Abstract. Sex determining region Y-box 18 (SOX18) has been found to be overexpressed in several types of tumor. However, the molecular mechanism underlying the biological function of SOX18 in osteosarcoma remains to be elucidated. The present study aimed to elucidate the roles of SOX18 in regulating the biological behavior of osteosarcoma cells. First, SOX18 mRNA expression was analyzed in osteosarcoma tissues using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The results demonstrated that the expression of SOX18 was elevated in osteosarcoma tissue, compared with normal bone tissue. In addition, the knockdown of SOX18 in U2OS or MG63 osteosarcoma cells inhibited cell proliferation and significantly increased the population of cells in the S-phase of the cell cycle, as measured by the CCK-8 assay and flow cytometric analysis, respectively. Additionally, suppression of the expression of SOX18 in the osteosarcoma cells significantly induced cell apoptosis as evaluated by annexin V/propidium iodide staining and flow cytometric analysis. The downregulation of SOX18 was found to significantly inhibit cell adhesion and invasion. The mRNA and protein expression levels of transforming growth factor-β, platelet-derived growth factor-A (PDGF-A), PDGF-B and RhoA were also reduced by SOX18 silencing, as assessed by RT-qPCR and western blot analysis, respectively. These results indicated that SOX18 may function as an oncogene, and may provide a novel and promising therapeutic strategy for osteosarcoma.

Introduction

Osteosarcoma is the most common type of primary bone tumor worldwide and exhibits a peak incidence in the second and third decades of life (1). Osteosarcoma can arise in any bone, however, it is most common in the metaphyses of long bones (1). Although survival rates have increased between 20 and 75% due to the combination of radical surgery and neoadjuvant chemotherapy (2-4), for patients who present with metastatic disease or present with tumor recurrence, the survival rates remain <30 and <20%, respectively (5). This emphasizes the requirement for the development of novel therapeutic targets and approaches for the treatment of osteosarcoma.

Sex determining region Y-box 18 (SOX18) is a member of the sex-determining region of the Y chromosome-related high mobility group box (SOX) family of transcription factors. It selectively interacts with the common SOX target sequence (A/T)ACAA(A/T)G, and activates transcription via a transactivation domain adjacent to the high mobility group domain (6,7). Previous studies (8,9) have suggested that the expression level of SOX18 may affect tumor growth. It has been reported that the expression levels of SOX18 are increased in gastric cancer tissues, compared with normal gastric tissues (10). Furthermore, the expression of SOX18 is correlated with poor survival rates (10). The expression of SOX18 has also been correlated with poor clinical outcome in patients with non-small cell lung cancer (11), ovarian cancer (12) and invasive ductal breast carcinoma (13). However, the expression pattern and biological functions of SOX18 in osteosarcoma remain to be fully elucidated.

The present study aimed to investigate the role of SOX18 in osteosarcoma. Initially, the expression levels of SOX18 were analyzed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis in osteosarcoma tissue samples obtained from 25 patients. Subsequently, the biological function of SOX18 in osteosarcoma cell lines was investigated using RNA interference (RNAi). The present study also aimed to elucidate whether SOX18 is involved in cell proliferation, cell cycle progression, apoptosis, adhesion and invasion, and whether SOX18 is involved in these processes by regulating the expression of transforming growth factor-β1 (TGF-β1), platelet-derived growth factor-A (PDGF-A), PDGF-B and Ras homolog family member A (RhoA).
Materials and methods

Patients and tissue samples. Between 2010 and 2012, 25 patients (16 men and 9 women) with conventional (occurring in the metaphyses of the long bones) osteosarcoma, who were admitted to the Department of Orthopedics, Shanghai Tenth People's Hospital (Tongji University, Shanghai, China), were enrolled in the present study. Complete clinical and pathological follow-up data were obtained for all patients. The patients ranged in age from 7 to 49 years with a median age of 18 years. Osteosarcoma tissues (0.1-0.2 g) were obtained from femur or tibia of these 25 patients and normal bone tissues were also collected as negative controls. These normal bone tissues were resected within at least 5 cm of the tumor margin when the patients underwent definitive surgery. Ethical approval for the present study was provided by the independent ethics committee of Shanghai Tenth People's Hospital, Tongji University (Shanghai, China). Informed and written consent was obtained from all patients or their advisers, according to the ethics committee guidelines.

Antibodies. The following primary antibodies were used in the present study: Mouse polyclonal SOX18 (Ab66145; 1:1,000), rabbit polyclonal TGF-β (Ab92486; 1:400) and rabbit polyclonal RhoA (Ab68826; 1:2,000) (Abcam, Cambridge, MA, USA), rabbit polyclonal PDGF-A (BA0408; 1:200) and rabbit polyclonal PDGF-B (BA0519-2; 1:200) (Wuhan Boster Biological Technology, Ltd. (Wuhan, China) and rabbit monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #5174; 1:2,000), Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Beyotime Institute Biotechnology (Shanghai, China).

Cell culture. MG63, HOS, 143B, Saos2, U2OS and HEK293T cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The MG63, HOS, Saos2, 143B and HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc.). All cell lines were maintained at 37˚C in a 5% CO₂ atmosphere.

Vector construction and virus transduction. Three shRNAs targeting human SOX18 mRNA (SOX18-Ri-3, AGGAAG CCGAACGCTGCGTTC; SOX18-Ri-2, AGGCTGCTCTT TCCCTCCTT; and SOX18-Ri-3, TACCCAGTGGCACTG GCCATT; Generay Biotech Co., Ltd., Shanghai, China) were cloned into a lentiviral vector (PLKO.1; Addgene, Inc., Cambridge, MA, USA). A non-specific scramble shRNA sequence (TTCTCCGAAAGTGTGACCT) was used as the negative control (NC). The constructs were then transfected into the HEK293T cells with lentiviral packaging vectors using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. The viruses were collected 48 h subsequent to transfection and used to transduce the U2OS cells and MG63 cells. After 48 h, the cells were processed for RT-qPCR and western blotting.

RT-qPCR. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed using a First Strand cDNA Synthesis kit (K1612; Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. RT-qPCR was performed using a SYBR Green PCR kit (Thermo Fisher Scientific, Inc.) on an ABI 7300 Real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA) using the following cycling parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec. GAPDH served as an internal control. The gene expression was calculated using the ΔΔCt method (14). All data represent the average of three replicates. The primers used (Generay Biotech Co., Ltd.) were as follows: SOX18 (NM_018419.2), forward 5'-CGGTTGTGATGTGGGTTC-3' and reverse 5'-ATGTAACCTGGCAATCCT-3'; TGF-β1 (NM_000660.4), forward 5'-GACTACTACGCCAAGGAGGTC-3' and reverse 5'-GAGGAGCAACACGGTGTCAG-3'; PDGF-A (NM_002607.5), forward 5'-CTGAGGGTGGAGTCTTGTT-3' and reverse 5'-AATATGACCGTCCTGTCCTTG-3'; PDGF-B (NM_002608.2), forward 5'-CTCTGGATCGCTCCCTTGTAG-3' and reverse 5'-AGGAAUTGGGTGTTTGTTG-3'; RhoA (NM_001664.2), forward 5'-GAGTGGTTCAGCAAGACACAAAG-3' and reverse 5'-TTCGACAGTTTTTCACACG-3'; GAPDH (NM_001256799.1), forward 5'-CACCCACT CCTCCTCACTTTG-3' and reverse 5'-ACACCCACCCCTTGTTGTAG-3'.

Western blotting. The treated and untreated MG63 and U2OS cells were harvested and washed twice with phosphate-buffered saline (PBS) and lysed in ice-cold radioimmunoprecipitation assay buffer (JRDUIN Biotechnology Co., Ltd., Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The cells were then incubated on ice for 30 min. The cell lysates were centrifuged at 16,000 x g for 10 min at 4°C. Protein concentration was measured using the Bicinchoninic Acid Assay kit (Thermo Fisher Scientific, Inc.) and the supernatant (20-30 µg protein) was run on a 15% SDS-PAGE gel and transferred electrophoretically onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Subsequent to blocking with 5% skimmed milk, the membranes were incubated with the primary antibodies, followed by the corresponding horseradish peroxidase-conjugated secondary antibodies (Beyotime Institute of Biotechnology). The blots were then visualized using enhanced chemiluminescence (EMD Millipore).

Cell proliferation assay. Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; Dojin Molecular Technologies, Inc., Kumamoto, Japan) according to manufacturer's protocol. In brief, the U2OS and MG63 cells (~1.5x10⁴) were seeded into 96-well plates. At 0, 24, 48 and 72 h, CCK-8 solution (10 µl in 100 µl DMEM) was added into each well, followed by incubation at 37°C for 1 h. The optical density values were measured at a wavelength of 450 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Inc.,
Hercules, CA, USA). All experiments were performed in triplicate and repeated a minimum of three times.

**Cell cycle distribution analysis.** Propidium iodide (PI; Sigma-Aldrich) staining was performed to analyze the DNA content in the cells to determine cell cycle distribution. The cells were harvested 48 h following transduction, and were labeled with PI, as previously described (15). In brief, the cells were resuspended in PBS and fixed with 70% ethanol. Cells were washed twice with PBS and then suspended at a concentration of 1x10^6 cells/ml. Following treatment with ribonuclease (Sigma-Aldrich) for 15 min at 37°C, PI (0.05 mg/ml) was added to the cells, followed by incubation at room temperature in the dark for 30 min. DNA content was then analyzed using a FACScan instrument equipped with FACStation running CellQuest software, version 3.3 (BD Biosciences, San Jose, CA, USA).

**Cell apoptosis assay.** The percentages of the cells actively undergoing apoptosis were determined by double staining with annexin V-fluorescein isothiocyanate (FITC) and PI. The adherent and floating virally transduced or control cells were harvested after 48 h, and double-labeled with annexin V-FITC and PI (BD Biosciences), according to the manufacturer's protocol. The cells were analyzed using a FACScan instrument equipped with FACStation running CellQuest software.

**Cell adhesion assay.** To determine cell adhesion, the assay was performed in 12-well plates. The plates were pre-coated with 1 ml fibronectin (5 µg/ml) for 2 h at room temperature. The cells were transduced, as described above, 48 h prior to the assay. The cells were seeded into the coated plates at a density of 10^5 cells/well and allowed to adhere at 37°C for 1 h. Non-adherent cells were washed off with PBS and the cells were fixed in 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and stained with 0.2% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.). The number of adherent cells was determined in five randomly selected fields under a microscope (Eclipse E600; Nikon Corporation, Tokyo, Japan), as previously described (16).

**In vitro invasion assay.** The upper well of a Transwell chamber (Corning Incorporated, NY, USA) was coated with Matrigel (BD Biosciences) at 37°C in a 5% CO_2 incubator for 1 h. The virus-treated and untreated cells were serum starved for 24 h, then 500 µl cell suspension containing 10^5 cells/ml were placed in the upper compartment of the chamber. Culture medium supplemented with 10% FBS (750 µl) was added into the lower well of the chamber. The cells were allowed to invade through the Matrigel membrane for 48 h, and non-invasive cells were removed from the upper membrane. The invasive cells on the underneath were washed with PBS, fixed in 4% paraformaldehyde and stained with 0.2% crystal violet. The invading cells were observed under a microscope. Cells were counted in the central field of the membranes in triplicate.

**Statistical analysis.** All data are presented as the mean ± standard deviation. Statistical significance was determined using Student's two-tailed t-test with SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**SOX18 is overexpressed in osteosarcoma.** The mRNA levels of SOX18 were measured in the osteosarcoma and adjacent normal tissues of 25 patients using RT-qPCR. As presented in Fig. 1A, SOX18 was overexpressed in 88% (22/25) of the osteosarcoma tissues assessed. Statistical analysis using Student's t-test indicated that SOX18 mRNA was significantly overexpressed in osteosarcoma tissues, compared with normal tissues (P<0.001).

**Knockdown of SOX18 suppresses the proliferation of osteosarcoma cells.** The expression levels of SOX18 in five osteosarcoma cell lines, Saos2, U2OS, HOS, MG63 and 143B, were assessed using RT-qPCR and western blotting. The results demonstrated that two of these cell lines, MG63 and U2OS, exhibited higher mRNA and protein expression levels of SOX18, compared with the remaining Saos2, HOS and 143B cell lines, which exhibited lower mRNA and protein expression levels of SOX18 (Fig. 1B).

To investigate the effect of SOX18 on osteosarcoma, SOX18 was knocked down in osteosarcoma cells using RNAi. U2OS and MG63 cells were selected for the RNAi experiment due to the fact that they expressed higher levels of SOX18. Three pairs of shRNA (SOX18-Ri-3, SOX18-Ri-2 and SOX18-Ri-3) targeting human SOX18, and a non-specific scramble shRNA (NC) were designed and cloned into a lentiviral plasmid. The recombinant lentivirus was then packaged into the HEK293T cells and used to transduce the U2OS and MG63 cells. The silencing effect of the shRNA was evaluated by western blotting and RT-qPCR (Fig. 1C and D). The results indicated that SOX18-Ri-3 was the most efficient shRNA, with a knockdown efficiency of ~70%. Therefore, SOX18-Ri-3 was selected for the following assays.

The effect of SOX18 RNAi on the proliferation of osteosarcoma was then assessed. Knockdown of SOX18 by transduction of the SOX18-shRNA virus into the U2OS or MG63 cells resulted in a reduced cell growth rate, compared with the corresponding control (Fig. 1E and F), whereas a similar growth rate was observed between the WT cells and the NC cells. These results indicated that SOX18 may promote the proliferation of osteosarcoma cells.

**Silencing of SOX18 induces S-phase arrest and apoptosis in osteosarcoma cells.** The potential effects of SOX18 knockdown on cell cycle progression were then investigated. PI staining and flow cytometry analysis revealed that knockdown of SOX18 in the U2OS (Fig. 2A) and MG63 cells (Fig. 2B) resulted in an increase in the number of cells in the S-phase and a corresponding reduction in the number of cells in the G_0/G_1-phase. These results suggested that silencing of SOX18 prevented the osteosarcoma cells from entering the G_0/M-phase.

The apoptotic function of SOX18 in U2OS and MG63 cells was also assessed using the annexin V-FITC/PI staining assay. As shown in Fig. 2C and D, flow cytometric analysis
demonstrated that knockdown of SOX18 in the U2OS or MG63 cells significantly induced cell apoptosis, compared with the corresponding scramble shRNA (U2OS cells, 23.03±0.46, vs. 3.80±0.23%; MG63 cells, 34.43±1.32, vs. 3.93±0.18%). These results indicated that the proliferation-promoting function of SOX18 may be mediated via the promotion of cell cycle...
progression between the S-phase and G2/M-phase, inhibiting apoptosis.

**Knockdown of SOX18 inhibits the metastasis of osteosarcoma cells.** Metastasis begins with the invasion of tumor cells into the surrounding host tissue. The invasive tumor cells must first alter cell-to-cell adhesion and adhesion to the extracellular matrix (17). The effects of SOX18 on the adherent ability of osteosarcoma cells were evaluated in the present study (Fig. 3A and B). The number of adherent SOX18-Ri-3 cells was 29.5% of that of the NC cells when U2OS cells were used. Similar results were obtained with the MG63 cells. These data suggested that the adherent ability to fibronectin was significantly inhibited in osteosarcoma cells by SOX18 knockdown.

Whether SOX18 affected the invasive ability of osteosarcoma cells was also investigated using a Transwell assay. As
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Figure 3. Silencing of SOX18 inhibits the metastasis of osteosarcoma cells and reduces tumor growth in vivo. The U2OS and MG63 cells were transduced with the indicated virus. Adhesion ability was analyzed using a cell adhesion assay. (A) Representative images and (B) quantitative results of the cell adhesion assay. The U2OS and MG63 cells were transduced with the indicated virus and cell invasion was analyzed in Matrigel-coated Transwell chambers. (C) Representative images and (D) quantitative results of the cell adhesion assay. Data are representative of a minimum of three independent experiments and are presented as the mean ± standard deviation (**P<0.01, vs. NC); magnification, x100. SOX18, sex-determining region Y-box 18; WT, wild-type; NC, scrambled shRNA virus transduction; SOX18-Ri-3, SOX18-shRNA-3 virus transduction.

Figure 4. Expression levels of TGF-β1, PDGF-A, PDGF-B and RhoA are downregulated by SOX18 RNAi. The protein and mRNA levels of the indicated genes were evaluated using (A and B) western blotting and (C and D) reverse transcription-quantitative polymerase chain reaction in the U2OS and MG63 cells. Data are presented as the mean ± standard deviation (**P<0.01, vs. NC). TGF-β1, transforming growth factor-β1; PDGF-A, platelet-derived growth factor-A; RhoA, Ras homolog family member A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SOX18, sex-determining region Y-box 18; WT, wild-type; NC, scrambled shRNA virus transduction; SOX18-Ri-3, SOX18-shRNA-3 virus transduction.
shown in Fig. 3C and D, transduction of the SOX18-shRNA virus into U2OS or MG63 cells significantly reduced the cell invasion ability, compared with the scramble shRNA (NC). These data suggested that SOX18 promoted osteosarcoma cell invasion.

**Expression levels of TGF-β1, PDGF-A, PDGF-B and RhoA are downregulated by SOX18 RNAi.** A previous study demonstrated that TGF-β1 is a promoter of tumor progression and invasion (18). The classic PDGFs, PDGF-A and PDGF-B, are regarded to be associated with metastasis in various types of human cancer (19-21). It is well known that small GTPase RhoA promotes the invasion of tumor cells (22-24). In order to investigate the molecular mechanisms underlying the role of SOX18 in osteosarcoma cells, the mRNA and protein expression levels of TGF-β1, PDGF-A, PDGF-B and RhoA were determined (Fig. 4). The expression levels of all the genes examined were markedly reduced following the downregulation in the expression of SOX18, which suggested that the biological function of SOX18 in osteosarcoma may be associated with these genes.

**Discussion**

In the present study, it was found that SOX18 was overexpressed in osteosarcoma. Knockdown of the expression of SOX18 markedly inhibited the transforming ability of osteosarcoma cells. These data indicated the diagnostic and therapeutic value of SOX18 in osteosarcoma.

The involvement of SOX18 in several types of cancer has been an area of investigation, and it has been reported that SOX18 is overexpressed in several types of cancer tissue (10-13), and that SOX18 may promote cellular proliferation (9,25). Garcia-Ramirez et al (25) found that SOX18 is co-localized with the proliferating cell nuclear antigen protein in vascular smooth muscle cells of human coronary atherosclerotic lesions, and that inhibiting the expression of SOX18 results in a reduced proliferation rate in these cells. The expression of dominant-negative SOX18 also reduces the proliferation of human MCF-7 breast cancer cells (9). In the present study, the knockdown of SOX18 in U2OS and MG63 osteosarcoma cells significantly reduced the cell growth rate (Fig. 1). In addition, cell cycle analysis revealed that SOX18 knockdown induced S-phase arrest and apoptosis of osteosarcoma cells (Fig. 2), which may explain the inhibited proliferation of the SOX18-knockdown cells.

Previously, SOX18 was reported to be associated with cell migration and tumor metastasis, and dominant-negative SOX18 was reported to impair the migration of MCF-7 cells (9). Duong et al (26) reported that tumor metastasis is inhibited in SOX18-deficient mice. In line with these observations, the present study found that reduction in the expression of SOX18 in osteosarcoma cells by RNAi significantly reduced their adhesive and invasive capabilities (Fig. 3), indicating that SOX18 may be important in promoting metastasis of osteosarcoma.

The exact pathway that SOX18 may regulate in osteosarcoma remains unclear. TGF-β1 has been considered as a promoter of tumor progression and invasion (18). Additionally, PDGFs have been found to induce tumor growth (27,28), and their expression may be useful as a diagnostic and prognostic marker for several types of cancer (29-31). The classic PDGFs, PDGF-A and PDGF-B, are associated with the metastasis of various types of human cancer (19-21). In the present study, SOX18 RNAi significantly downregulated the expression levels of TGF-β1, PDGF-A and PDGF-B, which indicated that SOX18 may execute its functions through regulating the expression of these genes.

It is well known that small GTPase RhoA promotes the invasion of tumor cells (22-24). The expression levels of RhoA may be positively correlated with the progression of carcinoma, suggesting that RhoA may be important in tumorigenesis and tumor progression (32-35). The malignant phenotype in gastric cancer cells (36) and breast cancer cells (37) can be reversed by inhibiting the expression of RhoA. In the present study, it was observed that SOX18 knockdown impaired the expression of RhoA (Fig. 4). Therefore, it was hypothesized that SOX18 may perform its biological function through regulating the expression of RhoA.

In conclusion, the present study demonstrated high expression levels of SOX18 in osteosarcoma, which suggested that SOX18 may be a diagnostic marker for osteosarcoma. The present study suggested for the first time, to the best of our knowledge, that SOX18 is key in the proliferation, apoptosis and metastasis of osteosarcoma cells. In addition, SOX18 may regulate these biological processes through TGF-β1, PDGF-A, PDGF-B and RhoA, thus providing potentially useful information for the targeted therapy of osteosarcoma.

**References**


