

Downregulation of SOX3 leads to the inhibition of the proliferation, migration and invasion of osteosarcoma cells

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Abstract. Sex determining region Y-box protein 3 (SOX3) is involved in embryonic development and tumorigenesis. However, the expression and precise role of SOX3 in osteosarcoma remain unclear. In this study, we reported that SOX3 expression was upregulated in osteosarcoma tissues compared with non-cancerous bone cyst tissues. To elucidate the cellular and molecular function of SOX3, we examined the consequences of SOX3 knockdown in osteosarcoma cells. We found that the downregulation of SOX3 inhibited the proliferation, migration and invasion of osteosarcoma cells. SOX3 downregulation also increased the cell population in the G1 phase and induced cell apoptosis. SOX3 knockdown-mediated cell cycle arrest and cell apoptosis were associated with decreased levels of Cdc25A, cyclin D1, proliferating cell nuclear antigen (PCNA) and Bcl-2, as well as an increased Bax expression. We also found that the downregulation of SOX3 decreased the expression of Snail, Twist and matrix metalloproteinase-9 (MMP-9), and increased E-cadherin expression, resulting in the inhibition of cell migration and invasion. Taken together, our data indicate that SOX3 may serve as an oncogene in osteosarcoma, and SOX3 downregulation may prove to be a novel approach for the inhibition of osteosarcoma progression.

Introduction

Osteosarcoma is the most common primary malignant bone tumor arising from long bones in children and adolescents (1). During the past decades, treatment strategies combining surgery, chemotherapy and in some cases radiotherapy, have increased the 5-year survival rates of patients with localized tumor from 20% to approximately 60% (2). However, the

prognosis of patients with advanced osteosarcoma remains unfavorable due to recurrence, distant metastasis and resistance to treatment (3). Therefore, novel diagnostic biomarkers and therapeutic targets for patients with osteosarcoma are required.

Sex determining region Y-box protein (SOX)3 is a member of SOX family transcription factors, which selectively interact with the common target sequence (A/T)ACAA(A/T)G and activate gene transcription. SOX3, together with SOX1, SOX2 and SOX21, belongs to the subgroup of the SOX B family (4). SOX3 is believed to play a critical role during embryonic development (5-7). In recent years, evidence has been provided to suggest the involvement of SOX3 in tumorigenesis. Xia *et al* reported that SOX3 was able to induce the oncogenic transformation of chicken embryo fibroblasts (8). Yang *et al* and Cai *et al* indicated that SOX3 acted as an oncogene in human epithelial ovarian cancer (9) and esophageal squamous cell carcinoma (ESCC) (10). Although other members of the SOX family, including SOX2 (11,12), SOX9 (13) and SOX18 (14), have been found to be involved in the development of osteosarcoma, the expression and biological function of SOX3 in osteosarcoma remain unclear.

In this study, we demonstrated that SOX3 was upregulated in osteosarcoma tissues in comparison with non-cancerous bone cyst tissues. To determine whether SOX3 is involved in the development and progression of osteosarcoma, we carried out the functional characterization of SOX3 in human osteosarcoma cell lines in which SOX3 was silenced. We further investigated the mechanisms underlying the effects of SOX3 knockdown on osteosarcoma. Our results indicate that SOX3 may act as an oncogene in osteosarcoma by regulating cell proliferation, migration and invasion.

Materials and methods

Patients and tumor sample preparations. This study was approved by the Ethics Committees of Shanghai Sixth People's Hospital, Shanghai, China. Written consent was obtained from all the enrolled patients for the use of tissue specimens. A total of 70 patients with primary osteosarcoma and 20 patients with bone cysts admitted to Department of Orthopedic Surgery, Shanghai Sixth People's Hospital were enrolled in this study. All collected primary osteosarcoma and non-cancerous bone cyst tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

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RNA extraction and RT-qPCR. TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA according to the manufacturer's instructions. Total RNA was then reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI, USA). Quantitative (real-time) PCR was carried out on an ABI 7300 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The sequences of the primers used were as follows: SOX3, forward, 5'-TGAAGTCAAGAACCCCGTAGG-3' and reverse, 5'-GCTGCGTTCGCACTACTCT-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-CAC CCACTCCTCCACCTTTG-3' and reverse, 5'-CCACCA CCCTGTTGCTGTAG-3'. The abundance of SOX3 mRNA was expressed relative to GAPDH mRNA.

Cell culture, lentiviral production and infection. The MG63 and U2OS osteosarcoma cells, and the 293T cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in a humidified incubator at 37°C/5% CO₂. The MG63 and 293T cells were grown in DMEM medium with 10% fetal bovine serum (FBS), while the U2OS cells was grown in RPMI-1640 medium (all from Invitrogen) with 10% FBS.

SOX3 shRNA (shSOX3) target sequence (CAAGGAGTT AGTTAAATGC) and a scramble shRNA (shNC) was cloned into the lentiviral vector PLKO.1 (Addgene, Cambridge, MA, USA). Lentivirus was produced by transfecting the 293T cells with the shRNA plasmids and packaging plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The viral supernatant was collected and filtered at 48-72 h following transfection. The cells were infected with the viruses in the presence of 8 µg/ml of Polybrene (Sigma, St. Louis, MO, USA). A U2OS stable cell line was established using puromycin (Sigma) selection.

Western blot analysis. Protein was extracted using ice-cold radio immunoprecipitation assay buffer (Beyotime, Shanghai, China). Equal amounts of protein from each sample were electrophoretically resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). After blocking with 5% skim milk for 1 h at room temperature, the blots were probed with specific primary antibodies at 4°C overnight. The protein expression levels were determined by incubating the membranes with horseradish peroxidase-conjugated secondary antibody (Beyotime) and enhanced chemiluminescence reagent (Millipore). Each sample was examined in triplicate and the protein expression levels were expressed relative to GAPDH. Antibodies used included anti-SOX3 (Abcam, Cambridge, MA, USA; ab183606; 1:500), anti-cyclin D1 (Abcam; ab16663; 1:200), anti-Bax (Abcam; ab32503; 1:2,000), anti-Bcl-2 (Abcam; ab692; 1:500), anti-Twist (Abcam; ab50581; 1:2,000), anti-matrix metalloproteinase-9 (MMP-9) (Abcam; ab119906; 1:500), anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA; #5174; 1:1,500), anti-Cdc25A (Cell Signaling Technology; #3652; 1:1,000), anti-proliferating cell nuclear antigen (PCNA) (Cell Signaling Technology; #13110; 1:1,000), anti-Snail (Cell Signaling Technology; #3879S; 1:1,000) and anti-E-cadherin (Cell Signaling Technology;

#14472; 1:1,000). Goat anti-mouse (A0216; 1:1,000) and goat anti-rabbit (A0208; 1:1,000) secondary antibody (both from Beyotime). The protein expression levels were determined by incubating the membranes with horseradish peroxidase conjugated secondary antibody (Beyotime) at room temperature for 1 h. Enhanced chemiluminescence reagent (Millipore) was applied to examine the protein expression. Western blot analysis was repeated three times and quantification of the blots was performed by using ImageJ software (NIH, Bethesda, MD, USA) with GAPDH as loading control.

Cell proliferation assay. Cell proliferation was examined using the Cell Counting kit-8 CCK-8; Beyotime) following the manufacturer's instructions. Briefly, the MG63 or U2OS cells were plated in 96-well plates (3x10³ cells/well). Following overnight incubation, the cells were infected with shNC or shSOX3 lentivirus. Cell proliferation was determined every 24 h with CCK-8 solution. Each experiment was performed in triplicate.

Tumor xenograft model. All animal experiments were approved by the Ethics Committee of the Department of Orthopedic Surgery, Shanghai Sixth People's Hospital. A total of 12 female athymic nude mice (4 to 5 weeks old, 15-20 g) (SLAC Animal, Shanghai, China) was used in this study. U2OS stable cells (2x10⁶) were injected subcutaneously into the left flanks (six mice were injected with shNC-transfected cells and six mice were injected with shSOX3-transfected cells. Tumors were measured every 3 days using a Vernier caliper, and tumor volumes were calculated using the following formula: V=0.5 x (length x width²). At 24 days following implantation, all mice were sacrificed, and the tumors were isolated, weighed and protein expression in the tumor samples was examined by western blot analysis.

Cell cycle distribution analysis. At 24 h following infection, the cells were harvested and washed with phosphate-buffered saline (PBS), followed by fixation with ice-cold 70% ethanol at -20°C overnight. After washing with PBS, the cells were resuspended in PBS containing 0.05 mg/ml propidium iodide (PI; Sigma) and 100 U/ml RNase A in the dark at room temperature for 30 min. Samples were analyzed for DNA content on a flow cytometer (BD Biosciences, San Jose, CA, USA).

Analysis of cell apoptosis. At 24 h following infection, the cells were harvested and washed with PBS, followed by staining with the Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis assay kit (Beyotime) as instructed by the manufacturer. Samples were analyzed for cell apoptosis on a flow cytometer. Cells undergoing early and late apoptosis were defined by Annexin V⁺/PI staining and Annexin V⁺/PI⁺ staining, respectively.

Cell migration and invasion assays. Cell migration assays were performed in Boyden chambers (8-µm pore size; Corning, Corning, NY, USA). The cells (5x10⁴ cells/well) transfected with the shRNAs in serum-free medium were seeded into the upper chamber. Medium with 10% FBS was added to the lower chamber. Following 24 h of incubation, the cells in the upper chamber were completely removed with a cotton swab. The cells attached to the bottom of the

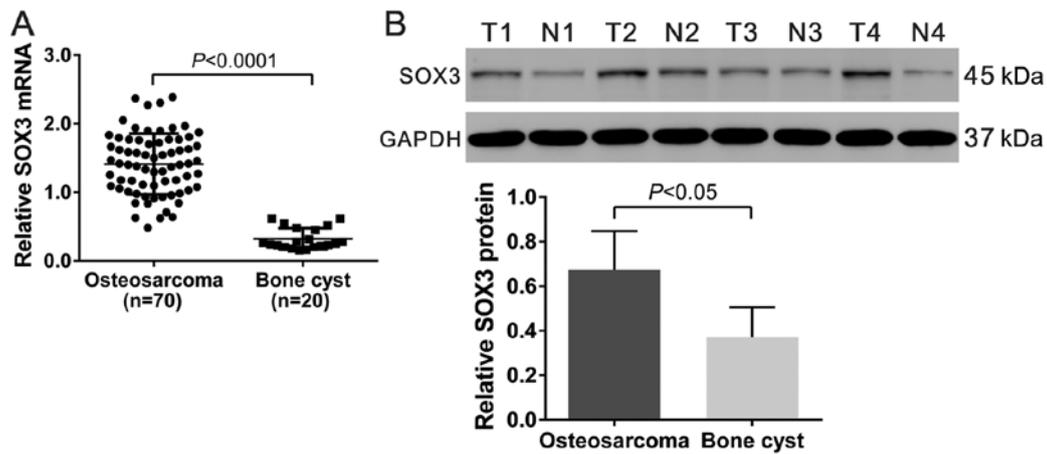


Figure 1. Sex determining region Y-box protein 3 (SOX3) mRNA and protein expression in osteosarcoma and bone cyst tissues. (A) Expression levels of SOX3 mRNA in osteosarcoma tissues (n=70) and non-cancerous bone cyst tissues (n=20) detected by RT-qPCR. (B) Expression levels of SOX3 protein in osteosarcoma tissues and non-cancerous bone tissues by western blot analysis. T1, T2, T3 and T4 refer to osteosarcoma tissues. N1, N2, N3 and N4 refer to noncancerous bone cyst tissues.

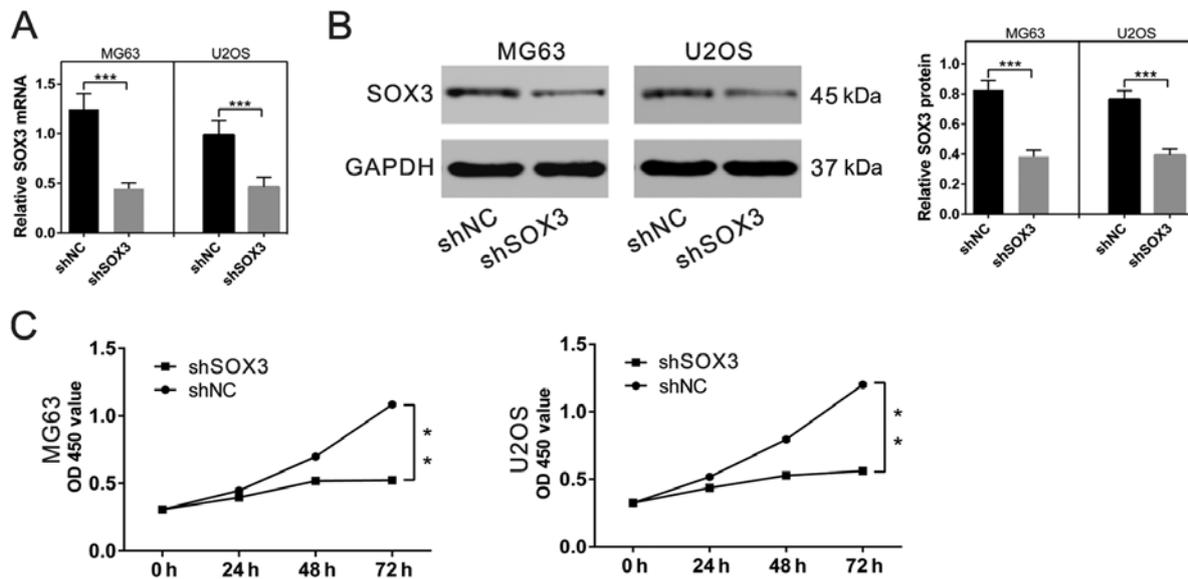


Figure 2. Effects of sex determining region Y-box protein 3 (SOX3) knockdown on the proliferation of osteosarcoma cells *in vitro*. (A) RT-qPCR and (B) western blot analysis of SOX3 mRNA and protein expression at 48 h after lentiviral infection of the MG63 and U2OS cells. (C) Cell proliferation after SOX3 knockdown in osteosarcoma cells was measured by cell counting kit-8 (CCK-8) assay at 0, 24, 48 and 72 h after viral infection. The assay was performed in triplicate. **P<0.01 and ***P<0.001.

membranes were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The migrated cells were counted in 5 randomly selected fields (x200) under a microscope (Nikon, Tokyo, Japan).

Cell invasion assays were performed in the same manner as the migration assay, with the difference that the upper chamber was pre-coated with 30 μ l Matrigel (BD Biosciences). Experiments were performed in triplicate.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism software (version 6.0; GraphPad Prism Software, San Diego, CA, USA). The Student's t-test was used for statistical analysis between 2 independent groups. P-values <0.05 were considered to indicate statistically significant differences.

Results

Upregulation of SOX3 in human osteosarcoma tissues. As shown in Fig. 1A, the relative level of SOX3 mRNA in the osteosarcoma tissues (1.41 ± 0.05) was significantly higher than that in non-cancerous bone cyst tissues (0.32 ± 0.03 ; $P < 0.0001$). Similar results were obtained at the protein level (osteosarcoma tissues, 0.67 ± 0.09 ; bone cyst tissues, 0.37 ± 0.07 ; $P < 0.05$; Fig. 1B).

SOX3 knockdown inhibits the proliferation of osteosarcoma cells *in vitro*. To perform the functional analysis of SOX3, we knocked down SOX3 expression in two human osteosarcoma cells (MG63 and U2OS) by using a lentivirus system. At 48 h following infection, the effects of shSOX3

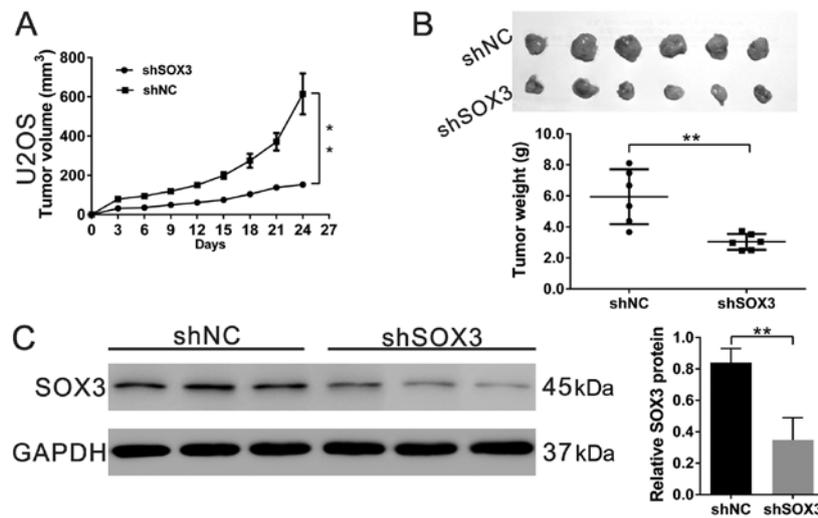


Figure 3. Effects of sex determining region Y-box protein 3 (SOX3) knockdown on the proliferation of U2OS cells *in vivo*. (A) Growth curve of xenograft tumors derived from U2OS cells stably transfected with SOX3 shRNA (shSOX3) or shNC (n=6). (B) After 24 days, xenograft tumors [from mice injected with shNC-transfected cells (n=6) and shSOX3-transfected cells (n=6)] were dissected, photographed (upper panel) and weighed (lower panel). (C) The protein expression of SOS3 in tumor xenografts was assessed by western blot analysis. **P<0.01.

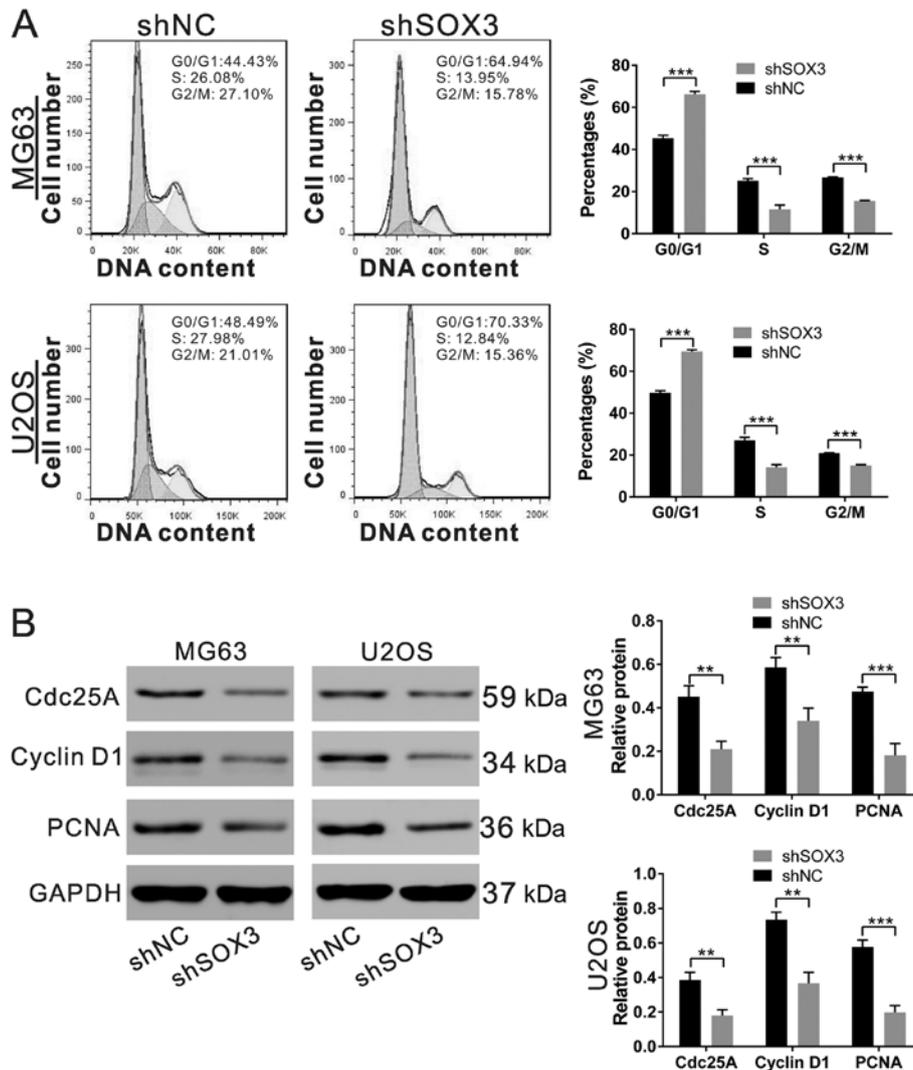


Figure 4. Effects of sex determining region Y-box protein 3 (SOX3) knockdown on cell cycle. (A) Cell cycle analysis of shNC- and SOX3 shRNA (shSOX3)-infected cells at 24 h after infection. DNA content was measured by propidium iodide (PI) staining and flow cytometry analysis. (B) Western blot analysis was performed to compare expression levels of cell-cycle-regulated proteins, Cdc25A, cyclin D1 and PCNA between shNC- and shSOX3-infected cells. **P<0.01 and ***P<0.001.

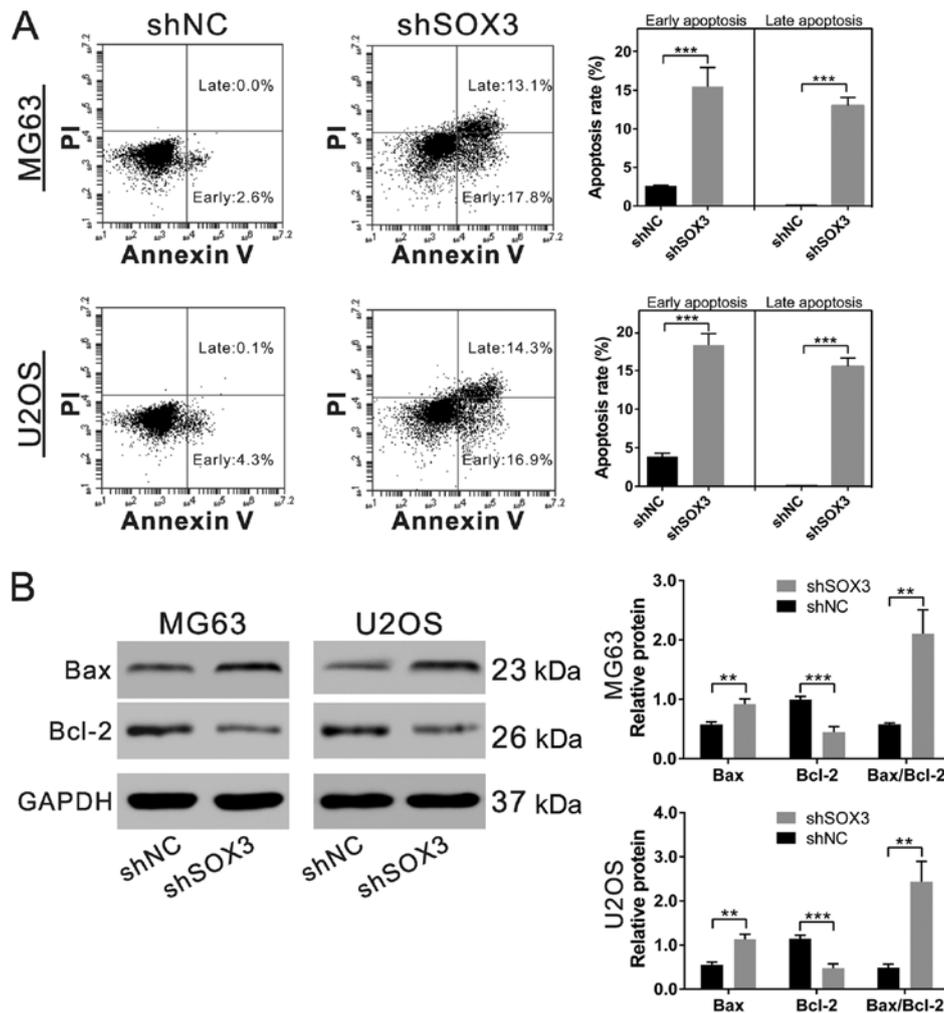


Figure 5. Effects of sex determining region Y-box protein 3 (SOX3) knockdown on cell apoptosis. (A) Cell apoptosis of shNC- and SOX3 shRNA (shSOX3)-infected cells at 24 h after infection was determined by Annexin V/propidium iodide (PI) staining and flow cytometry analysis. (B) Western blot analysis was performed to compare expression levels of cell apoptosis-related proteins, Bax and Bcl-2 between shNC- and shSOX3-infected cells. **P<0.01 and ***P<0.001.

on endogenous SOX3 expression were evaluated. As shown in Fig. 2A and B, SOX3 mRNA and protein expression was markedly inhibited due to shSOX3 infection in both osteosarcoma cells (P<0.001).

To determine the proliferation rates following the down-regulation of SOX3 in the osteosarcoma cell lines, CCK-8 assays were performed over a 3-day period. We found that both cell lines in which SOX3 was silenced exhibited significant growth inhibition compared with the shNC-infected cells (P<0.01; Fig. 2C).

SOX3 knockdown suppresses tumor growth in a xenograft mouse model. To investigate whether SOX3 knockdown in osteosarcoma cells suppresses tumor growth *in vivo*, U2OS cells stably transfected with shSOX3 or shNC were subcutaneously implanted into nude mice. As shown in Fig. 3A, the growth rates of xenografts formed from shSOX3 stably transfected cells were much slower than those from shNC cells (P<0.01). After 24 days, the volumes and weights of the tumors formed from shSOX3-transfected cells were significantly decreased compared with those of tumors derived from shNC-transfected cells (P<0.01; Fig. 3B). Moreover, SOX3 protein expression decreased by 58.5% in the xenografts formed

from shSOX3-transfected cells, as compared to the xenografts formed from shNC-transfected cells (P<0.01; Fig. 3C). These data suggested that the knockdown of SOX3 inhibited tumor growth in nude mice.

SOX3 knockdown induces G1 phase arrest of osteosarcoma cells. To clarify the mechanisms underlying the growth inhibitory effects of the knockdown of SOX3 by shRNA on osteosarcoma cell lines, cell cycle distribution was analyzed by flow cytometry in the cells stained with PI (Fig. 4A). At 24 h following infection, a higher proportion of shSOX3-infected cells was observed in the G1 phase (MG63, 66.34±1.29%; U2OS, 49.68±1.04%; P<0.001) in comparison with that of shNC-infected cells (MG63, 45.40±1.39%; U2OS, 69.52±0.72%). Concomitant decreases were observed in the proportions of cells in S and G2/M phases.

To explore the potential molecular mechanisms responsible for shSOX3-induced G1 arrest in osteosarcoma cells, we explored the effects of shSOX3 on the expression of cell cycle-regulated proteins. Western blot analysis revealed that the protein levels of Cdc25A, cyclin D1 and PCNA were significantly decreased in the shSOX3-infected cells, compared with the shNC-infected cells (Fig. 4B).

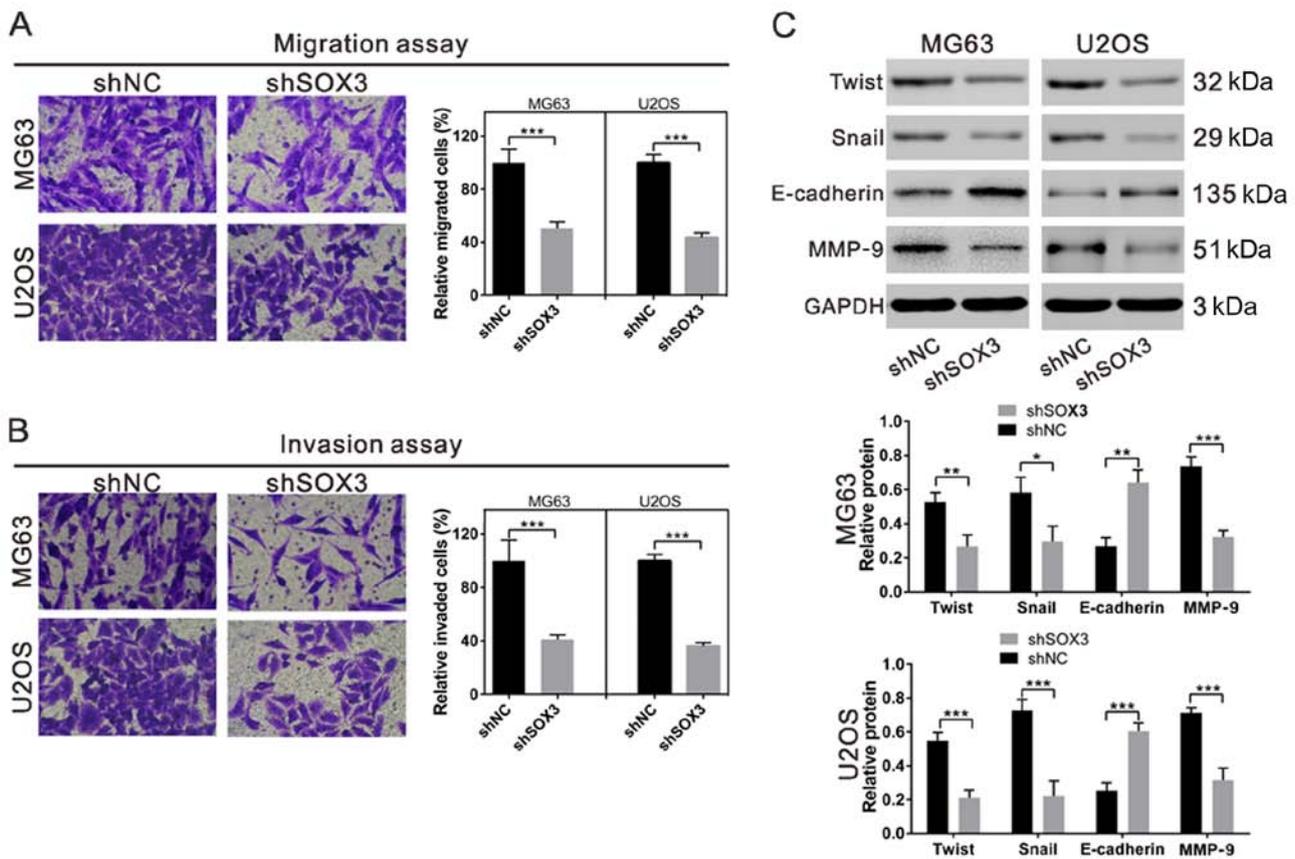


Figure 6. Effects of sex determining region Y-box protein 3 (SOX3) knockdown on cell migration and invasion. (A and B) MG63 or U2OS cells infected with either shNC or SOX3 shRNA (shSOX3) lentivirus were subjected to (A) cell migration and (B) cell invasion assay. The migration or invasion of cells transfected with shNC was set as 100%. (C) Western blot analysis was performed to compare expression levels of epithelial-mesenchymal transition (EMT)-related proteins, Twist, Snail, E-cadherin and MMP-9 between shNC- and shSOX3-infected cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

SOX3 knockdown induces the apoptosis of osteosarcoma cells.

To determine whether apoptosis occurred in shSOX3-infected cells, we performed flow cytometry analysis on the cells stained with Annexin V/PI. As shown in Fig. 5A, a significant increase in the percentages of cells undergoing apoptosis was observed in the MG63 cells in which SOX2 was knocked down (15.50 ± 2.46 and $13.00 \pm 1.05\%$ for early and late apoptosis, respectively) compared with the shNC-infected cells (2.60 ± 0.10 and $0.03 \pm 0.06\%$ for early and late apoptosis, respectively). Similar results were obtained in the U2OS cells.

To explore the possible molecular mechanisms responsible for the shSOX3-induced apoptosis of osteosarcoma cells, we examined the effects of the silencing of SOX3 by shSOX3 on the expression of cell apoptosis-related proteins. Western blot analysis revealed that the protein levels of Bax, a pro-apoptotic protein (15), were significantly increased in the shSOX3-infected cells, compared with the shNC-infected cells, whereas the expression of Bcl-2, an anti-apoptotic protein (15), was decreased in the shSOX3-infected cells compared with the control cells (Fig. 5B).

SOX3 knockdown suppresses the migration and invasion of osteosarcoma cells.

To determine whether SOX3 affects the migration and invasion of osteosarcoma cells, Transwell assay was performed (Fig. 6A). The shSOX3-infected cells (both cell lines) exhibited a significant decrease in migration and invasion compared with the shNC-infected cells.

It has been noted that epithelial-mesenchymal transition (EMT) is a critical event during tumor invasion and metastasis (16). The effect of SOX3 knockdown on the expression of EMT-related proteins was also explored. The protein levels of EMT-promoting proteins [Twist, Snail and MMP-9 (17)] were significantly decreased in the cells in which SOX3 was knocked down, whereas the expression of the main factor of EMT [E-cadherin (17)] was markedly elevated in comparison with the shNC-infected cells.

Discussion

Previous studies have reported that SOX3 expression is often increased in human esophageal squamous cell carcinoma (ESCC) (18) and epithelial ovarian cancer (9). In the current study, we found that SOX3 expression was upregulated in osteosarcoma, suggesting the oncogenic role of SOX3. Furthermore, we performed the functional characterization of SOX3 in osteosarcoma cell lines by lentivirus-mediated RNA interference. Previous studies have suggested the promoting effect of SOX3 on the proliferation of ESCC (10) and epithelial ovarian cancer cell lines (9). In this study, we found that the silencing of SOX3 inhibited cell proliferation, migration and invasion. SOX3 plays an oncogenic role and may be a potential target for osteosarcoma treatment.

In general, deregulated cell cycle and apoptosis are main causes for uncontrolled proliferation (19). Several members

of SOX family proteins, including SOX2 (20), SOX7 (21) and SOX21 (22), have been shown to regulate cell cycle and apoptosis in diverse cell lines. In this study, flow cytometric analysis revealed that SOX3 knockdown in osteosarcoma cells induced G1 phase arrest and apoptosis. We demonstrated that decreased protein levels of Cdc25A, cyclin D1, PCNA and Bcl-2, and an increased protein level of Bax were associated with SOX3 knockdown. These results were consistent with the findings of G1 phase arrest and increased cell apoptosis observed in the shSOX3-infected osteosarcoma cells. It has been reported that Src (23) activates the transcription of cyclin D1. Cyclin D1 (24) and Cdc25A (25) are direct TCF/ β -catenin transcriptional targets. SOX3 targets Src kinase in epithelial ovarian cancer cell lines (9), whereas the overexpression of *Xenopus* SOX3 was found to inhibit β -catenin activity (26,27). Thus, further studies are warranted to whether SOX3 functions in the cell cycle through Src or Wnt signals in osteosarcoma cells.

We further elucidated that SOX3 knockdown suppressed cell migration and invasion. We demonstrated that SOX3 knockdown led to decreased protein levels of Twist, Snail and MMP-9, and increased protein level of E-cadherin. It has been reported that other SOX family members, such as SOX2 and SOX4, promote cell migration and invasion in breast, prostate and liver cancer cells via regulating EMT (28-31). SOX family members have been shown to regulate the expression of EMT-related protein, including Snail, ZEB1, Twist and E-cadherin (28,32,33), being consistent with our finding. In chicken embryo, SOX3 is found to repress Snail expression (7). The discrepancy between this finding and our data may be due to different species and biology processes. Further studies are required to clarify the mechanism how SOX3 regulates the expression of EMT-related proteins.

In conclusion, we revealed that SOX3 expression was frequently upregulated in osteosarcoma. Moreover, we also showed that silencing of SOX3 expression inhibited cell proliferation through cell cycle arrest and apoptosis in osteosarcoma cells, as well as suppressed cell migration and invasion. To the best of our knowledge, we have demonstrated, for the first time, SOX3 acts as an oncogene in osteosarcoma, and SOX3 inhibitors or downstream effectors may be interesting targets for osteosarcoma therapy.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YG and XY conceived and designed the study. YG, JY and MT performed the experiments. YG, JY and XY wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

For the use of human sample, approval was obtained by the Ethics Committees of Shanghai Sixth People's Hospital. Written consent was obtained from all enrolled patients for the use of tissue specimens. All animal experiments were approved by the Ethics Committee of Department of Orthopedic Surgery, Shanghai Sixth People's Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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