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ORIGINAL RESEARCH

Interleukin-6 upregulates SOX18 expression in osteosarcoma

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Aim: SOX18 is a potential oncogene in osteosarcoma via controlling osteosarcoma cell proliferation and metastasis. Interleukin-6 (IL-6), a major activator of Janus kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) signaling, plays an important role in the growth of carcinoma cells. The present study aims to investigate the correlation between IL-6 and SOX18 in osteosarcoma.

Materials and methods: Protein expression and mRNA expression were determined by Western blot and real-time polymerase chain reaction (PCR) analysis, respectively. Cell proliferation and apoptosis were identified by Cell Counting Kit-8 assay and flow cytometry analysis, respectively.

Results: We found that SOX18, IL-6 and p-STAT3 were elevated in osteosarcoma compared with bone cyst tissues. A positive correlation between the mRNA levels of IL-6 and SOX18 was observed in osteosarcoma tissues. IL-6 stimulation dose dependently induced the mRNA and protein levels of SOX18 in U-2OS and MG63 cells. Furthermore, IL-6 significantly rescued the inhibitory and induction effects of SOX18 knockdown on osteosarcoma cell proliferation and apoptosis, respectively. The changes in cell proliferation (PCNA) and apoptosis-related proteins (Bcl-2, Bax and Cleaved-Caspase 3) were in line with the results of cell proliferation and apoptosis assays.

Conclusion: Our data suggest that IL-6 is a possible upstream regulator for SOX18 in osteosarcoma.

Keywords: IL-6, SOX18, osteosarcoma, proliferation, apoptosis

Introduction

Osteosarcoma is the most common malignant bone-forming tumor.[1] Unlike other tumors, osteosarcoma usually occurs during the second and third decades of life. Despite the advances in surgery and chemotherapy for the past 2 decades, the survival time has hardly been improved.[2] As such, understanding the molecular mechanisms involved in the development of osteosarcoma is crucial for developing new molecular targets of diagnosis and effective therapy.

SOX18 belongs to sex-determining region on the Y chromosome-related high-mobility group box (SOX) gene family.[3,4] SOX genes are highly conserved across species. SOX genes encode a group of transcription factors participating in developmental processes.[5] Recently, several studies have revealed the role of SOX18 in carcinogenesis. SOX18 expression is elevated in gastric cancer[6] and pancreatic ductal adenocarcinoma (PDAC).[7] Overexpression of SOX18 indicates poor prognosis for a wide spectrum of human malignancies, such as gastric cancer,[6] invasive ductal breast carcinoma,[8] ovarian cancer[9] and non-small cell lung cancer.[10] In our previous paper, we have showed that SOX18 is upregulated in osteosarcoma and served as a potential

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oncogene in osteosarcoma via regulating osteosarcoma cell proliferation and metastasis.[11] However, little is known about the upstream regulators of SOX18.

Interleukin-6 (IL-6), a proinflammatory cytokine, is a major activator of Janus kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) signaling.[12] STAT3 is a vital transcription factor that participates in the regulation of cell proliferation[13] and cell apoptosis.[14] It has been identified as an oncogene in a variety of tumor types.[15] The total and phosphorylated forms of STAT3 are elevated in osteosarcoma cell lines and tissues. The increased level of phosphorylated STAT3 is associated with the poor prognosis in patients with osteosarcoma.[16] Expression of other SOX genes, such as SOX2,[17,18] is mediated by STAT3. However, whether IL-6/STAT3 regulates SOX18 expression in osteosarcoma is unknown.

In the present study, upregulation of IL-6 and SOX18 was observed in human osteosarcoma. The mRNA level of SOX18 had a positive correlation with that of IL-6 in osteosarcoma tissues. Cell proliferation and apoptosis assays suggested that the IL-6 was a possible upstream regulator for SOX18.

Materials and methods

Tissue samples

From 2011 to 2014, 50 patients with osteosarcoma and 20 patients with bone cyst admitted to Shanghai Tenth People's Hospital were enrolled in this study. This study was approved by the ethics committee of Shanghai Tenth People's Hospital, Tongji University. Written informed consent was obtained from every participant and complied with the guidelines of the ethics committee. Osteosarcoma and bone cyst samples were collected and immediately frozen in liquid nitrogen and stored at −80°C until use.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) assay

The mRNA levels of SOX18 were evaluated by real-time quantitative RT-PCR assay as previously described.[11] In brief, total RNA was extracted from tissue samples or cell lines with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and then reverse transcribed into complementary DNA (cDNA) by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) per the manufacturer's instructions. Real-time PCR was carried out with SYBR Green PCR kit (Thermo Fisher Scientific) by ABI 7300 instrument (Thermo Fisher Scientific). SOX18 expression levels were determined by normalization to internal control, GAPDH.

Western blot

Tissue samples (~200 mg) were ground into fine powder in liquid nitrogen using a mortar. Frozen tissue powder and cell lines were lysed in radio immunoprecipitation assay buffer (Beyotime, Shanghai, China) containing proteinase inhibitor cocktail (Sigma-Aldrich Co., St Louis, MO, USA), placed on ice for 30 min and then centrifuged at 13,000 rpm for 20 min at 4°C. The collected supernatant was quantitated by BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein (30 μg) was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). To block nonspecific binding, the membrane was incubated with 5% skim milk at room temperature for 30 min. Following incubation with primary antibodies at 4°C overnight, the membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Beyotime). Protein expression was then analyzed using enhanced chemiluminescence (ECL; EMD Millipore) and ImageJ software (National Institutes of Health, Bethesda, MA, USA). GAPDH was detected as loading control. Sources of primary antibodies were as follows: 1) SOX18, p-STAT3, PCNA and Cleaved-Caspase 3 (Abcam, Cambridge, MA, USA); 2) STAT3 and GAPDH (Cell Signaling Technology, Danvers, MA, USA) and 3) Bcl-2 and Bax (Santa Cruz Biotechnology Inc., Dallas, TX, USA).

Cell culture

U-2OS, MG63 and HEK293T cells were obtained from American Type Culture Collection (Manassas, VA, USA). U-2OS cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific), while MG63 and HEK293T cells were grown in the Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific). Both media were supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% antibiotic (penicillin/streptomycin). All cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

Silencing of SOX18 by short hairpin RNA (shRNA)

shRNA targeting SOX18 (RNA interference [RNAi], TACC ACGTGGCACTGGCCATT) and a nonspecific scramble shRNA sequence (NC, TTCTCCGAACGTGTCACGTTT) was cloned into PLKO.1 (Addgene, Cambridge, MA, USA).

Recombinant lentiviruses were produced by transfecting the lentiviral construct and package plasmids into HEK293T cells as described[11] and collected to transduce U-2OS and MG63 cells.

Cell Counting Kit-8 (CCK-8) assay

Cell proliferation was evaluated by using CCK-8 Assay Kit (Beyotime) following the manufacturer's protocol. U-2OS and MG63 cells seeded in 96-well plates (1,000–1,500 cells/well) were transduced with shRNAs and treated with or without 50 ng/mL IL-6 (Sigma-Aldrich Co.). After incubating for 0, 24, 48 and 72 h, CCK-8 solution was added to each well and then incubated for 1 h. Optical density (OD) values at the wavelength of 450 nm were measured with a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). All experiments were conducted in triplicate and repeated at least three times.

Cell apoptosis assay

Cell apoptosis rate was assessed by Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining kit (BD Biosciences, Franklin Lakes, NJ, USA). U-2OS and MG63 cells were transduced with shRNAs and treated with or without 50 ng/mL IL-6 (Sigma-Aldrich Co.) for 24 h. At the end of culture period, both adherent and floating cells were harvested and then labeled with Annexin V-FITC and PI. Cell apoptosis was then analyzed using a FACScan instrument (BD Biosciences).

Statistical analysis

All data are presented as mean ± SD. Statistical significance was determined by one-way analysis of variance (ANOVA). A P-value <0.05 was considered as statistically significant.

Results

SOX18 expression was positively correlated with IL-6 expression in osteosarcoma tissues

The mRNA expression of SOX18 and IL-6 was determined in osteosarcoma (n=50) and control bone cyst tissues (n=20). Comparing with bone cyst tissues, SOX18 and IL-6 mRNA levels increased by 127.5% and 71.4%, respectively (Figure 1A). Pearson correlation analysis was then performed to assess whether any relationship existed between the mRNA levels of SOX18 and IL-6 in osteosarcoma tissues. As shown in Figure 1B, the SOX18 mRNA level was positively correlated with the IL-6 level (r=0.7446, $P<0.001$). The protein levels of SOX18, IL-6 and p-STAT3 were also evaluated in osteosarcoma and bone cyst tissues. Osteosarcoma tissues had higher protein levels of SOX18, IL-6 and p-STAT3 as compared to bone cyst tissues (Figure 1C). These data indicated an association between IL-6/STAT3 and SOX18 during osteosarcoma progression.

IL-6/STAT3 induced SOX18 expression

To determine whether IL-6 affected SOX18 expression in osteosarcoma, we treated U-2OS and MG63 cells with recombinant IL-6 and found that IL-6 concentration dependently enhanced the mRNA and protein levels of SOX18 (Figure 2A). These data suggested that IL-6 may induce SOX18 expression at the transcriptional level. Moreover, IL-6 treatment increased the levels of p-STAT3 in a concentration-dependent manner (Figure 2B). Additional

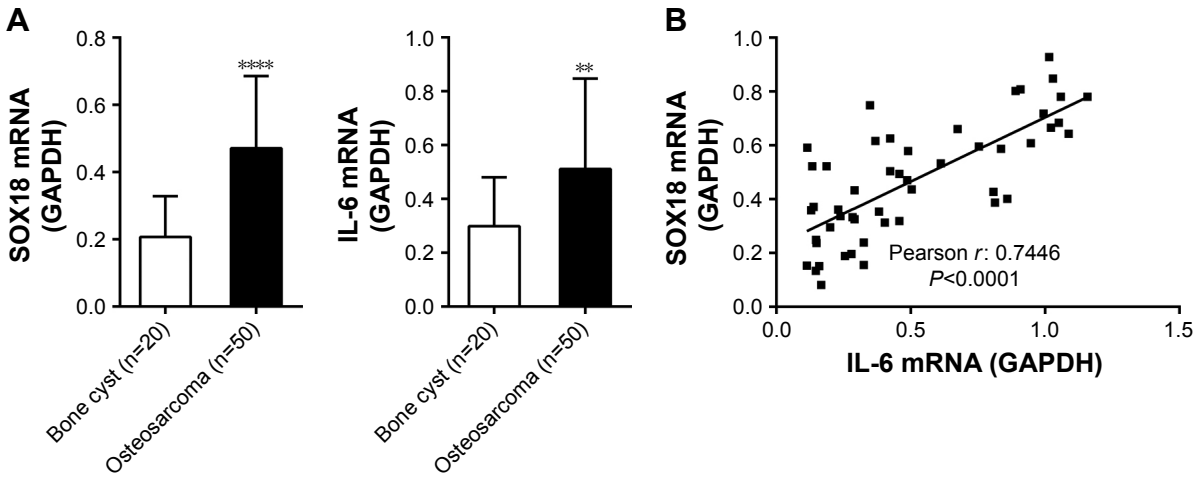

Figure 1 (*Continued*)

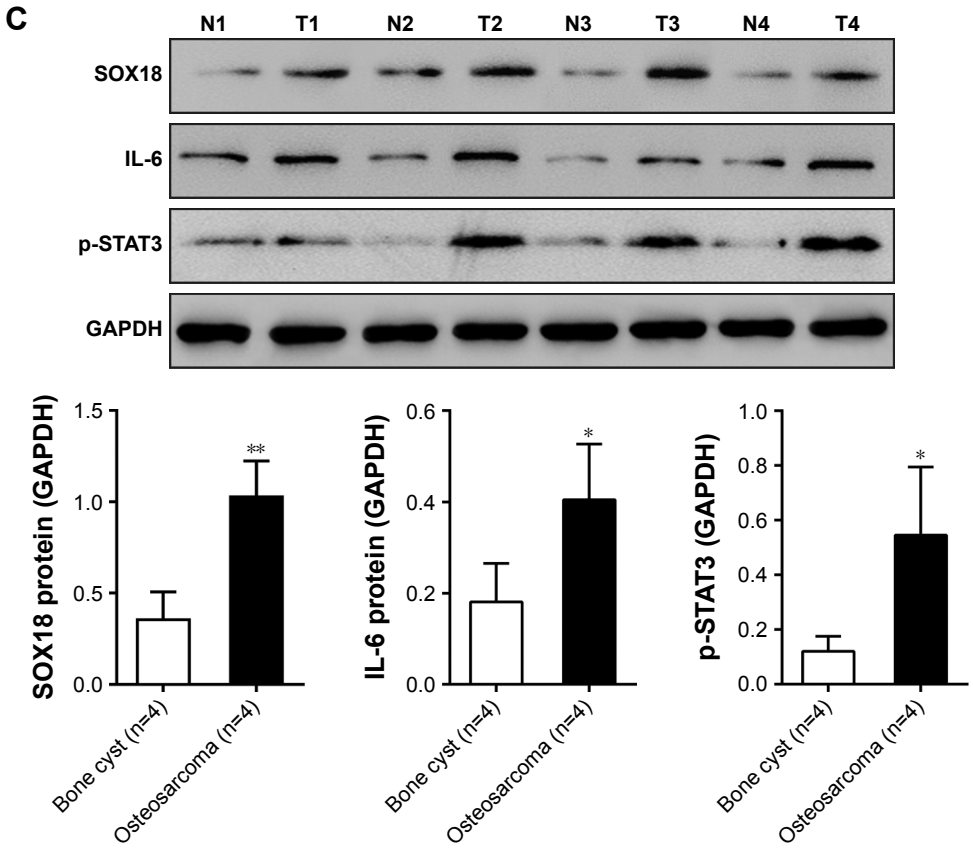

Figure 1 SOX18 mRNA expression was correlated with IL-6 mRNA expression in osteosarcoma tissues.
Notes: (**A**) mRNA levels of SOX18 and IL-6 were significantly higher in osteosarcoma tissues (n=50) than in bone cyst tissues (n=20). (**B**) Pearson correlation scatter plot of IL-6 and SOX18. (**C**) Western blot analysis of SOX18, IL-6 and p-STAT3. Representative blots and protein levels relative to GAPDH are shown. *$P<0.05$, **$P<0.01$ and ****$P<0.0001$ as compared with bone cyst tissues.
Abbreviation: IL-6, interleukin-6.

AG490 (a STAT3 inhibitor) exposure significantly attenuated IL-6-induced SOX18 expression (Figure 2C). These results suggested that IL-6/STAT3 induced SOX18 transcription in osteosarcoma cells.

IL-6 promoted osteosarcoma cell proliferation via SOX18

Several investigators have reported that IL-6 can modulate the proliferation of carcinoma cells.[19–21] To investigate whether IL-6 exerted functions through SOX18, we knocked down SOX18 expression in two osteosarcoma cell lines, U-2OS and MG63, by RNAi as previously reported (Figure 3A).[11] As displayed in Figure 3B and C, IL-6 exposure remarkably induced cell proliferation and such effect was notably attenuated by SOX18 knockdown.

IL-6 exposure attenuated the induction effects of SOX18 knockdown on osteosarcoma cell apoptosis

Cell apoptosis was evaluated by Annexin V-FITC/PI staining assay. As shown in Figure 4A and B, knockdown of SOX18 in U-2OS or MG63 cells significantly induced cell apoptosis in comparison with corresponding scramble shRNA. The presence of IL-6 significantly reduced SOX18 knockdown-induced cell apoptosis.

Expression of PCNA, Bcl-2, Bax and Cleaved-Caspase 3

We detected the protein levels of a cell proliferation marker (PCNA[22]) and cell apoptosis-associated proteins (Bcl-2, Bax and Cleaved-Caspase 3[23,24]). Consistent with the results of CCK-8 and Annexin V-FITC/PI staining assays, IL-6 stimulation enhanced the protein expression of PCNA and Bcl-2, which was reduced by SOX18 knockdown (Figure 5). The reversed effects were observed in Bax and Cleaved-Caspase 3 expression.

Discussion

Our recent study has reported that SOX18 is overexpressed in osteosarcoma and served as a potential oncogene in osteosarcoma via regulating osteosarcoma proliferation and metastasis.[11] Several studies have concerned the upstream

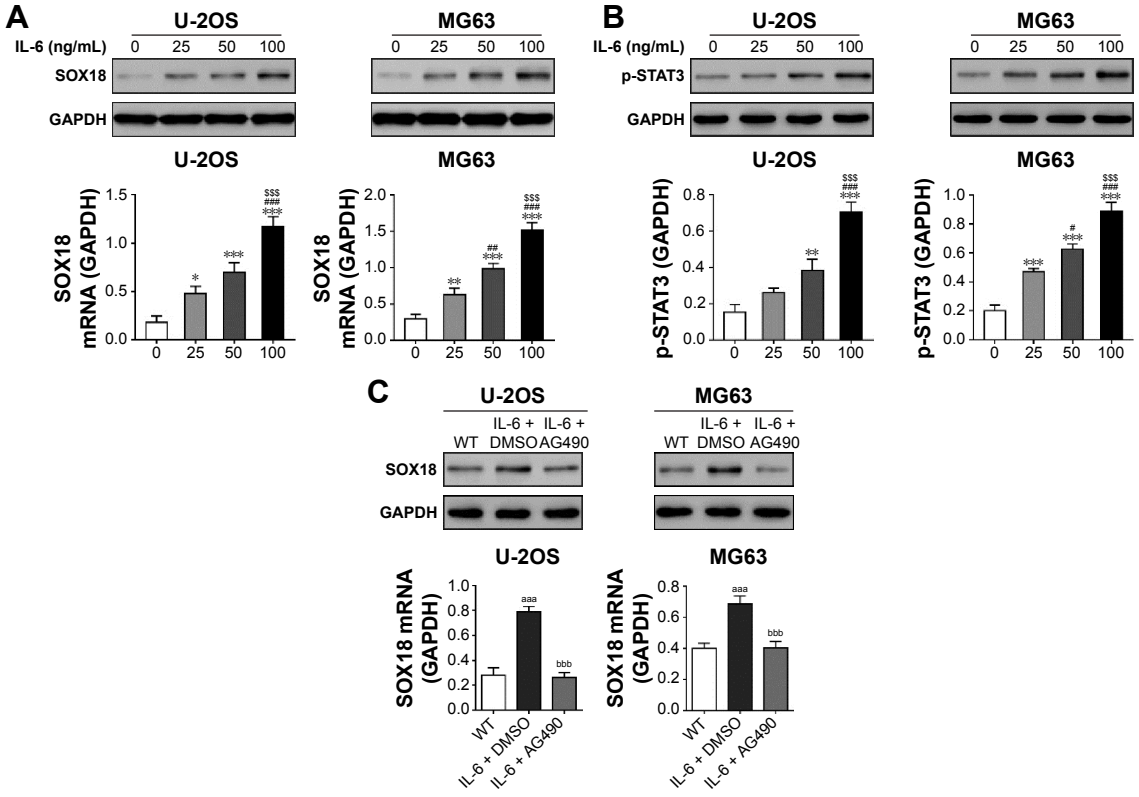

Figure 2 IL-6 increased SOX18 expression.

Notes: (**A** and **B**) U-2OS and MG63 cells were exposed with increasing dose of IL-6 (0, 25, 50 and 100 ng/mL) for 24 h. Expression of SOX18 protein (upper panel) and GAPDH (lower panel) was analyzed by Western blot and real-time PCR, respectively (**A**). Western blot analysis of p-STAT3 is shown (**B**). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs 0 ng/mL group; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs 25 ng/mL group and $^{\#}P<0.05$ and $^{\$\$\$}P<0.001$ vs 50 ng/mL group. (**C**) U-2OS and MG63 cells were pretreated with 30 μM AG490 (Selleck Chemicals, Houston, TX, USA) or DMSO for 1 h and then exposed to 50 ng/mL IL-6 for 24 h. Expression of SOX18 protein (upper panel) and GAPDH (lower panel) was analyzed. $^{aaa}P<0.001$ vs WT and $^{bbb}P<0.001$ vs IL-6 + DMSO.

Abbreviations: PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; WT, without any treatment; IL-6, interleukin-6.

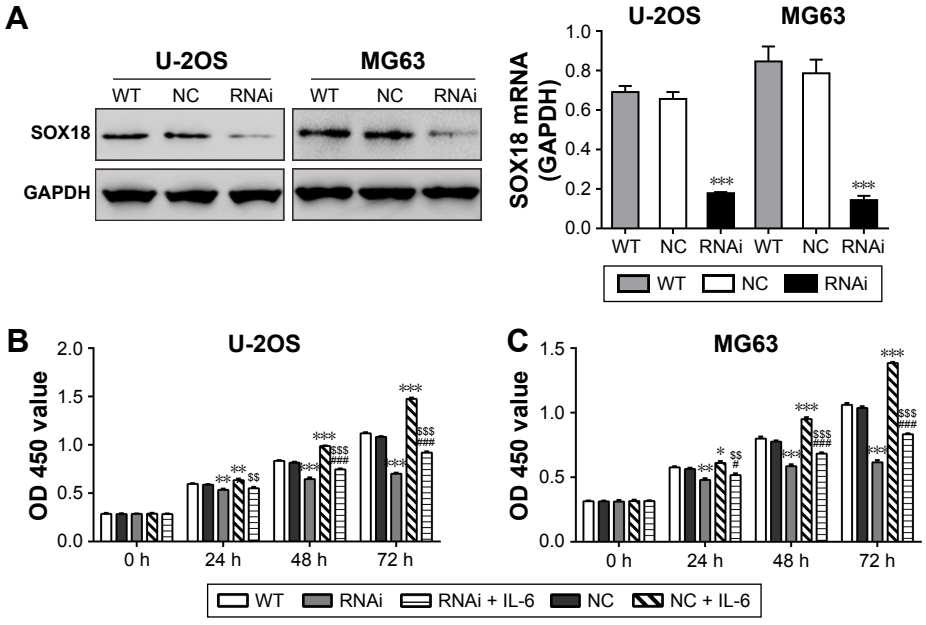

Figure 3 IL-6 promoted osteosarcoma cell growth via SOX18.

Notes: (**A**) U-2OS and MG63 cells were transduced with SOX18 shRNA (RNAi) or control shRNA (NC). At 48 h after transduction, expression of SOX18 protein (upper panel) and GAPDH (lower panel) was analyzed. (**B**) U-2OS and (**C**) MG63 cells seeded in 96-well plates were transduced with shRNAs and treated with or without 50 ng/mL IL-6. After incubating for 0, 24, 48 and 72 h, CCK-8 assay was performed to determine cell proliferation. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs WT; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ vs RNAi and $^{\$\$}P<0.01$ and $^{\$\$\$}P<0.001$ vs NC + IL-6.

Abbreviations: RNAi, RNA interference; CCK-8, Cell Counting Kit-8; WT, without any treatment; OD, optical density; IL-6, interleukin-6.

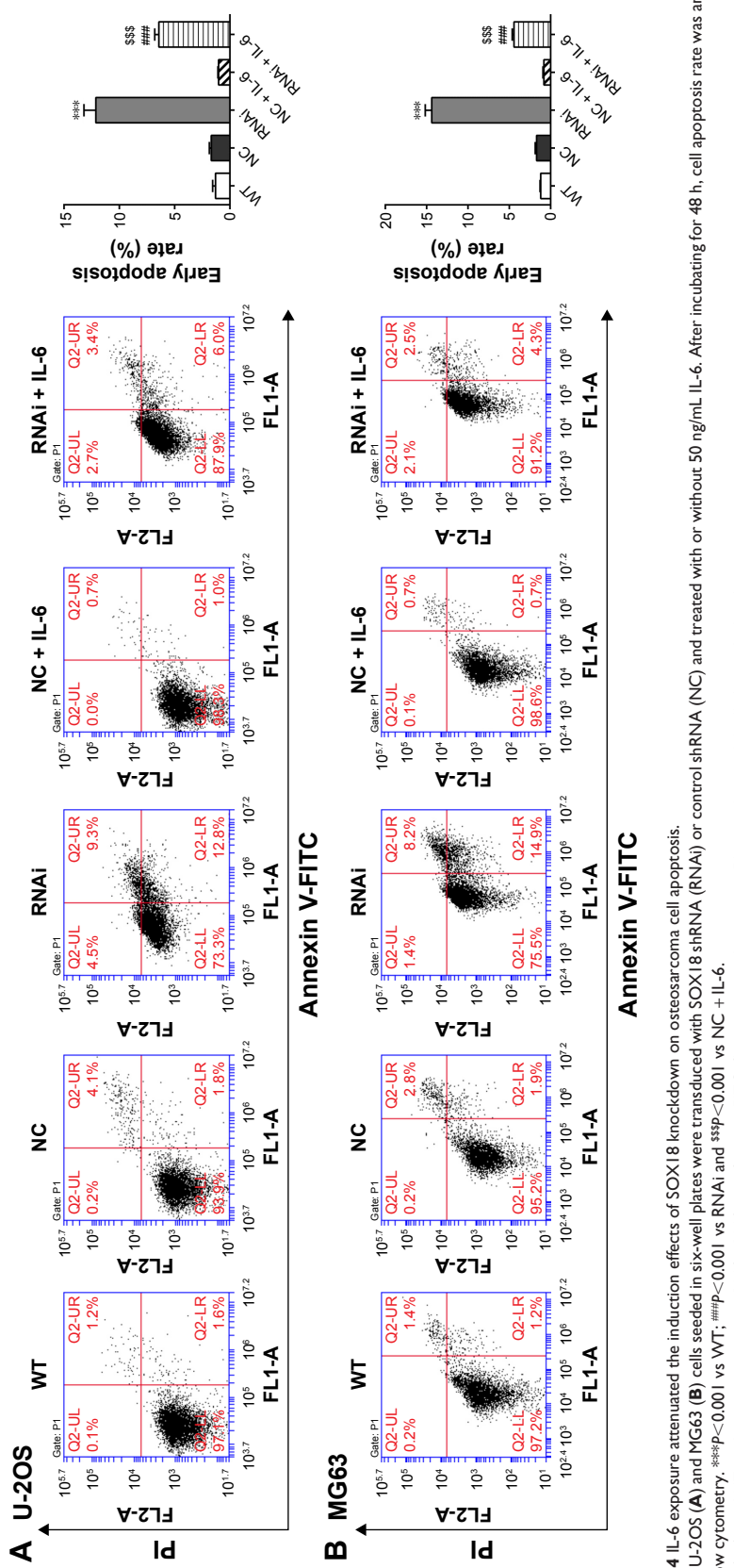

Figure 4 IL-6 exposure attenuated the induction effects of SOX18 knockdown on osteosarcoma cell apoptosis.
Notes: U-2OS (**A**) and MG63 (**B**) cells seeded in six-well plates were transduced with SOX18 shRNA (RNAi) or control shRNA (NC) and treated with or without 50 ng/mL IL-6. After incubating for 48 h, cell apoptosis rate was analyzed using flow cytometry. ***$p<0.001$ vs WT; ###$p<0.001$ vs RNAi and $$$$p<0.001$ vs NC + IL-6.
Abbreviations: WT, without any treatment; RNAi, RNA interference; FITC, fluorescein isothiocyanate; PI, propidium iodide; IL-6, interleukin-6.

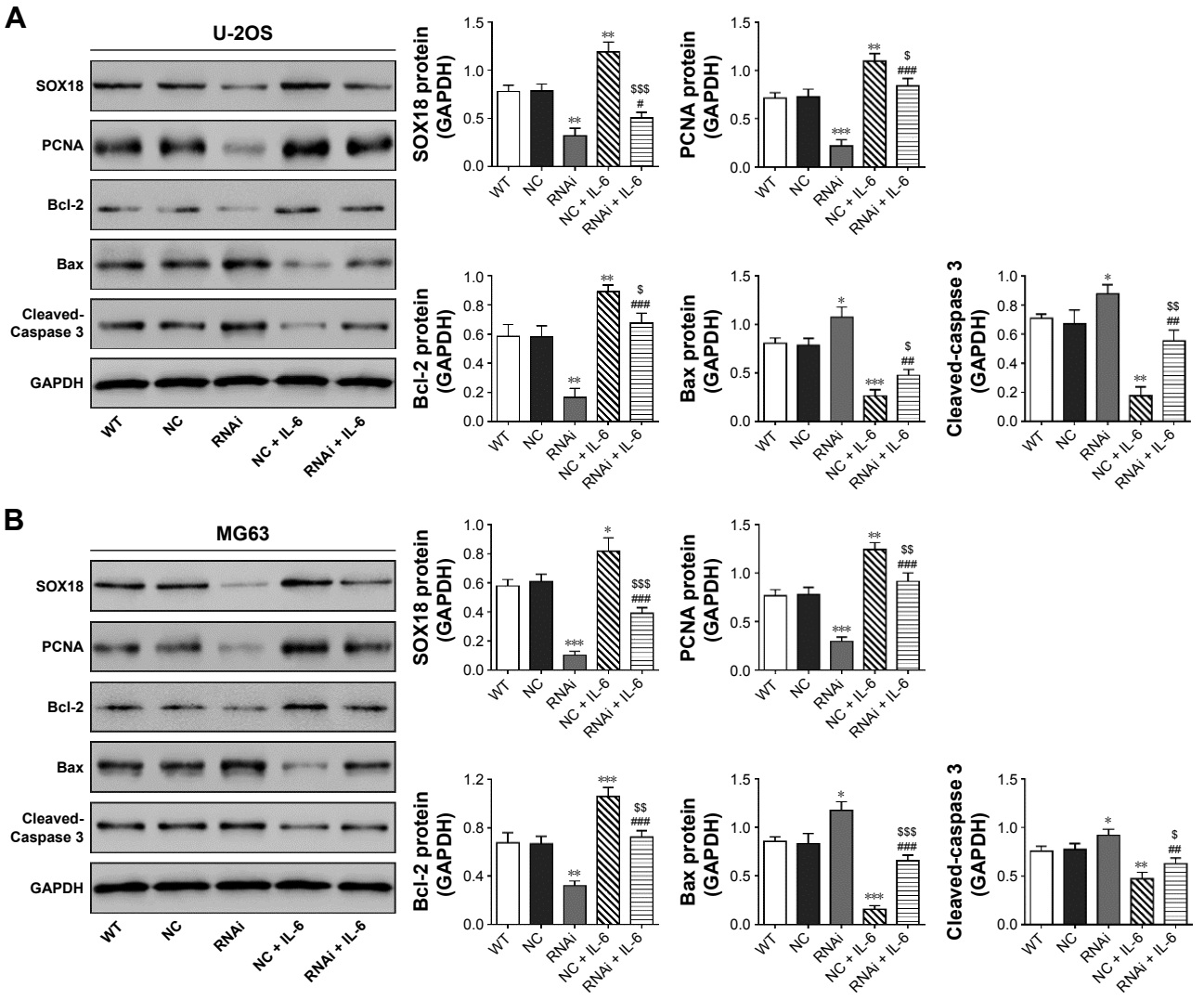

Figure 5 Expression of PCNA, Bcl-2, Bax and Cleaved-Caspase 3.

Notes: U-2OS (**A**) and MG63 (**B**) cells seeded in six-well plates were transduced with SOX18 shRNA (RNAi) or control shRNA(NC) and treated with or without 50 ng/mL IL-6. After incubating for 48 h, the protein levels of indicated genes were evaluated. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs WT; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs RNAi and $^{\$}P<0.05$, $^{\$\$}P<0.01$ and $^{\$\$\$}P<0.001$ vs NC + IL-6.

Abbreviations: WT, without any treatment; RNAi, RNA interference; IL-6, interleukin-6; PCNA, cell proliferation.

regulators for SOX genes. For instance, SOX4 was induced by TGF-β in Th2 cells.[25] SOX2 expression was induced by STAT3 in the neural precursor cell and breast cancer cells.[17,18] IL-6 expression was significantly higher in human osteosarcoma tissues than in the normal bone.[26] As a major activator of JAK2/STAT3 signaling,[12] IL-6 has been reported to modulate the proliferation of carcinoma cells.[19–21] Thus, we tried to investigate the effects of IL-6 treatment on SOX18 expression. In the present study, elevated IL-6 and SOX18 expression were observed in osteosarcoma tissues as compared with bone cyst tissues at both mRNA and protein levels. The increased level of phosphorylated STAT3 is associated with poor prognosis of osteosarcoma.[16] Presently, phosphorylated STAT3 was elevated in osteosarcoma tissues. Moreover, SOX18 mRNA expression was strongly correlated with IL-6 expression in osteosarcoma tissues. IL-6 exposure to osteosarcoma cells significantly increased the mRNA and protein expression of SOX18 and the phosphorylation of STAT3 in a dose-dependent manner. Our study suggested that IL-6/STAT3 regulated SOX18 expression in osteosarcoma, although the detailed mechanisms remain to be elucidated.

Furthermore, the involvement of SOX18 in IL-6-affected osteosarcoma cell proliferation and apoptosis was also studied. CCK-8 assays showed that IL-6 treatment significantly promoted osteosarcoma cell proliferation. Knockdown of SOX18 significantly suppressed the effects of IL-6 on cell proliferation. Although IL-6 had little effect on cell

apoptosis, it could significantly reduce SOX18 knockdown-induced cell apoptosis. These findings revealed that IL-6 is a possible upstream regulator for SOX18. PCNA is a well-accepted marker for cell proliferation.[22] Bcl-2 family proteins can either stimulate cell survival (eg, Bcl-2) or induce cell apoptosis (eg, Bax).[23] Cleaved-Caspase 3 is a marker for cell apoptosis.[24] Here, the change trends of these four proteins were consistent with the results of CCK-8 and cell apoptosis assays.

Conclusion

IL-6 treatment significantly enhanced SOX18 expression in osteosarcoma. IL-6 rescued the inhibitory and induction effects of SOX18 knockdown on osteosarcoma cell proliferation and apoptosis, respectively. Our study may provide insights to advance our understandings on the occurrence and development of osteosarcoma.

Disclosure

The authors report no conflicts of interest in this work.

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