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Original Article

Loss of SOX5 protein expression by RNAi in osteosarcoma cells suppresses cell proliferation and invasion

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Abstract: Sex determining region Y-box protein 5 (SOX5) is involved in the regulation of embryonic development and associated with various types of cancers. However, the expression and biological function of SOX5 in osteosarcoma remains to be investigated. Here, we found that SOX5 mRNA was significantly elevated in osteosarcoma tissues compared with bone cyst tissues. *In vitro* experiments demonstrated that knockdown of SOX5 by RNA interference in two osteosarcoma cell lines, MG63 and U2OS cells, inhibited cell proliferation, G1/S cell cycle transition, migration and invasion. In addition, knockdown of SOX5 in osteosarcoma cells reduced the expression of proteins associated with G1/S cell cycle transition (CDK2 and CDC25A) and invasion (MMP-2, MMP-9, Twist1 and Snail1). Our results indicate for the first time that SOX5 is a potential diagnostic biomarker and a therapeutic target for osteosarcoma.

Keywords: SOX5, osteosarcoma, proliferation, invasion

Introduction

Osteosarcoma is the most common primary bone tumor in children and adolescents, predominantly arising from long bones [1]. Recent advances in the treatment protocols combining chemotherapy and radical surgery have increased the 5-year overall survival for patients with osteosarcoma to around 50-60% [2]. However, for patients presenting with metastases or recurrent patients, the survival rate was still unfavorable [3]. Therefore, a better understanding of molecular pathogenesis of osteosarcoma will shed light on tumorigenesis and tumor development, and subsequently develop novel strategies for the treatment of osteosarcoma.

Sex determining region Y-box protein 5 (SOX5) is a member of the SOX family of transcription factors [4] and plays an important role in the regulation of embryonic development [5]. Recent studies suggested that SOX5 was associated with various types of cancers including human glioma [6], nasopharyngeal carcinoma [7], prostate cancer [8], breast cancer [9], pituitary tumor [10] and hepatocellular carcinoma [11]. Several studies have been performed to explore the expression and/or functions of other member of Sox family, including SOX2 [12], SOX9 [1] and SOX18 [13] in osteosarcoma. However, the expression and biological function of SOX5 in osteosarcoma remains unknown.

In this current study, we found that SOX5 was frequently up-regulated in osteosarcoma tissues. Functional research implied that knockdown of SOX5 inhibited cell proliferation, migration and invasion. SOX5 might be involved in these progresses by regulating the expression of cell cycle, apoptosis and metastasis-related proteins. Our results indicate that SOX5 may act as an oncogene and a candidate therapeutic target for osteosarcoma.

Materials and methods

Patients and tumor sample preparations

This study was approved by the independent ethics committee, Zhongnan Hospital of Wu-

han University (Wuhan, China). Written informed con-sent was obtained from all patients according to the guidelines of the ethics committee. A total of 35 patients with primary osteosarcoma and 15 patients with bone cysts admitted to Department of Orthopedics, Zhongnan Hospital of Wuhan University was enrolled in this study. All collected tissues were snap frozen in liquid nitrogen and store at -80°C until use.

RNA extraction and real-time PCR

Total RNA was extracted from tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was then synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega, Madison, WI, USA) and random hexamer primers (Generay, Shanghai, China). Real-time PCR was carried out using a standard SYBR Green PCR kit (Thermo, Rockford, IL, USA) on an ABI 7300 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 45 s. The expression level of the samples was normalized with that of GAPDH. The primer pairs used for SOX5 were 5'-GTGATGGGACTGCTTATGTAG-3' and 5'-ACTTTAGGGTGGTGTTTCG-3'; and for GAPDH were 5'-CACCCACTCCTCCACCTTTG-3' and 5'-CCACCACCCTGTTGCTGTAG-3'.

Cell lines and culture conditions

MG63, Saos2, SW1353, U2OS and HOS cells were purchased from American Type Culture Collection (Rockville, MD, USA) and maintained in corresponding medium (Invitrogen) with 10% fetal bovine serum (FBS, Invitrogen) and 1% antibiotic (penicillin/streptomycin, Invitrogen) in a humidified incubator at 37°C/5% CO_2. MG63, Saos2, SW1353 and HOS cells were grown in DMEM Medium, while U2OS cells was grown in RPMI 1640 medium.

Knockdown of SOX5 by small interfering RNA (siRNA) transfection

Three siRNAs targeting human SOX5 mRNA (siSOX5-1: 5'-CUCCAGGCUUCAGCUAUAA-3'; siSOX5-2: 5'-CCCACAUAAAGCGUCCAAU-3'; siSOX5-3: 5'-GGAUGAUCCAGAUGUAGAU-3') and a non-specific scramble siRNA sequence (siNC: 5'-UUGUACUACACAAAAGUACUG-3') were synthesized by Genepharma (Shanghai, China). MG63 and U2OS cells were transiently transfected with the siRNAs by using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. mRNA and protein expression of SOX5 was detected 48 h after transfection.

Western blotting

Cells transfected with siRNAs were washed with ice-cold phosphate buffer saline (PBS) and lysed in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). Aliquots with equal protein (25 μg) were electrophoretically resolved on 10% SDS-PAGE gels, transferred onto nitrocellulose membranes (Millipore, Bredford, MA, USA) and blocked with 5% skim milk for 1 h at room temperature. After incubating with specific primary antibodies at 4°C overnight, the membrane were incubated with HRP-conjugated secondary antibody (Beyotime) and the protein bands were developed using enhanced chemiluminescence system (ECL, Millipore). The sources of primary antibodies were as follows: Anti-SOX5, anti-CDK2, anti-MMP-2, anti-MMP-9, anti-Twist1 were obtained from Abcam (Cambridge, MA, USA). Anti-CDC25A, anti-Snail1 and anti-GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell proliferation

Cell proliferation was determined by using Cell Count Kit-8 (CCK-8, Beyotime) according to manufacturer's instructions. Briefly, 3×10^3 of MG63 or U2OS cells were plated in 96-well plates and incubated overnight. MG63 and U2OS cells were transiently transfected with the siRNAs. After varying periods of time (0, 24, 48 and 72 96 h), CCK-8 solution was added to each well and incubated for 1 h. Optical density values (OD) was detected at a wavelength of 450 nm with a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Cell cycle distribution analysis

The cell cycle distribution was evaluated by using propidium iodide (PI) staining and flow cytometric analysis. Cells were collected at 48

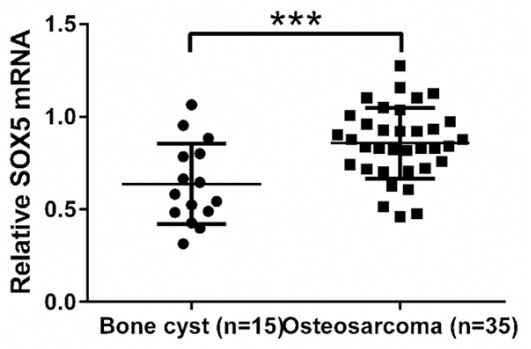


Figure 1. SOX5 was overexpressed in osteosarcoma tissues. The mRNA level of SOX5 in osteosarcoma and bone cyst tissues collected from patients admitted to Department of Orthopedics, Zhongnan Hospital of Wuhan University was detected by real-time PCR. SOX5 mRNA was significantly higher in osteosarcoma tissues than in bone cyst tissues (***P<0.001).

h after siRNA transfection by trypsinized, fixed with ice-cold 70% ethanol at -20°C overnight, and washed with PBS. The cells were then incubated with 0.05 mg/ml PI (Sigma) and 100 U/ml ribonuclease A (Sigma) at room temperature in the dark for 30 min. DNA content was analyzed on a flow cytometer (BD Biosciences, San Jose, CA, USA). Independent experiments repeated three times and at least 3×10^4 cells were analyzed per sample.

In vitro migration and invasion assays

In vitro cell migration and invasion assays were performed using Boyden chambers (8-μm pores, Corning Incorporated, NY, USA). For invasion assay, the upper wells of the Boyden chambers were pre-coated with Matrigel (BD Biosciences). Cells transfected with siRNAs were plated in the upper chamber with serum-free medium at a density of 5×10^4 cells per well. Medium containing 10% FBS was added to the lower chamber as chemoattractant. After 24 h of culture, cells on the upper surface of the membrane were completely removed and the migrated cells were fixed in 4% paraformaldehyde, stained with 0.5% crystal violet and counted in five randomly selected fields (×200) under a microscope (Nikon, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± standard deviation (SD) of at least triplicates of three independent experiments. Significance of statistical analysis was done using two-tailed, unpaired Student's *t* test. *P*-values less than 0.05 were considered significant.

Results

SOX5 mRNA level was elevated in human osteosarcoma tissues

We first analyzed SOX5 mRNA levels in osteosarcoma tissues and bone cyst tissues by real-time PCR. As shown in **Figure 1**, SOX5 expression was significantly up-regulated in osteosarcoma tissues (n=35), as compared with control bone cyst tissues (n=15, P<0.001).

SOX5 knockdown in osteosarcoma cells

We assessed the mRNA and protein expression of SOX5 in 5 osteosarcoma cell lines by real-time PCR and western blot, respectively. MG63 and U2OS cells showed higher mRNA and protein expression of SOX5 than the other three cell lines, Saos2, SW1353 and HOS cells (**Figure 2A**).

To investigate the functions of SOX5 in osteosarcoma, we knockdown its expression in MG63 and U2OS cells, which expressed high levels of SOX5 by siRNA transfection. Three siRNAs targeting human SOX5 (siSOX5-1, siSOX5-2 and siSOX5-3) and a non-specific scramble siRNA (siNC) were synthesized. The efficiency of siRNA-mediated knockdown of SOX5 in MG63 and U2OS cells was evaluated at both mRNA and protein levels by real-time PCR and western blot, respectively (**Figure 2B** and **2C**). All the three siRNAs targeting SOX5 (siSOX5-1, siSOX5-2 and siSOX5-3) were able decreased the SOX5 mRNA and protein levels in both cell lines. siSOX5-3 was the most effective one among the tested siRNAs with a knockdown ratio of greater than 75% in both cell lines. Therefore, siSOX5-3 was selected for the following experiments.

SOX5 knockdown suppressed the proliferation of osteosarcoma cells

The effect of SOX5 knockdown on osteosarcoma proliferation was investigated by CCK-8 assay. As shown in **Figure 3**, SOX5 knockdown in both MG63 and U2OS cells resulted in a significant reduction of cell viability at 24 h, 48 h and 72 h compared with cells without knockdown of SOX5 (siNC). These results indicated

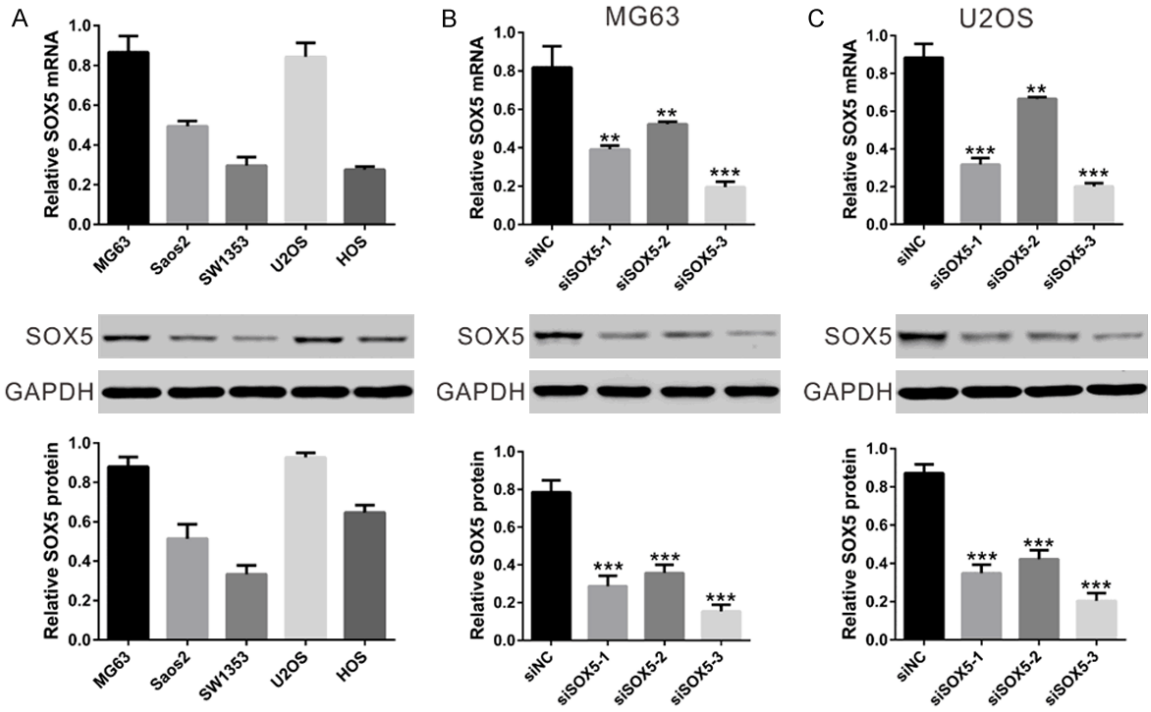


Figure 2. Knockdown of SOX5 in osteosarcoma cells. A. SOX5 expression in 5 osteosarcoma cell lines was analyzed by real-time PCR (upper panel) and Western blot (middle and lower panels). B, C. Knockdown of SOX5 in MG63 and U2OS cells by real-time PCR (upper panel) and Western blot (middle and lower panels). siNC: non-specific scramble siRNA transfected cells; siSOX5-1, siSOX5-2 and siSOX5-3: SOX5-siRNA-1, -2 and -3 transfected cells.

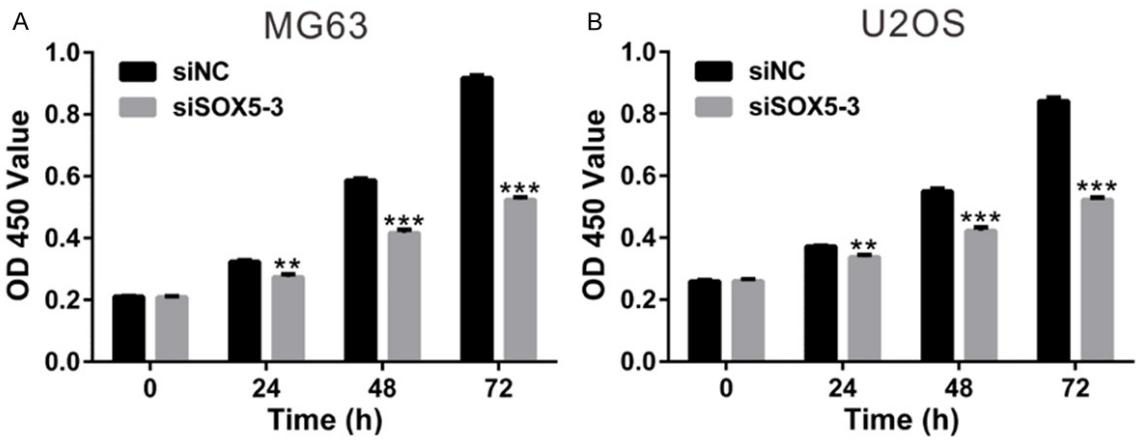


Figure 3. Effects of SOX5 knockdown on osteosarcoma cell proliferation in vitro. Proliferation of MG63 (A) and U2OS (B) cells with knockdown of SOX5 (siSOX5-3) was significantly slower than proliferation of cells without knockdown of SOX5 (siNC). (**P<0.01, ***P<0.001 as compared with siNC).

that SOX5-siRNA exerted inhibitory effects in the proliferation of osteosarcoma cells.

SOX5 knockdown induced G1 phase arrest of osteosarcoma cells

To investigate whether SOX5 influenced cell cycle progression of osteosarcoma cells, cell cycle distribution was determined by PI staining and flow cytometry analysis (**Figure 4A** and **4B**). SOX5 knockdown in MG63 cells increased the proportion of G0/G1 phase cells from 52.80 ± 1.43% to 66.71 ± 0.62%, while decreased the proportion of S phase cells from 30.72 ± 1.72% to 17.27 ± 3.15%. Similar results were obtained in U2OS cells.

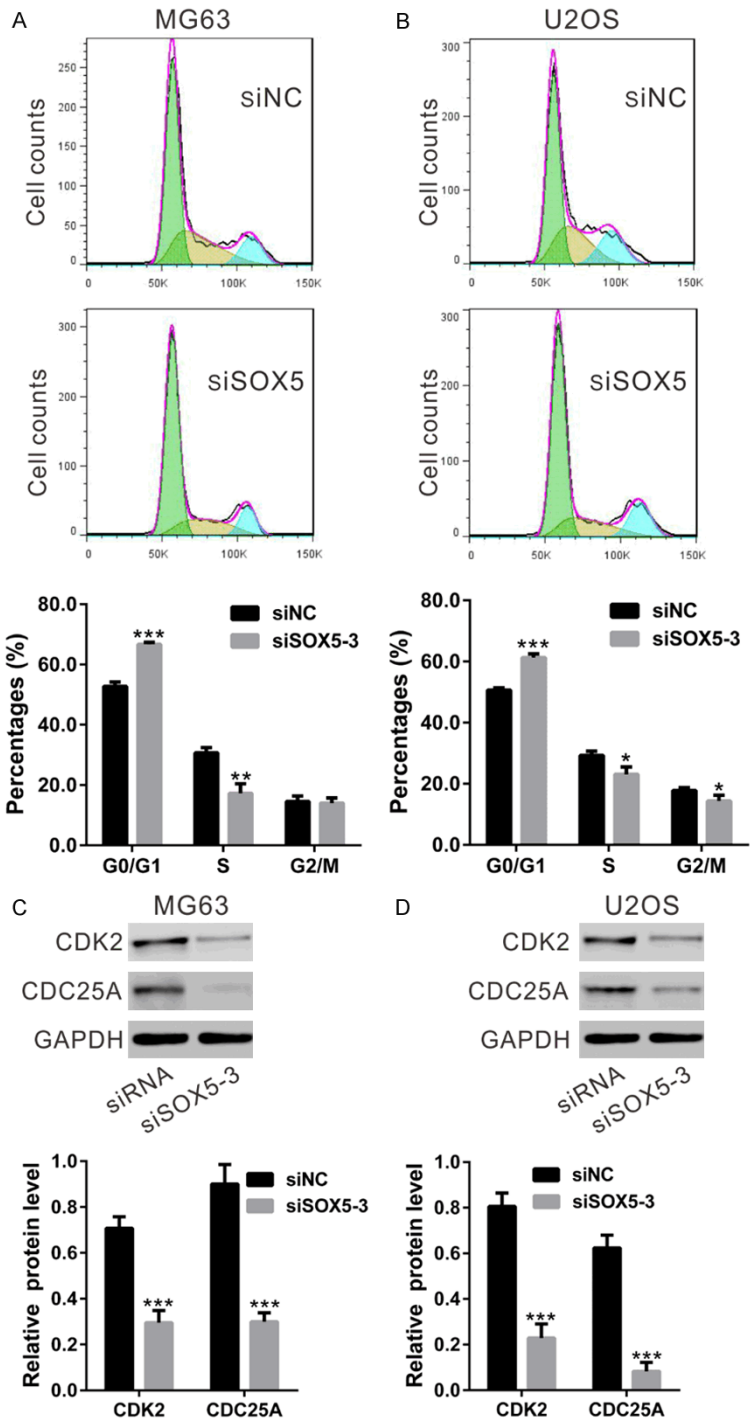


Figure 4. Effects of SOX5 knockdown on osteosarcoma cell cycle distribution. A, B. The percentage of G0/G1 phase population in MG63 and U2OS cells with knockdown of SOX5 (siSOX5-3) was higher than those in cells without knockdown of SOX5 (siNC). C, D. Expression of CDK2 and CDC25A was evaluated by Western blot. (*P<0.05, **P<0.01, ***P<0.001 as compared with siNC).

Moreover, the expression levels of cell cycle regulating proteins were then estimated by Western blot. SOX5 knockdown in MG63 and U2OS cells significantly reduced the expression of CDK2 and CDC25A (**Figure 4C** and **4D**), indicating that SOX5 played an important role in the cell cycle progression of osteosarcoma cells.

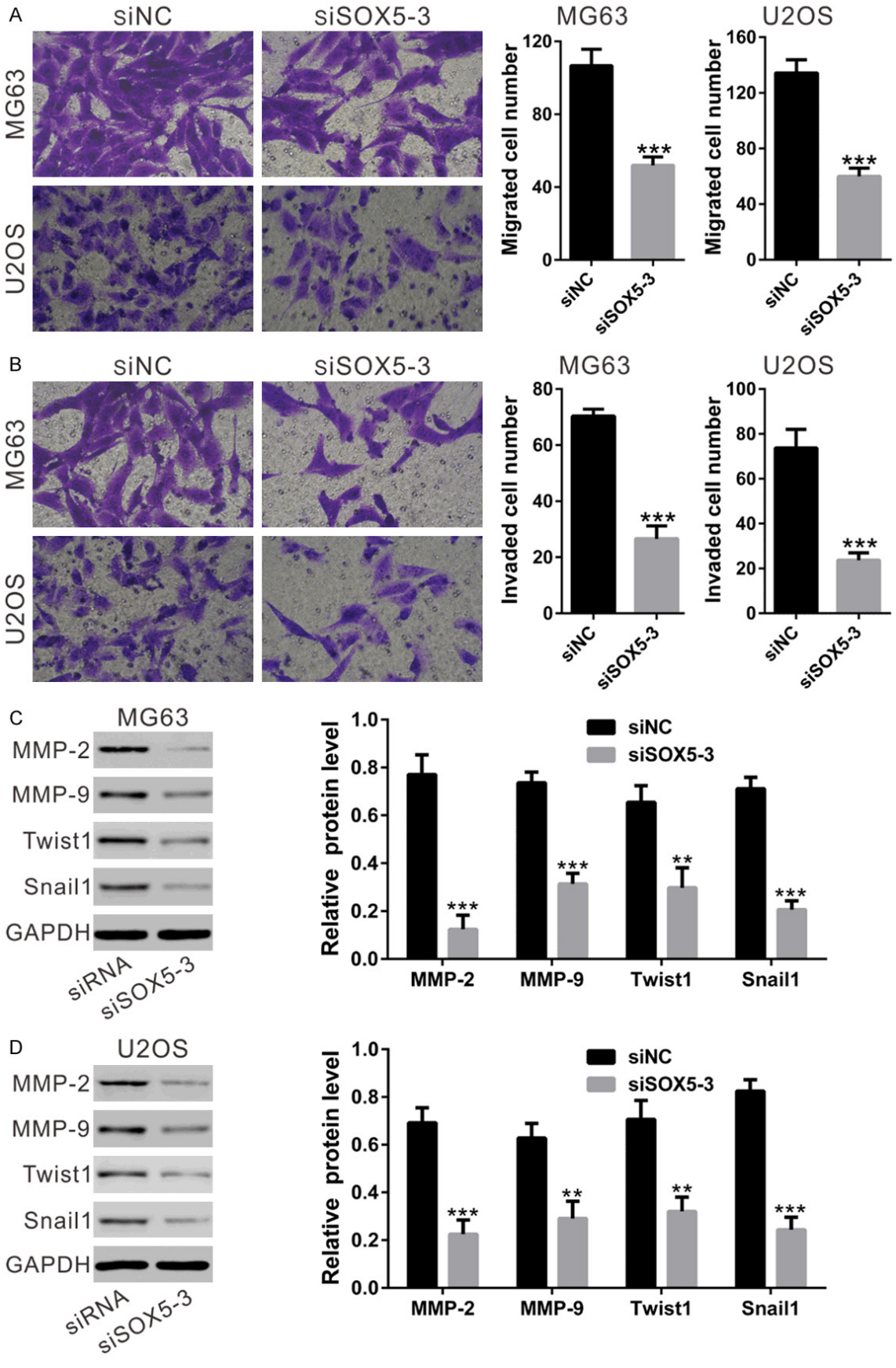
SOX5 knockdown suppressed the migration and invasion ability of osteosarcoma cells

To explore the possible function of SOX5 in the metastasis, the migration and invasion ability of MG63 and U2OS cells were evaluated by Transwell assay (**Figure 5**). SOX5 knockdown significantly reduced the migrated cell number of both osteosarcoma cells compared with control cells (MG63 cells: siNC, 107 ± 9; siSOX5-3, 52 ± 5; U2OS cells: siNC, 134 ± 9; siSOX5-3, 60 ± 6). The number of invaded knockdown cells was about 37.9% and 32.1% of that of the control cells in MG63 and U2OS cells, respectively.

Moreover, the effect of SOX5 knockdown on the expression levels of important factors to regulate invasion was also explored. The protein levels of MMP-2, MMP-9, Twist1 and Snail1 were significantly reduced by SOX5 knockdown in both osteosarcoma cells. These results further demonstrated the role of SOX5 in the invasion of osteosarcoma cells.

Discussion

The SOX family of transcription factors plays important roles in embryogenesis, cell differentiation, and proliferation [14]. SOX5, together with SOX6 and SOX13, belongs to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MMP-2
MMP-9
Twist1
Snail1

Figure 5. Effects of SOX5 knockdown on the migration and invasion of osteosarcoma cells. A. Migration ability was assessed using Boyden chambers. Migrated cell number in MG63 and U2OS cells with knockdown of SOX5 (siSOX5-3) was reduced as compared with that in cells without knockdown of SOX5 (siNC). B. Invasive ability was measured using Boyden chambers pre-coated with Matrigel. Invaded cell number in MG63 and U2OS cells with knockdown of SOX5 (siSOX5-3) was reduced as compared with that in cells without knockdown of SOX5 (siNC). C, D. Expression of MMP-2, MMP-9, Twist1 and Snail1 was evaluated by western blot. (**P<0.01, ***P<0.001 as compared with siNC).

Subgroup D of the SOX family. Previous studies have reported the functions of SOX5 during skeletogenesis [15], neural crest development [16] and gliogenesis [17]. Recently, SOX5 has been linked to various cancers. It may serve as a diagnostic and prognostic marker for glioma patients [6]. SOX5 plays an important role in the regulation of nasopharyngeal carcinoma progression through down-regulating SPARC expression [7]. Furthermore, SOX5 enhances proliferation, migration and invasion of breast cancer [9] and hepatocellular carcinoma cells [11]. In our study, we demonstrated that SOX5 was overexpressed in osteosarcoma (**Figure 1**) and contributed to cell proliferation (**Figure 3**), migration and invasion (**Figure 5**). Our results, consistent with the previous studies, suggest the oncogenic role of SOX5.

Aberrant cell proliferation in most malignancies is mostly due to the inhibition of cell cycle progression. Martinez-Morales et al. has suggested the role of SOX5 in the timing of cell cycle exit of neural progenitors by WNT-catenin signaling [18]. Here, cell cycle distribution analysis (**Figure 4A** and **4B**) suggested that SOX5 knockdown in osteosarcoma cells induced G0/G1 cell arrest. We then detected protein expression of CDK2 [19] and CDC25A [20], which were important for cell cycle progression of G1 to S. Consistent with the data of cell proliferation and cell cycle analysis, expression of CDK2 and CDC25A were significantly down-regulated by SOX5 knockdown (**Figure 4C** and **4D**). Our data that SOX5 knockdown suppressed cell proliferation of osteosarcoma cells via inhibiting cell cycle progression.

Previous studies have shown the role of SOX5 in cell migration and invasion via regulating Twist1 expression [9, 11]. In the current study, our data demonstrated that knockdown of SOX5 significantly reduced the migration and invasion capacity of osteosarcoma cells (**Figure 5A** and **5B**). Matrix metalloproteinases, including MMP-2 and MMP-9, are responsible for the degradation of the extracellular matrix, thus involving in tumor invasion and metastasis [21]. Twist and snail, transcription factors involved in epithelial to mesenchymal transition (EMT), have significant role in the pathogenesis of osteosarcoma [22]. In this study, we showed that down-regulation of SOX5 down-regulated the expression of MMP-2, MMP-9 and Twist1 and snail (**Figure 5C** and **5D**). Thus, we proposed that SOX5 induces cell invasion by regulation of MMPs expression and EMT.

Taken together, we found that SOX5 was over-expressed in osteosarcoma tissues. Further in vitro experiments demonstrated that SOX5 knockdown suppressed cell proliferation and invasion of osteosarcoma cells. Our study suggests the far-reaching clinical implications of SOX5. SOX5 may serve as a potential diagnostic biomarker and a therapeutic target for osteosarcoma.

Disclosure of conflict of interest

None.

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