intermediates, two mechanisms exist for the biogenesis of circRNAs: the canonical spliceosome-induced splicing and noncanonical lariat-typed splicing [6, 7]. Accumulating studies have shown that circRNAs modulate diverse physiological and pathophysiological processes via sponging microRNAs (miRNAs), interacting with RNA-binding proteins, and modulating the epigenetic, transcriptional, or translational alterations of target genes [8-11]. Abnormal circRNAs expression has been found to correlate with the pathogenesis of various cancers and exert essential regulatory effects on gene expression, cell invasion, cell cycle, migration, apoptosis, and proliferation [12-14]. Moreover, circRNAs are thought to possess high diagnostic and therapeutic potential given their structural stability, evolutionary conservation, abundance and organ specificity [15, 16]. However, to date, the roles of circRNAs in OS are not clearly known.

This study evaluated the expression profiles of circRNAs in OS as well as in the adjacent non-cancerous tissues using high-throughput sequencing. We found a novel circRNA, designated circ_001422, regulates progression of OS. Higher expression of circ_001422 was remarkably associated with more advanced clinical stage, large tumor size, greater metastases and poorer prognosis. Experimental results indicated that circ_001422 exerted pro-oncogenic effects on OS proliferation and metastasis via targeting the miR-195-5p/FGF2/PI3K/Akt axis. Our findings reveal that circ_001422 is a potential therapeutic target against OS.

Methods

Collection of patient samples

This study was approved by the Ethics Committee of the Affiliated Zhujiang Hospital of Southern Medical University. All participants consented to participate in the study. Paired OS and adjacent non-cancerous tissues were collected from biopsies of 55 patients before commencement of neoadjuvant chemotherapy or radiotherapy. The histological diagnosis of OS was performed independently by two experienced pathologists. Upon extraction, the tissues were immediately frozen in liquid nitrogen and kept at -80 °C. Table 1 shows the patients' clinical characteristics.

Cell culture

Human osteoblast cell line (hFOB1.19) and OS cell lines (143B, U-2 OS, MG-63, MNNG and Saos-2) were purchased from Jennio (Guangzhou, China). Osteoblastic hFOB1.19 cells were cultured in DMEM/F-12 medium (Gibco, USA) with 10% fetal bovine serum (FBS) (BI, Israel), 2.5 mM L-glutamine (Invitrogen, USA) and 0.3 mg/ml geneticin (Gibco, USA). OS cells were maintained in DMEM medium with 1% penicillin/streptomycin (Invitrogen, USA) and 10% FBS in a humidified incubator at 37 °C and 5% CO₂.

RNA sequencing

High-throughput sequencing was performed to identify circRNAs according to the following steps. Total cellular RNA was isolated from 3 matched OS tissues and adjacent non-cancerous tissues with the RNAiso Plus reagent kit (TaKaRa, Japan). Ribosomal RNA was eliminated based on the Ribo-Zero Magnetic Kit (Epicentre, USA), whereas linear RNA digestion was performed using RNase R (Epicentre, USA). The RNAs were broken into small pieces using a fragmentation buffer (Ambion, USA) to generate templates for reverse transcription. Then cDNA was purified with magnetic beads and eluted with EB buffer. Fragments of suitable sizes were selected following agarose gel electrophoresis before amplification to generate the cDNA library. Sequencing of the prepared libraries was conducted on the Illumina HiSeq 2500 platform.

Measurement of RNAs expression

For qRT-PCR analysis of circRNAs and mRNAs, cDNA was synthesized by reverse transcription using the PrimeScrip RT Reagent kit (TaKaRa, Japan). Real-time amplification was conducted using SYBR Premix Ex Taq II (TaKaRa, Japan) in the LightCycler 96 System (Roche, Germany). For miRNA analysis, reverse transcription was achieved using stem-loop RT primers specific for miRNA (GeneChem, China) based on the Evo M-MLV RT kit (Accurate Biology, China), followed by qPCR with SYBR Green Premix Pro Taq HS qPCR kit (Accurate Biology, China). U6 (for miRNA) and GAPDH (for circRNA and mRNA) served as the endogenous controls. Each experiment was repeated thrice. Expression results were analyzed based on the $2^{-\Delta\Delta C_t}$ method. Primer sequences are provided in the Additional file 1: Table S1.

RNase R digestion and nucleic acid electrophoresis

Total RNA (2 µg) was extracted and digested with RNase R (3 U/µg) for 15 min at 37 °C. The control group underwent the same processes as the experimental samples except for the addition of RNase R. cDNA and real-time PCR assay were performed as aforementioned. Additionally, circular and linear transcripts were amplified using specific divergent and convergent primers with or without RNase R. The PCR products with cDNA or genomic DNA (gDNA) as templates were analyzed using 2% agarose gel electrophoresis. DNA fragment bands were visualized with UV trans-illumination and images were taken.

Actinomycin D assay

Briefly, OS cells were maintained in six-well plates for 24 hours after which they were treated with new medium supplemented with 2 µg/mL actinomycin D (Sigma-Aldrich, USA). Total cellular RNA for qRT-PCR analysis was extracted at 0, 4, 8, 12 and 24 hours following actinomycin D treatment.

Nuclear-cytoplasmic fractionation

Extraction and purification of cytoplasmic and nuclear RNAs were performed with the PARIS kit (Life Technologies, USA) as per the manufacturer's protocols. Next, qRT-PCR was performed to quantify the

expression of linear and circular RNAs, with U6 and GAPDH serving as internal references for the nuclear and cytoplasmic RNA, respectively.

Fluorescence in situ hybridization (FISH)

RNA-FISH assay was performed to uncover the subcellular localization of circ_001422. Cy3-labeled circ_001422 probe was constructed by RiboBio (Guangzhou, China). The fluorescent signal was generated using the Fluorescent in Situ Hybridization kit (RiboBio, China) and a Nikon laser scanning confocal microscope was utilized to take pictures.

Oligonucleotides, plasmids, cell transfection and lentiviral infection

The miR-195-5p mimics/inhibitors, miR-195-5p agomir/antagomir and their negative controls (NC) were purchased from RiboBio (Guangzhou, China). The short hairpin RNAs (shRNAs) and NC vectors for circ_001422 and FGF2 silencing were constructed by Geneseeed (Guangzhou, China). The sequences of the most effective shRNAs (sh-circ_001422#2 and sh-FGF2#1) and their NC vectors were cloned into the recombinant lentiviral vectors and thereafter packaged into viral particles. For overexpression lentiviruses, the full-length cDNA sequences of circ_001422 and FGF2 were PCR amplified and cloned into the lentiviral overexpression vectors constructed by Geneseeed (Guangzhou, China). Transient transfections were performed using the EndoFectin Max reagent (Genecopoeia, USA). At 48 h post-lentivirus infection, OS cells were subject to puromycin (2 µg/mL, Invitrogen, USA) or geneticin (500 µg/ml, Gibco, USA) selection for 2 weeks to construct the stable cell lines. Knockdown or overexpression efficiency was verified using qRT-PCR.

Cell proliferation assays

Cell viability of 143B and Saos-2 cells after transfection was assessed using the 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay and colony formation assay. The EdU Apollo 488 kit (RiboBio, China) was utilized to conduct the EdU incorporation assay. Using a Nikon inverted fluorescence microscope, EdU-positive cells were stained green and total nuclei were stained blue.

To evaluate the ability of cells to form colonies, transfected cells were counted and seeded into 6-well plates at the density of 550 cells/well. After 10 days of cultivation, cells were stained using 0.1% crystal violet (Solarbio, China) and the colony numbers were counted.

Flow cytometry

Assessment of the cell cycle of transfected cells was performed by the following procedure. In brief, the cultured cells were harvested, washed twice in phosphate buffer (PBS) and fixed overnight at 4 °C using pre-cooled 75% ethanol. After staining with propidium iodide, cell cycle distribution was determined by a BD flow cytometer. The 4',6-diamidino-2-phenylindole (DAPI) and Annexin-V-allophycocyanin (APC) double staining kit (BestBio, China) were used for cell apoptotic analyses.

Transwell assays

The migratory and invasive abilities of OS cell lines were evaluated using Transwell migration chambers (Costar, USA) and Transwell invasion chambers pre-coated with 50 μ L of 2 mg/ml Matrigel (BD Biosciences, USA), respectively. Briefly, transfected cells (4×10^4 cells/well for migration, 8×10^4 cells/well for invasion) suspended in 200 μ L of serum-free DMEM medium were seeded into the upper chambers. Meanwhile, 600 μ L of DMEM medium supplemented with 10% FBS used as the attractant was introduced into the lower chambers. After a 24-h culture, cells adhering to the lower surface of the membranes were fixed with paraformaldehyde (4%) and stained using crystal violet (0.1%), whereas cotton swabs were used to wipe off cells on the upper surface. At least three random fields of cells that had migrated or invaded to the lower surface were photographed under an inverted light microscope.

Western blot

Total proteins of homogenized tissues or cell lysates were extracted using ice-cold RIPA solution (Fudebio, China) and protease inhibitors (Fudebio, China). The proteins were diluted to equal concentrations, denatured in boiling water bath, detached by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline-Tween (TBST) buffer and then incubated overnight at 4 °C with several primary antibodies against cleaved CASP3 (1:1000) (Affinity Biosciences, USA), CCND1 (1:1200) (Proteintech, China), CDK4 (1:2000) (Abcam, USA), BAX (1:1000) (Abcam, USA), BCL2 (1:800) (Abcam, USA), E-cadherin (1:2000) (Proteintech, China), N-cadherin (1:2000) (Proteintech, China), Vimentin (1:3000) (Proteintech, China), FGF2 (1:200) (Santa Cruz Biotechnology, USA), PI3K (1:1000) (Cell Signaling Technology, USA), phosphorylated PI3K (p-PI3K, 1:1000) (Cell Signaling Technology, USA), Akt (1:1000) (Cell Signaling Technology, USA), phosphorylated Akt (p-Akt, 1:1000) (Cell Signaling Technology, USA) and GAPDH (1:10000) (Proteintech, China). After four washes with TBST buffer, the membranes were re-incubated for 60 min at 25 °C with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000) (Bioss, China). The protein bands were visualized with a chemiluminescence imaging system (Bio-Rad, USA).

Animal models

The protocols for animal experiments were approved by the Medical Ethics Committee of Southern Medical University. Four weeks old female BALB/c athymic nude mice were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China) and reared under pathogen-free facility with adequate standard food and water. A subcutaneous xenograft tumor model was established by subcutaneously injecting 5×10^6 143B stable cells into the right limbs of nude mice ($n = 5$ per group). The tumor volume was measured every 3 days and calculated using the formula: volume = length $\times$ width² $\times$ 0.5. For the lung metastatic model, the abovementioned cells were injected into nude mice via the tail vein (2×10^6 143B cells/ per mouse, $n = 5$ per group). To evaluate the effect of miR-195-5p in vivo, the miR-195-5p antagomir/agomir or negative controls were injected intratumorally (for the subcutaneous xenograft tumor model) or intravenously via tail vein (for the lung metastatic model) twice a week for 2

weeks, following the manufacturer's recommendations (RiboBio, China). Tumor tissues were extracted from sacrificed mice four weeks after inoculation.

Hematoxylin and eosin (H&E) staining

Paraffin-embedded thin 4- μ m lung sections containing metastatic nodules were dewaxed using xylene and thereafter rehydrated using gradient alcohol. Then the sections were stained using hematoxylin and eosin for general histological examination by standard procedures.

Tissue expression of target proteins

The expression of target proteins in tissue samples of OS patients or xenograft animal models was determined using immunohistochemistry (IHC) as previously described [17]. The tissues were incubated with primary antibodies against FGF2 (1:100), Ki-67 (1:200), PCNA (1:300), N-cadherin (1:200), E-cadherin (1:200), Vimentin (1:300). Apart from FGF2 (Santa Cruz Biotechnology, USA) the rest of the antibodies were purchased from the same manufacturer (Proteintech, China). After washing the tissue sections, they were re-incubated in the presence of HRP-conjugated secondary antibodies (1:200) (Servicebio, China) and thereafter stained with Diaminobenzidine (Zhongshan Golden Bridge, China). The tissue images were observed and captured using an orthophoto microscope.

TUNEL assay

To assess apoptotic DNA fragmentation, xenograft tumor tissues were first fixed for 24 h with 4% paraformaldehyde and thereafter embedded in paraffin. Apoptosis in situ was evaluated based on the TUNEL Apoptosis Assay kit (Alexa Fluor 488) (Yeasen, China). Corresponding photos of the apoptotic cells were captured using a fluorescence microscope. The analyses were performed in at least three random fields for each sample.

Bioinformatics analysis

To predict the potential miRNAs binding with circ_001422, five online bioinformatics tools were used; starBase (<http://starbase.sysu.edu.cn/>), miRanda (<http://www.microrna.org/>), TargetScan (http://www.targetscan.org/vert_72/), RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>), and RNA22 (<https://cm.jefferson.edu/rna22/>). The potential downstream target mRNAs for miR-195-5p were predicted following analysis of miRanda, RNAhybrid, TargetScan and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>) databases.

RNA immunoprecipitation (RIP)

Transfected cells were washed twice in ice-cold PBS and thereafter lysed in RIP lysis solution containing RNase and protease inhibitors. Incubation of the cell lysates (200 μ l) was done overnight at 4 °C with immunoprecipitation buffer containing anti-Argonaute2 (anti-Ago2) conjugated magnetic beads

(Millipore, USA) or negative anti-IgG (Millipore, USA). Subsequently, the immunoprecipitated RNAs were extracted and purified to determine the abundance of target RNAs using qRT-PCR.

RNA pull-down assay

A control probe and a biotinylated circ_001422 probe were constructed by RiboBio (Guangzhou, China). The probes were coated with C-1 magnetic beads (Life Technologies, USA) following 2 h incubation at room temperature with the aforementioned beads. The transfected cells were harvested and treated with ice-cold lysis solution, and thereafter incubated overnight at 4 °C with circ_001422 or oligo probes. Finally, the pull-down products were extracted and purified using the RNeasy Mini kit (Qiagen, USA). The abundance of circ_001422 and miRNAs in the RNA complexes was evaluated by qRT-PCR.

Luciferase reporter assay

The circ_001422 or FGF2 fragments with the mutant-type (MUT) or wild-type (WT) miR-195-5p binding sites were subcloned downstream of the Renilla gene in psiCHECK-2 dual-luciferase reporter vector (Geneseeed, China). The 143B and Saos-2 cells in the logarithmic growth phase were co-transfected with reporter vectors and miR-195-5p mimics or NC mimics. After 48 h incubation, the Dual-Luciferase Reporter Assay System (Promega, USA) was utilized to measure the luciferase activity.

Statistical analysis

Continuous variables were presented as mean $\pm$ standard deviation. All analyses were performed using SPSS 20.0 software (IBM, USA). Difference between groups was analyzed using unpaired Student's t-test, or one-way analysis of variance (ANOVA) and Tukey's test. Survival was analyzed by Kaplan-Meier curves and log-rank test. The correlations among circ_001422, miR-195-5p and FGF2 were determined using Pearsons correlation analyses. $P < 0.05$ was considered significant.

Results

Identification and characterization of circ_001422 in OS

To investigate the potential involvement of circRNAs in OS, three matched OS samples and adjacent non-cancerous tissues were subjected for high-throughput sequencing analysis. Based on P -value < 0.05 and $|\text{fold change}| \geq 2$, 374 differentially expressed circRNAs candidates were identified, including 192 upregulated and 182 downregulated circRNAs in OS tissues relative to matched non-cancerous tissues. The top ten most upregulated and downregulated circRNAs are presented in the clustered heatmap (Fig. 1a). In combination with the circRNAs annotation in the circBase database (<http://www.circbase.org/>), circ_001422 was identified to locate on chromosome 4 (chr4: 1900626-1935262) with a molecular weight of 1703 bp and was generated by circularization of exons 2-7 of the host gene NSD2. The existence and structure of circ_001422 was validated through primer specific amplification and sequencing of the PCR products using the Sanger sequencing platform (Fig. 1b). In addition, qRT-PCR further demonstrated an

upregulated expression of circ_001422 in OS cells relative to normal osteoblast cells (Fig. 1c). Thus, circ_001422 was selected for further analyses.

CircRNAs are member of ncRNAs, characterized by stable structures due to absence of 5' cap or a 3' polyadenylated tail. Herein, circ_001422 and NSD2 mRNA were amplified using divergent and convergent primers respectively. After RNase R treatment, there was a sharp decrease in the level of linear NSD2 mRNA, whereas that of circ_001422 remained stable (Fig. 1d). Using cDNA and gDNA as templates, NSD2 mRNA was amplified by convergent primers in both cDNA and gDNA, whereas circ_001422 was amplified by divergent primers only in cDNA (Fig. 1e). In addition, actinomycin D based transcription inhibition revealed that circ_001422 displayed a longer half-life than NSD2 mRNA (Fig. 1f). Nuclear-cytoplasmic fractionation and FISH assays further revealed that circ_001422 was predominantly located in the cytoplasm (Fig. 1g and h).

Correlation of circ_001422 expression with clinical characteristics

The upregulated expression of circ_001422 was validated following analysis of cohort OS tissues and matched non-cancerous tissues ($n = 55$) (Fig. 2a). In particular, overexpression of circ_001422 was observed in 40 (72.7%) of the 55 OS tissues relative to their matched non-cancerous tissues (Fig. 2b). Further analyses revealed that overexpression of circ_001422 in OS tissues positively correlated with advanced clinical stage (I vs. III-IV, $P = 0.026$; II vs. III-IV, $P = 0.048$, Fig. 2c), large tumor size ($P = 0.003$, Fig. 2d) and distant metastasis ($P = 0.005$, Fig. 2e). No significant relationship was found for other parameters including age ($P = 0.520$), gender ($P = 0.150$) and primary tumor location ($P = 0.818$, Fig. 2f) (Table 1). Kaplan-Meier survival analysis and log-rank test of the OS patients in high and low circ_001422 expression groups (based on the median circ_001422 expression) revealed that patients with higher circ_001422 expression exhibited worse overall survival rate (Fig. 2g). Moreover, receiver operation curve (ROC) analysis was used to determine the diagnostic potential of circ_001422 based on the 55 paired tissues samples. As showed in Fig. 2h, the area under the ROC curve (AUC) was 0.752. This implies that circ_001422 is a relatively accurate diagnostic marker for OS.

Circ_001422 promotes proliferation and metastasis of OS cells

Loss-of-function assays were first performed through transfecting 143B and Saos-2 cell lines with three shRNAs designed to specifically target the junction sites of circ_001422. Among the shRNAs, sh-circ_001422#2 showed the most potent knockdown efficiency and was used in further analyses, named sh-circ_001422 (Additional file 2: Figure S1a). After knockdown of circ_001422, the DNA synthesis and colony formation abilities of 143B and Saos-2 cells were both dramatically repressed (Fig. 3a and b). Flow cytometry analyses further revealed that circ_001422 knockdown arrested the cell cycle at G0-G1 phase (Fig. 3c) and enhanced OS cell apoptosis (Fig. 3d). Moreover, the results of Transwell assays showed that circ_001422-knockdown cells had a robustly decreased capacity to migrate and invade (Fig. 3e). By western blot (Fig. 3f), we found that circ_001422 silencing could decrease the expression of CCND1, CDK4, BCL2, N-cadherin and Vimentin, but upregulated the expression of cleaved CASP3, BAX and E-cadherin. Given the physiological role of these proteins, these findings suggest that circ_001422

regulates cell cycle-, apoptosis- and epithelial-mesenchymal transition (EMT), all of which impact on OS development. For in vivo experiments, 143B cells transfected with stable sh-circ_001422 lentivirus or control lentivirus were injected subcutaneously (for the subcutaneous xenograft tumor model) or intravenously via tail vein (for the lung metastatic model) into the nude mice. Mice injected with circ_001422-knockdown cells exhibited significantly decreased tumor volume and weight (Fig. 3g-i), as well as decreased number of metastatic pulmonary colonies relative to mice in the control group (Fig. 3j and k). Immunohistochemistry analysis further revealed that circ_001422 knockdown reduced the expression of Ki-67, PCNA, Vimentin and N-cadherin proteins, but upregulated those of E-cadherin proteins (Fig. 3l). More apoptotic cells were detected in the circ_001422-knockdown tumors as compared to the control tumors by the TUNEL assay (Additional file 2: Figure S1b).

In addition, OS cell lines were also transfected with the circ_001422-overexpression vectors or the control vectors, and the overexpression efficiency was validated using qRT-PCR analysis (Additional file 3: Figure S2a). The results of gain-of-function assays consistently showed that overexpression of circ_001422 remarkably suppressed apoptosis but promoted proliferation and metastasis of OS cells (Fig. 4, Additional file 3: Figure S2b). Collectively, these findings demonstrate that circ_001422 regulates the oncogenic and metastatic properties of OS cells.

Circ_001422 sponges miR-195-5p

Accumulating studies have revealed that circRNAs possess abundant binding sites for miRNAs, thus may serve as miRNA sponges. Given that circ_001422 was primarily and stably located in the cytoplasm, we speculated that circ_001422 might be a competitive endogenous RNA. RIP assay for Ago2 was performed by transfecting 143B cells with the Ago2-overexpressing plasmid and the control vector. The qRT-PCR data demonstrated that compared to the control group, endogenous circ_001422 was immunoprecipitated more effectively in the 143B clones overexpressing Ago2 plasmids (Fig. 5a). This suggests that circ_001422 might bind to miRNAs via Ago2 protein. Following, 4 miRNA candidates were identified by overlapping the prediction results from 5 bioinformatics databases (TargetScan, miRanda, starBase, RNAhybrid and RNA22) (Fig. 5b). To validate our findings, we performed the pull-down assay using biotinylated probe specific for circ_001422. As presented in Fig. 5c and d, overexpression of circ_001422 markedly enhanced the pull-down efficiency, and only miR-195-5p was remarkably pull-downed by the biotinylated circ_001422 probe in both Saso-2 and 143B cells. After validating the transfection efficiencies of miR-195-5p mimics and inhibitors (Additional file 4: Figure S3), we performed dual-luciferase reporter assay, which demonstrated that miR-195-5p mimics suppressed the luciferase activity of wild-type reporter for circ_001422 (circ_001422-WT vector) as opposed to the mutant-type reporter vector (circ_001422-MUT vector) (Fig. 5e and f). Moreover, an anti-Ago2 RIP assay revealed that Ago2, circ_001422 and miR-195-5p were all efficiently immunoprecipitated in the presence of anti-Ago2 but not anti-IgG antibodies. There was a significant enrichment of circ_001422 and miR-195-5p in 143B and Saos-2 cells transfected with miR-195-5p mimics relative to the NC mimics group (Fig. 5g-i). The qRT-PCR further revealed exceptionally low levels of miR-195-5p in 55 OS samples relative to their paired non-cancerous tissues, and its expression was inversely related to circ_001422 expression (Fig. 5j and k).

Compared to normal osteoblast cells, miR-195-5p expression was also downregulated in OS cells (Fig. 5l).

The pro-oncogenic effect of circ_001422 depends on miR-195-5p

To determine whether circ_001422 facilitated OS malignant phenotype via targeting miR-195-5p, functional rescue experiments were subsequently performed. The miR-195-5p inhibitors were first transfected into the circ_001422-knockdown cells. According to the EdU incorporation and colony formation assays, circ_001422 knockdown dramatically decreased the EdU-positive cells and inhibited colony formation in both 143B and Saos-2 cells, whereas miR-195-5p silencing reversed these phenomena (Fig. 6a and b). Flow cytometric analyses revealed that reintroduction of miR-195-5p inhibitors abolished the increased proportions of G0-G1 and apoptotic cells induced by circ_001422 knockdown (Fig. 6c and d). The suppressive effects of circ_001422 knockdown on the migration and invasion of OS cells were effectively weakened by exogenous downregulation of miR-195-5p expression (Fig. 6e and f). Western blot further validated the downregulated expression of cycle-associated proteins (CCND1 and CDK4), anti-apoptotic protein (BCL2) and mesenchymal phenotype proteins (N-cadherin and Vimentin) following circ_001422 knockdown but miR-195-5p inhibition restored normal expression of these proteins. In contrast, miR-195-5p knockdown remarkably reversed the sh-circ_001422-mediated upregulated expression of pro-apoptotic proteins (cleaved-CASP3 and BAX) and epithelial cell marker (E-cadherin) (Fig. 6g). Furthermore, functional rescue experiments revealed that miR-195-5p mimics could markedly attenuate the promotive effects of circ_001422 on cell proliferation and metastasis (Additional file 5: Figure S4). Collectively, these findings demonstrate that circ_001422 aggravates the malignant phenotypes of OS cells, at least in part by miR-195-5p sponging.

Circ_001422 positively regulates FGF2 expression in OS cells via miR-195-5p sponging

Given above results, we further explored probable downstream molecule regulated by the circ_001422/miR-195-5p axis. Prior evidence demonstrates that circRNAs function as miRNAs sponge to terminate their regulatory effects on target genes. Meanwhile, the interaction between miRNA and target mRNA usually leads to the degradation or post-translational repression of the mRNA. Thus, we hypothesized that there would be a positive association between the expressions of circ_001422 and its target genes. We first analyzed the expression profile of mRNAs through sequencing of the differentially expressed genes (DEGs) in 143B cells with or without circ_001422 knockdown. A total of 2758 downregulated DEGs were screened out based on P -value < 0.05 and fold change ≤ -2 (Fig. 7a). Further bioinformatic analysis of four databases including TargetScan, miRanda, RNAhybrid and miRTarBase revealed 466 potential miR-195-5p target genes (Fig. 7b). After overlapping the mRNA-seq-identified downregulated genes ($n = 2758$) and the potential miR-195-5p target genes as predicted by bioinformatics databases ($n = 466$), we identified 71 intersection genes which were further subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. As presented in Fig. 7c, we found the “PI3K/Akt signaling pathway” was the most enriched pathway except for the “MicroRNAs in cancer”. All

genes included in the “PI3K/Akt signaling pathway” (CCND2, ITGA2, PRKAA1, FGF2, LAMC1 and GNB1) were considered as mRNA candidates that might be regulated by the circ_001422/miR-195-5p axis.

Subsequently, the abundance of these 6 candidate mRNAs in 143B and Saos-2 cells was determined using qRT-PCR. Compared to controls, circ_001422 knockdown remarkably altered the mRNA levels of FGF2 and LAMC1 in both 143B and Saos-2 cells (Fig. 7d and e). After introduction of exogenous circ_001422 into OS cells, both 143B and Saos-2 cells displayed a significant increase of FGF2 mRNA, whereas no significant change of LAMC1 mRNA was detected in 143B cells (Fig. 7f and g). Interestingly, FGF2 has been widely identified as an oncogene to participate in tumorigenesis and metastasis of multiple cancer types [18-20]. Thus, we presumed that circ_001422 might contribute to the malignant progression of OS via protecting FGF2 from degradation induced by miR-195-5p.

According to the western blot, circ_001422 silencing effectively reduced the FGF2 protein and inhibited the phosphorylation of PI3K and Akt (Fig 7h). On the other hand, overexpression of circ_001422 significantly enhanced the FGF2 expression and PI3K/Akt signaling pathway (Fig. 7i). Additionally, introduction of exogenous miR-195-5p mimics in 143B and Saos-2 cells downregulated the expression of FGF2, p-PI3K and p-Akt proteins. Conversely, miR-195-5p silencing exerted opposite effect in both cell lines (Fig. 7j and k). As expected, circ_001422-mediated the promotive effect on FGF2/PI3K/Akt axis could be attenuated following introduction of miR-195-5p (Fig. 7l and m). Next, the putative binding sites of FGF2 for miR-195-5p were revealed (Fig. 7n). Transfection of FGF2-WT reporter vector with miR-195-5p mimics but not scrambled oligonucleotide dramatically decreased the luciferase activity. Mutation in binding sequence however abolished the disruption of luciferase activity (Fig. 7o). Expressions of FGF2 mRNA and corresponding proteins were all upregulated in OS tissues relative to corresponding non-cancerous tissues (Fig. 7p and q, Additional file 6: Figure S5a). Besides, FGF2 expression showed significantly positive and negative corrections with circ_001422 expression and miR-195-5p expression in OS tissues, respectively (Additional file 6: Figure S5b and S5c).

Circ_001422 performs its function via the miR-195-5p/FGF2 axis

We performed rescue experiments to explore whether circ_001422 promoted the malignant properties of OS cells by competitively interacting with miR-195-5p and then upregulating expression of FGF2. Overexpression and knockdown efficiencies of FGF2 were verified by qRT-PCR (Additional file 7: Figure S6a and S6b). First, stable circ_001422-silenced cell lines were transfected with miR-195-5p inhibitors alone or together with sh-FGF2. The results of EdU assay were consistent with previous data that reduced proliferation abilities of sh-circ_001422 stable cells were significantly attenuated by the miR-195-5p inhibitors, whereas these rescued effects of miR-195-5p inhibitors on sh-circ_001422 stable cells could be effectively reversed by co-transfection with sh-FGF2 (Fig. 8a). Also, miR-195-5p silencing could abolish the cell arrest and cell apoptosis induced by circ_001422 knockdown, while reintroduction of sh-FGF2 successfully attenuated these effects of miR-195-5p silencing (Additional file 7: Figure S6c and S6d). Transwell assays revealed that the inhibitory effects of circ_001422 depletion on cell migratory and invasive capacities were dramatically weakened through exogenously downregulating miR-195-5p.

However, stable circ_001422-silenced cells after co-transfection with miR-195-5p inhibitors and sh-FGF2 restored the suppressive effects of sh-circ_001422 on cell motility (Fig. 8b, Additional file 7: Figure S6e). Similar trends were observed for the regulatory effects of the circ_001422/miR195-5p/FGF2 axis on the expression of cell cycle-, apoptosis-, EMT- and PI3K/Akt signaling pathway-associated proteins (Fig. 8c). For in vivo experiments, compared to controls, circ_001422 deficiency decreased subcutaneous tumor formation and lung metastases. Administration of miR-195-5p antagomir abolished the effects of circ_001422 silencing and accelerated growth and metastasis of OS cells, while knockdown of FGF2 further reversed the pro-oncogenic role of miR-195-5p antagomir (Fig. 8d-h). IHC analysis (Fig. 8i) and TUNEL assays (Additional file 7: Figure S6f) further validated the role of circ_001422/miR195-5p/FGF2 axis in OS. Moreover, functional rescue experiments were performed in stable circ_001422-overexpression cell lines. The results, as illustrated in Fig. 9 and Additional file 8: Figure S7, revealed that circ_001422 competitively interacted with miR-195-5p to upregulate FGF2 expression and activate PI3K/Akt signaling pathway, thus facilitating the metastatic and proliferative abilities of OS cells. A schematic diagram depicts the role of the circ_001422/miR-195-5p/FGF2 axis in OS (Fig. 10).

Discussion

OS is the most prevalent malignant bone tumor. It is highly metastatic, resulting in very poor survival rate [2]. Approximately 80% of OS patients exhibit subclinical pulmonary micrometastases at the time of diagnosis [21]. The lack of accurate biomarkers has further constrained efforts to improve the clinical outcome of OS. Recently, the dysregulation of ncRNAs in OS has generated significant interest from the science community. Using global miRNA expression microarrays, Duan et al. analyzed the miRNA expression profiles in drug-resistant and non-resistant OS cells, and revealed that miR-15b inhibitors might contribute to the treatment of drug-resistant OS when co-administered with doxorubicin [22]. In a separate research, it was found that upregulation of long non-coding RNA (lncRNA) TP73-AS1 was closely related to advanced Enneking stage, adverse pathological features and distant metastases of OS [23]. Unlike these two kinds of ncRNAs, circRNAs have emerged as more reliable and promising tumor biomarkers owing to their exceptionally stable structure. Advanced genome sequencing techniques have validated the roles of circRNAs in multiple cancer types including hepatocellular carcinoma [24], gastric cancer [25], colorectal cancer [26] and lung squamous carcinoma [27]. However, to date, the expression profile and role of circRNAs in OS are not well understood.

This study presents for the first time that circ_001422 contributes to the malignant progression of OS. It is widely accepted that circRNAs are unorthodox RNA species generated by the alternative splicing of pre-mRNA [28]. There are three main classes of circRNAs: exonic circular RNAs, exon-intron circular RNAs and intronic circular RNAs [29]. Sanger sequencing revealed that circ_001422 was generated via back-splicing and covalent bonding of 3' and 5' ends of exons 2-7 of NSD2. Interestingly, research shows that NSD2 is an important oncogene that drives development of multiple cancer types by catalyzing histone-lysine methylation and disruption of chromatin integrity [30-32]. Additionally, linear NSD2 regulates EMT and the expression of BCL2 and SOX2 proteins, which facilitates the cell survival, metastasis, and chemotherapy resistance in OS [33, 34]. Herein, we revealed the upregulated expression of circ_001422 in

OS tissues and cells using high-throughput sequencing and qRT-PCR. Clinicopathological characteristics of 55 OS patients revealed that higher circ_001422 positively correlated with advanced clinical stage, tumor size and distant metastasis. Functional analyses further validated the role of circ_001422 in not only promoting proliferation and metastasis of OS cells, but also modulating apoptosis of these cells both in vivo and in vitro. These findings underline the significant relationship between the alternative splicing forms of NSD2 transcripts and undesirable aspects of OS.

The subcellular distribution of RNA is intimately tied to its biological function [35]. Increasing evidence shows that cytoplasmic circRNAs are sponges for miRNAs, which repress translation or induces degradation of the corresponding mRNAs following binding of Ago2 protein [36, 37]. Herein, we found circ_001422 is mainly a cytoplasmic RNA of OS cells. In addition, circ_001422 can recognize and bind Ago2 protein, suggesting that circ_001422 might exert its regulatory functions by classically harboring miRNAs. Among 4 candidate miRNAs predicted by bioinformatics databases, only miR-195-5p was further validated to possess a high binding capacity with circ_001422. Even so, the involvement of miR-195-5p in the pathogenesis of multiple tumors is not a new phenomenon [38, 39]. Nevertheless, the function and mechanism underlying the involvement of both miR-195-5p and circRNAs in cancer is scarcely reported. Herein, we found that miR-195-5p was remarkably decreased in clinical OS tissue samples and was inversely correlated with circ_001422 expression. Functional rescue experiments further revealed that miR-195-5p inhibitors substantially reversed the inhibitory effects of circ_001422 depletion on OS proliferation and metastasis, whereas miR-195-5p mimics abolished the promotive effects of circ_001422 overexpression.

Meanwhile, we found FGF2 is a downstream target for miR-195-5p in OS cells. Consistent with the theory of competing endogenous RNA, our current study uncovered a positive correlation of FGF2 with circ_001422 and a negative correlation of FGF2 with miR-195-5p in clinical OS tissues. Bioinformatic analysis and functional experiments revealed that circ_001422 upregulated FGF2 expression by sponging miR-195-5p. This also triggered activation of PI3K/Akt pathway to accelerate OS progression, including suppressing apoptosis and promoting proliferation, migration and invasion. FGF2, also known as bFGF, was among the first angiogenic factors to be identified [40]. Evidence indicates that FGF2 is implicated in diverse biological processes, including neurodevelopment, immune homeostasis, angiogenesis and neoplastic transformation [41]. Although the role of FGF2 in malignancies remains controversial, FGF2 has been proposed as a pro-oncogenic regulator during the development of OS [42-44]. In the present study, abnormally elevated levels of FGF2 mRNA and protein were consistently observed in OS tissues and cells. Furthermore, the PI3K/Akt pathway was verified to involve in the carcinogenesis mediated by the circ_001422/miR-195-5p/FGF2 axis in OS. It is well established that PI3K/Akt cascade controls basic intracellular processes. Abnormal activation of this pathway is quite prevalent in various neoplasms [45, 46].

There are several limitations to note in our study. First, the subcutaneous xenograft and metastatic lung models used in this study may not fully mimic the natural OS microenvironment. As such, some of our findings may not be reproducible in the natural disease state. Second, less invasive or non-invasive

detections of highly specific biomarkers in body fluids would be more convenient and acceptable approaches. Previous evidence has demonstrated that some circRNAs have the potential to be stably detected by liquid biopsies [47, 48]. Thus, the expression profiles of circ_001422 in body fluids such as serum, plasma and urine warrant further investigation. Finally, we have only focused on the role of the circ_001422 in tumor proliferation and metastasis. More detailed researches are necessary to explore the impact of circ_001422 on other malignant biological behaviors of OS cells, including chemoresistance, angiogenesis and immune escape.

Conclusions

In summary, this research found that circ_001422 promotes progression and metastasis of OS via the miR-195-5p/FGF2/PI3K/Akt axis. Our findings elucidate a novel regulatory network that may offer new insight into the identification of potential biomarkers or therapeutic targets for OS treatment.

Abbreviations

circRNAs: Circular RNAs; OS: Osteosarcoma; circ_001422: Hsa_circ_001422; miR-195-5p: microRNA-195-5p; FGF2: Fibroblast growth factor 2; PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; ncRNAs: Non-coding RNAs; miRNAs: MicroRNAs; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; qRT-PCR: Quantitative real-time polymerase chain reaction; cDNA: Complementary DNA; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; gDNA: Genomic DNA; FISH: Fluorescence in situ hybridization; NC: Negative control; shRNAs: Short hairpin RNAs; EdU: 5-ethynyl-2'-deoxyuridine; PBS: Phosphate buffer; APC: Annexin-V-allophycocyanin; DAPI: 4',6-diamidino-2-phenylindole; TBST: Tris-buffered saline-Tween; CCND1: Cyclin D1; CDK4: Cyclin dependent kinase 4; CASP3: Caspase 3; BAX: B cell lymphoma/leukemia-2 associated X; BCL2: B cell lymphoma/leukemia-2; HRP: Horseradish peroxidase; H&E: Hematoxylin and eosin; IHC: Immunohistochemistry; PCNA: Proliferating cell nuclear antigen; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; RIP: RNA immunoprecipitation; Ago2: Argonaute2; WT: Wild-type; MUT: Mutant-type; NSD2: Nuclear receptor binding SET domain protein 2; KEGG: Kyoto Encyclopedia of Genes and Genomes; CCND2: Cyclin D2; ITGA2: Integrin subunit alpha 2; PRKAA1: Protein kinase AMP-activated catalytic subunit alpha 1; LAMC1: Laminin subunit gamma 1; GNB1: G protein subunit beta 1; EMT: Epithelial-mesenchymal transition; lncRNA: Long non-coding RNA

Declarations

Acknowledgements

Not applicable.

Authors' contributions

LJL and QZL conceived and designed the experiments. BSY, LTL and GT performed most of the experiments and data analysis. BSY and LTL drafted the manuscript. ZRZ, JYT, ZXS, ZWL and JZL performed the bioinformatics analysis and revised the work critically for important intellectual content. WWG, JPC and SSZ helped with the in vivo experiments. GFW, LL and SZ collected clinical sample and information. All authors read and approved the final manuscript.

Funding

The present study was supported by the Science and Technology Program of Guangzhou, China (grant no. 201704020129).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experiment protocols were approved by the Ethics Committee of the Affiliated Zhujiang Hospital of Southern Medical University and conducted following the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all participants or their legal guardians prior to sample collection. All animal experiments were approved by the Medical Ethics Committee of Southern Medical University and conducted according to the Guidelines for the Care and Use of Animals for Scientific Research.

Consent for publication

All authors have read and agreed to publish this manuscript.

Competing interests

The authors declare that they have no competing interests.

References

1. Ritter J, Bielack SS: Osteosarcoma. *Ann Oncol* 2010, 21 Suppl7:i320-i325.
2. Ottaviani G, Jaffe N: The epidemiology of osteosarcoma. *Cancer Treat Res* 2009, 152:3-13.
3. Miwa S, Shirai T, Yamamoto N, Hayashi K, Takeuchi A, Igarashi K, Tsuchiya H: Current and Emerging Targets in Immunotherapy for Osteosarcoma. *J Oncol* 2019, 2019:7035045.
4. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, et al: Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol* 2002, 20:776-790.

5. Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P, Wu M: CircRNA: functions and properties of a novel potential biomarker for cancer.*Mol Cancer* 2017, 16:94.
6. Chen LL, Yang L: Regulation of circRNA biogenesis.*Rna Biol* 2015, 12:381-388.
7. Zhou WY, Cai ZR, Liu J, Wang DS, Ju HQ, Xu RH: Circular RNA: metabolism, functions and interactions with proteins.*Mol Cancer* 2020, 19:172.
8. Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, Evantal N, Memczak S, Rajewsky N, Kadener S: circRNA biogenesis competes with pre-mRNA splicing.*Mol Cell* 2014, 56:55-66.
9. Du WW, Yang W, Liu E, Yang Z, Dhaliwal P, Yang BB: Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2.*Nucleic Acids Res* 2016, 44:2846-2858.
10. Su M, Xiao Y, Ma J, Tang Y, Tian B, Zhang Y, Li X, Wu Z, Yang D, Zhou Y, et al: Circular RNAs in Cancer: emerging functions in hallmarks, stemness, resistance and roles as potential biomarkers.*Mol Cancer* 2019, 18:90.
11. Li Z, Huang C, Bao C, Chen L, Lin M, Wang X, Zhong G, Yu B, Hu W, Dai L, et al: Exon-intron circular RNAs regulate transcription in the nucleus.*Nat Struct Mol Biol* 2015, 22:256-264.
12. Wang D, Yang S, Wang H, Wang J, Zhang Q, Zhou S, He Y, Zhang H, Deng F, Xu H, et al: The progress of circular RNAs in various tumors.*Am J Transl Res* 2018, 10:1571-1582.
13. Li Z, Yanfang W, Li J, Jiang P, Peng T, Chen K, Zhao X, Zhang Y, Zhen P, Zhu J, Li X: Tumor-released exosomal circular RNA PDE8A promotes invasive growth via the miR-338/MACC1/MET pathway in pancreatic cancer.*Cancer Lett* 2018, 432:237-250.
14. Zhao ZJ, Shen J: Circular RNA participates in the carcinogenesis and the malignant behavior of cancer.*Rna Biol* 2017, 14:514-521.
15. Rybak-Wolf A, Stottmeister C, Glažar P, Jens M, Pino N, Giusti S, Hanan M, Behm M, Bartok O, Ashwal-Fluss R, et al: Circular RNAs in the Mammalian Brain Are Highly Abundant, Conserved, and Dynamically Expressed.*Mol Cell* 2015, 58:870-885.
16. Cui X, Wang J, Guo Z, Li M, Li M, Liu S, Liu H, Li W, Yin X, Tao J, Xu W: Emerging function and potential diagnostic value of circular RNAs in cancer.*Mol Cancer* 2018, 17:123.
17. Su Z, Yang B, Zeng Z, Zhu S, Wang C, Lei S, Jiang Y, Lin L: Metastasis-associated gene MAPK15 promotes the migration and invasion of osteosarcoma cells via the c-Jun/MMPs pathway.*Oncol Lett* 2020, 20:99-112.
18. Giulianelli S, Riggio M, Guillardoy T, Pérez PC, Gorostiaga MA, Sequeira G, Pataccini G, Abascal MF, Toledo MF, Jacobsen BM, et al: FGF2 induces breast cancer growth through ligand-independent activation and recruitment of ER α and PRB Δ 4 isoform to MYC regulatory sequences.*Int J Cancer* 2019, 145:1874-1888.
19. Coleman SJ, Chioni AM, Ghallab M, Anderson RK, Lemoine NR, Kocher HM, Grose RP: Nuclear translocation of FGFR1 and FGF2 in pancreatic stellate cells facilitates pancreatic cancer cell invasion.*Embo Mol Med* 2014, 6:467-481.

20. de Aguiar RB, Parise CB, Souza CR, Braggion C, Quintilio W, Moro AM, Navarro MF, Buchpiguel CA, Chammas R, de Moraes JZ: Blocking FGF2 with a new specific monoclonal antibody impairs angiogenesis and experimental metastatic melanoma, suggesting a potential role in adjuvant settings. *Cancer Lett* 2016, 371:151-160.
21. Jaffe N: Osteosarcoma: review of the past, impact on the future. The American experience. *Cancer Treat Res* 2009, 152:239-262.
22. Duan Z, Gao Y, Shen J, Choy E, Cote G, Harmon D, Bernstein K, Lozano-Calderon S, Mankin H, Hornicek FJ: miR-15b modulates multidrug resistance in human osteosarcoma in vitro and in vivo. *Mol Oncol* 2017, 11:151-166.
23. Chen X, Zhou Y, Liu S, Zhang D, Yang X, Zhou Q, Song Y, Liu Y: LncRNA TP73-AS1 predicts poor prognosis and functions as oncogenic lncRNA in osteosarcoma. *J Cell Biochem* 2018.
24. Xu J, Ji L, Liang Y, Wan Z, Zheng W, Song X, Gorshkov K, Sun Q, Lin H, Zheng X, et al: CircRNA-SORE mediates sorafenib resistance in hepatocellular carcinoma by stabilizing YBX1. *Signal Transduct Target Ther* 2020, 5:298.
25. Niu Q, Dong Z, Liang M, Luo Y, Lin H, Lin M, Zhong X, Yao W, Weng J, Zhou X: Circular RNA hsa_circ_0001829 promotes gastric cancer progression through miR-155-5p/SMAD2 axis. *J Exp Clin Cancer Res* 2020, 39:280.
26. Wu M, Kong C, Cai M, Huang W, Chen Y, Wang B, Liu X: Hsa_circRNA_002144 promotes growth and metastasis of colorectal cancer through regulating miR-615-5p/LARP1/mTOR pathway. *Carcinogenesis* 2020.
27. Harrison EB, Porrello A, Bowman BM, Belanger AR, Yacovone G, Azam SH, Windham IA, Ghosh SK, Wang M, McKenzie N, et al: A Circle RNA Regulatory Axis Promotes Lung Squamous Metastasis via CDR1-Mediated Regulation of Golgi Trafficking. *Cancer Res* 2020, 80:4972-4985.
28. Jia GY, Wang DL, Xue MZ, Liu YW, Pei YC, Yang YQ, Xu JM, Liang YC, Wang P: CircRNAFisher: a systematic computational approach for de novo circular RNA identification. *Acta Pharmacol Sin* 2019, 40:55-63.
29. Ruan Y, Li Z, Shen Y, Li T, Zhang H, Guo J: Functions of circular RNAs and their potential applications in gastric cancer. *Expert Rev Gastroenterol Hepatol* 2020, 14:85-92.
30. Aytes A, Giacobbe A, Mitrofanova A, Ruggero K, Cyrta J, Arriaga J, Palomero L, Farran-Matas S, Rubin MA, Shen MM, et al: NSD2 is a conserved driver of metastatic prostate cancer progression. *Nat Commun* 2018, 9:5201.
31. Bennett RL, Swaroop A, Troche C, Licht JD: The Role of Nuclear Receptor-Binding SET Domain Family Histone Lysine Methyltransferases in Cancer. *Cold Spring Harb Perspect Med* 2017, 7.
32. Wang JJ, Zou JX, Wang H, Duan ZJ, Wang HB, Chen P, Liu PQ, Xu JZ, Chen HW: Histone methyltransferase NSD2 mediates the survival and invasion of triple-negative breast cancer cells via stimulating ADAM9-EGFR-AKT signaling. *Acta Pharmacol Sin* 2019, 40:1067-1075.
33. Lu MH, Fan MF, Yu XD: NSD2 promotes osteosarcoma cell proliferation and metastasis by inhibiting E-cadherin expression. *Eur Rev Med Pharmacol Sci* 2017, 21:928-936.

34. He C, Liu C, Wang L, Sun Y, Jiang Y, Hao Y: Histone methyltransferase NSD2 regulates apoptosis and chemosensitivity in osteosarcoma. *Cell Death Dis* 2019, 10:65.
35. Buxbaum AR, Haimovich G, Singer RH: In the right place at the right time: visualizing and understanding mRNA localization. *Nat Rev Mol Cell Biol* 2015, 16:95-109.
36. Dai X, Guo X, Liu J, Cheng A, Peng X, Zha L, Wang Z: Circular RNA circGRAMD1B inhibits gastric cancer progression by sponging miR-130a-3p and regulating PTEN and p21 expression. *Aging (Albany NY)* 2019, 11:9689-9708.
37. Dragomir MP, Knutsen E, Calin GA: SnapShot: Unconventional miRNA Functions. *Cell* 2018, 174:1038.
38. Lin X, Wang S, Sun M, Zhang C, Wei C, Yang C, Dou R, Liu Q, Xiong B: miR-195-5p/NOTCH2-mediated EMT modulates IL-4 secretion in colorectal cancer to affect M2-like TAM polarization. *J Hematol Oncol* 2019, 12:20.
39. Shen S, Li K, Liu Y, Liu X, Liu B, Ba Y, Xing W: Silencing lncRNA AGAP2-AS1 Upregulates miR-195-5p to Repress Migration and Invasion of EC Cells via the Decrease of FOSL1 Expression. *Mol Ther Nucleic Acids* 2020, 20:331-344.
40. Powers CJ, McLeskey SW, Wellstein A: Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer* 2000, 7:165-197.
41. Kwabi-Addo B, Ozen M, Ittmann M: The role of fibroblast growth factors and their receptors in prostate cancer. *Endocr Relat Cancer* 2004, 11:709-724.
42. Maloof P, Wang Q, Wang H, Stein D, Denny TN, Yahalom J, Fenig E, Wieder R: Overexpression of basic fibroblast growth factor (FGF-2) downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells. *Breast Cancer Res Treat* 1999, 56:153-167.
43. Cheng Y, Lin KH, Young TH, Cheng NC: The influence of fibroblast growth factor 2 on the senescence of human adipose-derived mesenchymal stem cells during long-term culture. *Stem Cells Transl Med* 2020, 9:518-530.
44. Shimizu T, Ishikawa T, Iwai S, Ueki A, Sugihara E, Onishi N, Kuninaka S, Miyamoto T, Toyama Y, Ijiri H, et al: Fibroblast growth factor-2 is an important factor that maintains cellular immaturity and contributes to aggressiveness of osteosarcoma. *Mol Cancer Res* 2012, 10:454-468.
45. Ediriweera MK, Tennekoon KH, Samarakoon SR: Role of the PI3K/AKT/mTOR signaling pathway in ovarian cancer: Biological and therapeutic significance. *Semin Cancer Biol* 2019, 59:147-160.
46. Alzahrani AS: PI3K/Akt/mTOR inhibitors in cancer: At the bench and bedside. *Semin Cancer Biol* 2019, 59:125-132.
47. Necula L, Matei L, Dragu D, Neagu AI, Mambet C, Nedeianu S, Bleotu C, Diaconu CC, Chivu-Economescu M: Recent advances in gastric cancer early diagnosis. *World J Gastroenterol* 2019, 25:2029-2044.
48. Pardini B, Sabo AA, Birolo G, Calin GA: Noncoding RNAs in Extracellular Fluids as Cancer Biomarkers: The New Frontier of Liquid Biopsies. *Cancers (Basel)* 2019, 11.

Tables

Table 1 Correlation between circ_001422 expression and clinicopathological characteristics of OS

Parameters	Group	Cases	Circ_001422 expression	<i>P</i> value
Age (years)				0.520
	≤ 18	30	0.001479 ± 0.000728	
	> 18	25	0.001606 ± 0.000711	
Gender				0.150
	Male	32	0.001655 ± 0.000751	
	Female	23	0.001372 ± 0.000644	
Clinical stage				0.019
	I	19	0.001333 ± 0.000727	
	II	20	0.001399 ± 0.000508	
	III-IV	16	0.001951 ± 0.000790	
Tumor size (cm)				0.003
	≤ 5	24	0.001217 ± 0.000420	
	> 5	31	0.001784 ± 0.000802	
Distant metastasis				0.005
	Absent	39	0.001367 ± 0.000617	
	Present	16	0.001951 ± 0.000790	
Primary tumor location				0.818
	Arm/hand	20	0.001610 ± 0.000757	
	Leg/foot	31	0.001508 ± 0.000733	
	Others	4	0.001394 ± 0.000405	

Figures

Figure 1

Identification and characterization of circ_001422 in OS a. Clustered heatmap showing the top ten most upregulated and downregulated circRNAs in 3 paired OS tissues and adjacent non-cancerous tissues using high-throughput sequencing. b. Schematic illustration of circ_001422 formation and the results of Sanger sequencing. c. Relative expression level of circ_001422 in OS cells and normal osteoblast cells. d. Relative expression level of circ_001422 and NSD2 mRNA in OS cells under the treatment of RNase R. e.

Linear and back-splicing products were amplified with convergent and divergent primers with and without RNase R treatment and subjected to PCR assay. f. Relative abundance of circ_001422 and NSD2 mRNA after treatment with actinomycin D at the indicated time points. g & h. Nuclear-cytoplasmic fractionation and FISH assays revealed subcellular location of circ_001422 in OS cells. Nuclei were stained with DAPI, and circ_001422 probes were labeled with Cy3. Scale bar, 50 μ m. n.s., not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2

Correlation of circ_001422 expression with clinical characteristics a. The expression level of circ_001422 was detected by qRT-PCR in 55 paired OS tissues and adjacent non-cancerous tissues. b. The fold changes (log2) of circ_001422 in each paired sample were arranged from high to low. c. Correlation between circ_001422 expression and clinical stage. d. Correlation between circ_001422 expression and tumor size. e. Correlation between circ_001422 expression and distant metastasis. f. Correlation between circ_001422 expression and primary tumor location. g. OS cases were divided into two groups according

to the median value of circ_001422 expression. Overall survival was analyzed by Kaplan-Meier survival analysis using the log-rank test. h. ROC curve showing the diagnostic sensitivity and specificity of circ_001422 in OS. n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3

Knockdown of circ_001422 inhibits the proliferation and metastasis of OS cells a & b. EdU (scale bar, 100 μ m) and colony formation assays detected the effect of circ_001422 knockdown on cell proliferation

ability. c & d. Flow cytometry detected the effect of circ_001422 knockdown on cell cycle distribution and apoptosis. e. Transwell migration and Matrigel invasion assays detected the effect of circ_001422 knockdown on cell migration and invasion. Scale bar, 100 μ m. f. Western blot detected the effect of circ_001422 knockdown on the expression of cell cycle-, apoptosis-, and EMT-associated biomarkers. g. Representative images of xenograft tumors in nude mice after subcutaneous implantation of 143B cells stably infected with circ_001422-knockdown lentivirus or control lentivirus. h & i. Effect of circ_001422 knockdown on tumor growth and tumor weight in nude mice. j & k. Representative images of lung tissues and lung sections stained with H&E in the lung metastatic mice model. Scale bar, 1000 μ m. l. Tumor tissues were sectioned and stained for Ki-67, PCNA, E-cadherin, N-cadherin and Vimentin by IHC. Scale bar, 100 μ m. n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4

Overexpression of circ_001422 promotes the proliferation and metastasis of OS cells a & b. EdU (scale bar, 100 μ m) and colony formation assays detected the effect of circ_001422 overexpression on cell proliferation ability. c & d. Flow cytometry detected the effect of circ_001422 overexpression on cell cycle distribution and apoptosis. e. Transwell migration and Matrigel invasion assays detected the effect of circ_001422 overexpression on cell migration and invasion. Scale bar, 100 μ m. f. Western blot detected

the effect of circ_001422 overexpression on the expression of cell cycle-, apoptosis-, and EMT-associated biomarkers. g. Representative images of xenograft tumors in nude mice after subcutaneous implantation of 143B cells stably infected with circ_001422-overexpression lentivirus or control lentivirus. h & i. Effect of circ_001422 overexpression on tumor growth and tumor weight in nude mice. j & k. Representative images of lung tissues and lung sections stained with H&E in the lung metastatic mice model. Scale bar, 1000 μ m. l. Tumor tissues were sectioned and stained for Ki-67, PCNA, E-cadherin, N-cadherin and Vimentin by IHC. Scale bar, 100 μ m. n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5

Circ_001422 sponges miR-195-5p a. RIP assay for circ_001422 levels in 143B cells transfected with Ago2-overexpression vectors or control vectors. b. Schematic illustration showing overlapping of the target miRNAs of circ_001422 predicted by TargetScan, miRanda, starBase, RNAhybrid and RNA22. c. Lysates prepared from OS cells transfected with circ_001422-overexpression vectors or control vectors were subject to RNA pull-down assay, and the efficiency was confirmed by qRT-PCR. d. The relative levels of 4 miRNA candidates in the OS cell lysates were examined by qRT-PCR. e. The putative binding sites between miR-195-5p and circ_001422. f. The luciferase activities were detected in OS cells after co-transfection with circ_001422-WT or circ_001422-MUT and miR-195-5p mimics or NC mimics. g-i. Anti-Ago2 RIP assay was conducted in OS cells after transfection with miR-195-5p mimics or NC mimics, followed by western blot and qRT-PCR analyses to detect the expression levels of Ago2, circ_001422 and miR-195-5p. j. The expression level of miR-195-5p was detected by qRT-PCR in 55 paired OS tissues and adjacent non-cancerous tissues. k. Pearson correlation analysis of circ_001422 and miR-195-5p expression in 55 OS tissues. l. Relative expression level of miR-195-5p in OS cells and normal osteoblast cells. n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6

Knockdown of miR-195-5p rescues sh-circ_001422-mediated the inhibitory effect on the proliferation and metastasis in OS a & b. OS cells were transfected with sh-circ_001422-NC or sh-circ_001422 or co-transfected with sh-circ_001422 and miR-195-5p inhibitors. EdU (scale bar, 100 μ m) and colony formation assays detected the cell proliferation ability of OS cells in each group. c & d. Flow cytometry evaluated the effect of miR-195-5p silencing on sh-circ_001422-mediated the cell cycle distribution and apoptosis. e

& f. Transwell migration and Matrigel invasion assays evaluated the effect of miR-195-5p silencing on sh-circ_001422-mediated the migration and invasion ability. Scale bar, 100 μ m. g. The protein expressions of CCND1, CDK4, cleaved CASP3, BAX, BCL2, E-cadherin, N-cadherin and Vimentin were analyzed by western blot and normalized to GAPDH. n.s., not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 7

Circ_001422 positively regulates FGF2 expression in OS cells via miR-195-5p sponging a. Clustered heatmap showing the significantly differentially expressed mRNAs between 143B cells transfected with sh-circ_001422 and with sh-circ_001422-NC. b. Venn diagram showing overlapping of the target mRNAs of miR-195-5p predicted by TargetScan, miRanda, RNAhybrid and miRTarBase. c. The intersection mRNAs between the downregulated mRNAs and target mRNAs of miR-195-5p were subjected to KEGG enrichment analysis. d & e. QRT-PCR showed the mRNA levels of 6 candidate genes in OS cells transfected with sh-circ_001422 or sh-circ_001422-NC. f & g. QRT-PCR showed the mRNA levels of 6 candidate genes in OS cells transfected with OE-circ_001422 or OE-circ_001422-NC. h & i. Western blot showed the protein levels of FGF2, p-PI3K, t-PI3K, p-Akt and t-Akt in OS cells transfected with sh-circ_001422 or OE-circ_001422 or their negative controls. j & k. Western blot showed the protein levels of FGF2, p-PI3K, t-PI3K, p-Akt and t-Akt in OS cells transfected with miR-195-5p mimics or miR-195-5p inhibitors or their negative controls. l & m. Western blot evaluated the effect of miR-195-5p silencing (or miR-195-5p overexpression) on sh-circ_001422 (or OE-circ_001422)-mediated the protein level change of FGF2, p-PI3K, t-PI3K, p-Akt and t-Akt. n. The putative binding sites between miR-195-5p and FGF2. o. The luciferase activities were detected in OS cells after co-transfection with FGF2-WT or FGF2-MUT and miR-195-5p mimics or NC mimics. p. Representative images of IHC analysis for FGF2 expression in OS tissues and paired non-cancerous tissues. Scale bar, 100 μ m. q. FGF2 protein expression in 12 OS tissues (T) and paired non-cancerous tissues (N). n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 8

Loss-of-function assays confirm the involvement of circ_001422/miR-195-5p/FGF2 axis in OS progression and metastasis a. sh-circ_001422 stable OS cells were transfected with miR-195-5p inhibitors (inh) or co-transfected with miR-195-5p inhibitors and sh-FGF2. EdU (scale bar, 100 μ m) assay evaluated the effect of the circ_001422/miR-195-5p/FGF2 axis on the cell proliferation. b. Transwell migration and Matrigel invasion assays evaluated the effect of the circ_001422/miR-195-5p/FGF2 axis on the migration

and invasion ability of 143B cells. Scale bar, 100 μ m. c. The protein expressions of CCND1, CDK4, cleaved CASP3, BAX, BCL2, E-cadherin, N-cadherin, Vimentin, p-PI3K, t-PI3K, p-Akt and t-Akt were analyzed by western blot and normalized to GAPDH. d. Nude mice were distributed into 4 groups: control, sh-circ_001422, sh-circ_001422 + miR-195-5p antagomir (anta), and sh-circ_001422 + miR-195-5p antagomir + sh-FGF2. Representative images of xenograft tumors in 4 groups. e & f. Effect of the circ_001422/miR-195-5p/FGF2 axis on tumor growth and tumor weight in nude mice. g & h. Representative images of lung tissues and lung sections stained with H&E in the lung metastatic mice model. Scale bar, 1000 μ m. i. IHC detection of Ki-67, PCNA, E-cadherin, N-cadherin and Vimentin expression in tumor samples from nude mice in 4 groups. Scale bar, 100 μ m. n.s., not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 9

Gain-of-function assays confirm the involvement of circ_001422/miR-195-5p/FGF2 axis in OS progression and metastasis a. OE-circ_001422 stable OS cells were transfected with miR-195-5p mimics (mim) or co-transfected with miR-195-5p mimics and OE-FGF2. EdU (scale bar, 100 μ m) assay evaluated the effect of the circ_001422/miR-195-5p/FGF2 axis on the cell proliferation. b. Transwell migration and Matrigel invasion assays evaluated the effect of the circ_001422/miR-195-5p/FGF2 axis on the migration

and invasion ability of 143B cells. Scale bar, 100 μm . c. The protein expressions of CCND1, CDK4, cleaved CASP3, BAX, BCL2, E-cadherin, N-cadherin, Vimentin, p-PI3K, t-PI3K, p-Akt and t-Akt were analyzed by western blot and normalized to GAPDH. d. Nude mice were distributed into 4 groups: control, OE-circ_001422, OE-circ_001422 + miR-195-5p agomir (ago), and OE-circ_001422 + miR-195-5p agomir + OE-FGF2. Representative images of xenograft tumors in 4 groups. e & f. Effect of the circ_001422/miR-195-5p/FGF2 axis on tumor growth and tumor weight in nude mice. g & h. Representative images of lung tissues and lung sections stained with H&E in the lung metastatic mice model. Scale bar, 1000 μm . i. IHC detection of Ki-67, PCNA, E-cadherin, N-cadherin and Vimentin expression in tumor samples from nude mice in 4 groups. Scale bar, 100 μm . n.s., not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 10

Schematic illustration of the biological role of the circ_001422/miR-195-5p/FGF2 axis in OS progression and metastasis

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1TableS1.docx](#)
- [Additionalfile2FigureS1.tif](#)
- [Additionalfile3FigureS2.tif](#)
- [Additionalfile4FigureS3.tif](#)
- [Additionalfile5FigureS4.tif](#)
- [Additionalfile6FigureS5.tif](#)
- [Additionalfile7FigureS6.tif](#)
- [Additionalfile8FigureS7.tif](#)