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ORIGINAL ARTICLE

Knockdown of *Sox2* Inhibits OS Cells Invasion and Migration via Modulating Wnt/β-Catenin Signaling Pathway

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Abstract

Osteosarcoma (OS) was a prevalent malignant bone tumor which threatens people's health worldwide. Wnt/β catenin signaling pathway had been proved significant in various cancers, indicating its possible function in OS as well. *Sox2*, a crucial member among SOX family could regulate cells biologically. How *Sox2* modulated Wnt/β catenin signaling pathway in OS remained to be discussed. The study aimed to investigate the effects of *Sox2* on the invasion and migration of OS cells and the related molecular mechanisms. Twenty-four human OS and adjacent tissue samples were involved in this study. Human OS cell lines MG63 and HOS were selected for further investigation. The liposome carrier si-*Sox2* which could interfere with the expression of *Sox2* gene was built to transfect MG63 and HOS cells). QRT-PCR assay and western blot were utilized to analyze the expression of mRNA and proteins of *Sox2*. Transwell assay and wound healing assay were conducted to test the invasion and migration level of cells. The expression of GSK3, β-catenin, cyclin D1 and c-myc proteins were detected by western blot assay after transfection with si-*Sox2*. Compared with normal tissues and cells, the expression of *Sox2* in OS tissues and cells was significantly higher. The mRNA and protein levels of *Sox2* significantly decreased after transfection with si-*Sox2*. The invasion and migration of OS cells were down-regulated significantly through the inhibition of *Sox2* by inactivating Wnt/β-catenin signaling pathway related proteins. Knockdown of *Sox2* could inhibit invasion and migration of OS cells via modulating Wnt/β-catenin signaling pathway.

Keywords Osteosarcoma · *Sox2* · Wnt/β-catenin

Introduction

Osteosarcoma (OS) is a most prevalent primary malignant tumor of bone which mainly affects children and adolescents [1–3]. OS often arises at rapid bone growth sites, like the metaphysis of long bones [4]. Aggressive proliferation, high rate of recurrence and early systemic metastasis are the marks of OS [5]. The early symptoms of OS cancer are not notable, causing a delay in early diagnosis, therefore making the OS treatment challenging [6]. The advent of chemotherapy promoted the long-term cure rate of non-metastatic OS, but failed to increase the survival rate (less than 20%) of metastatic OS over the last 30 years [7]. The introduction of combination therapy using biologic agents such as muramyl tripeptide and cytotoxic chemotherapy has no definite effects on patients with OS as well [4, 8, 9]. Although it seems biologically reasonable that activated pathways during bone growth may contribute to development of OS [10, 11], to increase the understanding of the pathogenesis of OS and the use of preclinical models to test novel biological agents is critical to improvement of outcomes of treatment.

Wnt/β-catenin signaling pathway plays a vital role in numerous types of cancer [12]. For example, Wnt/β-catenin signaling pathway activated by PRC1 has effects on lung adenocarcinoma development [13]. Wnt/β-catenin signaling pathway can be regulated by miR-148a therefore inhibits the invasion of pancreatic cancer cells [14]. It also has effect on bone development through modulating proliferation, differentiation and motility of cells [15]. Previous studies indicated that the expression levels of ligands, receptors and co-

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receptors related with Wnt/β-catenin signaling pathway in OS cells are higher than that in normal cells [5, 16, 17]. Therefore a number of novel therapies for OS have been developed through targeting the Wnt/β-catenin signaling pathway [18]. However, the mechanism of Wnt/β-catenin signaling pathway affecting cancer development remains unknown.

The SRY-related HMG-box (*Sox*) family is a potential regulator of embryonic development, stem cell maintenance, tissue homeostasis and carcinogenesis [19, 20], among which *Sox2* is a crucial member [21]. The main function of *Sox2* is connected with stem cell biology regulation, cellular reprogramming, disease initiation and maintenance in a number of cancers [19]. *Sox2* is also an independent prognostic factor for long-term survival in oesophageal adenocarcinoma [22]. It is also involved in chemoresistance to conventional lung cancer therapies [23]. Previous studies showed that the knockdown of *Sox2* led to an osteopenic phenotype in mice [7], while there are few studies about the effect of *Sox2* knockdown on human OS.

More studies need to be done to explore the role of *Sox2* in modulating Wnt/β-catenin signaling pathway. In this study, we found that knockdown of *Sox2* could inhibit invasion and migration of OS cells and restrain teh expression of Wnt/β-catenin signaling pathway. These results suggested that *Sox2* could be a target of therapeutic protocols of OS.

Materials and Methods

Tissue Samples and Cell Lines

OS tissues and normal adjcacent tissues were obtained from 24 patients between 2014 and 2016 at Shanghai Ninth People's Hospital. This study had already gotten approval from the institutional review board and the ethics committee of Shanghai Ninth People's Hospital and all subjects had also given written informed consent.

Normal human osteoblast cells hFOB1.19 and human OS cell lines HOS, U2OS, SAOS2, MG63 were purchased from BeNa Culture Collection (Beijing, China). *Sox2* siRNA and negative control siRNA were purchased from Genepharma (Shanghai, China).

Cell Culture and Transfection

Cells were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, Missouri, USA) containing 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA) and antibiotics composed of 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were cultured at room temperature in a humid atmosphere containing 5% CO_2. Cells in logarithmic growth phase were placed in a 6-well plate and incubated until 70–80% confluent. Then Lipofectamine 2000 (Invitrogen, Gaithersburg, MD, USA) was used to transfect cells according to manufacturer's instructions. All the cells were assigned to three groups: non-transfection group (control), negative control group (si-NC) and *Sox2* siRNA transfected group (si-*Sox2*).

QRT-PCR Assay

Total RNA was extracted from cell lines and tissues with Trizol reagent, and 2.0 μg of total RNA was used for reverse transcription by TaqMan RT Kit according to the manufacturer's instructions (Applied Biosystems, USA). Reverse transcription cDNA was amplified by PCR with 20 μl reaction system using Go Tag Green Master Mix / Platinum SYBR Super Mix (Invitrogen, Gaithersburg, MD, USA). PCR was performed as follows: 95 °C pre-denaturation for 5 min, 95 °C denaturation 15 s, 60 °C for 3 s, followed by 40 cycles of 72 °C for 30s and 72 °C for 10 min. GAPDH acted as an internal control. Primer sequences designed for qRT-PCR were shown in Table 1.

Western Blot Assay

Total proteins were extracted and measured by the bicinchoninic acid (BCA) protein concentration assay Kit (Biyuntian, Beijing, China). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was applied to protein isolation. Then we transferred the proteins to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA) and incubated the membrane with 5% skimmed milk at room temperature for 1 h. After the PVDF membrane was dealt with Tris buffer saline-tween (TBST) for 10 min, 3times, it was incubated with TBST diluted primary antibodies (Rabbit anti-Human *Sox2*, GSK3, β-catenin, cyclin D1, anti-c-myc, Proteintech, USA) at 4 °C overnight. The membrane was washed with TBST for 10 min, 3times. Then we added the TBST diluted infrared fluorescent marked secondary antibodies in the membrane and incubated it without light at 37 °C for 1 h. The PVDF membrane was dealt with TBST in no-light condition for 10 min, 3times. Odyssey two-color infrared fluorescence scanning system (LI-COR Biosciences, Cambridge, UK) was used to obtain and analyze the images of samples.

Table 1 The primers designed for qRT-PCR

	Sequence (5′-3′)
Sox2 forward	GCCGAGTGGAAACTTTTGTCG
Sox2 reverse	GGCAGCGTGTACTTATCCTTCT
GAPDH forward	GAAGGTGAAGGTCGGAGTC
GAPDH reverse	GAAGATGGTGATGGGATTTC

Transwell Assay

The Transwell chambers (Corning Inc., NY, USA) were prepared early. The bottom membrane of Transwell chambers was coated with 50 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), which was diluted to 1: 2 of concentration using serum-free 1640 medium (Gibco Life Technologies, Grand Island, NY, USA) and incubated at 37 °C for 2 h. Trypsin was used to digest the cells after 24 h transfection. The cell suspension was prepared by resuspending the serum-free DMEM (Sigma, St. Louis, MO, USA) medium. Transwell chambers were placed in 24-well plates. The upper chambers were added with 200 μL of serum-free cell suspension and the lower chambers were supplemented with DMEM containing 10% FBS. After 36 h incubation, the chambers were washed 3 times with cool PBS. 4% methanal was utilized to fix the cells for 20 min, before the cells were stained with 1% crystal violet for 20 min. Then we rinsed the cells and photographed 5 random fields of every group under microscope (100 times) to count the cell numbers.

Wound Healing Assay

Cells in logarithmic growth phase were placed in 6-well plates with a density of 2×10^5 cells per well and incubated until 80–90% confluent. 200 μl sterile pipette tips were used to scratch the culture surface. Then we washed the serum-free medium twice to remove the unattached cells with pre-warmed PBS and cultured them with DMEM containing 2% FBS. Photographs were taken at 0 h, 24 h and the location and migration of cells was measured by the wound area.

Statistical Analysis

SPSS 21.0 (Chicago, Illinois, USA) was used for statistical analyses. All data were expressed as mean ± standard deviation. Student's *t*-test or one-way ANOVA was used for assessing the comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The Expression Level of *Sox2* in OS Tissues and Cells is High

We analyzed the expression levels of *Sox2* in OS tissues by qRT-PCR and western blot. QRT-PCR results suggested that the mRNA expression levels were higher in OS tissues than in normal tissues ($P < 0.05$, Fig. 1a). The results of Western blot indicated that the expression levels of *Sox2*-

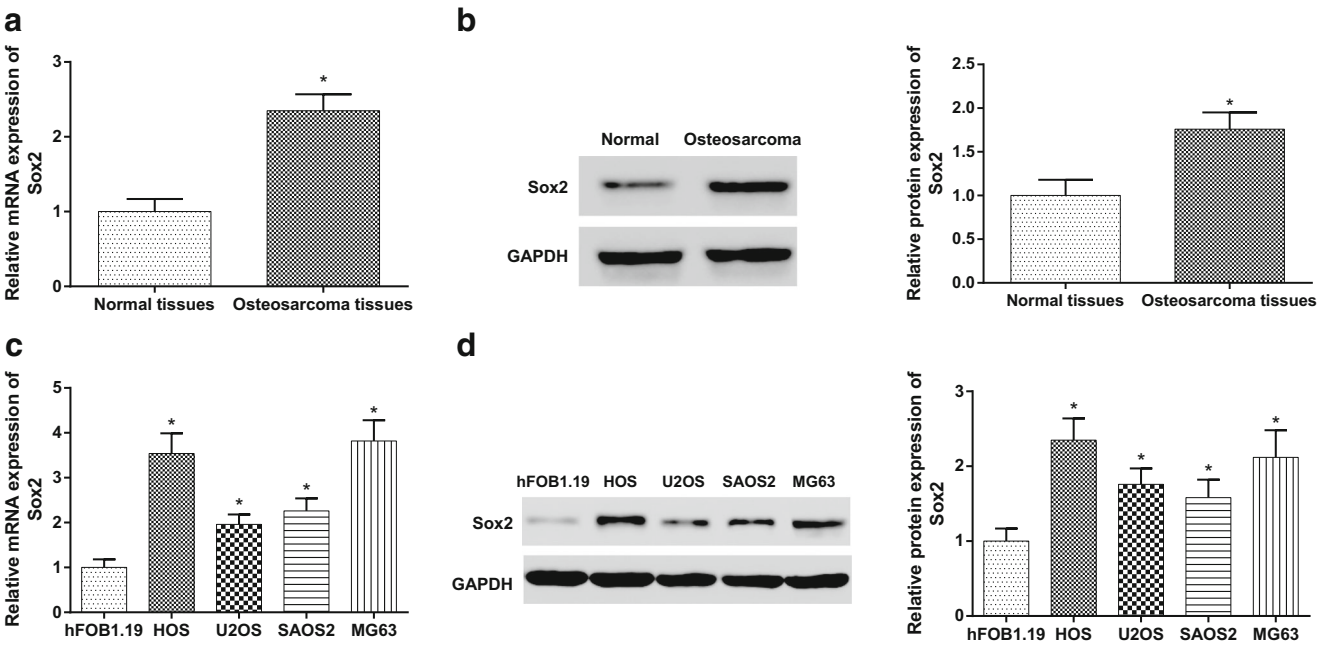

Fig. 1 *Sox2* mRNA and protein were highly-expressed in OS tissues and cells. **a** *Sox2* mRNA level was higher in OS tissues than in normal tissues. **b** *Sox2* protein level was higher in OS cells than in normal tissues. GAPDH was used as the internal control. $^*P < 0.05$, compared with normal tissues. **c** *Sox2* mRNA expression was higher in OS cell lines HOS, U2OS, SAOS2 and MG63 than in normal cell line hFOB1.19. $^*P < 0.05$, compared with hFOB1.19 cells. **d** *Sox2* protein expression was higher in cell lines HOS, U2OS, SAOS2 and MG63 than in hFOB1.19. GAPDH was used as the internal control. $^*P < 0.05$, compared with hFOB1.19 cells

related proteins were also higher ($P < 0.05$, Fig. 1b). Besides, expressions of *Sox2* mRNA and proteins in human OS cell lines HOS, U2OS, SAOS2 and MG63 were overexpressed as compared with normal human osteoblast cell line hFOB1.19 ($P < 0.05$, Fig. 1c-d). *Sox2* was most significantly expressed in cell lines HOS and MG63, therefore, they were selected for following experiments.

Knockdown of *Sox2* Inhibits the Invasion and Migration of OS Cells

For the transfected cell lines (HOS, MG63), we found that their expression of *Sox2*-related mRNA and proteins was reduced dramatically compared with non-transfection group and negative control group. The qRT-PCR and western blot results suggested a successful transfection ($P < 0.05$, Fig. 2). Figure 3 detected the invasion capability of MG63 and HOS cells, which indicated that the invasion cell numbers in si-*Sox2* group were significantly smaller ($P < 0.05$). The wound healing results showed that because of the inhibition of *Sox2*, the migration capacity in transfected group was down-regulated compared with control groups considering the wider wound area ($P < 0.05$, Fig. 4). The results proved that the knockdown of *Sox2* could inhibit the invasion and migration of OS cells.

Knockdown of *Sox2* Inactivates Wnt/β-Catenin Signaling Pathway

Sox2 had an antagonistic effect of fibroblast growth factor on Wnt/β-catenin signaling pathway, which regulated the bone formation. In this study, pathway related proteins GSK3, β-catenin, cyclin D1 and c-myc were detected by western blot. Compared with the control group and the si-NC group, expressions of all proteins in si-*Sox2* group were significantly down-regulated. Therefore, the knockdown of *Sox2* could restrain the expression of Wnt/β-catenin signaling pathway by affecting related proteins (Fig. 5).

Discussion

The current study revealed that the knockout of *Sox2* significantly impeded MG63 and HOS migration and invasion by inactivating Wnt/β-catenin axis. Our study brought out an improved understanding of mechanisms in OS cells.

Sox family has been proved to be related with the embryonic development, stem cell maintenance, tissue homeostasis and carcinogenesis of OS [24]. For instance, the expression level of *Sox9* in OS tissues was found higher than that in adjacent tissues [25]. The suppression of *Sox7* promoted the invasion and migration of OS cells [26]. These studies suggested that the expressions of *Sox* family played vital but different roles in the regulation of OS cells. Our study found that *Sox2* was up-regulated in OS tissues and cells. It was detected that the expression level of *Sox2* was high in several OS cell lines [7]. In addition, Mansukhani et al. found that *Sox2* could inhibit osteoblast differentiation at transcriptional level [27]. We thus speculated that the mutation of *Sox2* might influence carcinogenesis via some signaling pathway. Zou et al. demonstrated that *Sox2* could be directly regulated by miR-34a thereafter promoted invasion of OS cells [28]. Ren et al. reported that the inhibition of *Sox2* could induce cell apoptosis in Ewing's sarcoma through the PI3K/Akt pathway [29]. A previous study also showed that OS cells with reduced *Sox2* expression failed to form tumors in xenograft assays [7]. Besides of that, *Sox2* could inhibit Wnt/β-

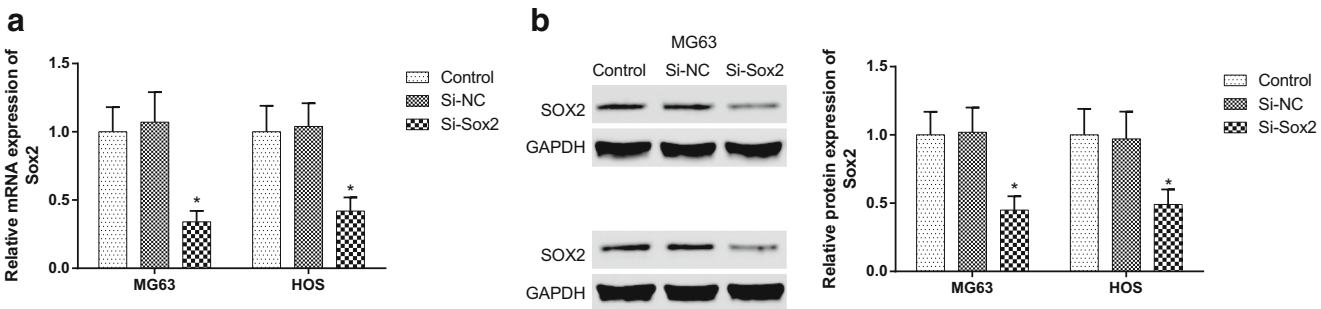

Fig. 2 *Sox2* mRNA and protein expression were down-regulated in MG63 and HOS cells transfected with si-*Sox2*. **a** After si-*Sox2*]transfection, relative *Sox2* mRNA expression was significantly down-regulated in both HOS and MG63 cell lines compared with control group and si-NC group. *$P < 0.05$, compared with control group. **b** After si-*Sox2* transfection, relative *Sox2* protein expression was significantly down-regulated in both HOS and MG63 cell lines compared with control group and si-NC group. GAPDH was used as the internal control. *$P < 0.05$, compared with control group

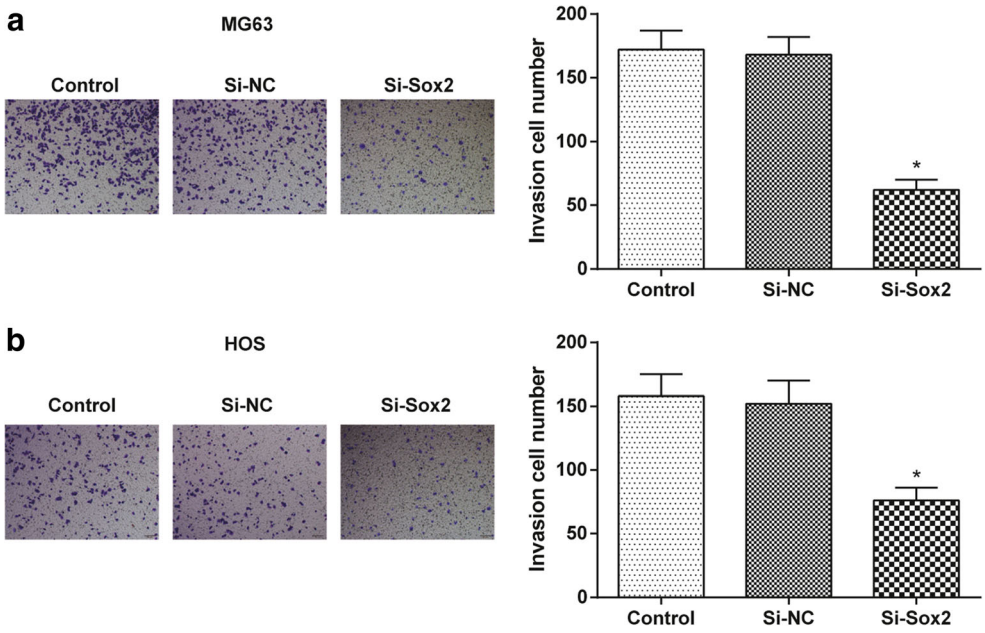

Fig. 3 *Sox2* knockdown reduced the invasion of OS cells MG63 and HOS. *$P < 0.05$, compared with control group. **a** The invaded MG63 cell number in si-*Sox2* group was significantly smaller than in control group. *$P < 0.05$, compared with control group. **b** The invaded HOS cell number in si-*Sox2* group was significantly smaller than in control group. *$P < 0.05$, compared with control group

catenin signaling in cisplatin-resistant lung adenocarcinoma cells [23]. In our study, we found that the knockdown of *Sox2* resulted in the silence of Wnt/β-catenin signaling in OS cells, indicating that the knockdown of *Sox2* may lead to the reduction of cell growth and invasion. We thus came to the conclusion that the knockdown of *Sox2* could inhibit the invasion and migration of OS cells possibly via Wnt/β-catenin signaling pathway.

Active Wnt/β-catenin signaling pathway was seen in OS cells [30, 31], and an increased level of active β-catenin was

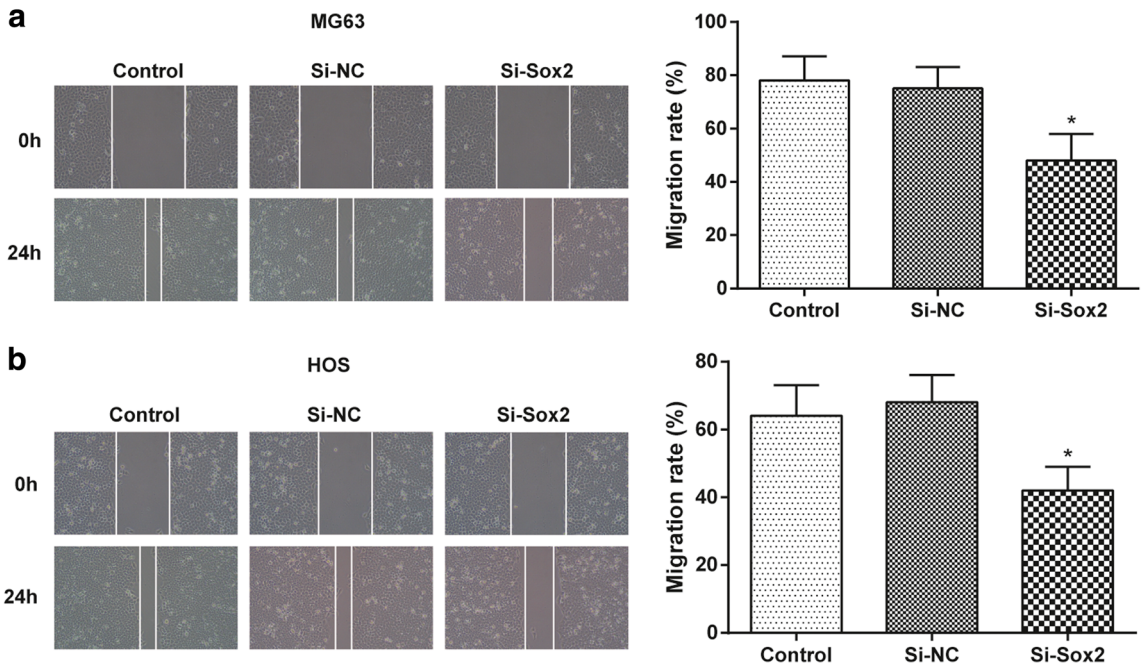

Fig. 4 *Sox2* knockdown reduced the migration rate of cell lines MG63 and HOS. *$P < 0.05$, compared with control group. **a** The migration rate of MG63 cells in si-*Sox2* group was significantly smaller than in control group. *$P < 0.05$, compared with control group. **b** The migration rate of HOS cells in si-*Sox2* group was significantly smaller than in control group. *$P < 0.05$, compared with control group

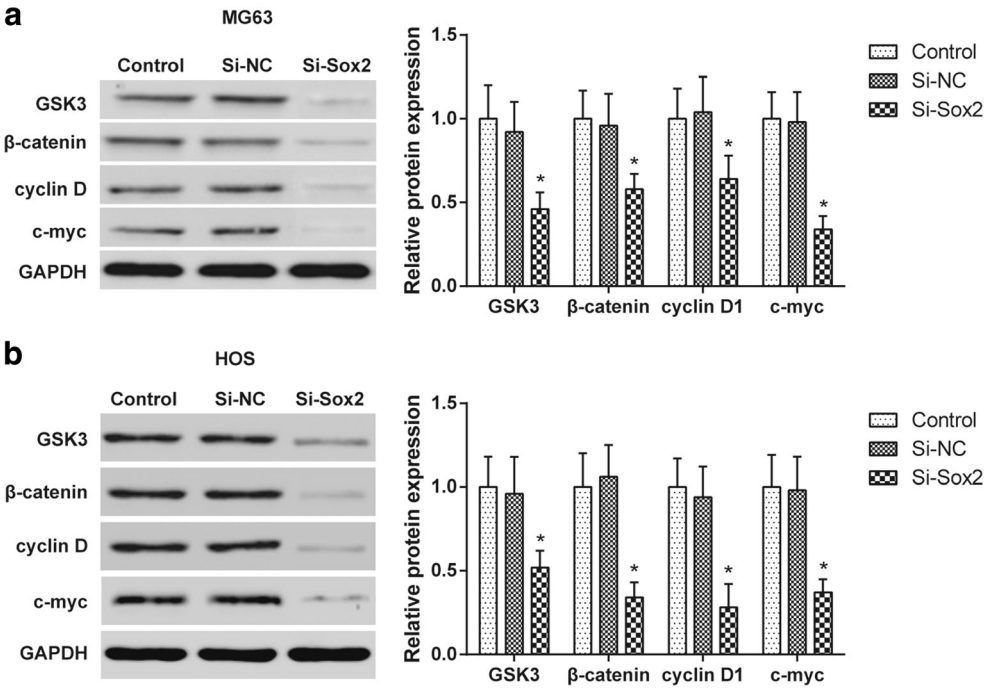

Fig. 5 Expression levels of Wnt/β-catenin signaling pathway-related proteins in OS cells were significantly down-regulated after *Sox2* knockdown. **a** The expression levels of GSK3, β-catenin, cyclin D1 and c-myc in MG-63 cells transfected with si-*Sox2* were significantly down-regulated. **b** The expression levels of GSK3, β-catenin, cyclin D1 and c-myc in HOS cells transfected with si-*Sox2* were significantly down-regulated. GAPDH was used as the internal control. $^*P < 0.05$, compared with control group

observed in *Sox2* shRNA transfected cells as well [7]. The accumulation of β-catenin in nuclear and cytoplasm occurred in OS cells was thought to be associated with the pathogenesis of OS [5]. We also found that the knockdown of *Sox2* led to suppression of GSK-3, β-catenin, cyclin-D and c-myc, i.e. the inactivation of Wnt/β-catenin signaling pathway. Our finding indicated that the knockdown of *Sox2* may inhibit e invasion and migration of OS cells possibly via Wnt/β-catenin signaling pathway. Massive evidence indicated that Wnt/β-catenin signaling pathway promoted osteoblast differentiation and function [32]. A previous study also revealed that the inhibition of XRCC6 could result in OS proliferation reduction by regulating Wnt/β-catenin signaling pathway [15]. All in all, Wnt/β-catenin signaling pathway suppression could result in less aggressiveness of OS.

However, we should take the limitation of this study into consideration. For example, only 24 human tissues were tested, making the result less convincing. Although the 24 patients were randomly selected, more OS patients should be involved in further studies. In vivo experiments should also be considered in further studies to confirm the effects of *Sox2* knockdown on Wnt/β-catenin signaling pathway activation in vivo.

To sum up, we have proved that *Sox2* is overexpressed in OS cells. And the knockdown of *Sox2* could inhibit the invasion and migration of OS cells through modulating the Wnt/β-catenin signaling pathway. Our study provided a deeper understanding of the mechanism, which improved the development of diagnosis and therapeutic strategies in OS cancer.

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Compliance with Ethical Standards

Conflict of Interest No conflict of interest exits in the submission of this manuscript and this manuscript has been approved by all authors for publication.

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