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Down-regulation of microRNA-31-5p inhibits proliferation and invasion of osteosarcoma cells through Wnt/β-catenin signaling pathway by enhancing AXIN1

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ABSTRACT

Objectives: Recently, the role of microRNA-31-5p (miR-31-5p) in gene expression regulation has been reported in various cancers. Studies have shown that Wnt/β-catenin signaling pathway is involved in the proliferation and invasion of osteosarcoma (OS) cells. Therefore, this study aims to probe into the regulatory role of miR-31-5p targeting AXIN1 in OS cells through Wnt/β-catenin signaling pathway.

Methods: Firstly, microarray expression profiles were used to screen differentially expressed miRNAs associated with OS. Next, OS and normal fibrous connective tissues as well as OS cell lines were obtained for investigating the role of miR-31-5p on OS. Then, the putative binding sites between miR-31-5p and AXIN1 were predicted and verified. The regulatory effects of miR-31-5p on proliferation and invasion as well as tumorigenic potential of OS cells targeting AXIN1 were also analyzed. Besides, the relationship between miR-31-5p and Wnt/β-catenin signaling pathway was assessed by immunofluorescence staining.

Results: The microarray dataset GSE63939 showed that miR-31-5p and AXIN1 were involved in OS. miR-31-5p expression increased while the expression of AXIN1 decreased in OS tissues and cells. AXIN1 was identified as a target gene of miR-31-5p, intense expression of which inhibited the transcription of AXIN1. Down-regulated miR-31-5p suppressed proliferation, invasion and tumorigenicity of OS cells through promoting AXIN1. Decreased miR-31-5p activated Wnt/β-catenin signaling pathway, as reflected by increased β-catenin translocation into nuclei, through up-regulating the transcription of AXIN1.

Conclusions: All in all, repression of miR-31-5p targets AXIN1 to activate the Wnt/β-catenin signaling pathway, thus suppressing proliferation, invasion and tumorigenicity of OS cells.

1. Introduction

Osteosarcoma (OS) is the most common malignant bone tumor featuring an aggressive clinical process and is the third most prevailing cancer found in children and young adults (Zhang et al., 2010). Local pain, limitation of joint movement and localized swelling are the three typical symbols and symptoms of OS, and in scarce situations, a pathological fracture can be the first sign of OS for those patients with osteolytic tumors (Ritter and Bielack, 2010). The incidence data shows that from 5 to 9 years old, there are 2.4 cases per million every year; at the age of 10 to 14 years, there are 7.6 cases per million every year; and from 15 to 19 years, there are 8.2 cases per million every year (Kager et al., 2010). OS primarily affects adolescents and young adults, and it is lethal if it is not cured (Broadhead et al., 2011). Modern treatment protocols of OS combine neoadjuvant chemo-therapy, surgery, adjuvant chemotherapy, and sometimes radiotherapy (Yuan et al., 2017). The 5-

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year survival rate for patients diagnosed with no metastatic disease is about 60%–70%; nevertheless, the survival rate of those patients diagnosed with metastatic disease is far < 30%, with pulmonary metastasis as the predominant site of OS relapse and the main cause of death (Osaki et al., 2011). Accumulating evidence has shown that microRNAs (miRNAs) play a crucial role in tumor proliferation, progression and metastasis through the regulation of genes (Duan et al., 2011).

miRNAs were reported to play a critical role in cellular courses such as proliferation, apoptosis, invasion and metastasis (Yan et al., 2012). miR-31-5p expression is remarkably high in tumor tissue and down regulation of miR-31-5p contributes to the treatment of RAS metastatic colorectal cancer (Mlcochova et al., 2015). From the dual luciferase reporter gene assay of the present paper, AXIN1 is the target gene of miR-31-5p. It is acknowledged that missense AXIN1 mutations are one of the most common mutations found in human cancers, polymorphism of which is remarkably over-represented in those medulloblastoma patients and can confirm the occurrence of occasional point mutations (Baeza et al., 2003). As a tumor-suppressor protein, AXIN1 was proved to play an essential role in the regulation of Wnt signaling pathway through controlling the forming process of a multiprotein complex that regulates the phosphorylation of β-catenin (Noutsou et al., 2011). Previous studies have found that activating Wnt/β-catenin signaling pathway invariably occurs in different kinds of tumors which are involved in cell proliferation, differentiation and migration (Jia et al., 2010; Zhou et al., 2012). Suppressing the Wnt/β-catenin signaling pathway can strength apigenin-inhibited invasion and proliferation in OS cells (Liu et al., 2015). From all that information above, it is implied that miR-31-5p, AXIN1 as well as Wnt/β-catenin signaling pathway may work in therapy of OS. Nevertheless, the relations of miR-31-5p, AXIN1 and OS remain unclear, so the evidences of this study furnish a new potential role of miR-31-5p, AXIN1 and Wnt/β-catenin signaling pathway in modulating proliferation and invasion of OS cells.

2. Materials and methods

2.1. Ethics statement

All sample collections were approved by the Ethics Committee of the Second Hospital of Jilin University and the informed written consent was obtained from each patient. Nude mice were used for *in vivo* studies and were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

2.2. Microarray analysis

National Center of Biotechnology Information (NCBI) is a public platform for storing gene expression data sets, original sequence and records, from which GSE63939 chip was obtained by retrieving OS chips. The chip data were downloaded from Gene Expression Omnibus (GEO) and discovered that GSE63939 included 3 OS samples (GSM1560896, GSM1560897, GSM1560898) and 3 control samples (GSM1560902, GSM1560903, GSM1560904). The Bioconductor-based 'limma' package in the R language and the empirical Bayesian method were used to select important differentially expressed genes. Finally, differentially expressed genes were annotated by the "annotate" package. $p < .05$ was considered statistically significant.

2.3. Prediction of the relevant signaling pathway and miR

The signaling pathway prediction site (https://www. wikipathways. org/) was applied to predict the AXIN1-related signaling pathway. The miR target prediction site (microRNA. org, http://mirwalk. umm. Uniheidelberg. de/, http://ophid.utoronto.ca/mirDIP/) was used to predict the miRNAs that regulates AXIN1, the candidate gene, and the intersection of the predicted results was obtained.

2.4. Specimen collection and cell culture

Twenty-five tumor samples from patients (aged 19.08 ± 6.47 years) who underwent OS orthotopic resection at the Second Hospital of Jilin University from January 2017 to June 2017 were enrolled in this study. The OS and fibrous connective tissues were retained at the ratio of 3: 2 (male to female). The tissue specimens were immediately subpackaged into the eppendorf (EP) cryopreservation tube after resection. All tumor specimens were confirmed by post-operative histopathological analysis. No lymph node metastasis or distant metastasis occurred in the patients who were all diagnosed as primary OS with osteoblasts as main types. A total of 12 cases of tumors were located in femur, 13 cases were located in tibia, all in stage IIB according to Enneking stage system. Human OS cell lines HOS and U2OS were obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China) and cultured in 90% Dulbecco's modified eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA). The cells were assigned into 5 groups, blank group (cells were not transfected), control inhibitor group (cells were transfected with control inhibitor), miR-31-5p inhibitor group (cells were transfected with miR-31-5p inhibitor), miR-31-5p inhibitor + si-AXIN1 group (cells were co-transfected with miR-31-5p inhibitor and si-AXIN1), miR-31-5p inhibitor + si-negative control (NC) group (cell co-transfected with miR-31-5p inhibitor and si-NC) (Wang et al., 2015).

2.5. Plasmid construction

The 3′-untranslated region (UTR) sequence of human AXIN1 was obtained by polymerase chain reaction (PCR) amplification. After the PCR product was recovered, the 3′-UTR sequence of AXIN1 was inserted into the pGL3 plasmid (Promega Corporation, Madison, WI, USA) through *Xba*I enzyme digestion to obtain a pGL3-wild type (WT)-SFRP1–3′-UTR plasmid (WT-AXIN1). At the same time, the miR-31-5p binding site on the AXIN1–3′UTR sequence was mutated to the restriction enzyme cutting site, and the control plasmid pGL3-mutant type (MUT)-SFRP1–3′-UTR (MUT-SFRP1) was obtained. The PCR reaction conditions were as follows: pre-denaturation at 95 °C for 5 min, a total of 25 cycles at 95 °C for 30 s, at 58 °C for 30 s, and at 72 °C for 30 s, and finally extension at 72 °C for 5 min. The sequences used were as follows: AXIN1 3′-UTR 5′-end: 5′-UGUACGCGCCACCGAGUGCCUUC; 3′-end: 5′-CCGUAAGUGGCGCACGGAAU; Wnt1 3′-UTR 5′-end: 5′-CTAGACCC GGACCCCCTCCCTCCCTCTCTCCCGGCGG; 3′-end: 5′-CTAGAGGGAGG ACCCATCCTCCCTCTCTCT. The conditions of enzyme digestion were at 37 °C for 6 h. The miR-31-5p inhibitor (5′-AGGCAAGAUGCUGGCAUA GCU-3′) and control inhibitor were ordered directly from GenePharma (Shanghai, China), si-AXIN1 and si-NC were ordered from Invitrogen (Carlsbad, CA, USA) (Wang et al., 2015; Huang et al., 2014).

2.6. Reverse transfection quantitative polymerase chain reaction (RT-qPCR)

After 72 h of cell culture, the cells were washed three times with phosphate buffered saline (PBS), and total RNA was extracted in accordance with the RNAiso Plus kit (TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China). Reverse transcription was employed with the PrimeScript RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China). Fluorescence quantitative test was conducted with SteponePlus (ABI Company, Oyster Bay, NY, USA) PCR instrument (Wang et al., 2015). The PCR system was as follows: 1.6 μL of cDNA solution, 5 μL of 2 × STBR GREEN Taq PCR MIX (TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China), PCR forward and reverse primers (10 μM) each 0.2 μL, and 3 μL of distilled water. The PCR reaction conditions were: pre-denaturation at 95 °C for 5 min, a total of 60 cycles at 95 °C for 10 s, at 58 °C for 10 s, and at 72 °C for 10 s, finally extension at 72 °C for 10 min. The detection genes were AXIN1 (Gene ID

Table 1
The primer sequences for RT-qPCR.

Genes	Primer sequence (5′-3′)	
AXIN1	GACCTGGGGTATGAGCCTGA	GGCTTATCCCATCTTGGTCATC
miR-31-5p	ACGCGGCAAGATGCTGGCA	CAGTGCTGGGTCCGAGTGA
β-actin	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

Note: RT-qPCR, reverse transfection quantitative polymerase chain reaction; miR-31-5p, microRNA-31-5-p.

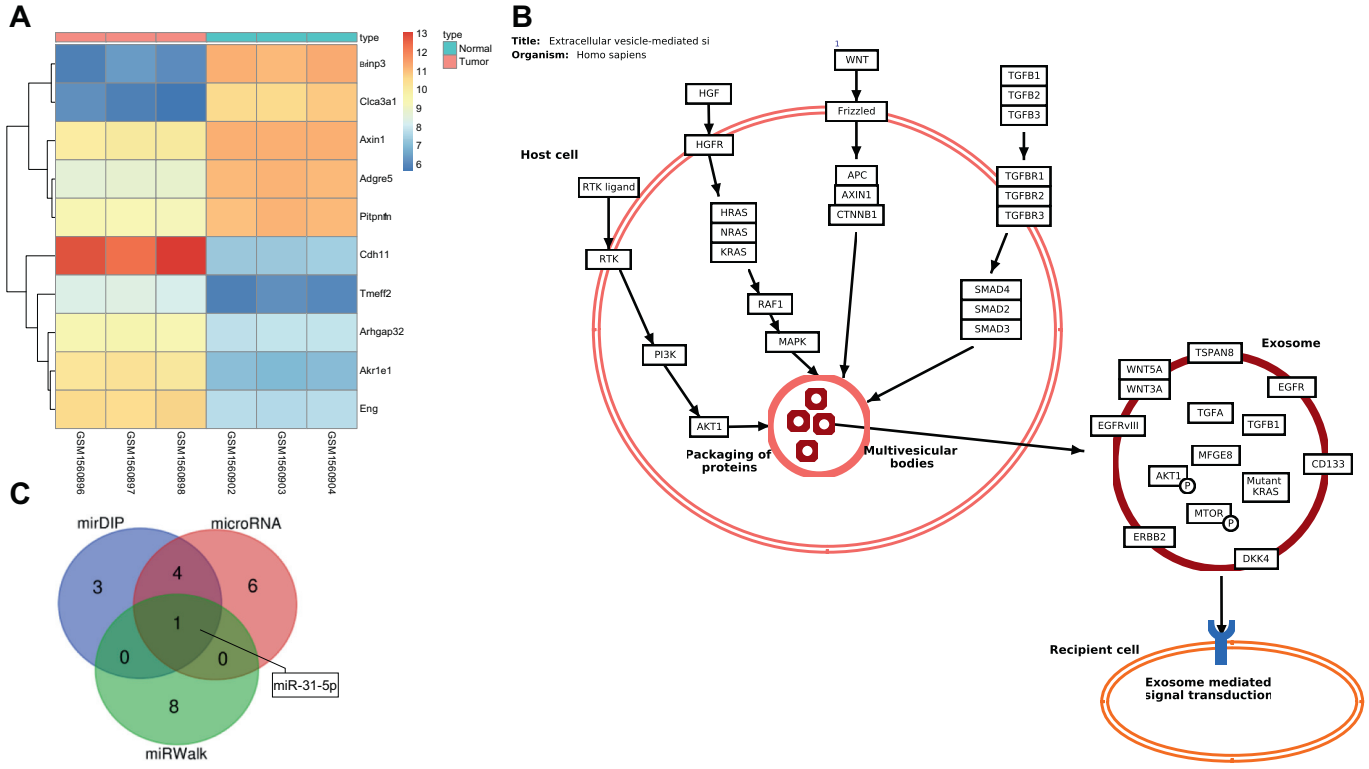

Fig. 1. AXIN1 and miR-31-5p are predicted to be involved in OS. A, the heat map of differentially expressed genes in GSE63939 chip, in which the abscissa represents the sample number, the ordinate represents the gene name, and the upper dendrogram shows the sample type clustering. The color gradation histogram on the right side indicates the gene expression, in which red indicates high expression, blue indicates low expression, each box in the figure represents the expression of a gene in one sample, and the left dendrogram represents the clustering of gene expression levels. B, the website https://www.wikipathways.org prediction of the AXIN1-associated signaling pathway. C, the Venn analysis of AXIN1 regulatory miRNAs; blue indicates the prediction results of the mirDIP database, red indicates the microRNA database prediction results, and green indicates the miRWalk database prediction results; miR-31-5p: micro RNA-31-5-p; OS: osteosarcoma. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

6422), miR-31-5p, and the internal reference was *β-actin* (Gene ID 2597). Primer sequences are shown in Table 1 (Bao et al., 2014).

2.7. Western blot analysis

After the culture for 24 h, cells were washed, homogenized by adding radioimmunoprecipitation assay lysis buffer (Gibco, Carlsbad, CA, USA) and protease inhibitor (Sigma-Aldrich Chemical Company, St Louis, MO, USA), and then centrifuged at 12000 × *g* for 10 min at 4 °C with the supernatant taken as a protein sample. A bicinchoninic acid (BCA) protein assay kit method (Bio-Rad, Hercules, CA, USA) was used to measure the protein concentration, and then the protein was stored at −80 °C. Protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein sample (20 μg) was added to each well. Primary antibodies of rabbit polyclonal antibody to AXIN1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-Vimentin (ab3974, Abcam Inc., Cambridge, MA, USA), rabbit monoclonal ant-E-Cadherin (ab40772, Abcam Inc.), rabbit polyclonal anti-TIMP-1 (ab61224, Abcam Inc.), rabbit polyclonal anti-TIMP-2 (ab180630, Abcam Inc.) and β-catenin (ab16051, Abcam Inc.) were added for incubation overnight at 4 °C. The secondary antibody peroxidase (POD)-conjugated goat anti-rabbit (1:5000) was added for culture at room temperature for 30 min, and then cells were developed in horseradish peroxidase (HRP; Bio-Rad, Hercules, CA, USA). The results were analyzed with Image Quant 350 and Image Quant TL-1 (GE Healthcare, Fairfield, CT, USA), using β-actin (ab8227, Abcam Inc.), Lamin A/C (ab227176, Abcam Inc.) and GAPDH (ab181602, Abcam Inc.) as internal reference (Guo et al., 2014).

2.8. Dual-luciferase reporter gene assay

For the dual-luciferase reporter gene assay, the cells were classified into four groups and transfected with WT-AXIN1 + miR-31-5p inhibitor, MUT-AXIN1 + miR-31-5p inhibitor, WT-AXIN1 + control inhibitor, MUT-AXIN1 + control inhibitor. The transfection of WT-Wnt1 + control inhibitor, WT-Wnt1 + miR-31-5p inhibitor, MUT-Wnt1 + control inhibitor, MUT-Wnt1 + miR-31-5p inhibitor was conducted using the lipofectamineRNAiMAX Kit (Thermo Fisher Scientific, Waltham, MA, USA). After 48 h, the Dual-Luciferase Reporter Assay

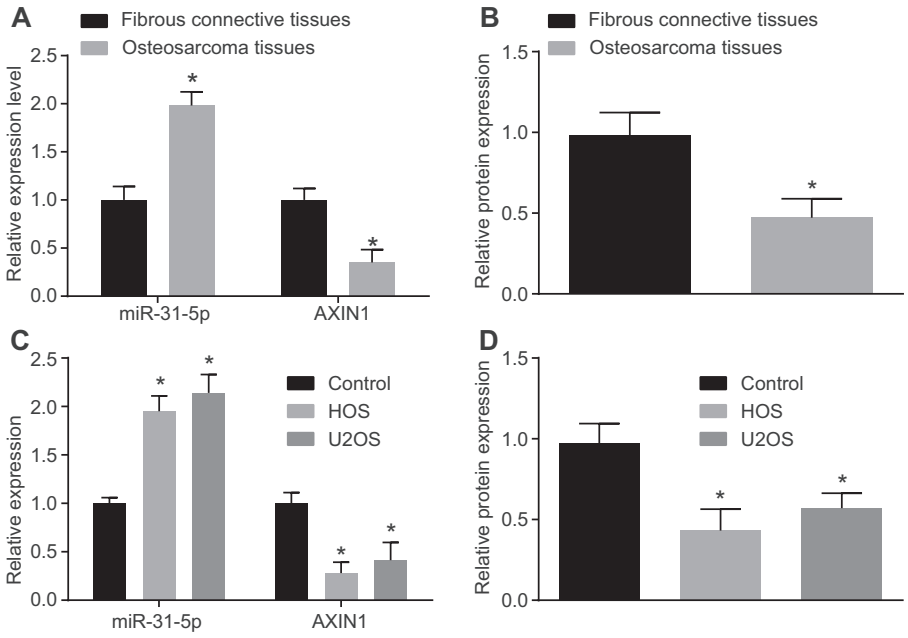

Fig. 2. The expression of miR-31-5p increased while that of AXIN1 diminished in OS tissues and cells. A, miR-31-5p expression and mRNA expression of AXIN1 in OS tissues and fibrous connective tissues determined by RT-qPCR; B, the protein expression of AXIN1 in OS tissues and fibrous connective tissues measured by Western blot analysis; C, the miR-31-5p expression and mRNA expression of AXIN1 in HOS and U2OS cells; D, the protein expression of AXIN1 in HOS and U2OS cells. *$p < .05$ *vs.* the adjacent normal fibrous connective tissues; the data in the figure are all measurement data, which are expressed by mean ± standard deviation. The values of Fig. A and B were analyzed by paired *t*-test, and the values of Figures C and D were analyzed by one-way ANOVA. The experiment was repeated three times; miR-31-5p: microRNA-31-5-p; RT-qPCR, reverse transfection quantitative polymerase chain reaction; OS: osteosarcoma; ANOVA: analysis of variance.

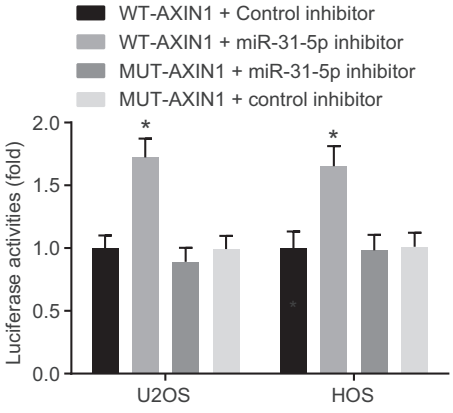

Fig. 3. AXIN1 is a target gene of miR-31-5p. *$p < .05$ *vs.* the WT-AXIN1 + control inhibitor group; the data in the figure are all measurement data, which are expressed by mean ± standard deviation. The values of each group are analyzed by one-way analysis of variance, and the experiment is repeated three times; miR-31-5p: microRNA-31-5-p; WT, wild type; MUT: mutant.

System (Promega Corporation, Madison, WI, USA) was employed to analyze the luciferase activity (Wang et al., 2015).

2.9. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

HOS and U2OS cells were plated in a 96-well plate at 1×10^5 cells per well, and transfected with miR-31-5p inhibitor and control inhibitor (GenePharma, Shanghai, China) respectively. Each group has 4 duplicated wells. After 12 h of culture, cell proliferation was measured at the 12th h, 24th h, 48th h and 72nd h using a Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) (Bao et al., 2014).

2.10. Transwell assay

The upper surface of Transwell invasion assay filters (Costar, Beijing, China) was coated with Matrigel. After incubation for 30 min at 37 °C, 600 μL of conditioned medium derived from tumor cells placed in the bottom compartment of the chamber. Harvested cells (5×10^4) in serum-free medium were added to the upper compartment of the chamber. After incubation at 37 °C for 24 h, the serum-free medium was removed and the noninvaded cells on the upper side of the chamber were scraped off. The cells were then fixed with 100% methanol and

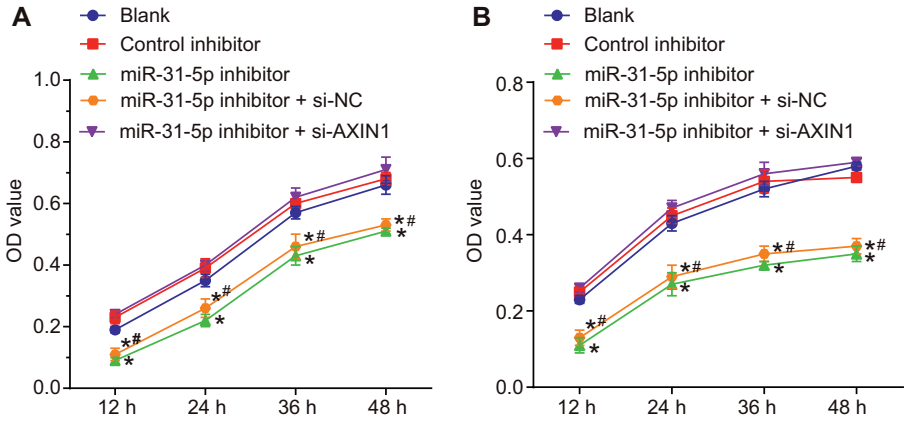

Fig. 4. MiR-31-5p downregulation suppresses proliferation of OS cells through promoting AXIN1. A, HOS cell proliferation detected by MTT assay; B, U2OS cell proliferation detected by MTT assay; *$p < .05$ *vs.* the control inhibitor group; #$p < .05$ *vs.* the miR-31-5p inhibitor + si-AXIN1 group; the data in the figure are all measurement data, expressed by mean ± standard deviation. The values of each group were analyzed by two-way variance and the experiment was repeated three times; miR-31-5p: microRNA-31-5-p; OS: osteosarcoma; MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

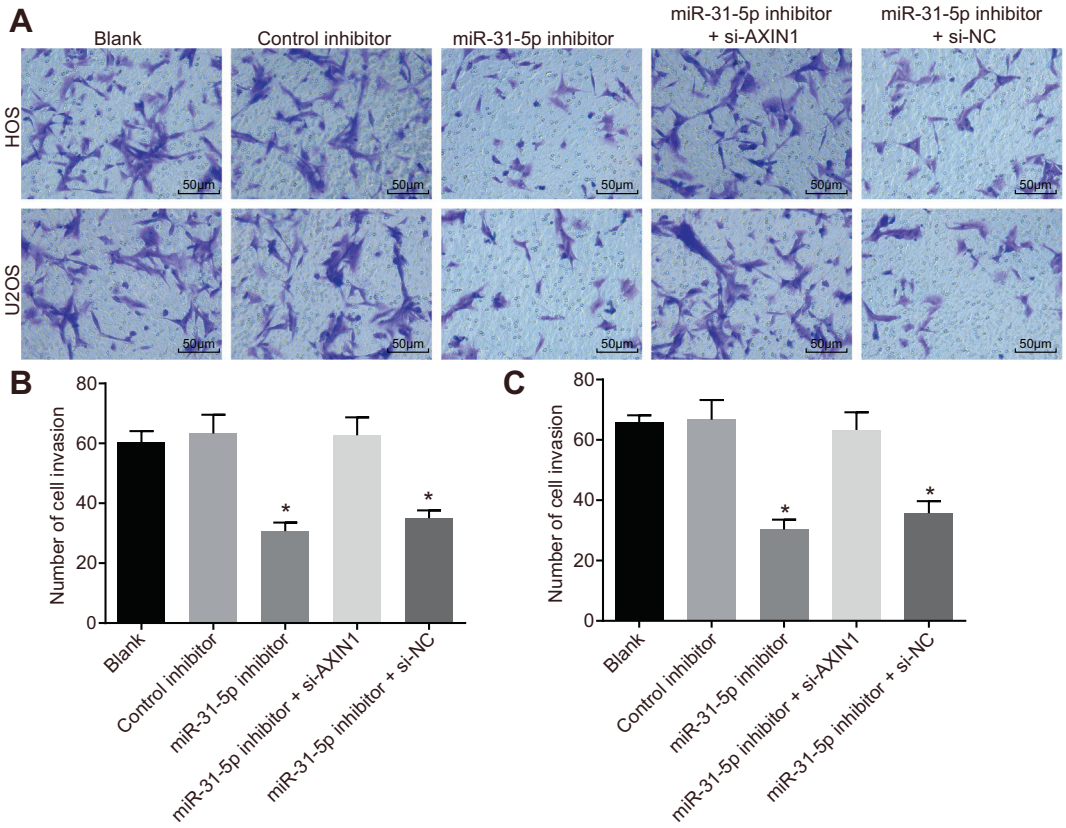

Fig. 5. MiR-31-5p downregulation inhibits the invasion of OS cells through enhancing AXIN1. A, invasion ability of HOS and U2OS cells after transfection; B—C, quantification of results from A. *$p < .05$ *vs.* the control inhibitor group; #$p < .05$ *vs.* the miR-31-5p inhibitor + si-AXIN1 group; the data in the figure are all measurement data, expressed by mean ± standard deviation. The values of each group were analyzed by one-way ANOVA and the experiment was repeated three times; miR-31-5p: microRNA-31-5-p; OS: osteosarcoma.

stained with crystal violet. The number of cells invading through the matrigel was counted using three randomly selected visual fields (Wang et al., 2015).

2.11. Immunofluorescence staining

A cover slip coated with 0.1% gelatin was placed in a six-well plate and cells were cultured on that cover slip. After the cell density reached 80%–90%, the cell solution was discarded, cells were washed three times with phosphate-buffered saline/tween (PBST), fixed with 4% paraformaldehyde for 20 min, then blocked with PBST containing 10% goat serum for 1 h at room temperature, and incubated with primary antibody for 30 min at room temperature at 4 °C overnight. The primary antibody was rabbit polyclonal antibody to β-catenin (Abcam, Cambridge, MA, USA). After washing three times with PBST, the secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (1: 5000) was added into cells. 6-diamidino-2-phenylindole (DAPI) reagent was used to stain the cell nuclei, and the cells were visualized using a confocal microscope (Wang et al., 2015).

2.12. Xenograft model in nude mice

Totally, 60 male C57/BL6 mice (age of 8 weeks) were divided into 5 groups (12 mice in each group): blank group (only subcutaneous injection with HOS cells without injection of any miRNA or siRNA into caudal veins), control inhibitor group (subcutaneous injection with HOS cells and injection of control inhibitor into caudal veins, 80 mg/kg, one time every other day for 35 d), miR-31-5p inhibitor group (subcutaneous injection with HOS cells and injection of miR-31-5p inhibitor into caudal veins, 80 mg/kg, one time every other day for 35 d), miR-31-5p inhibitor + si-AXIN1 group (subcutaneous injection with HOS cells, and injection of miR-31-5p inhibitor and si-AXIN1 into caudal veins, 80 mg/kg + 80 mg/kg, one time every other day for 35 d), miR-31-5p inhibitor + si-NC group (subcutaneous injection with HOS cells, and injection of miR-31-5p inhibitor and si-NC into caudal veins, 80 mg/kg + 80 mg/kg, one time every other day for 35 d). A total of 3×10^6 cells was subcutaneously injected into each mouse. The length (a, cm) and width (b, cm) of tumors were measured on the 7th, 14th, 21st, 28th, and 35th d after inoculation of HOS cells using calipers. Tumor volume was calculated using the formula $V = 1/2 \times a \times b \times b$. Six weeks later, the mice were euthanized and tumors were resected and weighed. The expression of related genes in tissues was evaluated by Western blot.

2.13. Statistical analysis

The data in the present research were analyzed by SPSS 21 (IBM Corp. Armonk, NY, USA) statistical software. All data were tested by normality and homogeneity of variance. The measurement data in accordance with the normal distribution were expressed as mean value ± standard deviation. The data of fibrous connective tissues and OS tissues were compared by paired *t*-test. The data with heterogeneity of variance were corrected by Welch's *t*-test. Data at different time points were analyzed by repeated measures analysis of variance (ANOVA); one-way ANOVA was used for comparison among multiple groups. Tukeys was applied for *post hoc* test. And data in skewed distribution were tested by rank sum test. $p < .05$ indicated that the difference was statistically significant.

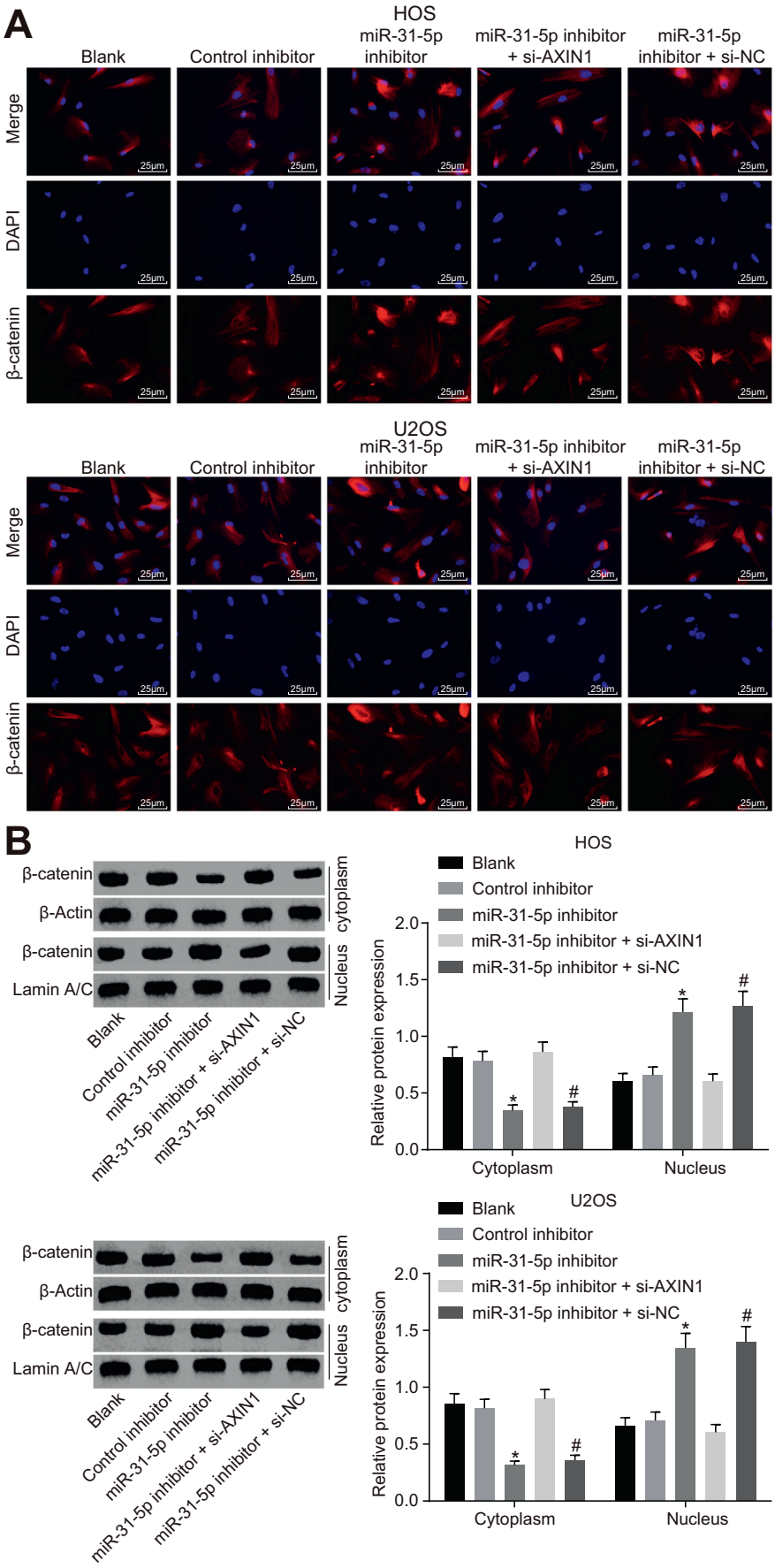

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Fig. 6. MiR-31-5p can regulate the Wnt/β-catenin signaling pathway through inhibiting the transcription of AXIN1. A, immunofluorescence staining of β-catenin in HOS and U2OS cells; B, the protein expression of β-catenin in cytoplasm and nucleus of HOS and U2OS cells assessed by Western blot analysis. *$p < .05$ *vs.* the control inhibitor group; #$p < .05$ *vs.* the miR-31-5p inhibitor + si-AXIN1 group; the data in the figure are all measurement data, expressed by mean ± standard deviation. The values of each group were analyzed by one-way ANOVA and the experiment was repeated three times; miR-31-5p: microRNA-31-5-p.

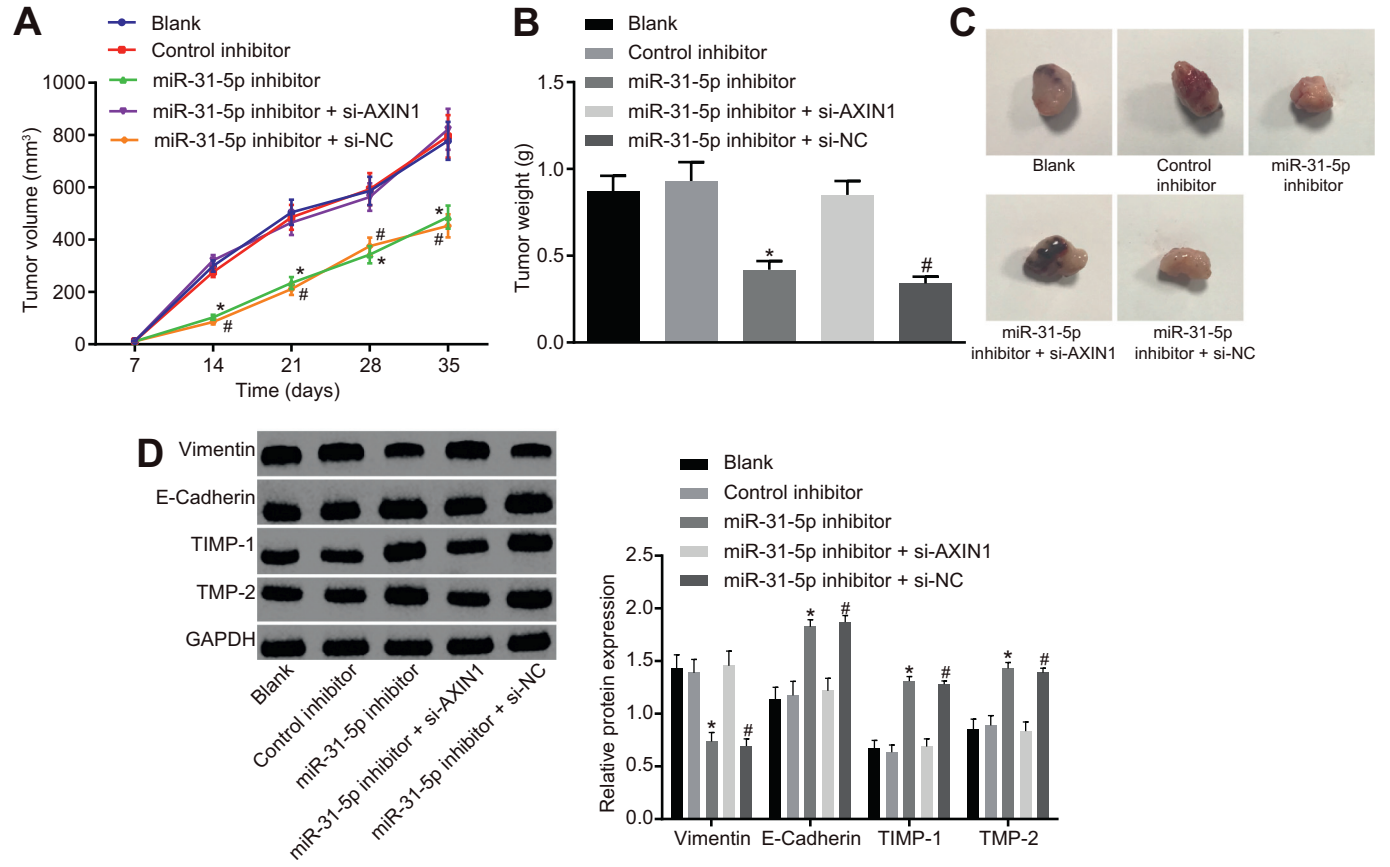

Fig. 7. MiR-31-5p downregulation suppresses xenograft tumor growth *in vivo* by targeting AXIN1. A, subcutaneous transplanted tumor volume of each group; B, subcutaneous transplanted tumor weight of each group; C, representative images of subcutaneous transplanted tumors in each group; D, expression of Vimentin, E-Cadherin, TIMP-1, TMP-2 in subcutaneous transplanted tumor tissues of mice measured by Western blot analysis. *$p < .05$ *vs.* the control inhibitor group; #$p < .05$ *vs.* the miR-31-5p inhibitor + si-AXIN1 group; the data in the figure are all measurement data, expressed by mean ± standard deviation. The values of each group were analyzed by one-way ANOVA and the experiment was repeated three times; n = 12; miR-31-5p: microRNA-31-5-p.

3. Results

3.1. AXIN1 and miR-31-5p are involved in OS

The gene expression dataset GSE63939 was downloaded from the GEO database, and a total of 7433 differentially expressed genes were obtained from the GSE63939 chip. According to the literature, the AXIN1 gene codes the regulators of G-protein signaling (RGS) domain and the DIX domain. The protein encoded by AXIN1 interacts with adenomatous polyposis, catenin β-1, and glycogen synthase kinase 3β. Mutations in AXIN1 are associated with hepatocellular carcinoma, hepatoblastoma, ovarian endometrial adenocarcinoma and medulloblastoma (Koyama-Nasu et al., 2015; Pu et al., 2014; Pu et al., 2017; Zhou et al., 2015). AXIN1 plays a carcinogenic role in many human cancers. However, the exact function of AXIN1 in human OS is unclear. As the candidate gene of our study, and we aimed to predict function and clinical significance of AXIN1 in OS. Moreover, AXIN1 ranked highly in differentially expressed genes. Fig. 1A is a heat map of selected 10 differentially expressed genes, illustrating that AXIN1 was poorly expressed in OS. Through the signaling pathway prediction site, we found that a protein encoded by AXIN1 as a negative regulator of the Wnt/β-catenin signaling pathway, could induce apoptosis. Furthermore, the AXIN1 gene was involved in the mucus components and tumor size of colorectal cancer in the Wnt/β-catenin signaling pathway. This indicated that the AXIN1 gene could regulate the Wnt/β-catenin signaling pathway (Korkmaz et al., 2016; Picco et al., 2017) (Fig. 1B). In order to study the upstream of the differentially expressed gene AXIN1, the regulatory miRNAs of AXIN1 was predicted through the bioinformatics website and the Venn map. Only one miRNA was in the intersection, miR-31-5p (Fig. 1C).

3.2. Overexpression of miR-31-5p and down regulation of AXIN1 are identified in OS

Next, RT-qPCR was applied to test the miR-31-5p expression and mRNA expression of AXIN1 in OS tissues and cells. The results showed that the expression of miR-31-5p in OS tissues increased, while the mRNA expression of AXIN1 decreased compared with the fibrous connective tissues ($p < .05$). The results of Western blot analysis showed that the protein expression of AXIN1 in OS reduced compared with the fibrous connective tissues ($p < .05$). This result demonstrates that the expression of miR-31-5p is enriched while the expression of AXIN1 is diminished in the OS tissues. The results of RT-qPCR showed that the expression of miR-31-5p was increased in the HOS and U2OS cell lines,

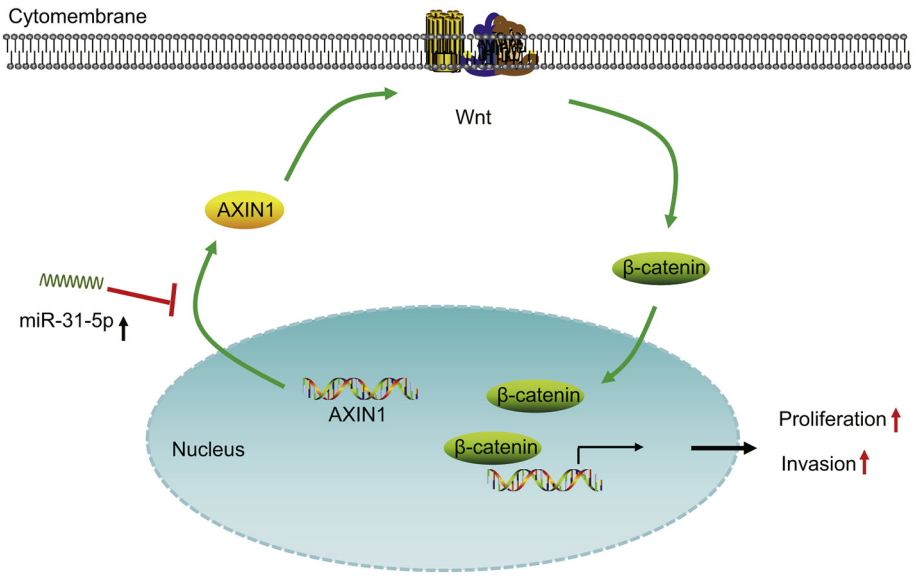

Fig. 8. Schematic diagram of the regulation of miR-31-5p in OS. AXIN1 is a target gene of miR-31-5p. Down-regulation of miR-31-5p suppresses OS cell proliferation and invasion through activation of the Wnt/β-catenin signaling pathway by promoting AXIN1; miR-31-5p: microRNA-31-5-p; OS: osteosarcoma.

while the mRNA expression of AXIN1 was inhibited in HOS and U2OS cell lines compared with fibrous connective tissues ($p < .05$). The results of Western blot analysis showed that the protein expression of AXIN1 in HOS and U2OS cell lines decreased compared with the fibrous connective tissues ($p < .05$) (Fig. 2A–D). This result demonstrates that expression of miR-31-5p is increased while the expression of AXIN1 is decreased in OS tissues and cell lines.

3.3. AXIN1 is the target gene of miR-31-5p

Dual luciferase reporter gene assay was applied to detect the relationship between miR-31-5p and AXIN1. The luciferase activity was measured after HOS and U2OS cells were transfected (Fig. 3). The results showed that the luciferase activity of HOS and U2OS cells transfected with WT-AXIN1 + control inhibitor was 1, and there was no significant change of luciferase activity in the MUT-AXIN1 + miR-31-5p inhibitor and MUT-AXIN1 + control inhibitor groups. Nevertheless, the luciferase activity in the WT-AXIN1 + miR-31-5p inhibitor group significantly enhanced ($p < .05$). These results indicate that AXIN1 mRNA 3′-UTR is a specific functional target of miR-31-5p in OS cells.

3.4. Down-regulation of miR-31-5p suppresses proliferation of OS cells through promoting AXIN1

Cell viability was measured by MTT assay every 24 h after cell transfection for 24 h. At the 12th h, 24th h, 36th h and 48th h after culture, the proliferation of HOS and U2OS cells transfected with miR-31-5p inhibitor decreased compared with cells transfected with control inhibitor (all $p < .05$) (Fig. 4A–B). The proliferation of HOS and U2OS cells transfected with miR-31-5p inhibitor + si-AXIN1 had no difference from cells transfected with control inhibitor (all $p > .05$). The proliferation of HOS and U2OS cells transfected with miR-31-5p inhibitor + si-NC was lower than cells transfected with control inhibitor and miR-31-5p inhibitor + si-AXIN1 (both $p < .05$). The above data show that decreased miR-31-5p suppresses proliferation of OS cells through promoting AXIN1.

3.5. Down-regulation of miR-31-5p inhibits the invasion of OS cells through enhancing AXIN1

Cell invasion was detected by Transwell assay at the 24th h after transfection of HOS and U2OS cells. The number of invasion of HOS and U2OS cells transfected with miR-31-5p inhibitor decreased compared with cells transfected with control inhibitor (all $p < .05$) (Fig. 5A–B). The number of invasion of HOS and U2OS cells transfected with miR-31-5p inhibitor + si-AXIN1 had no difference from cells transfected with the control inhibitor (all $p > .05$). The number of invasion of HOS and U2OS cells transfected with miR-31-5p inhibitor + si-NC diminished relative to cells transfected with the control inhibitor and the miR-31-5p inhibitor + si-AXIN1 (all $p < .05$). The results demonstrate that down-regulated miR-31-5p inhibits the invasion of OS cells through enhancing AXIN1.

3.6. miR-31-5p can regulate the Wnt/β-catenin signaling pathway through inhibiting the transcription of AXIN1

After the transfection of HOS and U2OS cells, immunofluorescence staining was applied to evaluate the activity of Wnt/β-catenin signaling pathway. It turned out that β-catenin did not translocated into the nucleus of the HOS and U2OS cells in the blank group and the control inhibitor group, but the expression of β-catenin in the HOS and U2OS cells transfected with miR-31-5p inhibitor increased and β-catenin translocated into the nucleus. However, the expression of β-catenin in HOS and U2OS cells transfected with si-AXIN1 + miR-31-5p inhibitor did not increase significantly and β-catenin did not translocate into the nucleus; whereas the expression of β-catenin in HOS and U2OS cells transfected with si-NC + miR-31-5p inhibitor elevated and β-catenin translocated into the nucleus (Fig. 6A). We further measured the expression of β-catenin in the cytoplasm and nucleus by Western blot analysis. The results showed that the expression of β-catenin in HOS and U2OS cells treated with miR-31-5p inhibitor increased significantly in the nucleus, but decreased significantly in the cytoplasm (Fig. 6B). Whereas the involvement of si-AXIN1 in the presence of miR-31-5p inhibitor would reverse the trend. We also observed that changes in miR-31-5p expression did not affect the transcriptional expression of Wnt1 (Supplementary Fig. 1A–B). These results suggest that miR-31-5p can regulate the Wnt/β-catenin signaling pathway through the inhibition of AXIN1 transcription and the translocation of β-catenin into the nucleus.

3.7. Down-regulation of miR-31-5p suppresses xenograft tumor growth by targeting AXIN1 in vivo

To confirm whether miR-31-5p and AXIN1 affect OS tumorigenesis *in vivo*, nude mice were injected with miR-31-5p alone or with si-AXIN1 through caudal veins, in addition to subcutaneous injection of HOS cells. From the 7th d after injection, the volume of subcutaneously transplanted tumor was measured every 7 d. On the 35th d, the mice were euthanized, the tumors were photographed and weighed (Fig. 7A–C). In comparison with the control inhibitor group, the volume and weight of the transplanted tumors in the mice injected with miR-31-5p inhibitor was remarkably repressed ($p < .01$). Meanwhile, no significant difference was observed in the volume and weight of transplanted tumor between mice injected with miR-31-5p inhibitor and si-AXIN1 and those with control inhibitor ($p > .05$). However, the volume and weight of transplanted tumor of mice injected with miR-31-5p inhibitor and si-NC were significantly downregulated relative to those with control inhibitor and those with miR-31-5p inhibitor plus si-AXIN1 ($p < .01$). Moreover, the expression of genes associated with invasion and metastasis (Vimentin, *E*-Cadherin, TIMP-1, TMP-2) was measured so as to determine the role of miR-31-5p *in vivo*. The miR-31-5p inhibitor group presented significantly decreased Vimentin expression and promoted E-Cadherin, TIMP-1 and TIMP-2 expression, *versus* the control inhibitor group. No significant difference was identified in the expression of Vimentin, E-Cadherin, TIMP-1 and TMP-2 between the miR-31-5p inhibitor + si-AXIN1 group and the control inhibitor group. Nevertheless, the combined injection of miR-31-5p inhibitor and si-NC led to upregulated E-Cadherin, TIMP-1 and TIMP-2 expression and downregulated Vimentin expression, relative to the injection of miR-31-5p inhibitor and si-AXIN1 (Fig. 7D). Taken together, these results indicate that miR-31-5p downregulation inhibits tumor growth *in vivo* by targeting AXIN1.

4. Discussion

OS is the most prevailing primary bone cancer featuring local relapse and metastatic potential, and is usually found in the long bones of children, adolescents and young adults (Kobayashi et al., 2012). miRNAs play a crucial role in controlling putative target genes in OS and defining OS's expression identity (Jones et al., 2012). Nevertheless, the role of miR-31-5p functioning in OS has not been discovered, and the evidences of this study probe into a new potential role of miR-31-5p, AXIN1 and Wnt/β-catenin signaling pathway in regulating proliferation and invasion of OS cells. As a consequence, this study demonstrates that down-regulation of miR-31-5p suppresses OS cell proliferation and invasion through activation of the Wnt/β-catenin signaling pathway by promoting AXIN1.

Initially, our results demonstrated that overexpression of miR-31-5p and down-regulation of AXIN1 were determined in patients with OS tissues. Similar to our study, Mlcochova et al. found that miR-31-5p was highly expressed in colorectal tumor tissues (Mlcochova et al., 2015). In addition, Zhong and his team revealed that the high expression of mature miR-31-5p was detected in non-small cell lung cancer cells (Zhong et al., 2013). A previous study found that AXIN1 was known as a tumor inhibitor, and its expression was down-regulated in several kinds of solid tumors, including breast cancer, and hepatocellular carcinoma (Mazzoni and Fearon, 2014; Zhang et al., 2012), suggesting its important role in cancers.

Next, we found that down-regulation of miR-31-5p inhibits proliferation and invasion of OS cells as well as tumor growth *in vivo* through Wnt/β-catenin signaling pathway by enhancing AXIN1. And AXIN1 is the target gene of miR-31-5p. Accumulating evidence indicated key role of miRNAs played in regulation of tumor cell invasion and metastasis (Nicoloso et al., 2009). It has been demonstrated that down-regulation of miR-31 promotes chemotherapy efficacy (5-fluoracil) at an earlier stage, and inhibits proliferation, invasion and migration of colorectal cancer cells (Nosho et al., 2014; Wang et al., 2010), which was partly consistent with our results. Interestingly, a very recent study proved that down-regulation of miR-26a inhibited proliferation, migration and invasion of OS Saos-2 cells through regulation of Wnt/β-catenin signaling pathway (Qu et al., 2016). Interestingly, miR-31-5p was reported to target the key genes of the Wnt pathway (Liu et al., 2013). The Wnt/β-catenin signaling pathway modulates various genes that in turn regulate a diversity of cell functions such as proliferation, differentiation and morphogenesis, and β-catenin was proved to enhance progression, invasion and tumorigenesis in cancers (Liu et al., 2015). A previous study suggested that inactivation of the Wnt/β-catenin signaling pathway play a crucial role in suppressing the chemotherapeutic responses, tumorigenesis and metastasis of OS (Ma et al., 2015). In addition, down-regulation of Wnt/β-catenin signaling pathway was demonstrated to inhibit the invasion and proliferation of OS cells and suppress the tumorigenesis of OS stem cells (Dai et al., 2017; Liu et al., 2015). AXIN1 is primarily served as regulator of Wnt pathway through assembling the β-catenin destruction complex (Korkmaz et al., 2016). What's more, AXIN1 has direct interaction with β-catenin, and down-regulating AXIN1 in Wnt-activated cells is likely to cause β-catenin stabilization (Li et al., 2012). AXIN is featured as a tumor inhibitor in various cancers; for instance, AXIN1 serves as a potential target gene for testicular germ cell tumor therapy (Xu et al., 2017). Additionally, abundance of AXIN1 can lead to apoptosis of BRAF ^{V600E} tumor cells in human melanoma, which provides potential approaches for vemurafenib therapy (Biechele et al., 2012). Also, it was evidenced that down-regulation of miR-107 inhibits proliferation of hepatocellular carcinoma cell by promoting AXIN2 (Zhang et al., 2015). Besides, TIMP-1 is assumed to function importantly in diminishing invasive potential of the OS cells treated with gambogic acid (Xin et al., 2013). In the xenograft model in nude mice of the present study, we provided evidence that miR-31-5p downregulation inhibited the tumorigenic, invasive and metastatic potential of OS cells by targeting AXIN1, as proved by downregulated Vimentin and upregulated *E*-Cadherin, TIMP-1, and TIMP-2.

In line with the previous studies, we have confirmed that overexpression of miR-31-5p and down regulation of AXIN1 were determined in patients with OS tissues. We also demonstrate that AXIN1 is a target gene of miR-31-5p, and down-regulation of miR-31-5p inhibits proliferation and invasion of OS cells through Wnt/β-catenin signaling pathway by enhancing AXIN1 (Fig. 8). We speculate that miR-31-5p may be potential new direction in the progress of OS's therapeutic treatments. Nevertheless, further study is required to detect the mechanism through which miR-31-5p regulates the Wnt/β-catenin signaling pathway in cancer.

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Competing interests

The authors declare that they have no competing interests.

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