

## Original Article

# miR-761 inhibits human osteosarcoma progression by targeting CXCR1

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**Abstract:** microRNAs (miRs) are endogenous noncoding RNAs that participate in a variety of cellular processes by regulating multiple targets to promote or inhibit cell behaviors. Emerging evidence suggests that miR-761 plays important roles in human cancers to function as either a tumor suppressor gene or an oncogene. However, its biological role in osteosarcoma (OS) remains largely elusive. This study found the expression of miR-761 was significantly reduced in OS tissues and cell lines. CXCR1, an oncogene which was overexpressed in a variety of human cancers including OS, was identified as a novel target of miR-761 using bioinformatic, luciferase reporter, and western blot analyses. Cell function assays revealed that miR-761 could inhibit cell proliferation and invasion at least partially through targeting CXCR1. Collectively, our present study suggested that miR-761 may be a promising treatment biomarker for OS.

**Keywords:** miR-761, CXCR1, osteosarcoma, proliferation, invasion

## Introduction

The incidence of osteosarcoma (OS) is about 4-5 cases per million people worldwide [1]. Five-year overall survival for localized OS patients has improved to about 60% due to the development of multimodal treatment [2, 3]. However, to date, it is still a challenge to diagnose OS patients at early stages and therefore half of the patients develop metastases [4]. Thus, it is essential to understand molecular mechanisms of OS progression and initiation with the aim to improve the prognosis of patients with OS.

MicroRNAs (miRNAs) are a group of non-protein-coding RNAs that reported to regulate approximately 50% of human genes [5, 6]. Accumulating evidence demonstrated that miRNAs regulate cell behaviors including cell proliferation, cell migration, and cell invasion, through their influence on target genes [7-9]. The importance of miRNAs in the initiation and progression of human cancers including OS has been appreciated [10, 11]. It is reported

that miRNAs could function as either tumor suppressor or oncogene in human cancers, and have the potential to be used as diagnostic and therapeutic biomarkers [10, 11]. So far, miRNAs including miR-27a, miR-34a, and miR-505 has been demonstrated to be closely correlated with the progression of OS [12-14].

miR-761, located at chromosome 1p2, has been reported to serve a crucial role in the initiation and progression of several cancers including non-small cell lung cancer, hepatocellular carcinoma, and triple-negative breast cancer [15-17]. miR-761 participated in tumor progression by regulating the expression of tumor-associated proteins [15-17]. It was reported that miR-761 was overexpressed in non-small cell lung cancer, and direct target inhibitor of growth family, member 4 (ING4) and tissue inhibitor of metalloproteinase 2 (TIMP2), leading to cell proliferation and metastasis stimulation [17].

However, whether miR-761 has a role in the initiation or progression of OS remains unclear. In

this study, we, for the first time, revealed the downregulation status of miR-761 in OS and investigated the effects of miR-761 on cell proliferation and invasion. Moreover, we identified CXCR1 as a direct target of miR-761 involved in the progression of OS. In conclusion, we showed miR-761 was a tumor suppressive RNA in OS and may be a potential therapeutic target for OS.

### Materials and methods

#### *Human tissue specimens*

Fifty-four pairs of OS tumor tissues and matched adjacent non-tumor tissues were obtained from patients who received treatment at The Third Affiliated Hospital of Kunming Medical University (Tumor Hospital of Yunnan Province) between February 2014 and May 2015. All these tissues were snap-frozen in liquid nitrogen and stored at -80°C until further use. Written informed consents were obtained from all the enrolled patients. The study protocol was approved by the Ethics Committee of The Third Affiliated Hospital of Kunming Medical University (Tumor Hospital of Yunnan Province) (KY2014-01).

#### *Cell line and transfection*

The human OS cell lines (MG-63 and Saos-2) purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, Inc.). The normal osteoblast cell line NHOst purchased from ATCC was cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, Inc.). These cells were maintained in a 37°C humidified incubator containing 5% CO<sub>2</sub>.

For transfection, the cells were seeded in 6-well plate at a density of  $2 \times 10^5$  cells/well. The miR-761 agomir, miR-761 antagomir, and negative control miRNA (NC-miRNA) were purchased from GenePharm Co. Ltd. (Shanghai, China). The CXCR1 overexpression construct and empty pcDNA3.1 vector were purchased from GenScript (Nanjing, China). Transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) when the cells reached

about 90% confluence. Cells were harvested for the following experiments after 48 h transfection.

#### *Quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNA from tissues samples and cultured cell lines was extracted using Trizol reagent (Thermo Fisher Scientific, Inc.) in line with the provided protocols. The first-strand cDNA was synthesized using Primer Script 1<sup>st</sup> strand cDNA synthesis kit (Takara, Dalian, China). qRT-PCR was conducted using SYBR Green Premix Ex Taq (Takara) using ABI 7500 equipment (Applied Biosystems, Thermo Fisher Scientific, Inc.). The following qRT-PCR procedure was employed: 1 cycle, 95°C at 5 min; 40 cycles, 95°C at 15 sec, 60°C at 30 sec. The following primers were used: miR-761: forward 5'-ACAGCAGGCACAGAC-3' and reverse 5'-GAGCAGGCTGGAGAA-3'; U6 snRNA: forward: 5'-TCCGATCGTGAAGCGTTC-3' and reverse: 5'-GTGCAGGGTCCGAGGT-3'. U6 snRNA was used as an endogenous control.

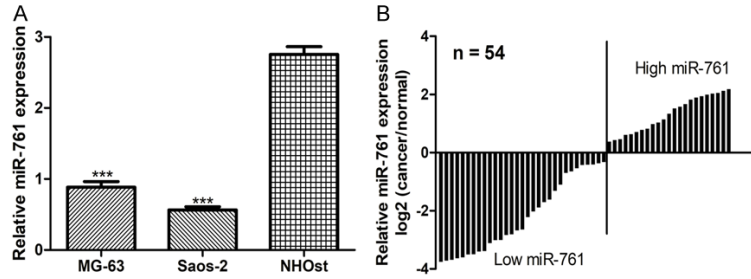
#### *Western blot*

Total protein from tissue samples and cultured cell lines was extracted using RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) according to the manufacture's instructions. The protein samples were separated using 10% SDS-PAGE and transferred to PVDF membrane (Beyotime). The membranes were blocked with 5% non-fat milk and then incubated with primary antibodies (CXCR1: ab124344 or GAPDH: ab9485; Abcam, Cambridge, MA, USA) at 4°C for overnight. After washing three times with TBST, the membrane was incubated with HRP conjugated secondary antibody (ab6742; Abcam) at room temperature for 1 h. The band was detected using ECL reagent (Beyotime) and quantified using Image J 1.42 software (NIH, Bethesda, MD, USA).

#### *Luciferase reporter gene assay*

MG-63 and Saos-2 cells co-transfected with miR-761 agomir or NC-miRNA and firefly luciferase reporter plasmid contained either the wild-type (wt) or mutant (mut) 3'-UTR of CXCR1 were seeded in a 24-well plate. After transfection for 48 h, the relative luciferase activity was analyzed using a dual-luciferase reporter assay (Promega, Madison, WI, USA).

## Tumor suppressive role of miR-761 in osteosarcoma



**Figure 1.** miR-761 expression was downregulated in OS. A. The expression of miR-761 in the OS cell lines (MG-63 and Saos-2) and normal osteoblast cell line NHOst was measured by qRT-PCR. B. The expression of miR-761 in OS tissues was measured by qRT-PCR and U6 snRNA was used as internal control. (\*\*\*)  $P < 0.001$ . miR-761: microRNA-761; OS: osteosarcoma; qRT-PCR: quantitative real-time polymerase chain reaction; snRNA: small nuclear RNA.

**Table 1.** Correlations of miR-761 expression and different clinicopathological features in osteosarcoma patients

Clinicopathological variable	No. of cases	miR-761 expression		P value*
		Low (n=31)	High (n=23)	
Age				
≥ 60	28	16	12	0.691
< 60	26	15	11	
Gender				
Male	27	17	10	0.248
Female	27	14	13	
Tumor size				
≥ 5	32	19	13	0.026
< 5	22	12	10	
Tumor stage				
I-II	20	11	0	0.016
III	34	20	14	

\*Chi-square test. miR-761: microRNA-761.

### Cell proliferation assay

The cells to be examined were seeded in a 96-well plate at a density of  $4 \times 10^3$  cells/well. Next, 10  $\mu$ l MTT solution at a concentration of 5 mg/ml was added to each well and further incubated for 4 h. Then, 100  $\mu$ l formazan solution was added to each well and incubated until dissolving of the purple crystal. The optical density was measured at 570 nm using microplate reader (Thermo Fisher Scientific, Inc.).

### Cell invasion assay

Cells to be examined were plated into the upper chamber in 100  $\mu$ l of RPMI-1640 medium at a density of  $2 \times 10^5$  cells/ml. The lower chamber was filled with 400  $\mu$ l basal medium with 10% FBS in 24-well plates. After incubation for 24 h,

the insert membrane was stained with 0.1% crystal violet. The invaded cell numbers were counted under an inverted microscope.

### Statistical analysis

Data are presented as mean  $\pm$  SD and analyzed by Student's t test (for two groups), or one-way analysis of variance (ANOVA) and Tukey post-hoc test (for three or above groups). The correlations between miR-761 and CXCR1 was analyzed using Pearson correlation analysis. Correlations between miR-761 and clinicopathological features were analyzed by Chi-square test. P value lower than 0.05 was considered significant.

### Results

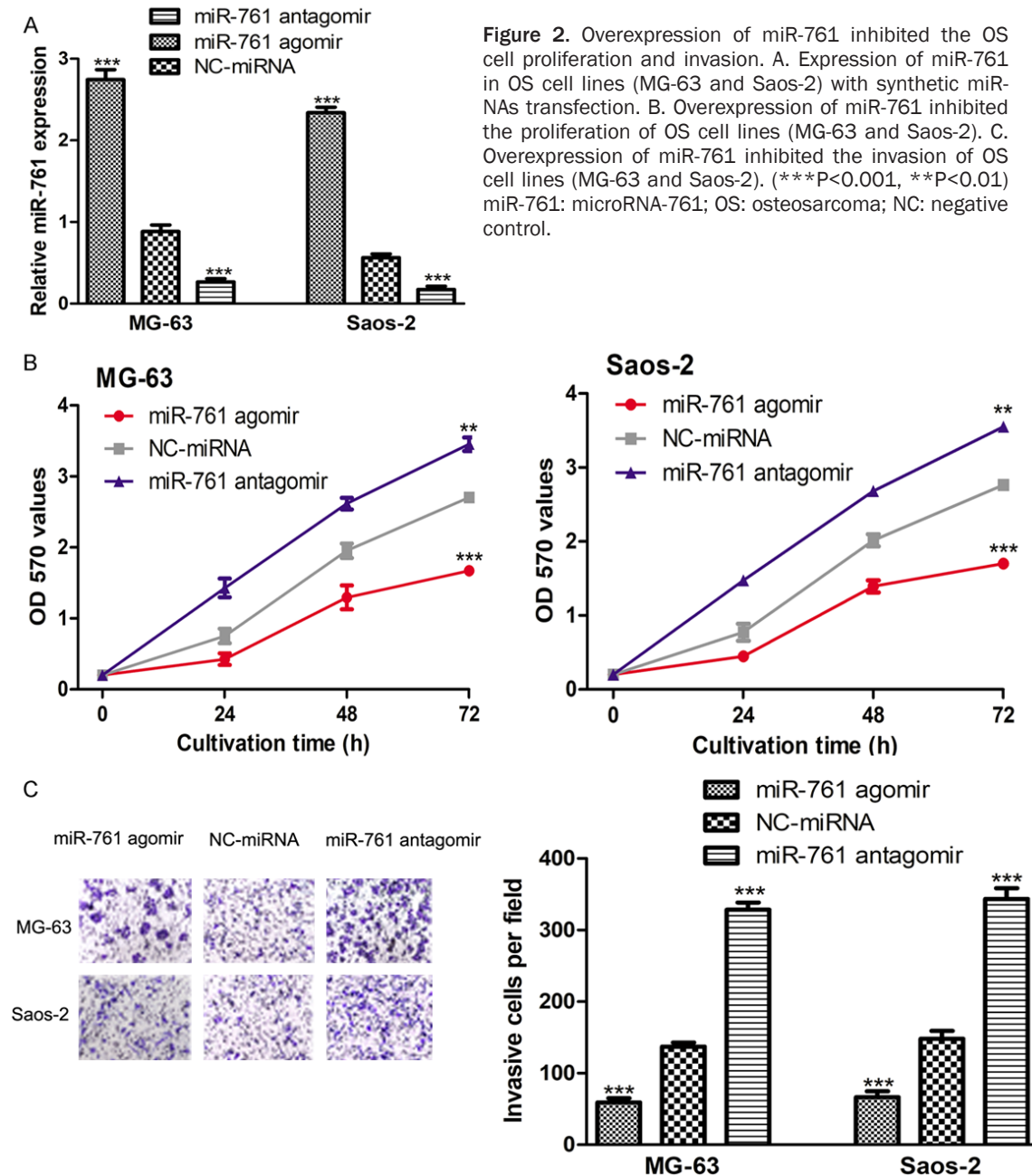
#### Downregulation of miR-761 in OS tissues and cell lines

We measured the expression of miR-761 in OS to investigate the role of miR-761. We found expression levels of miR-761 in OS cell lines were significantly lower than those in normal osteoblast cell line NHOst (**Figure 1A**). This result was further confirmed by ex-

amining the levels of miR-761 in OS tissues and adjacent noncancer tissues. Results showed miR-761 expression was significantly reduced in OS tissues compared to adjacent noncancer tissues (**Figure 1B**). Next, we classified these patients into high or low miR-761 expression groups using the relative expression levels of miR-761 in OS tissues. We found low expression of miR-761 was closely correlated with tumor size ( $P=0.026$ ) and tumor stage ( $P=0.016$ ) but not associated with age ( $P=0.691$ ) and sex ( $P=0.248$ ) (**Table 1**).

#### miR-761 suppresses proliferation and invasion of OS cells in vitro

To explore the biologic roles of miR-761 in the progression of OS, we transfected the miR-761 agomir, miR-761 antagomir, and NC-miRNA into



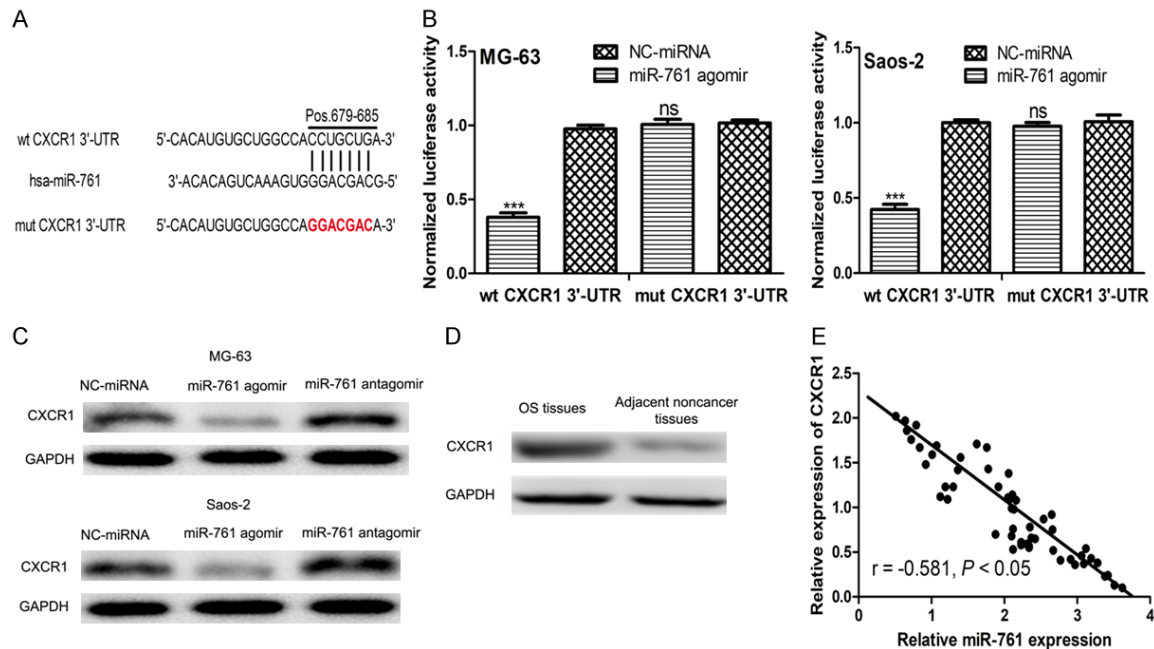
OS cells. As shown in **Figure 2A**, miR-761 expression was increased by miR-761 agomir and was obviously decreased by miR-761 antagomir. Following, MTT assay revealed that OS cell proliferation ability was significantly decreased by miR-761 agomir and was clearly increased by miR-761 antagomir (**Figure 2B**). Furthermore, the effects of miR-761 on the invasion of OS cells were analyzed using transwell invasion assay. The results showed that miR-761 agomir significantly inhibited OS cell

invasion, while miR-761 antagomir markedly promoted cell invasion (**Figure 2C**). Taken together, our results demonstrated that miR-761 functions as a tumor suppressor by inhibiting cell proliferation and invasion.

#### miR-761 directly targets CXCR1 in OS

To elucidate the molecular mechanism of miR-761 on OS progression, we predicted targets of miR-761 using the miRNA target online prediction algorithm using TargetScan. We identified





**Figure 3.** CXCR1 is a direct target of miR-761. **A.** The putative binding site of miR-761 and CXCR1 was presented. **B.** Ectopic expression of miR-761 decreased the luciferase activity of wt CXCR1 3'-UTR, but not in the mut CXCR1 3'-UTR in the MG-63 and Saos-2 cells. **C.** Elevated expression of miR-761 inhibited the protein expression of CXCR1 in the MG-63 and Saos-2 cells. **D.** The protein expression of CXCR1 in OS tissues and adjacent noncancer tissues was measured by western blot. **E.** miR-761 expression was negatively associated with the expression of CXCR1 in OS tissues. (\*\*\*)  $P < 0.001$ , ns not significant) miR-761: microRNA-761; OS: osteosarcoma; NC: negative control; wt: wild-type; mut: mutant; UTR: untranslated region; CXCR1: CXCR1 receptor-1.

CXCR1 contains a conserved miR-761-binding site in its 3'-UTR (**Figure 3A**). Subsequently, we measured the luciferase activity in OS cells co-transfected with miR-761 agomir or NC miRNA and wt or mut CXCR1 3'-UTR. The results demonstrated that miR-761 agomir suppressed the luciferase activity of the cells with wt CXCR1 3'-UTR construct transfection but did not change the luciferase activity of those with mut CXCR1 3'-UTR construct transfection (**Figure 3B**). To confirm CXCR1 as a target of miR-761 in OS cells, we measured the expression of CXCR1 in OS cells with miR-761 agomir or NC miRNA transfection using western blot. As shown in **Figure 3C**, the protein expression of CXCR1 was significantly suppressed by miR-761 agomir. The relationship between miR-761 and CXCR1 was further analyzed by analyzing the expression of miR-761 and CXCR1 in OS tissues. We found CXCR1 expression in OS tissues was significantly higher than in the adjacent normal tissues (**Figure 3D**). Then, we found the expression of miR-761 and CXCR1 was inversely correlated in OS tissues ( $r = -0.581, P < 0.05$ , **Figure 3E**). Taken together, these results sup-

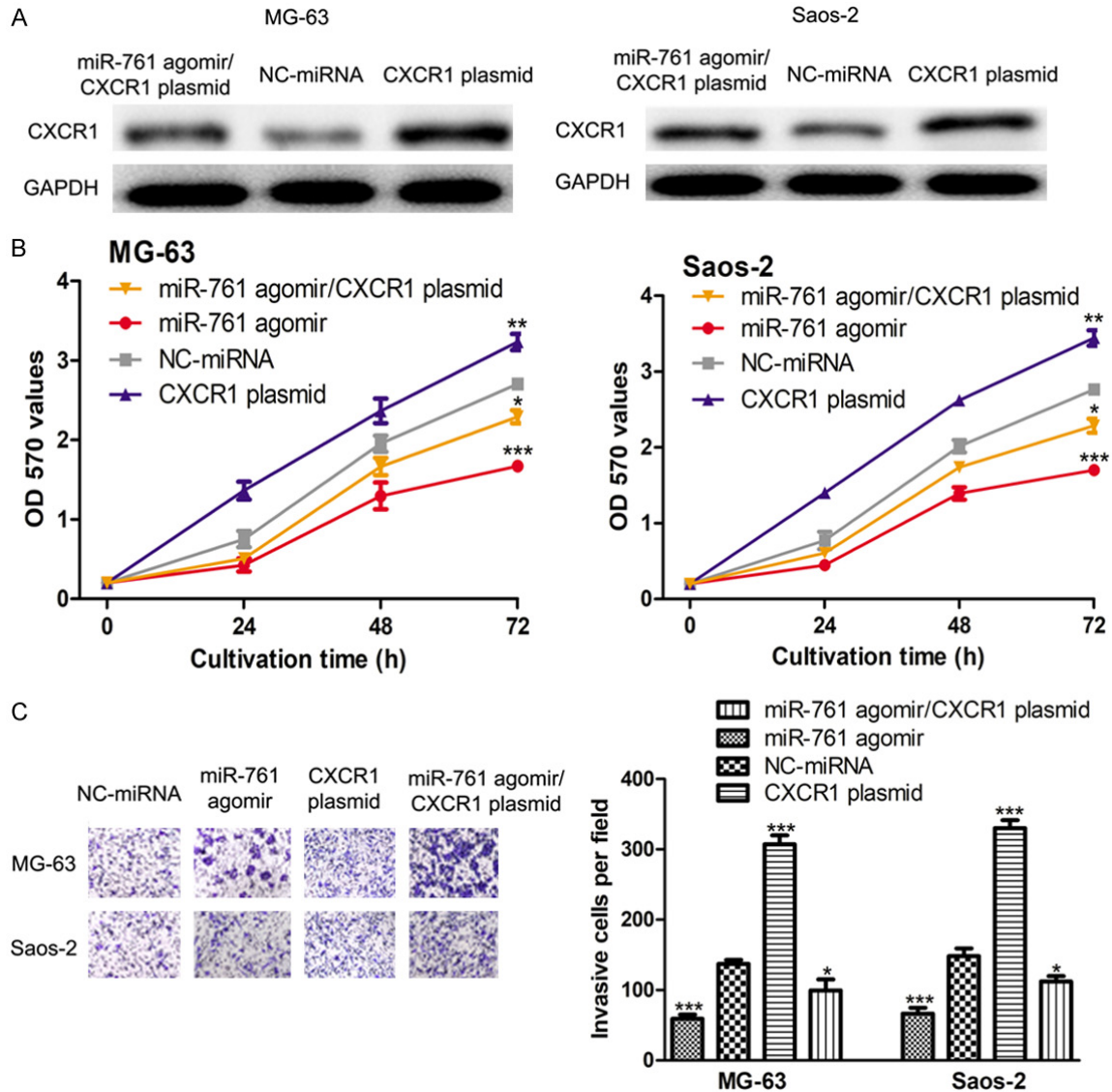
ported that CXCR1 is a direct target of miR-761.

#### Overexpression of CXCR1 rescued miR-761-induced inhibition proliferation and invasion of OS cells in vitro

To validate CXCR1 as a functional target of miR-761 in OS, CXCR1 plasmid was used to elevate the expression of CXCR1. We found levels of CXCR1 were higher in the miR-761 agomir and CXCR1 plasmid co-transfected cells than in miR-761 agomir-only cells (**Figure 4A**). Furthermore, we found cell proliferation and invasion inhibition induced by miR-761 agomir could be partially reversed by CXCR1 plasmid (**Figure 4B** and **4C**). In conclusion, our results revealed that CXCR1 is a functional target of miR-761 in OS.

#### Discussions

Emerging evidence has demonstrated that chemokines and their receptors play crucial roles in tumorigenesis [18, 19]. CXCR1, a ligand of interleukin-8, is abnormally expressed in sev-



**Figure 4.** miR-761 suppressed OS cell proliferation and invasion by targeting CXCR1. A. Overexpression of CXCR1 rescued miR-761 overexpression-induced CXCR1 inhibition. B. Overexpression of CXCR1 rescued miR-761 overexpression-induced MG-63 and Saos-2 cell proliferation inhibition. C. Overexpression of CXCR1 rescued miR-761 overexpression-induced MG-63 and Saos-2 cell invasion inhibition. (\*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ ) miR-761: microRNA-761; OS: osteosarcoma; NC: negative control; CXCR1: CXC receptor-1.

eral cancer types [20-22]. Zhu et al. reported that the high expression level of CXCR1 in non-metastatic clear-cell renal cell carcinoma and is a negative prognosticator for patients' overall survival and recurrence-free survival [20]. Another study conducted by Cao et al. found CXCR1 high expression indicated poorer overall survival of gastric cancer patients and has the potential to be used as an indicator for prognosis prediction accompanied by the current TNM staging system [21]. In OS, Han et al. found CXCR1 high expression is associated with che-

motherapy resistance through the IL-8/CXCR1/Akt signaling pathway [22]. However, how CXCR1 expression level is altered in cancers still remains to be elucidated.

Studies have demonstrated that miRNAs function as tumor suppressive gene or oncogene through targeting the tumor-related genes [5, 6, 8, 10]. miR-761 has been reported to be capable to regulate many target genes, such as ING4, TIMP2, Mitofusin-2, and TRIM29, that shape the cellular behaviors [15-17]. Therefore,

we were interested to investigate whether miR-761 might be a regulator for CXCR1 in OS. First, we found miR-761 expression levels in OS tissues and cell lines was lower than those in adjacent normal tissues and normal cell lines. Next, by using in silico algorithm prediction, luciferase activity assay, and western blot assay, we validated CXCR1 was a direct target of miR-761 in OS. Importantly, we revealed the expression of CXCR1 in OS tissues was higher than in adjacent normal tissues, which is the same as in previously published results [22]. Furthermore, by using cell proliferation and invasion assays, we revealed that overexpression of miR-761 inhibits cell proliferation and invasion in vitro and validated CXCR1 was a functional target of miR-761 to participate the cell proliferation and migration inhibition induced by miR-761. As we all know, the abnormal status of cell behaviors including sustaining proliferative signaling, resisting cell death, inducing angiogenesis, and activating invasion and metastasis etc. are crucial for the development and progression of cancers [23]. Therefore, our study demonstrated that miR-761 functions as a tumor suppressor in OS through regulating CXCR1.

To provide more evidence to validate miR-761 as a potential prediction or therapeutic target, we investigated the clinical significance of miR-761 in OS. We analyzed the correlations between miR-761 expression and the clinicopathological features. We found low expression of miR-761 was closely correlated with tumor size and metastasis, two indicators for the malignancy of cells.

In summary, for the first time, our data implied the downregulation status of miR-761 was in OS tissues and cell lines. Especially, we demonstrated that miR-761 overexpression inhibits cell proliferation and invasion through regulating CXCR1 expression. Thus, our results validated the tumor suppressive role of miR-761 in OS and might promote the development of novel means to treat human cancers.

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## Disclosure of conflict of interest

None.

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