

## Original Article

# Effects of long non-coding RNA SPRY4-IT1 on osteosarcoma cell biological behavior

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**Abstract:** Recent findings indicate that long noncoding RNAs (lncRNAs) were dysregulated in many kinds of tumors including osteosarcoma (OS). SPRY4-IT1 has been recently revealed as oncogenic regulator in various cancers, while its clinical value and potential function in OS are still unknown. To investigate the role of SPRY4-IT1 in OS, we evaluated the expression SPRY4-IT1 in OS tissues and cell lines, and investigated the effect of SPRY4-IT1 siRNA on cell proliferation, migration and invasion of OS *in vitro*. Our result showed that SPRY4-IT1 was upregulated in OS tissues. Further experiments revealed that SPRY4-IT1 knockdown significantly inhibited OS cells proliferation by causing G1 arrest and promoting apoptosis. Furthermore, inhibitory effects of SPRY4-IT1 on cell migration and invasion were partly associated with EMT process. In conclusion, these data suggest that SPRY4-IT1 could be an oncogene for OS, and may be served as a candidate target for new therapies in human OS.

**Keywords:** SPRY4-IT1, long noncoding RNA, osteosarcoma, proliferation, apoptosis, migration, EMT

## Introduction

Osteosarcoma (OS) is the most common histological form of primary bone cancer, which shows most prevalent in children and adolescents [1]. Although about 90% of patients are able to have limb-salvage surgery, many patients experience disease relapse and manifest pulmonary metastasis [2]. In addition, only 20% of patients can survive three years after relapse [3, 4]. Thus, there is a need for effective diagnosis and identification of molecular therapeutic targets that may help in treatment of OS patients.

Long non-coding RNAs (lncRNAs, > 200 nucleotides in length) are important new members of the family of ncRNAs with limited or no protein-coding capacity [5, 6]. Emerging evidence suggests that lncRNAs have important biological functions are closely related to human cancer [7-9]. lncRNAs also function as a competing endogenous RNA and sponge miRNAs, thus regulating the expression of target mRNA. Recently, increasing evidence has shown that *SPRY4* intronic transcript 1 (SPRY4-IT1), a

lncRNA derived from an intron within *SPRY4* gene, was shown to be upregulated in various cancer [10, 11]. Knockdown of SPRY4-IT1 expression contributes to tumor invasion inhibition, and elevated rates of apoptosis [12, 13]. However, the roles of SPRY4-IT1 in OS progression remain unclearly defined. It is also necessary to reveal the underlying molecular mechanisms by which SPRY4-IT1 is involved in OS tumorigenesis and cancer progression.

## Materials and methods

### Clinical tissue samples

Fifty-six tumor tissues and matched adjacent normal tissues were obtained from enrolled patients with OS who underwent surgery at The First Affiliated Hospital of Soochow University between 2010 and 2015. All tissue samples were immediately frozen in liquid nitrogen after operation and stored at -80°C until RNA extraction. No patient previously received chemotherapy, radiotherapy, and blood transfusion. Clinical characteristics of these patients were collected including sex, age, smoking, drinking,

alkaline phosphatase (ALP), tumor size, tumor site, tumor stage, post-operative chemotherapy, and initial metastasis. This study was approved by the Research Ethics Committee of The First Affiliated Hospital of Soochow University. Written informed consent was obtained from all of the patients.

#### *Cell culture*

Human OS cell lines (HOS, Saos-2, U2OS, and MG-63) and normal osteoblast cells (NH0st) were obtained from the Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. They were all placed in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

#### *RNA isolation and quantitative real-time reverse transcription-PCR (qRT-PCR)*

Total RNA was extracted from cells, frozen OS tissues and their corresponding non-neoplastic tissues using TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Total RNA was then converted to cDNA by reverse transcription using oligodT primers and SuperScript II reverse transcriptase (Invitrogen). For qRT-PCR, three replicates of each sample were amplified in a 20-µL reaction mixture containing SYBR Green reaction mix (Qiagen, Germany) and 0.5 mM of primer, and analyzed using a Roche Light-Cycler (Roche, Basel, Switzerland). The sequence of the primers were as following: SPRY4-IT1 (Forward: 5'-AGCCACATAAATTCAGCAGA-3', Reverse: 5'-CGATGTAGTAGGATTCCTTTCA-3') and GAPDH (Forward: 5'-GACTCATGACCACAGTCC-ATGC-3', Reverse: 5'-AGAGGCAGGGATGATGTTCTG-3'). An ABI 7500 was used to carry out the qPCR and data collections.

#### *Small interfering RNA and plasmids DNA transfections*

The cells were transiently transfected with si-RNAs after being sowed into the 6-well plates overnight. A scrambled negative control, a plasmid overexpressing SPRY4-IT1, and an empty vector, were cultured as well using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) and FuGENE® HD Transfection Reagent (Roche, Germany) according to the

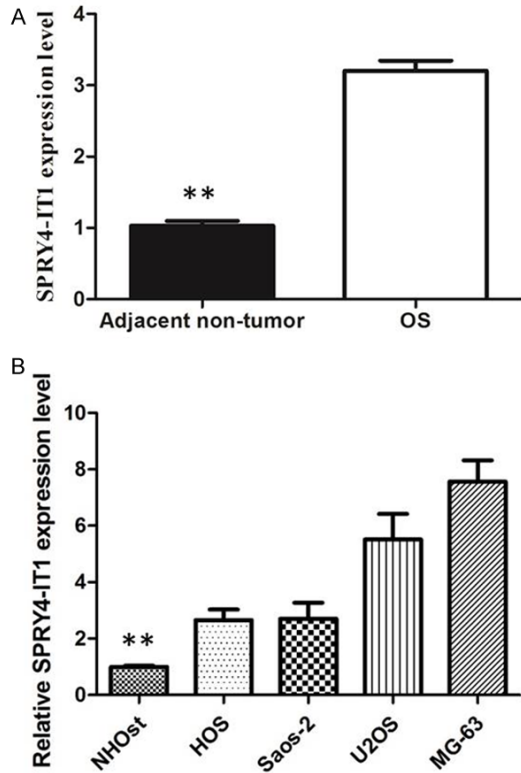
manufacturer's instructions, respectively. Forty eight hours after transfection, the cells were harvested to detect the overexpression or knockout efficiency via quantitative real-time PCR (qRT-PCR). For RNAi-mediated knockdown of SPRY4-IT1, two different Stealth siRNAs against SPRY4-IT1 were provided by Invitrogen. The target sequences for the si-SPRY4-IT1 included: si-SPRY4-IT1-1 (CCCAGAATGTTGACAGCTGCCTCTT) and si-SPRY4-IT1-2 (GCTTTCTGATTCCAAGGCCTATTAA) with the later having the highest inhibition efficiency. In order to ectopically express the SPRY4-IT1, the synthetic SPRY4-IT1 sequence (708 bp) was sub-cloned into the pEGFP-N1 plasmid vector. After the SPRY4-IT1 sequence was inserted into the vector, a sequencing analysis was conducted to make sure that this vector could specifically express SPRY4-IT1 (constructed by Invitrogen Inc.).

#### *Cell proliferation and colony formation assays*

MTT assays were performed to assess cell proliferation. Briefly, cells were seeded at a concentration of 10<sup>4</sup> cells/well in a 96-well plate. si-SPRY4-IT1 and si-NC were transfected into the cells using Lipofectamine 2000 the following day according to the manufacturer's protocol. At indicated time points (24, 48, and 72 h), the culture medium was removed and replaced with culture medium containing 10 µl of sterile MTT dye (5 mg/ml) and cultured for 4 h at 37°C. Then, 150 µl dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added into each well. The absorbance was determined in a microtiter plate reader (Molecular Devices, Menlo Park, CA, USA) at 570 nm wavelength. All experiments were performed in triplicate. For the colony formation assays, the transfected cells were collected and seeded at a density of 1000 cells/well in six-well plates and incubated at 37°C and 5% CO<sub>2</sub> in a humidified incubator for 2 weeks. Then, the colonies were stained with 1.0% crystal violet for 30 s after fixation with 10% formaldehyde for 5 min. The colony number in each well was counted and calculated in light microscope.

#### *Cell cycle and cell apoptosis assay*

Transfected cells were harvested using trypsinization, washed in ice-cold phosphate-buffered saline (PBS), and fixed in icecold ethanol in PBS. Then, bovine pancreatic RNase (Sigma-Aldrich) was added to a final concentration of 2

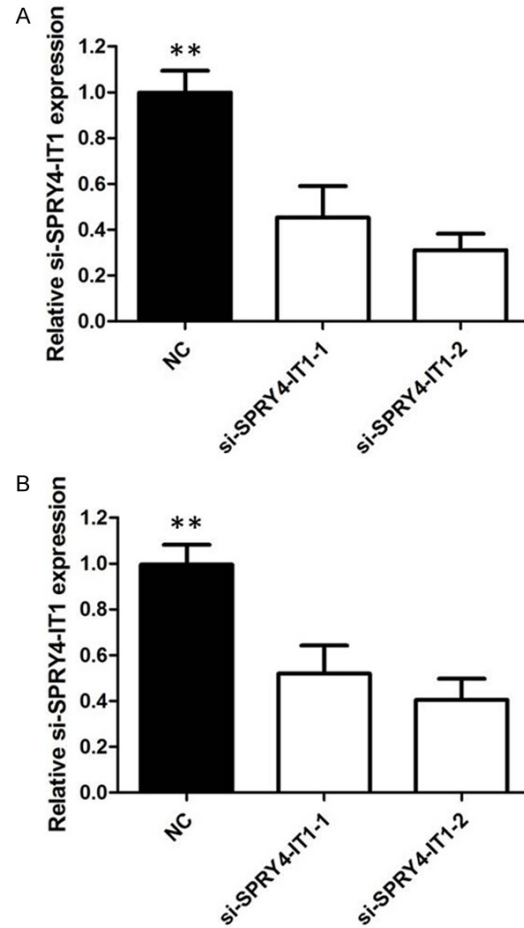


**Figure 1.** Expression of SPRY4-IT1 were increased in OS tissues and cell lines. A: Relative SPRY4-IT1 levels in OS tissues and adjacent non-tumor tissues. B: Relative SPRY4-IT1 levels in OS cell lines (HOS, Saos-2, U2OS, and MG-63) and human osteoblastic cell line (NHOst), \*P < 0.05; \*\*P < 0.01.

mg/ml and cultured for 30 min at 37°C. To detect the cell cycle distribution, the cells were stained with 20 mg/ml propidium iodide (PI; Sigma-Aldrich) for 20 min at room temperature. For the cell apoptosis assays, the cells were labeled by the Annexin VFITC Apoptosis Detection Kit (Invitrogen) according to the manufacturer's instructions. The cell cycle distribution and the cell apoptosis rates were quantified using a FACSCalibur flow cytometer (BD Biosciences, Mansfield, MA, USA).

*Cell migration and invasion assays in vitro*

The invasive and migratory potential of cells were analyzed using Transwell chambers (8 μm pore; BD Biosciences, San Jose, CA USA). For invasion assays, cells in 100 μl of serum-free medium were added to the upper chamber coated with Matrigel. Medium supplemented with 10% FBS was added to the lower chamber. After incubation for 24 h, non-invading cells on the upper surface were removed with a cotton swab and cells invading to the lower chamber



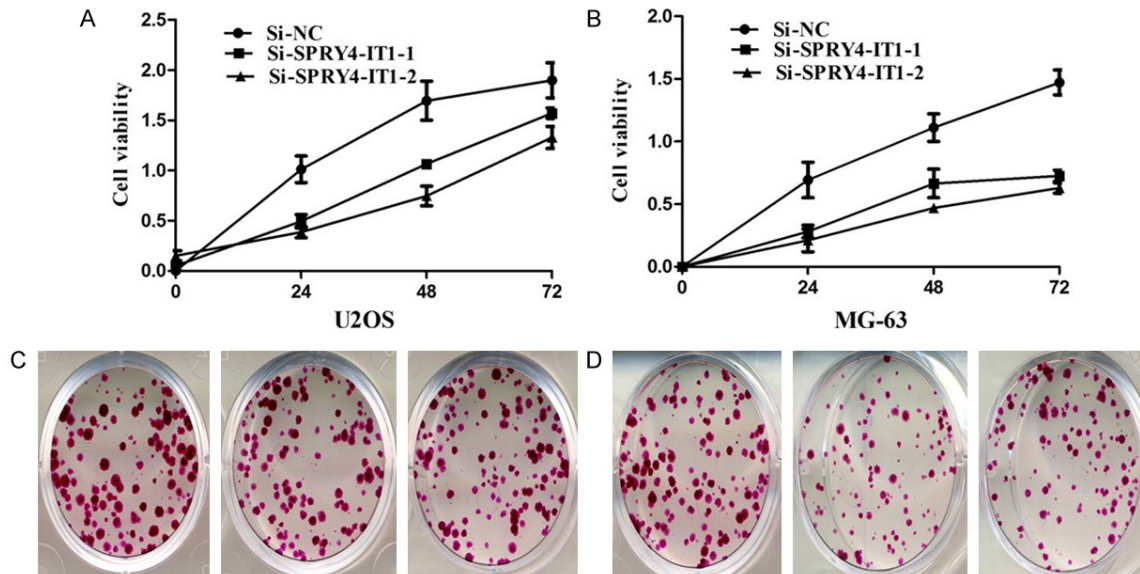
**Figure 2.** A: The qRT-PCR assay revealed that SPRY4-IT1 was efficiently downexpression by transfected with siRNA in U2OS cells; B: The qRT-PCR assay revealed that SPRY4-IT1 was efficiently downexpression by transfected with siRNA in MG-63 cells.

were fixed with methanol, stained with 0.1% crystal violet, dried, and photographed. For migration assays, cells in 100 μl serum-free medium were placed in the top chamber without Matrigel, and 500 μl of 10% FBS containing medium was added to the lower chamber. After 16 h, cells on the upper surface were removed and cells migrated to the lower chamber were fixed and stained as described above. The number of invading or migrating cells was counted under the microscope in five representative fields and expressed as the average per field.

*Western blot assay*

Cells were lysed in lysis buffer containing protease inhibitor cocktail. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA). Equivalent amounts of proteins were separated by SDS-

## SPRY4-IT1 and osteosarcoma



**Figure 3.** A: MTT assay showing knockdown of SPRY4-IT1 inhibited cell proliferation of U2OS cells compared to the negative control; B: MTT assay showing knockdown of SPRY4-IT1 inhibited cell proliferation of MG-63 cells compared to the negative control; C: Colony-formation assays showed that silencing of SPRY4-IT1 significantly increased the colony-forming ability of U2OS cells compared to the negative control; D: Colony-formation assays showed that silencing of SPRY4-IT1 significantly increased the colony-forming ability of MG-63 cells compared to the negative control.

PAGE, and then transferred onto polyvinylidene difluoride membranes (Bio-Rad). After being blocked in Tris-buffered saline (TBS) containing 5% non-fat milk, the membranes were incubated with specific primary antibody against E-cadherin, N-cadherin, vimentin, (1:1000; Abcam Inc., Cambridge, MA, USA) and  $\beta$ -actin (1:2000, Santa Cruz Biotechnology) antibodies at 4°C for 12 h and then with horseradish peroxidase-conjugated anti-rabbit antibody for 2 h at room temperature. Proteins were visualised using ECL (Pierce, Rockford, IL, USA) and detected using Biolmaging Systems (UVP Inc., Upland, CA, USA).

### Statistical analysis

Statistical analyses were performed using SPSS Statistics 16.0 (IBM Chicago, IL, USA). The chi-square test ( $X^2$  test), Fisher's exact test for non-parametric variables, and Student's t test for parametric variables were used (two tailed). All tests were two-sided, and a  $P < 0.05$  was considered statistically significant.

### Result

#### Different expression of SPRY4-IT1 in OS tissues and normal tissues

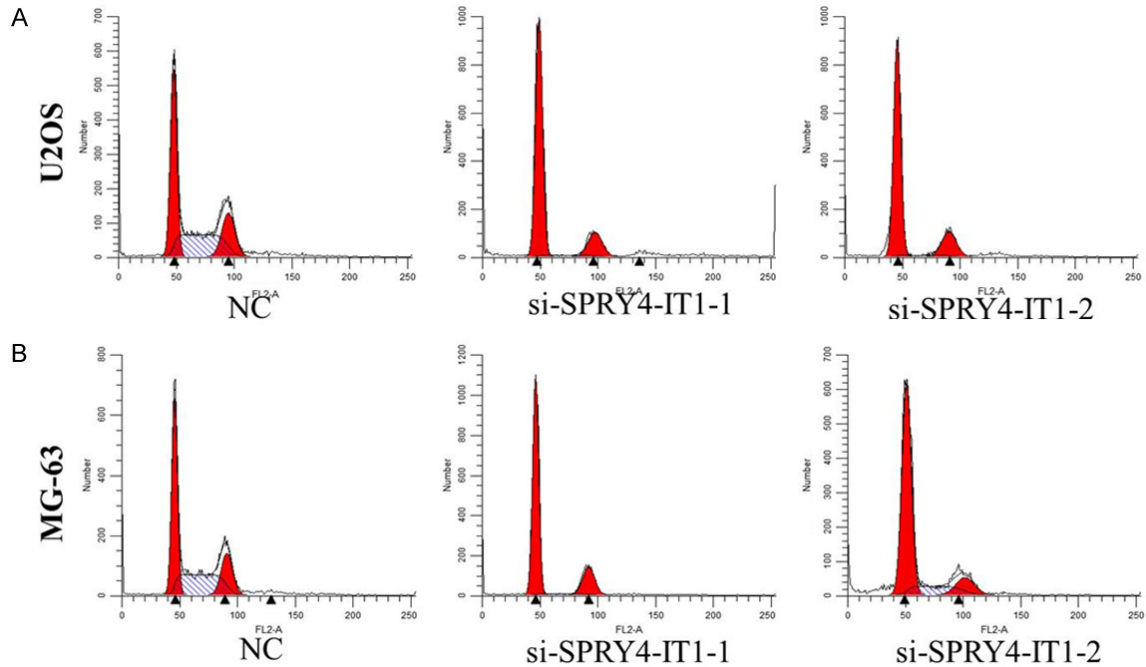
The qRT-PCR assay was used to measure SPRY4-IT1 expression levels in 56 paired pri-

mary cancerous and adjacent noncancerous tissues from OS patients. Our results indicated that the relative expression of SPRY4-IT1 in tumor tissues was significantly higher than that in the adjacent normal tissues ( $P=0.000$ , **Figure 1A**). To evaluate the association between SPRY4-IT1 expression and clinical features, the OS patients were divided into high and low expression group on the basis of their average expression of SPRY4-IT1. Then, the 56 patients were classified into two groups based on the median value of relative SPRY4-IT1 expression. The high SPRY4-IT1 group ( $n=30$ ) had SPRY4-IT1 expression levels  $>$  median value and the low SPRY4-IT1 group ( $n=26$ ) had SPRY4-IT1 expression levels  $<$  median value. Our results found that overexpression of SPRY4-IT1 were expression was positive correlated with the metastases, recurrence and tumor maximum diameter, suggesting that the expression level of SPRY4-IT1 might be associated with the development of the OS.

#### Different expression of SPRY4-IT1 in OS cell lines and normal cell lines

We then examined the expression of SPRY4-IT1 in four human OS cell lines, namely HOS, Saos-2, U2OS, and MG-63 cells, and in the normal osteoblast cell line NH0st. Significantly higher

## SPRY4-IT1 and osteosarcoma



**Figure 4.** A: Knockdown of SPRY4-IT1 led an increase in the number of U2OS cells in the G1-G0 phase and a decrease in the percentage of cells in the S phase, \* $P < 0.05$ ; \*\* $P < 0.01$ ; B: Knockdown of SPRY4-IT1 led an increase in the number of MG-63 cells in the G1-G0 phase and a decrease in the percentage of cells in the S phase, \* $P < 0.05$ ; \*\* $P < 0.01$ .

expression of SPRY4-IT1 was found in OS cells, compared with NHOst cells ( $P < 0.01$ ; **Figure 1B**). These results are consistent with previous findings in other cancer. Therefore, SPRY4-IT1 was depleted in U2OS and MG-63 cells, which exhibit a higher expression of SPRY4-IT1. The knockdown of SPRY4-IT1 in OS cell lines were confirmed by qRT-PCR (**Figure 2A** and **2B**).

### *Knockdown of SPRY4-IT1 inhibited cell proliferation*

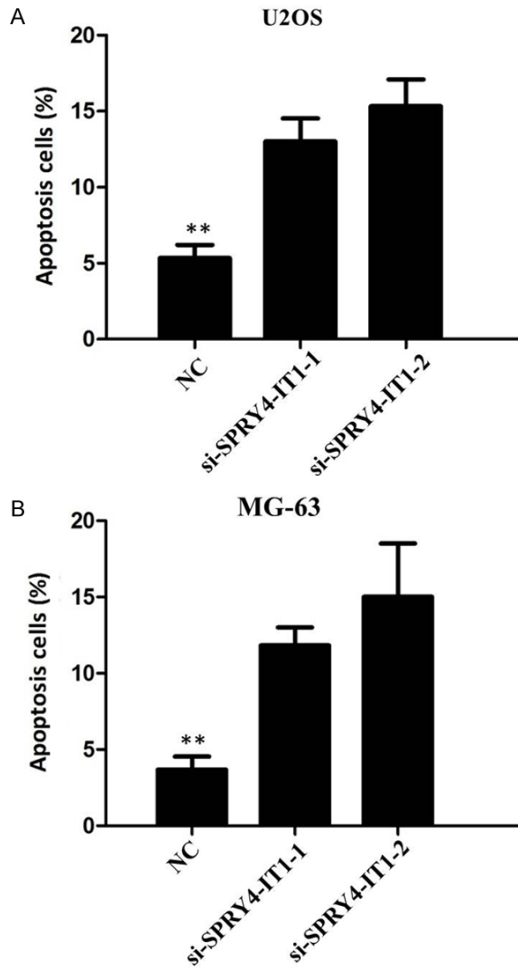
To further study the potential role of SPRY4-IT1 in cell proliferation of OS cells, MTT assay was performed after the transfection of si-SPRY4-IT1 into U2OS and MG-63 cells, which exhibit naturally high SPRY4-IT1 expression levels. As demonstrated in **Figure 3A** and **3B**, the si-SPRY4-IT1 group displayed a lower cell viability rate than si-NC group ( $P < 0.05$ ). Furthermore, cell proliferation was also measured using a colony formation assay. Compared with the control cells, SPRY4-IT1 knockdown in U2OS and MG-63 cells resulted in markedly decreased colony formation abilities ( $P < 0.05$ ; **Figure 3C** and **3D**), indicating that downregulation of SPRY4-IT1 could reduce proliferation of OS cells.

### *Downregulation of SPRY4-IT1 promotes G1 arrest and causes apoptosis in OS cells*

To probe the potential mechanisms of SPRY4-IT1 in the proliferation of OS cells, we examined the cell cycle in U2OS and MG-63 cells through flow cytometry. After treatment with si-SPRY4-IT1 or si-NC for 48 h, SPRY4-IT1 knockdown led to a significant accumulation of cells at the G1/G0 phase and a decreased number of cells in S phase ( $P < 0.05$ ; **Figure 4A** and **4B**). We then investigated the effects of SPRY4-IT1 knockdown on apoptosis. The results of flow cytometry showed that knockdown of SPRY4-IT1 resulted in a higher number of apoptotic cells compared with the controls (**Figure 5A** and **5B**). These data suggest that SPRY4-IT1 has exerts a critical effect on OS cell apoptosis.

### *SPRY4-IT1 regulates OS cell migration and invasion*

We then performed the transwell assay to investigate the role of SPRY4-IT1 in the regulation of cell migration and invasion in OS cells. Transwell invasion assays showed that the migratory activity of si-SPRY4-IT1 infected U2OS and MG-63 cells was reduced, and suppression of SPRY4-IT1 in both cell lines with si-

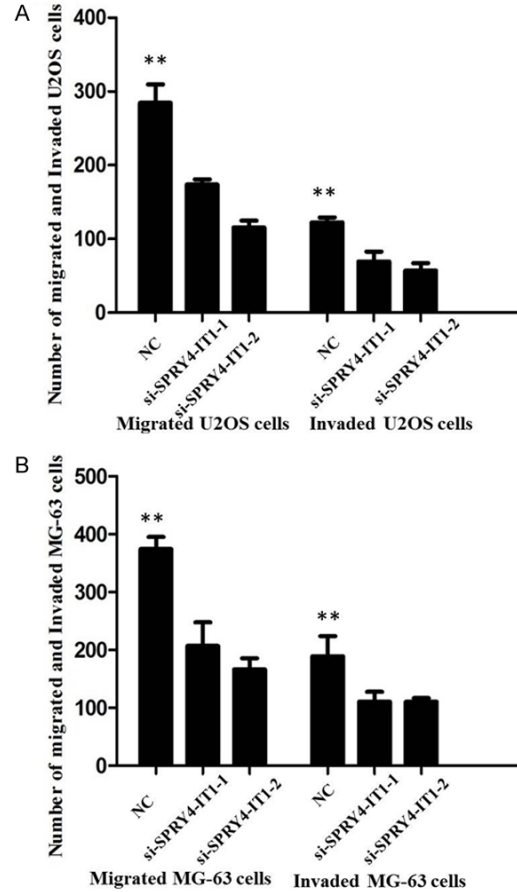


**Figure 5.** Flow cytometry for the apoptosis of OS cell lines transfected with SPRY4-IT1 siRNA and negative control. A: The proportion of apoptotic cells following SPRY4-IT1 siRNA treatment was increased in U2OS cells; B: The proportion of apoptotic cells following SPRY4-IT1 siRNA treatment was increased in MG-63 cells.

SPRY4-IT1 also decreased invasion in the Matrigel substrate, indicating that SPRY4-IT1 may promote the migration and invasion of OS cell lines (Figure 6A and 6B). Because EMT is vital for cell invasion, we next examined whether silencing SPRY4-IT1 expression inhibited mesenchymal features. As expected, SPRY4-IT1 knockdown decreased the expression of Vimentin and Snai1 and increased E-cadherin expression (Figure 7A and 7B). The results displayed that downregulation of SPRY4-IT1 obviously blocked the EMT process.

**Discussion**

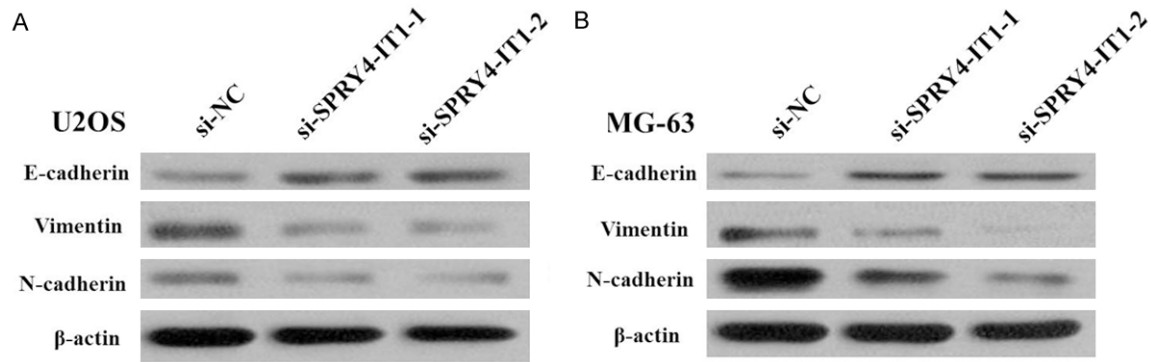
OS is the most common type of cancer that develops in bone, mainly arising from the



**Figure 6.** Effects of the SPRY4-IT1 on cell migration and invasion in OS cell line. A: Inhibition of migration and invasion of U2OS cells by SPRY4-IT1 siRNA; B: Inhibition of Migration and Invasion of MG-63 cells by SPRY4-IT1 siRNA.

metaphysis of the long bones [14]. Although great efforts have been made to understand the underlying mechanisms of OS carcinogenesis, the prognosis of advanced OS still remains poor [15]. Therefore, novel diagnostic biomarkers and therapeutic targets for OS are urgently needed for the development of effective therapeutic strategy. Recently, many lncRNAs have been reported to play significant regulatory roles in human diseases [16]. Cancer specific lncRNAs have been proven to contribute in tumor progress and serve as prognostic factors in many types of cancer.

lncRNA SPRY4-IT1 is a novel lncRNA, which was firstly reported to be associated with molecular etiology in human melanoma [17]. It is a 687nt unspliced, polyadenylated transcript, localized at chromosome 5q31.3. Moreover, lncRNA SPRY4-IT1 has been reported to be overexpressed and play an important role in



**Figure 7.** A: Knockdown of SPRY4-IT1 reverses EMT in U2OS cells; B: Knockdown of SPRY4-IT1 reverses EMT in MG-63 cells.

various types of cancers [18-20]. In this study, we firstly detected the expression of *SPRY4-IT1* in 56 paired OS tissues and normal bone tissues using qRT-PCR. Our study validated the upregulation of *SPRY4-IT1* in OS tissues and cell lines. Our results are consistent with previous results, which found that *SPRY4-IT1* is over-expressed in gastric cancer cell lines, and that the suppression of SPRY4-IT1 expression in gastric cancer cell line MKN-45 significantly reduced cell proliferation, colony formation, and cell migration/invasion [21].

We then determined whether SPRY4-IT1 expression influences tumor-like characteristics such as proliferation. Previous study reported that knockdown of SPRY4-IT1 attenuated esophageal squamous cell proliferation *in vitro* and markedly abrogated tumorigenicity *in vivo* [22]. Our results showed that knockdown of *SPRY4-IT1* by siRNA in U2OS and MG-63 cells, which exhibit naturally high SPRY4-IT1 expression levels, was shown to significantly inhibit cell proliferation, suggesting that SPRY4-IT1 may affect OS progression by affecting cell proliferation. Moreover, depletion of SPRY4-IT1 knockdown led to a significant accumulation of cells at the G1/G0 phase and a decreased number of cells in S phase.

Previous reports indicated that SPRY4-IT1 regulates the growth and metastasis of many cancers. Although *SPRY4-IT1* is involved in metastasis of different cancers, little is known about the underlying molecular mechanism. EMT is an essential process for tumor invasion and metastasis [23]. In the present study, we identified that knockdown of SPRY4-IT1 expression can suppress migratory and invasive pheno-

type of U2OS and MG-63 cells. In accordance with this, knockdown of SPRY4-IT1 expression increased the expression levels of E-cadherin and meanwhile greatly decreased the expression of Vimentin, indicating that SPRY4-IT1 affects OS metastasis partly via the EMT.

In conclusion, our findings indicated that the expression level of SPRY4-IT1 has the potential to be an oncogene for OS. Our results provide new insights into the function of lncRNAs in the development of OS and suggest that SPRY4-IT1 represents a potential therapeutic target and prognostic biomarker for OS.

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#### Disclosure of conflict of interest

None.

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## SPRY4-IT1 and osteosarcoma

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