



# microRNA-377-3p inhibits osteosarcoma progression by targeting *CUL1* and regulating Wnt/ $\beta$ -catenin signaling pathway

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## Abstract

**Objective** Emerging studies highlight the crucial effects of microRNAs on cancer initiation and malignant progression of various tumors. This study focused on the biological effect of miR-377-3p on *CUL1* and epithelial–mesenchymal transition (EMT) and Wnt/ $\beta$ -catenin pathways in osteosarcoma (OS).

**Methods** We performed quantitative real-time polymerase chain reaction (qRT-PCR) to analyze miR-377-3p and *CUL1* expression levels in OS tissues and MG-63 cells. Then, cell counting kit (CCK)-8 and Transwell assay were used to examine the functions of miR-377-3p in OS cell growth and metastasis abilities. Meanwhile, luciferase reporter assay was used to validate *CUL1* as direct target of miR-377-3p. qRT-PCR and Western blot were then carried out to detect the impact of miR-377-3p on EMT and Wnt/ $\beta$ -catenin pathways. Tumor xenograft models were established to further examine the effects of miR-377-3p on OS tumorigenesis in vivo.

**Results** miR-377-3p downregulation was frequently identified in OS tissues and cells, which was associated with worse prognosis of OS patients. Functional experiments showed miR-377-3p restoration could dramatically repress OS cell growth and migration by regulation of EMT and Wnt/ $\beta$ -catenin pathways. Moreover, luciferase reporter assay revealed that *CUL1* acted as a functional target of miR-377-3p. Additionally, the elevated *CUL1* expressions in OS tissues also indicated poor prognosis of OS patients. Furthermore, the OS tumor growth was also obviously inhibited by miR-377-3p overexpression in vivo.

**Conclusions** Collectively, all the above findings revealed that miR-377-3p exerted anti-OS functions via *CUL1* and EMT and Wnt/ $\beta$ -catenin pathways. These results may contribute to the development of clinical OS treatment.

**Keywords** Osteosarcoma · MiR-377-3p · *CUL1* · Wnt/ $\beta$ -catenin · Epithelial-to-mesenchymal transition

## Introduction

Osteosarcoma (OS) is the most common malignancy affecting children, adolescents, and young adults, accounting for 20% of all bone tumors and approximately 5% of all pediatric

tumors [1, 2]. The overall incidence worldwide is 3.4 per million per year [3]. Currently, the most common treatment for osteosarcoma is complete surgical resection combined with chemotherapy. The implementation of neoadjuvant and adjuvant chemotherapy has increased the overall survival of patients diagnosed with non-metastatic osteosarcoma to 70%; however, this has not significantly improved the prognosis of patients with metastasis or recurrence [4]. Current treatment for 5-year survival rate of metastatic or recurrent osteosarcoma patients can be achieved which ranges from 20 to 30% [5]. However, despite multiple attempts to use different chemotherapy regimens over the past 20 years, survival rates have remained essentially unchanged and there have been no successful targeted therapies for osteosarcoma to date [6, 7].

Accumulating evidence indicates that the occurrence, development and prognosis of osteosarcoma are closely related to molecular mechanisms [8]. MicroRNAs (miRNAs)

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are small non-coding RNAs that can affect mRNA expression by binding to their 3' untranslated regions (3' UTRs) [9]. MiRNAs emerged as post transcriptional regulators in regulating biogenesis and cancer progression, with the potential to be prognostic biomarkers for OS [10, 11]. Many studies have shown that miR-377 may affect the progression of different osteosarcoma by acting as a suppressor gene [12, 13]. In addition, miR-377 was found to have decreased expression in hepatocellular carcinoma, ovarian cancer, suggesting that miR-377 plays an important role in tumorigenesis and progression [14, 15].

Cullin (*CUL*) 1 is one of the targets of miR-377-3p. *CUL1* is an essential component of the *SCF* E3 ubiquitin ligase complex and plays an important role in tumorigenesis and progression [16]. One key role of cull1 as an oncogene is to promote cell cycle progression [32811853]. Increased *CUL1* expression is strongly associated with poor prognosis in osteosarcoma patients [17]. However, the specific mechanisms by which miR-377-3p and *CUL1* affect osteosarcoma progression have not been documented.

Therefore, the present study investigated the in vitro and in vivo functions of miR-377-3p in osteosarcoma. In addition, a miRNA–mRNA functional network was also constructed to elucidate the underlying mechanism of osteosarcoma progression.

## Materials and methods

### Osteosarcoma patients

This study was approved by the Research Ethics Committee of Nanxishan hospital of Guangxi Zhuang Autonomous Region (Guangxi, China). All participants were aware of the content of this study and signed an informed consent. We collected 10 osteosarcoma tissue samples during the surgical procedure. Normal bone samples were obtained from 10 participants who succumbed to mortality in traffic accidents. The characteristics of the patients are shown in Table S1.

### Cell culture

The human OS cell lines (MG-63), human embryonic kidney cell line (HEK293T) and immortalized human osteoblast cells hFOB (hFOB1.19) were purchased from Shanghai Institutes for Biological Sciences Cell Resource Center (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM; Hyclone, MA, USA) supplemented with 10% fetal bovine serum and 1% antibiotic and antimycotic solution. Cells were put into the incubator for culture at 37 °C and 5% CO<sub>2</sub>.

## Construction and transfection of miR-377-3p

Human miR-377-3p mimics (5'-UACAG UACUGUGAU AACUGAACAGUUAUCACAGUACUGU AUU-3') and the scramble mimics were synthesized in GenePharma (Shanghai, China). About the transfection, 80 nm miR-377-3p mimics and scramble mimics were transfected into MG-63 using Lipofectamine® 2000 (Thermo, MA, USA) according to the manufacturer's instructions. These cells were then incubated for 48 h.

### Luciferase reporter assays

For the luciferase reporter plasmids, the full-length 3'-UTR of *CUL1*, and 3'-UTR mutant for the miR-377-3p-binding site 3'-UTRs of the gene were cloned individually into pMIR-REPORT luciferase reporter vectors (Ambion, TX, USA). Then, vectors were co-transfected with miR-377-3p mimics into HEK293T through Lipofectamine 2000 reagent. The cells were collected and measured luciferase activities after 24 h using Dual-Luciferase reporter assay kit (Promega, WI, USA).

### Quantitative real-time polymerase chain reaction

The total RNA from tissue samples and cultured cells was extracted using Trizol kit (Thermo). Total RNA was reverse transcribed to cDNA utilizing a PrimeScript™ RT reagent Kit (Invitrogen, USA) or miRNA 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the SYBR Green qPCR Mix (Invitrogen). The results from qRT-PCR were normalized to *GAPDH* or *U6*. The primers listed below: miR-377-3p, 5'-TGGTGTCTGGAGTCG-3' and 5'-ACACTCCAGCTGGGATCACACAAAGGCA-3'; *CUL1*, 5'-GCCGTGTCGCACGCAG-3' and 5'-GTCCCCG CCAAGTCTAGCTG-3'; *GAPDH*, 5'-CTTTGGTATCGT GGAAGGACTC-3' and 5'-GTAGAGGCAGGGATGATG TTCT-3'. *U6*, 5'-CTCGCTTCGGCAGCACA-3' and 5'-AAC GCTTCACGAATTTGCGT-3'. The genes relative expression was calculated using the 2<sup>-ΔΔCt</sup> method. All reactions were carried out in triplicate.

### Western blot analysis

Cells were thoroughly mixed with RIPA cell lysis buffer (Beyotime, Shanghai, China) on ice. Proteins were quantified, then separated using 10% SDS/polyacrylamide gels and transferred onto PVDF membranes (Millipore, USA). Membranes were blocked using 5% fat-free milk at room temperature. The membranes were incubated with antibodies against

GAPDH, Vimentin, Twist, N-cadherin, Snail,  $\beta$ -catenin, Wnt3a, and cyclin D1 (Abcam, MA, USA) overnight at 4 °C. The membranes were then incubated with the following secondary antibodies. Immunoreactivity was detected using an ECL Kit (Pierce). The results were analyzed using Image Lab™ Software (Bio-Rad Laboratories). Protein bands were detected using an ECL Kit (Beyotime).

### Cell Counting Kit (CCK)-8

MG-63 cells transduced with miR-377-3p mimics and scramble mimics were seeded in 96-well plate ( $2.5 \times 10^3$ /well), cells were cultured for 96-h at 37 °C in a 5% CO<sub>2</sub> incubator. Then, 10  $\mu$ l CCK-8 reagent was added to each well and incubated for 2 h. The absorbance of each well was detected at a wavelength of 450 nm.

### Apoptosis assay

Cells were washed with PBS followed by incubation with Annexin V PE-A and PC5.5-A using the Annexin V Apoptosis Detection Kit (BD Biosciences, NJ, USA) for 15–20 min at room temperature. Cell apoptosis was then analyzed by flow cytometry (FACSCalibur) at 480 nm. Data were analyzed using FlowJo version 10 (FlowJo LLC, Ashland, OR, USA).

### Transwell

The cells were seeded into the upper well of a Millicell hanging cell culture insert of a Transwell chamber (8- $\mu$ m pore size, Millipore), for the migration assays after serum-free culture. The culture medium with serum was added into the lower chamber and subsequently incubated for 24 h. The cells migrating to or invading the bottom of the membrane were fixed with methanol and stained with crystal violet.

### Animal experiments

To establish OS murine xenograft model, the 6-week-old BALB/c nude mice were used. MG6-3 cells ( $7 \times 10^6$  cells) transfected with miR-377-3p mimics or scramble mimics were subcutaneously injected into the armpit of the nude mice (10 mice each group). After injection for 7 days, tumor growth was measured every 3 days, and these mice were euthanized after 19 days. The tumor growth inhibition rate was then calculated. The length (L) and width (W) of tumors were measured and assessed using the formula:  $(L \times W^2)/2$ . All experimental procedures were approved by the Ethics Committee of Animal Research of Nanxishan hospital of Guangxi Zhuang Autonomous Region.

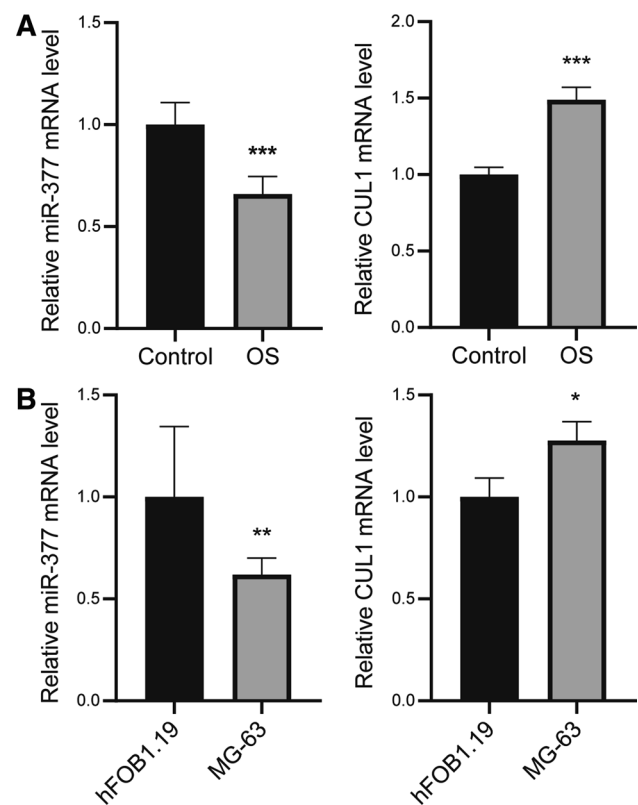
### Statistical analysis

Statistical analyses were performed through SPSS 20.0. Data are presented as the mean  $\pm$  standard deviation (SD). Statistical analyses were performed with Student's *t* test and the statistical significance level was set at  $\alpha=0.05$  (two-side). Values of  $P < 0.05$  were regarded as statistically significant.

## Results

### Expression of miR-377-3p and CUL1 in osteosarcoma tissues and cell lines

First, we examined the expression of miR-377-3p and *CUL1* using qRT-PCR in 10 osteosarcoma and 10 control samples. The results suggested that the level of miR-377-3p was lower in osteosarcoma tissues than in controls, whereas *CUL1* showed increased expression in osteosarcoma tissues (Fig. 1a). In addition, we also found that the expression level of miR-377-3p was decreased, while the expression of *CUL1*

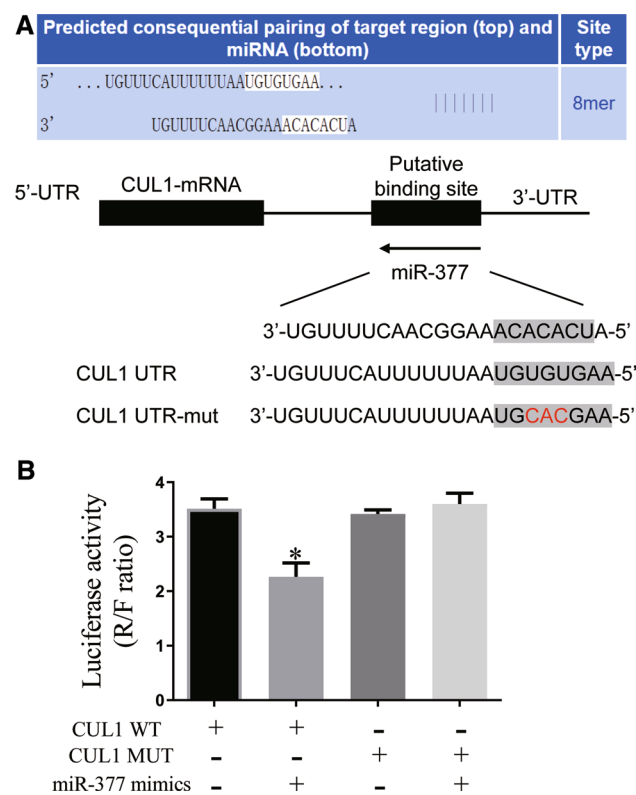


**Fig. 1** The expression levels of miR-377-3p and *CUL1* in osteosarcoma tissues and cell line. **a** The mRNA levels of miR-377-3p and *CUL1* in osteosarcoma tissues detected by qRT-PCR. OS, osteosarcoma. **b** The mRNA levels of miR-377-3p and *CUL1* in MG-63 cell detected by qRT-PCR. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$

was clearly increased in the osteosarcoma cell line MG-63, compared with osteoblast hFOB1.19 (Fig. 1b).

### miR-377-3p targets binding to CUL1

We predicted the binding sites of miR-377-3p and *CUL1* in the TargetScan online website (Fig. 2a). We suggested that miR-377-3p may be involved in the regulation of *CUL1* protein expression. miR-377-3p was predicted to interact with 8-mer seed match with the *CUL1*-3' UTR from position 417–422. To confirm whether miR-377-3p directly targets *CUL1*, a wild-type (WT) *CUL1* 3'-UTR luciferase reporter vector and a miR-377-3p homologous sequence mutant *CUL1* 3'-UTR luciferase reporter vector were constructed. The results showed that the luciferase activity of *CUL1*-WT cells with miR-377-3p mimics was significantly decreased (Fig. 2b). There was no significant change in luciferase reporter activity in the mutant *CUL1* 3'-UTR group. It suggested that the expression regulation of *cull1* was regulated by miR-377-3p targeting.



**Fig. 2** The miR-377-3p could regulate *CUL1* expression by targeting *CUL1*. **a** Though TargetScan online prediction, the sequence of the miR-377-3p-binding site in the 3'-UTR of *CUL1* was shown at the upper site. Mutated residues were shown at the lower site. **b** Luciferase activity of the wild-type 3'-UTR reporters and the mutant 3'-UTR reporters in 293 T cells treated with control mimics or miR-377-3p mimics was shown. \**P* < 0.05

### MiR-377-3p mimics inhibit proliferation and migration of MG-63 cells

To explore the effect of miR-377-3p on osteosarcoma, miR-377-3p mimics were transfected into MG-63 cells to overexpress miR-377-3p (Figure S1). *CUL1* expression in MG-63 cells decreased significantly after transfection with miR-377-3p mimic detected by qRT-PCR (Fig. 3a). Higher expression of miR-377-3p resulted in significantly lower proliferation of MG-63 cells compared with cells transfected with scramble mimics which detected using CCK-8 kit (Fig. 3b). In contrast, the level of apoptosis was clearly elevated with high expression of miR-377-3p (Fig. 3c). Cell migration was slightly decreased in cells treated with miR-377-3p mimics compared with cells of the scramble mimics group (Fig. 3d).

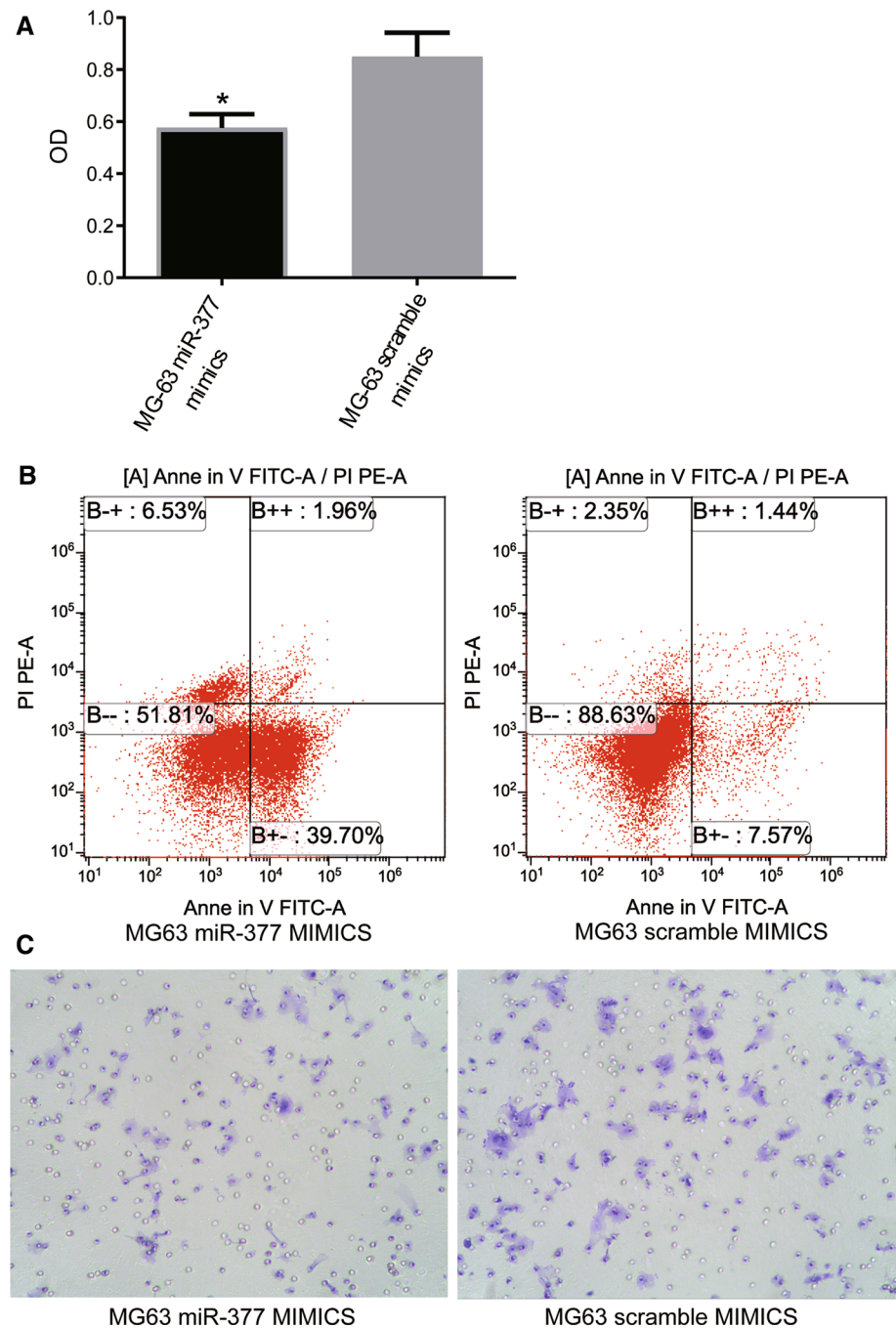
### MiR-377-3p inhibits EMT and Wnt/β-catenin signaling pathway in MG-63

Vimentin, Twist, N-cadherin, and Snail are markers of EMT [18], and β-catenin, Wnt3a, and cyclin D1 are three key components in the Wnt/β-catenin signaling pathway [19]. In KEGG website (<https://www.kegg.jp/entry/K03347>), we identified that *CUL1* participates in Wnt signaling pathway. To investigate the effect of miR-377-3p on EMT and the Wnt/β-catenin signaling pathway, we performed qRT-PCR and western blot assays on cells transfected with miR-377-3p mimics (Fig. 4a, b). The results showed that the EMT and Wnt/β-catenin signaling pathways may be suppressed in MG-63 cells with high expression of miR-377-3p. These results suggested that miR-377 may target *CUL1* to suppress EMT and Wnt/β-catenin signaling pathways in MG-63 cells.

### Tumor-suppressive effect of miR-377-3p

We further investigated the effect of miR-377-3p on the growth of MG-63 cells in nude mice in vivo. Tumor growth was slow in nude mice injected with MG-63 cells transfected with miR-377-3p mimics (Fig. 5a, b). Compared with the control group, nude mice overexpressing miR-377-3p had significantly smaller tumors. The body weight of nude mice overexpressing miR-377-3p was higher than that of control mice (Fig. 5c). On the 19th day, we calculated that the tumor growth inhibition rate was 78.64%. Using qRT-PCR assay, we found that the level of *CUL1* in the miR-377-3p overexpression group was significantly lower than that in the control group in tumor tissue (Fig. 5d).

**Fig. 3** MiR-377-3p overexpression affected the proliferation, apoptosis, and migration of cells. **a** The mRNA levels of *CUL1* after transfection of miR-377-3p mimic. **b** MG-63 cell growth curves were determined with CCK-8 assay. Transfection of miR-377-3p mimics significantly inhibited cell proliferation. \* $P < 0.05$ . *OD* optical density. **c** Flow cytometry results showed that miR-377-3p mimics increased the apoptosis rate of MG-63 cells. The apoptosis rate of MG-63 cells after transfection with miR-377-3p mimics was 39.70%, the apoptosis rate in which transfection with scramble mimics was 7.57%. **D.** miR-613 mimics decreased MG-63 migration detected by Transwell

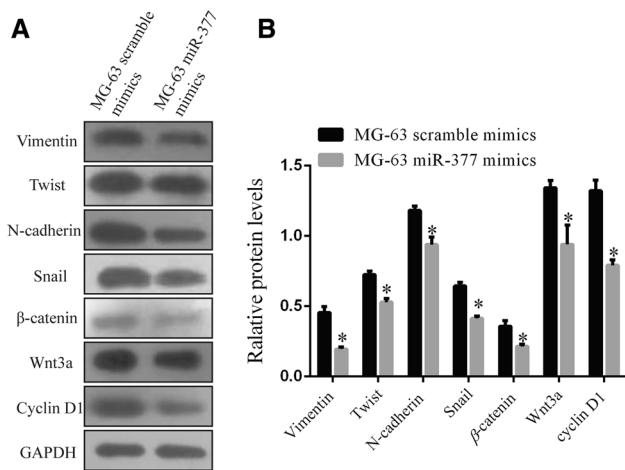


## Discussion

Although surgery combined with systemic chemotherapy is the gold standard for the treatment of osteosarcoma, the prognosis is poor due to the resistance to chemotherapy [20]. Therefore, it is urgent to find a new target for the treatment of osteosarcoma in order to achieve more effective treatment for osteosarcoma. In the present study, we designed a series of experiments to explore the role of miR-377-3p in osteosarcoma. Our experimental results showed that miR-377-3p was down-expressed in osteosarcoma samples and

osteosarcoma cell lines compared with normal controls. Overexpression of miR-377-3p in osteosarcoma cells inhibited cell proliferation and migration. In vivo experiments also confirmed that miR-377-3p overexpression inhibited the growth of tumors. These results suggest that miR-377-3p plays an inhibitory role in osteosarcoma progression.

However, studies on the genes regulated by miR-377-3p targets are currently lacking. A previous study showed that miR-377-3p targets to regulate *CUL4A* expression and is involved in ovarian cancer metastasis [15]. There are also studies confirming that miR-377-3p can target DNA



**Fig. 4** The inhibition of signal pathway in cells transfected with miR-377-3p mimics. **a** The mRNAs of key markers in EMT and Wnt/ $\beta$ -catenin signaling pathway were detected by qRT-PCR.  $*P < 0.05$ . **b** The EMT and Wnt/ $\beta$ -catenin signaling pathway was inhibited in MG-63 cells transfected with miR-377-3p mimics detected by western blot.  $*P < 0.05$

methyltransferase 1 to induce senescence in human dermal fibroblasts [21]. However, no study has yet determined whether miR-377-3p targets *CUL1*.

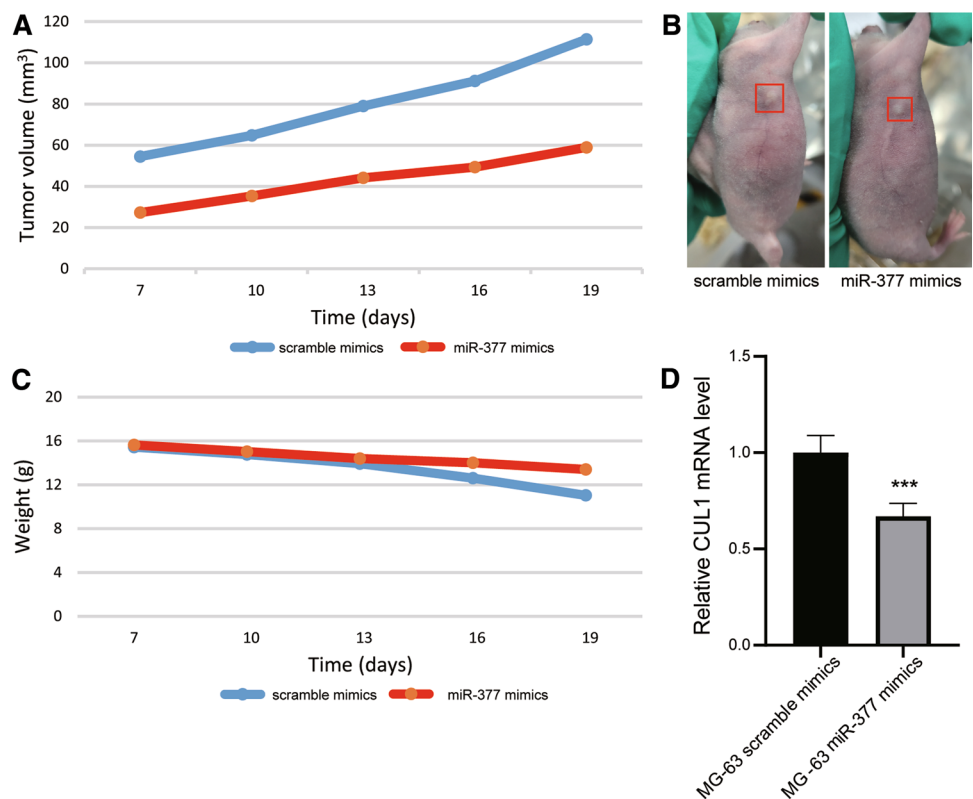
Cullin ubiquitin ligases affect a variety of important cellular functions such as cell cycle progression, signaling, and

DNA repair [22]. Among them, one key role of *CUL1* as an oncogene is to promote cell cycle progression [23]. *CUL1* is highly expressed in both lung adenocarcinoma and colorectal cancer and plays a tumor promoting role [24, 25]. In this study, through sequence complementation analysis and dual luciferase assay, we identified that *CUL1* was an inhibitory target of miR-377-3p and its expression was regulated by miR-377-3p. Upon miR-377-3p overexpression, a decrease in *CUL1* levels inhibits the proliferation and promotes the apoptosis of osteosarcoma cells.

In addition, to better understand the mechanism by which miR-377-3p regulates the phenotype of osteosarcoma cells, we examined the expression of epithelial–mesenchymal transition (EMT) and Wnt signaling pathway-related proteins. The results showed that transfection of miR-377-3p mimics in the osteosarcoma cell line MG-63 significantly decreased the expression of Vimentin, Twist, N-cadherin, Snail,  $\beta$ -catenin, Wnt3a, and cyclin D1. The downregulation of these proteins probably decreased the activity of the pathway.

EMT is considered an important factor in tumor invasion and metastasis. EMT is a dynamic multistep process that includes loss of intercellular adhesion, disruption of the cancer cell basement membrane and extracellular matrix, remodeling of the cytoskeleton and enhancement of cell motility [26]. In addition to driving tumor initiation and metastasis, EMT generates chemoresistance that helps tumor

**Fig. 5** A mouse osteosarcoma model showed that miR-377-3p has a tumor suppressive role. **a** Effects on tumor growth rate were assessed in each mouse on the day 0. miR-377-3p mimics groups are shown in red, control groups in blue.  $*P < 0.05$ . **b** Tumor size was observed after cells were injected for 7 days. **c** The body weight was assessed in each mouse on the day 0. miR-377-3p mimics groups are shown in red, control groups in blue.  $*P < 0.05$ .  $**P < 0.01$ . **D**. The levels of *CUL1* mRNA in the mice were determined by qRT-PCR.  $***P < 0.001$



cells evade destruction by the immune system [27]. This makes it difficult to treat cancer clinically. EMT is characterized by decreased expression of epithelial markers such as E-cadherin and  $\gamma$ -Catenin and increased expression of mesenchymal markers such as N-cadherin, vimentin, snail, and twist [28]. Translocation of  $\beta$ -Catenin from the cytoplasm to the nucleus regulates the activation of these proteins, thereby playing a key role in the EMT process [29]. B-Catenin is considered a key protein in Wnt signaling and an executor of the regulatory function of its downstream targets [30]. As miRNAs influence the Wnt/ $\beta$ -catenin signaling pathway, more and more attention has been paid to the interaction between miRNAs and EMT [31, 32]. The role of miRNAs as tumor suppressors in the regulation of EMT may facilitate the search for potential therapeutic targets.

Our experimental results suggest that miR-377-3p suppresses the proliferation of osteosarcoma cells and tumor growth in vivo. Therefore, we speculated that miR-377-3p could negatively regulate EMT and Wnt/ $\beta$ -catenin signaling pathways to inhibit the progression of osteosarcoma.

Our study also has certain limitations. First, the clinical patient's sample size is too small, which may affect the interpretation of the results. Second, there is a lack of findings that CUL1 directly regulates the EMT and Wnt pathways.

## Conclusion

In conclusion, this study suggests that miR-377-3p is a tumor suppressor gene in OS cancer, which promotes cancer cell apoptosis and inhibits tumor growth, inhibiting EMT and Wnt/ $\beta$ -catenin signaling pathways. These results provide a possible molecular mechanism for OS cancer initiation and progression. Overexpression of miR-377-3p inhibited the expression of *CUL1* and may serve as a novel therapeutic strategy for OS.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s12094-021-02633-6>.

**Author contributions** KL, LL and QL are responsible for the conception or design of the work. QO, LJ and GW contribute the acquisition, analysis, or interpretation of data for the work. All authors finally approved the manuscript version to be published.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** The study was approved by Ethical Committee of Nanxishan Hospital of Guangxi Zhuang Autonomous Region and conducted in accordance with the ethical standards.

**Informed consent** Subjects signed the informed consent.

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