Engineering 21 (2023) 187–193

Contents lists available at ScienceDirect

Engineering

journal homepage: www.elsevier.com/locate/eng

Research
New Technology of Tumor Diagnosis and Treatment—Article

Knockdown of a Specific Circular Non-Coding RNA Significantly Suppresses Osteosarcoma Progression

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ARTICLE INFO

Article history:
Received 12 October 2021
Revised 24 November 2021
Accepted 8 December 2021
Available online 4 January 2022

Keywords:
Non-coding RNA
Osteosarcoma
CircRNA
Molecular mechanism
Knockdown

ABSTRACT

Osteosarcoma (OS) is a malignant mesenchymal tissue tumor known to occur in children and adolescents, and pulmonary metastasis often leads to death in these patients. The mechanism underlying OS progression remains unclear. Therefore, identifying new therapeutic targets and treatment modalities for OS is urgently needed. Abnormally expressed non-coding circular RNAs (circRNAs) are crucial for the occurrence and development of OS. The purpose of this study was to explore the expression and role of a novel circRNA circ_000203 in OS and elucidate the underlying mechanism. circ_000203 was demonstrated highly expressed in OS cell lines and tissues, and circ_000203 knockdown significantly inhibited OS progression *in vitro* and *in vivo*. Furthermore, we found that circ_000203 is a sponge of miR-26b-5p, an upstream regulator of bone morphogenetic protein receptor 2 (BMPR2). Thus, the overexpression of BMPR2 could reduce the inhibitory effect on OS progression. This indicates that knockdown of circ_000203 suppresses OS progression through microRNA (miRNA)-mediated BMPR2 downregulation. Our findings provide important insights for understanding the occurrence and development of OS.

1. Introduction

Osteosarcoma (OS) is a malignant mesenchymal tissue tumor known to occur in children and adolescents [1–3]. Although the survival rate has significantly improved over the past several years, its prognosis remains unsatisfactory in numerous patients [4]. In addition, OS presents several challenges, such as multidrug resistance, pulmonary metastasis, and tumor recurrence [5]. Therefore, identifying new therapeutic targets and treatment modalities for OS is urgently needed.

Circular RNAs (circRNAs) have a unique circular structure, and abnormally expressed circRNAs are key genes in various tumors that regulate multiple biological processes [6–9]. For example, circ0001320 can inhibit cell growth and metastasis of lung cancer by regulating tumor necrosis factor (TNF)-α-induced protein 1 and tropomyosin 1 by sponge-like action of miR-558 [10]. circRNA 001306 reportedly promotes the growth of hepatocellular carcinoma (HCC) by increasing the expression of cyclin-dependent kinase 16 (CDK16) by sponge-like action of miR-584-5p [11]. Peng et al. [12] have recently reported that circCUL2 regulates autophagy activation via miR-142-3p/Rho-associated coiled-coil containing protein kinase 2 (ROCK2) to regulate malignant transformation and cisplatin resistance in gastric cancer. In addition, accumulating evidence has revealed that circRNAs play key roles in OS progression [13,14]. Shen et al. [15] have revealed that circular endothelin converting enzyme 1 (circECE1) regulates OS energy metabolism via oncogene *c-Myc*. Li et al. [16] have reported that circ_0000282 regulates OS cell proliferation via the miR-192/X-linked inhibitor of apoptosis protein (XIAP) axis. Pan et al. [17] have demonstrated that circ_0028171 can promote OS progression via the miR-218-5p/inhibitor of κB kinase β (IKBKB) axis. However, the expression and role of several circRNAs in OS remain elusive.

circ_000203 is a circRNA with a length of 685 nucleotides (nt), located on chr10:888871–931700 and spliced from exons of the La ribonucleoprotein 4B (*LARP4B*) gene [18]. *LARP4B* has been

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https://doi.org/10.1016/j.eng.2021.12.007

reported to play important roles in several tumor types [19]. Furthermore, abnormal expression of circ_000203 has been associated with cardiac hypertrophy and fibrosis in cardiac fibroblasts [18,20]. However, the expression and role of circ_000203 in OS are yet to be comprehensively elucidated. We observed that circ_000203 is overexpressed in OS tissues and cells when compared with tissues from healthy controls. Therefore, the purpose of this study was to explore the expression and role of circRNA (circ_000203) in OS and to elucidate the underlying mechanism.

2. Materials and methods

2.1. Study participants and sample collection

In total, 20 healthy bone tissues and 40 conventional OS tissues were collected from participants at Peking University People's Hospital. Written informed consent was obtained from all study participants, and the study was approved by the Ethics Committee of Peking University People's Hospital.

2.2. Cell culture and transfection

OS cell lines (143 B, KHOS, HOS, U2OS, SAOS2, and MG63) and healthy human osteoblast (hFOB) cells were purchased from American Type Culture Collection. 143 B and hFOB cells were cultured in Dulbecco's modified eagle medium, supplemented with 10% fetal bovine serum (FBS). Other cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS at 37 °C and 5% CO_2. miR-26b-5p mimic/inhibitor, short hairpin RNA (shRNA)–circ_000203, and overexpression vector targeting bone morphogenetic protein receptor 2 (BMPR2) were purchased from GenePharma (China) and transfected with lipo3000. shRNA–circ_000203 sequence: (F) 5′-GUCGUGUGUGAAUAUCCUACG-3′, (R) 5′-UAUAAUAAUCACACAGCACUG-3′; shRNA–negative control (NC) sequence: (F) 5′-TTCTCCGAACGTGTCACGTAA-3′, (R) 5′-TTACGTGACACGTTCGGAGAA-3′. miR-26b-5p mimic sequence: 5′-UUCAAGUAAUUCAGGAUAGGU-3′; miR-26b-5p inhibitor sequence: 5′-ACCUAUCCUGAAUUACUUGAA-3′.

2.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was isolated using the RNeasy Plus Universal Kit (Qiagen, Germany), and genomic DNA was removed with ribonuclease (RNase)-free deoxyribonuclease (DNase) (Qiagen). Next, we performed qRT-PCR analysis using the iTaq Universal One-Step RT-qPCR Kit (Bio-Rad, USA) according to the manufacturer's instructions. U6 small nuclear RNA and β-actin were separately used as endogenous controls for microRNA (miRNA) and other message RNAs (mRNAs). Data were analyzed using the Bio-Rad CFX96 software and presented as the average of three independent experiments conducted in triplicate. Primer sequences are listed in Table 1.

Table 1
Primers for qRT-PCR.

Primers	Dirction	Sequences (5′–3′)
circ_000203	F	AAGAGAAGTACAGATTGCTTCA
	R	CTCTTCTTTAACTTCTAATAATTC
BMPR2	F	CACCTCCTGACACAACACCACTC
	R	TGCTGCTGCCTCCATCATGTTC
β-actin	F	GTCAGGTCATCACTATCGGCAAT
	R	AGAGGTCTTTACGGATGTCAACGT
miR-26b-5p	F	TTCAAGTAATTCAGGATAGGT
	R	GTGCGTGTCGTGGAGTC
U6	F	CTCGCTTCGGCAGCACA
	R	AACGCTTCACGAATTTGCGT

2.4. Western blotting

Western blotting was performed as described previously [21]. Anti-human BMPR2 (1:1000; Abcam, UK) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000; Santa Cruz, USA) antibodies were employed. Band intensity was quantified using the National Institutes of Health ImageJ software (Bethesda, USA). All experiments were performed in triplicate. Data are presented as the means of three independent experiments.

2.5. Cell viability assay and colony formation

The transfected OS cells were seeded in a 96-well plate (5×10^3 cells per well). According to the manufacturer's instructions, cell-counting kit-8 (CCK-8; Dojindo, Japan) was used daily for three consecutive days to assess cell viability. For the colony formation test, transfected OS cells were cultured in complete medium for 5 d. After washing and fixing with phosphate-buffered saline (PBS), 0.1% crystal violet staining solution was used for cell staining. All experiments were repeated three times.

2.6. Transwell assay and wound healing assay

For transwell assays, 5×10^4 transfected OS cells were seeded in matrigel-coated or uncoated upper chambers (BD Biosciences, USA). After 24 h, the cells were washed with PBS three times and fixed using 4% paraformaldehyde. Then, the cells were stained with 0.1% crystal violet staining solution and observed under an inverted microscope. For the wound healing assay, transfected OS cells were inoculated into a 6-well plate, and a scratch was made on the plate. Cells were then cultured and photographed at 0 and 24 h. All experiments were performed in triplicate.

2.7. Dual-luciferase reporter assay

PmirGLO luciferase reporter vectors were cloned with the sequence of BMPR2 or circ_000203 3′-untranslated region containing the mutated or wild-type (WT) miR-206 binding sites, as previously described [22]. Then, vectors and miRNA mimic or NC were co-transfected into OS cells, and luciferase activity was evaluated. All experiments were performed in triplicate.

2.8. Immunohistochemistry

The experimental protocol was performed as previously described [2,21]. Anti-human BMPR2 (1:80) was used in this study, and the results were assessed by two independent pathologists. All experiments were performed in triplicate.

2.9. Northern blotting and RNase R experiments

For northern blotting, TRIzol (Invitrogen, USA) was used for RNA extraction from OS tissues. The circ_000203 and 18S probes were prepared using Biotin RNA Labeling Mix (Roche Applied Science, Germany) for northern blotting. For RNase R treatment, 10 μg of RNA was mixed with 40 unit (U) RNase R at 37 °C for 2 h. The expression level and stability of circ_000203 and LARP4B mRNA were evaluated using qRT-PCR. All experiments were performed in triplicate.

2.10. Tumor xenografts

Ten female BALB/c nude mice were randomly assigned to two groups (n = 5) to evaluate circ_000203 function in OS cells *in vivo*. Briefly, 5×10^6 shRNA–circ_000203 or shRNA-NC

transfected 143 B cells were injected subcutaneously into the left side of the mouse. Then, we used a Vernier caliper to measure the tumor size every 5 d for 15 d. Finally, all mice were sacrificed and used for further experiments. All animal experiments were approved by the Animal Care and Use Committee of Peking University People's Hospital.

2.11. Statistical analysis

GraphPad Prism 8 (GraphPad Software, Inc., USA) was used for data analysis, and all results are represented as mean ± standard deviation. The differences between groups were analyzed using the Student's *t*-test, and results were considered statistically significant at values $p < 0.05$ and $p < 0.01$.

3. Results

3.1. circ_000203 knockdown prevents OS progression by regulating miR-26b-5p/BMPR2

In brief, transfection of shRNA–circ_000203 into OS cells significantly downregulated the expression of circ_000203, and its sponged miR-26b-5p was released in large numbers. Overexpression of miR-26b-5p suppressed the expression of BMPR2, a known to induce the downregulation of tumor proliferation marker Ki67, tumor metastasis-associated protein N-cadherin, and neovascularization biomarker cluster of differentiation 31 (CD31) [23]. Therefore, circ_000203 knockdown ultimately inhibited the progression of OS (Fig. 1).

3.2. circ_000203 knockdown suppresses OS cell malignant progression in vitro

As shown in Fig. 2(a), circ_000203 originated from six exons of the *LARP4B* gene, with a mature length of 685 nt. The qRT-PCR results revealed that, compared with healthy tissues, circ_000203 was highly expressed in OS tissues (Fig. 2(b)). Next, we selected three pairs of OS tissue samples for northern blotting and observed that circ_000203 was markedly overexpressed in tumor tissues (Fig. 2(c)). Next, we used six different OS cell lines to examine circ_000203 expression using qRT-PCR (Fig. 2(d)). The selected OS cells expressed more circ_000203 than hFOB osteoblasts. Given that these OS cell lines were derived from patients with OS exhibiting different degrees of malignancy, the circ_000203 expression levels tended to vary. Specifically, 143 B and KHOS displayed the highest circ_000203 expression; therefore, these cell lines were selected for further experiments. Additionally, RNase R was used to verify the closed-loop structures of circ_000203. We found that circ_000203 was not digested by RNase R, whereas linear LARP4B was digested (Fig. 2(e) and Fig. S1 in Appendix A). Then, shRNA was used for circ_000203 knockdown in OS cells and evaluated by qRT-PCR (Fig. 2(f)). The CCK-8 assay (Fig. S2 in Appendix A) and colony formation assay (Fig. S3 in Appendix A) revealed that circ_000203 knockdown suppressed OS cell proliferation. Based on the findings of the wound healing assay, knockdown of circ_000203 inhibited the migration of 143 B and KHOS cells (Fig. 2(g) and Fig. S4 in Appendix A). Furthermore, the results of the transwell assays showed that circ_000203 knockdown inhibited both the migration and invasion of OS cells (Fig. 2(h) and Fig. S5 in Appendix A).

3.3. circ_000203 knockdown inhibits OS tumor progression in vivo

To evaluate the role of circ_000203 in OS tumorigenesis *in vivo*, shRNA–circ_000203 or shRNA-NC-transfected 143 B cells were used to establish a murine xenograft model. As shown in Figs. 3(a)–(c) and Fig. S6 in Appendix A, shRNA–circ_000203 showed significant anti-tumor behavior and retarded the tumor growth rate. Furthermore, expression levels of circ_000203, Ki67, N-cadherin, E-cadherin, and CD31 were evaluated in collected xenografts. Compared with the NC group, circ_000203 was significantly downregulated in circ_000203 knockdown tumors

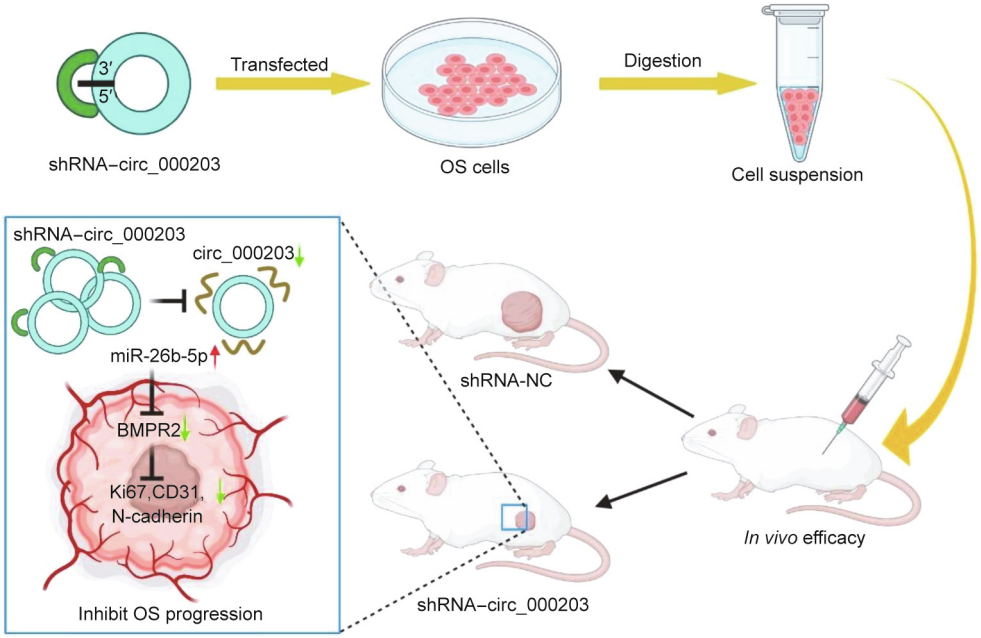

Fig. 1. Schematic representation of shRNA–circ_000203 knockdown prevents OS progression via miR-26b-5p/BMPR2 axis. After transfecting shRNA–circ_000203 into OS cells, circ_000203 knockdown or NC cells were digested and subcutaneously implanted into nude mice to form a xenograft model. Two weeks later, shRNA–circ_000203 knockdown significantly prevents OS progression. In OS cells, shRNA–circ_000203 significantly downregulates circ_000203, and its sponged miR-26b-5p is released in large numbers. Over-release of miR-26b-5p significantly downregulates the target gene *BMPR2*, further leading to the downregulation of tumor proliferation marker activity Ki67, tumor metastasis-associated protein N-cadherin, and neovascularization biomarker CD31, resulting in the inhibition of OS progression.

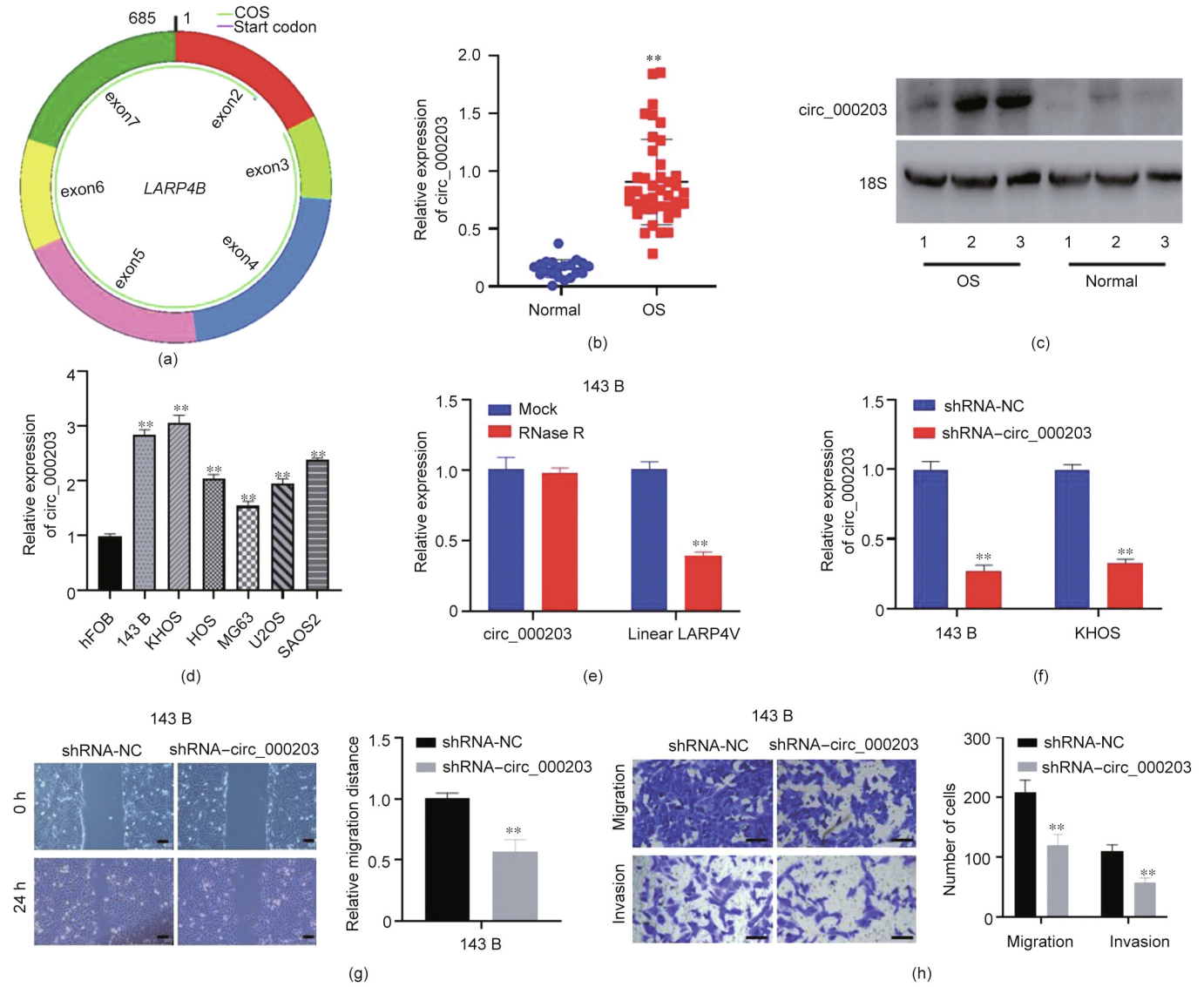

Fig. 2. circ_000203 is highly expressed in OS, and circ_000203 knockdown restrains OS cell malignant progression. (a) Schematic representation of circ_000203. (b) The expression of circ_000203. (c) Northern blotting. (d) OS cells expressed a higher level of circ_000203 than hFOB. (e) The closed-loop structure of circ_000203 was verified by RNase R. (f) circ_000203 knockdown in OS cells was detected by qRT-PCR. (g) The results of wound healing assay. (h) Transwell assay results. Scale bar = 100 μm; **: $p < 0.01$. CDS: coding domain sequence.

(Fig. 3(d)). Furthermore, the immunohistochemistry assay demonstrated that tumor metastasis-associated protein N-cadherin, tumor proliferation marker Ki67, and neovascularization biomarker CD31 were significantly inhibited in the circ_000203 knockdown group (Fig. 3(e)). However, the expression of E-cadherin was significantly upregulated (Fig. 3(e)). These results suggested that circ_000203 knockdown can markedly suppress the growth and metastasis of OS.

3.4. circ_000203 knockdown suppresses OS progression via miR-26b-5p release-mediated downregulation of BMPR2 expression

Using the DIANA-LncBase v2 tool[†], miR-26b-5p was predicted to be a potential downstream target of circ_000203. Then, the bioinformatics tool, The Encyclopedia of RNA Interactomes (ENCORI)[‡], predicted *BMPR2* as the target gene of miR-26b-5p. Dual-luciferase reporter assay demonstrated that miR-26b-5p inhibited the luciferase activity of circ_000203 or BMPR2 WT when compared with the mutant (MUT) group (Figs. 4(a)–(c)), which confirmed their direct interaction. Using qRT-PCR, miR-26b-5p was found to exhibit low expression in OS cells and tissues (Fig. S7 in Appendix A). In addition, we observed that the expression of miR-26b-5p and circ_000203 was negatively correlated (Fig. 4(d)). circ_000203 knockdown increased the expression of miR-26b-5p in OS cells (Fig. 4(e)), and BMPR2 was downregulated by miR-26b-5p mimics (Figs. 4(f) and (g)). In addition, double knockdown of miR-26b-5p and circ_000203 was achieved via the simultaneous transfection of a miR-26b-5p inhibitor (sequences shown in Section 2.2) and shRNA–circ_000203 into OS cells (Fig. S8 in Appendix A). Based on the qRT-PCR analysis, downregulated BMPR2 expression in shRNA–circ_000203-transfected OS cells could be reversed in double-knockdown cells (Fig. S8). These results undoubtedly indicated that circ_000203 regulates BMPR2 expression by sponging miR-26b-5p.

Furthermore, we transfected an overexpression vector targeting BMPR2 into OS cells (Fig. S9 in Appendix A) and performed functional experiments to further verify that circ_000203 pro-

[†] https://starbase.sysu.edu.cn/.
[‡] http://www.microrna.gr/LncBase.

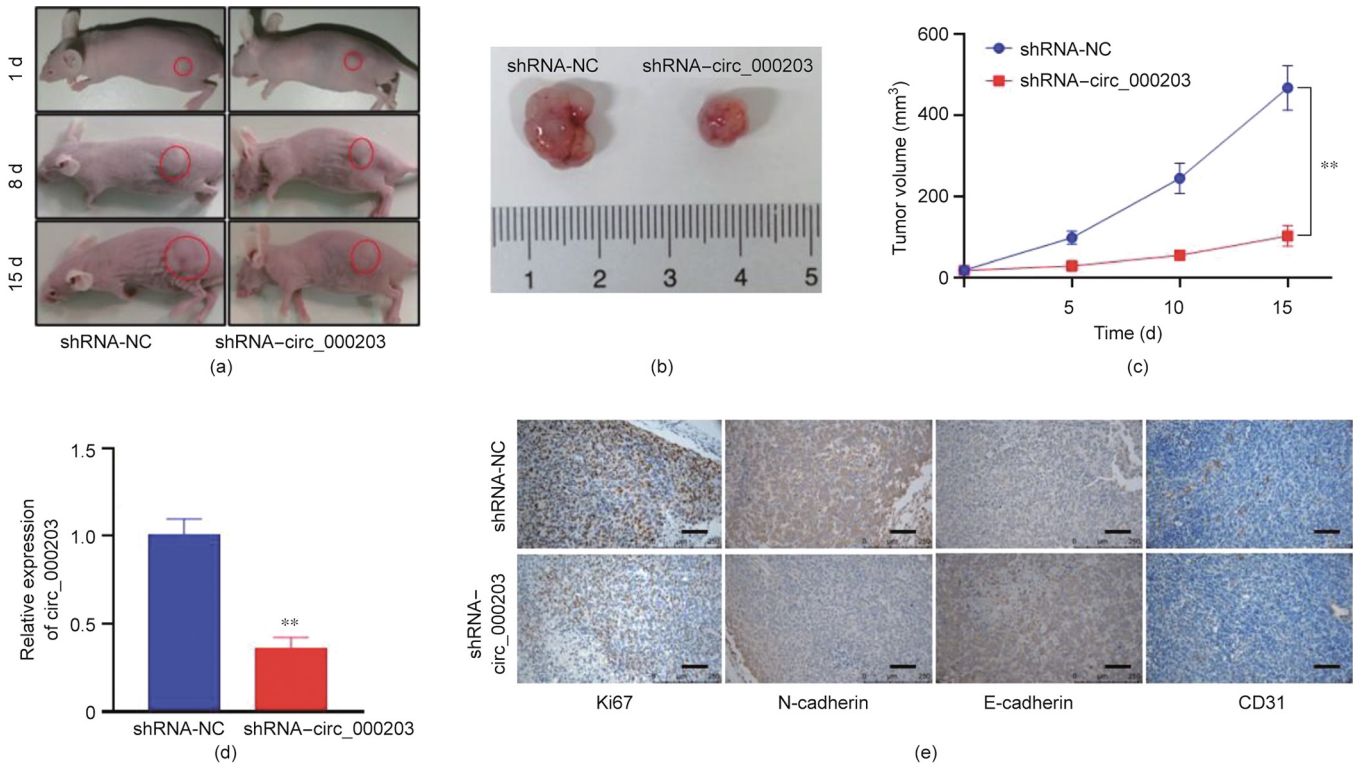

Fig. 3. circ_000203 knockdown inhibits OS tumor progression *in vivo*. (a) Photographs of mice with tumor xenografts in the left hind leg. Tumor sites are indicated by red circles. (b) Photographs of isolated subcutaneous xenograft tumors. (c) The growth curve of xenograft models. (d) The expression of circ_000203 in tumors. (e) Immunohistochemistry was used to evaluate the expression of tumor progression-associated protein markers in the xenograft samples. Scale bar = 100 μm. **: $p < 0.01$.

moted OS cell development via regulation of BMPR2 expression by sponging miR-26b-5p. The colony formation assay revealed that circ_000203 knockdown-induced inhibited proliferation could be reversed by increasing BMPR2 expression (Fig. S10 in Appendix A). Wound healing and transwell assays revealed that circ_000203 knockdown induced inhibited migration, partially relieved by BMPR2 overexpression (Figs. S11 and S12 in Appendix A). Combined with the results that miR-26b-5p overexpression can restrain OS cell malignancy progression (Fig. S13 in Appendix A), we confirmed that circ_000203 knockdown suppressed the development of OS via downregulation of BMPR2 by sponging miR-26b-5p.

We also detected miR-26b-5p and BMPR2 in collected xenografts and found that miR-26b-5p was significantly increased, and BMPR2 was decreased in circ_000203 knockdown tumors (Fig. S14 in Appendix A), suggesting that circ_000203 knockdown can prevent OS progression by targeting the miR-26b-5p/BMPR2 axis.

4. Discussion

Several studies have reported that circRNAs play crucial roles in the progression of various tumors, including OS. circ_000203 is a novel circRNA that has not been previously detected in tumors [18]. In the present study, we observed that circ_000203 is highly expressed in both OS tissues and cells when compared with healthy controls. Therefore, we aimed to explore the functional roles of circ_000203 and the mechanisms underlying its role in OS. We found that circ_000203 knockdown significantly inhibited the proliferation, migration, and invasion of OS cells.

Competitive endogenous RNA (ceRNA) is a key regulatory mechanism of circRNAs [24], and circRNAs can function as ceRNAs to sponge downstream target miRNAs, thus affecting free miRNA expression and achieving post-transcriptional regulation of target genes. Herein, we employed DIANA-LncBase v2 to predict miR-26b-5p as a potential downstream target of circ_000203. The bioinformatics tool ENCORI predicted *BMPR2* as the target gene of miR-26b-5p.

miR-26b-5p has been reported in several studies examining diverse tumors [25]. Han et al. [26] have found that in HCC, miR-26b-5p can boost T cell responses by regulating polymer of intrinsic microporosity (PIM)-2. Wu et al. [27] have revealed that miR-26a can restrain bladder cancer cell progression by targeting programmed cell death 10 (PDCD10). In addition, Niu et al. [28] have reported that miR-26b-5p can inhibit Burkitt lymphoma cell proliferation by targeting karyopherin α2 (KPNA2). However, the role of miR-26b-5p in OS remains unclear. In the present study, miR-26b-5p exhibited low expression in OS cells and tissues, and miR-26b-5p overexpression significantly suppressed the proliferation, migration, and invasion of OS cells.

BMPR2 is a crucial member of bone morphogenetic protein (BMP) signal transduction and is reportedly a key gene in tumor progression, including gastric cancer, prostate cancer, and mammary tumors [23,29-31]. We have previously reported that BMPR2 promotes invasion and metastasis via the Ras homolog gene family member A (RhoA)–Rho associated coiled-coil containing protein kinase (ROCK)–LIM domain kinase 2 (LIMK2) pathway in human OS cells [23]. In the present study, we further demonstrated that circ_000203 knockdown inhibited cell proliferation, migration, and invasion, which could be partially relieved by BMPR2 overexpression. These results indicate that circ_000203 promotes the development of OS cells by targeting the miR-26b-5p/BMPR2 axis. Collectively, these findings could be valuable in establishing a detailed understanding of the regulatory sub-network in OS progression.

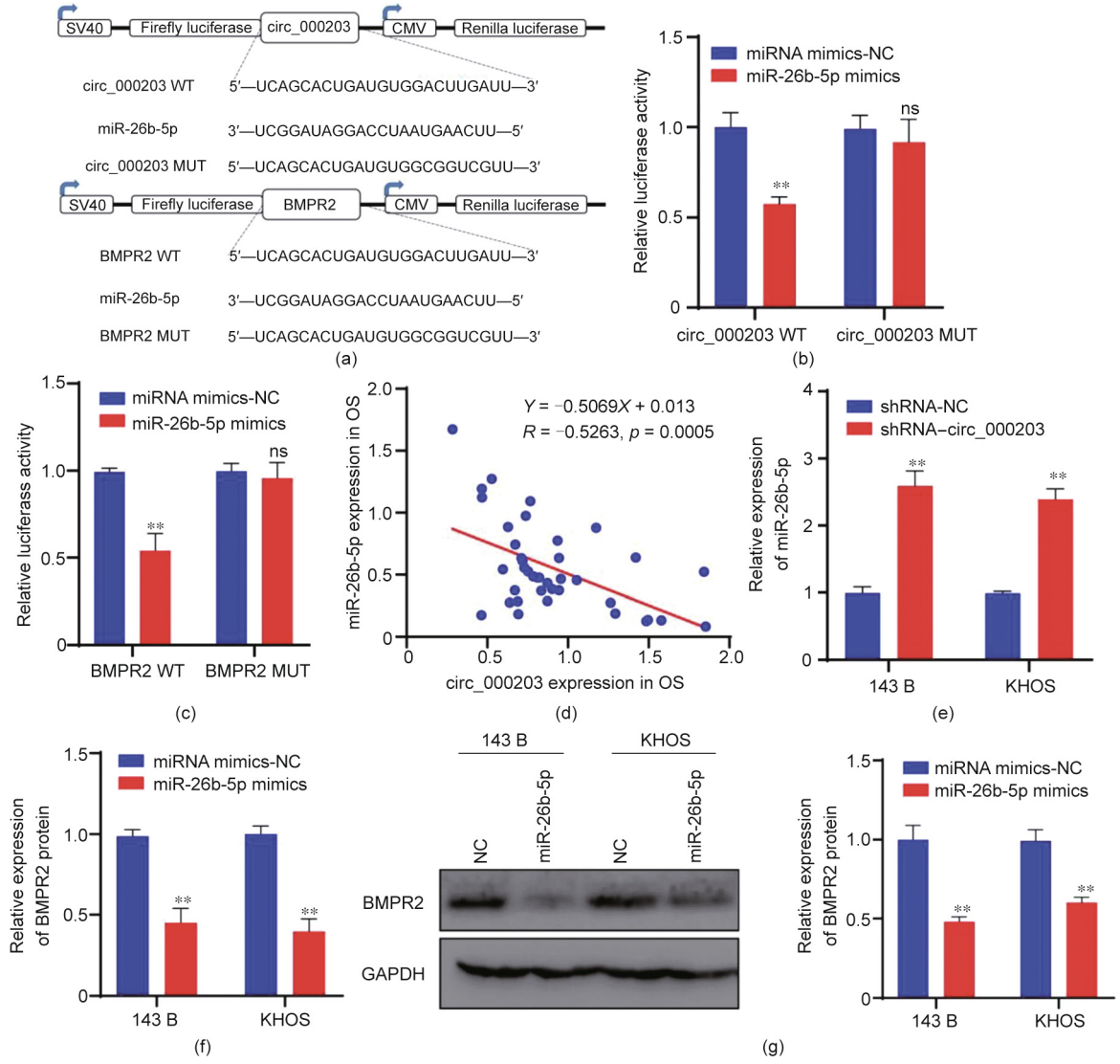

Fig. 4. circ_000203 knockdown suppresses OS progression through miR-26b-5p release-mediated downregulation of BMPR2 expression. (a) Schematic diagram of the binding site between miR-26b-5p and circ_000203/BMPR2. (b) Detection of dual-luciferase reporter gene for miR-26b-5p and circ_000203. (c) Detection of the dual-luciferase reporter gene for miR-26b-5p and BMPR2. (d) qRT-PCR results indicate that the expressions of circ_000203 and miR-26b-5p are negatively correlated in OS tissues. (e) qRT-PCR results show that miR-26b-5p is increased in circ_000203 knockdown OS cells. (f) miR-26b-5p downregulates BMPR2 expression. (g) Western blot for expression of BMPR2 protein. **: $p < 0.01$.

5. Conclusions

circRNAs are crucial in the progression of OS, but the underlying mechanisms remain unexplored. Herein, we revealed that circ_000203 could promote OS development by regulating the miR-26b-5p/BMPR2 axis. In OS cells, circ_000203 knockdown specifically induced the release of miR-26b-5p, an upstream inhibitor of BMPR2 expression, thereby reducing BMPR2 levels and inhibiting OS cell progression. In addition, we demonstrated the feasibility of suppressing OS xenografts *in vivo* by manipulating circRNAs. Our findings provide valuable insights into the molecular mechanism of OS and indicate the potential role of circ_000203 as a novel biomarker and therapeutic target for OS.

Acknowledgments

This study was supported by the Peking University People's Hospital Research and Development Funds (RDY2018-13), the National Key Research and Development Program of China (2020YFA0908900), the National Natural Science Foundation of China (22107097), and the Youth Innovation Promotion Association of Chinese Academy of Science (2021226).

Compliance with ethics guidelines

Shidong Wang, Hongliang Zhang, Bo Li, Chenglong Chen, Tingting Ren, Yi Huang, Kai Liu, Jingjing Li, and Wei Guo declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2021.12.007.

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