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Knockdown of SALL4 Inhibits Proliferation, Migration, and Invasion in Osteosarcoma Cells

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Sal-like protein 4 (SALL4) is a zinc finger transcription factor that has been reported to be aberrantly expressed in several human malignancies and identified as an oncogene. However, the potential role of SALL4 in osteosarcoma remains to be elucidated. In this study, we explored the biological functions of SALL4 in osteosarcoma. We found that SALL4 was overexpressed in osteosarcoma tissues and cell lines. Knockdown of SALL4 inhibited osteosarcoma cell proliferation, migration, and invasion in vitro. In addition, SALL4 knockdown suppressed osteosarcoma growth and metastasis in vivo. We also showed that SALL4 knockdown decreased the protein expression of Wnt3a and β -catenin in osteosarcoma cells. Taken together, our study showed that SALL4 plays an important role in regulating the proliferation, migration, and invasion of osteosarcoma cells. Thus, SALL4 may represent a potential therapeutic target in the treatment of osteosarcoma.

Key words: Sal-like protein 4 (SALL4); Proliferation; Invasion; Osteosarcoma

INTRODUCTION

Osteosarcoma, one of the most common bone tumors, is frequently found in children and adolescents^{1–3}. Because of the potential for malignancy and metastasis, osteosarcoma is characterized by aggressive growth and local recurrence^{4,5}. Great improvements have been made in osteosarcoma treatment, which include tumor resection and pre- or postoperative chemotherapy^{6–8}. However, patients with osteosarcoma still suffer from a low survival rate because of metastatic lesions and resistance to chemotherapy^{9–11}. Therefore, finding a new and effective therapeutic strategy is of great importance for improving the outcome of osteosarcoma patients.

Sal-like protein 4 (SALL4), a zinc finger transcription factor, was initially cloned in 2006 from *Drosophila*^{12,13}. As a player in embryo development and organ formation, SALL4 functions under a normal physiological state¹⁴. Moreover, SALL4 interacts with various transcription factors, epigenetic modulators, and signaling pathways to sustain pluripotency and self-renewal of hematopoietic and embryonic stem cells^{14–18}. SALL4 was first found to play an oncogenic role in leukemogenesis with constitutive expression in acute myeloid leukemia¹⁹. Gradually, SALL4 was studied by many researchers. Most of these

researchers identified a close association of SALL4 with tumorigenesis and the progression of different types of cancers. For example, Cheng et al. demonstrated that SALL4 promoted the proliferation, migration, and invasion of colorectal cancer cells and thus confirmed the oncogenic role of SALL4 in colorectal cancer²⁰. Liu and Shan investigated the biological significance of SALL4 in prostate cancer and found its promoting effect on the proliferation and colony formation of prostate cancer cells²¹. Although increasing evidence has revealed the involvement of SALL4 in carcinogenesis, the functional roles of SALL4 in osteosarcoma have not been clarified.

In this study, we aimed to make clear the biological functions of SALL4 in osteosarcoma. We found SALL4 to be overexpressed in osteosarcoma tissues and cell lines. Knockdown of SALL4 inhibited osteosarcoma cell proliferation, migration, and invasion in vitro. We established xenograft models and observed a suppressive effect of SALL4 knockdown on osteosarcoma growth and metastasis in vivo. We also found that SALL4 knockdown inhibited the Wnt/ β -catenin signaling pathway via decreasing the protein expression of Wnt3a and β -catenin. Finally, we suggest SALL4 as a promising target for osteosarcoma treatment.

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MATERIALS AND METHODS

Tissue Specimens

A total of 30 osteosarcoma patients from the Huaihe Hospital of Henan University participated in the study. Osteosarcoma tissues and their matching normal tissues were obtained from these patients with their written consent. No patient received any radiotherapy or chemotherapy before surgical resection. All tissue samples were collected and used with the approval of the ethics committee of the Huaihe Hospital of Henan University.

Cell lines and Cell Culture

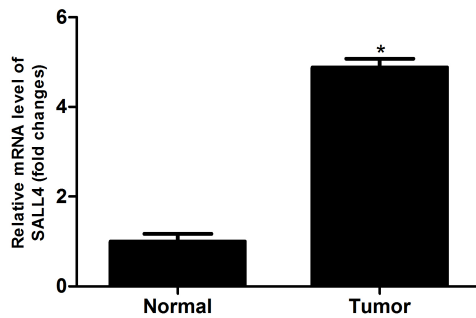
Human osteosarcoma cells (SAOS2 and 143B) and normal human osteoblast hFOB1.19 were purchased from

the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and incubated at 37°C in humidified air containing 5% CO₂.

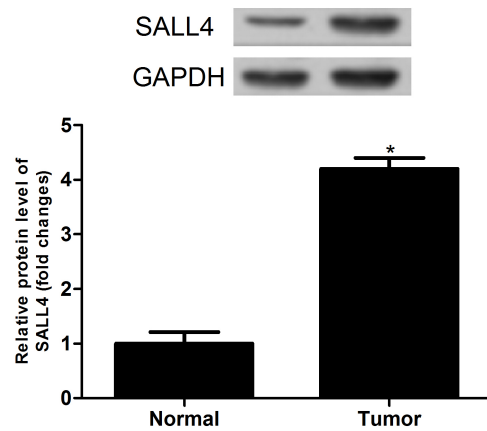
Quantitative Real-Time RT-PCR

Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA). The synthesis of cDNA was performed using the PrimeScript RT Reagent Kit (TaKaRa Biotechnology, Dalian, P.R. China). The following primers were used: SALL4, 5'-CCAGGGAATGACGAGGTGG-3' (forward) and 5'-GAACTCCGCACAGCATTTCTC-3' (reverse); GAPDH, 5'-GGAGCGAGATCCC

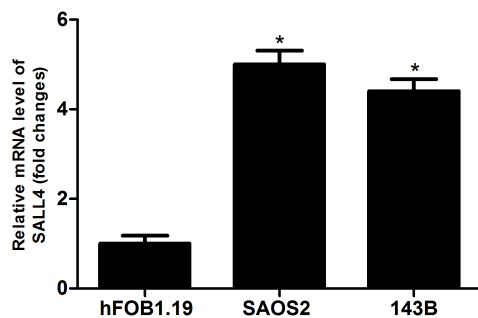
A



B



C



D

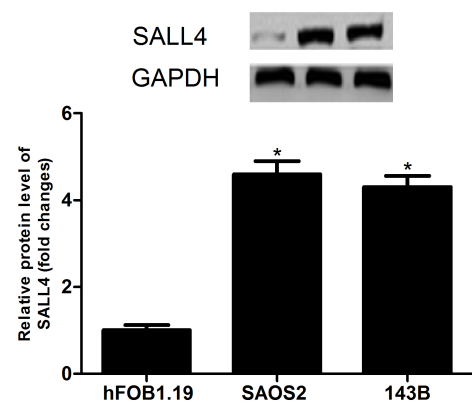


Figure 1. SALL4 is overexpressed in osteosarcoma tissues and cell lines. (A, B) Relative mRNA and protein expression of SALL4 in osteosarcoma tissues and normal bone tissues. (C, D) Relative mRNA and protein expression of SALL4 in two osteosarcoma cell lines (SAOS2 and 143B) and normal osteoblast hFOB1.19. * $p < 0.05$.

TCCAAAAT-3' (forward) and 5'-GGCTGTTGTCATAC TTTCATGG-3' (reverse). The reaction was carried out under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s. The relative mRNA expression was normalized to GAPDH and analyzed using the $2^{-\Delta\Delta CT}$ method²².

Western Blot

Tissues and cells were lysed in lysis buffer at 4°C for 20 min. Lysates were centrifuged for 10 min at 13,200 rpm to separate protein from cellular debris. The concentration of total protein was measured with a BCA assay kit (Invitrogen). The protein was resolved by 12% SDS-PAGE and then transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% bovine serum albumin, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-SALL4, anti-Wnt3a, anti- β -catenin, or anti-GAPDH. Subsequently, the membranes were washed with TBST three times and further incubated for 1 h at 37°C with horseradish peroxidase-conjugated secondary antibody. Antibodies in the study were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein bands were visualized via an enhanced chemiluminescence system (Bio-Rad). Protein expression was quantified with the Quantity One 4.6 software (Bio-Rad).

Cell Transfection

The shRNA targeting SALL4 (shSALL4) and the negative control shRNA (shControl) were purchased from Santa Cruz Biotechnology. The sequences were as follows: shSALL4, 5'-GCCTTGAAACAAGCCAAGC TA-3'; shControl, 5'-TTCTCCGAACGTGTACCGT-3'. SAOS2 and 143B cells were transfected with these shRNAs and harvested after 48 h. Western blot analysis was performed to measure the transfection efficiency.

Cell Proliferation Assay

The MTT assay was carried out to measure cell proliferation. In brief, cells were seeded in 96-well culture plates at a density of 2×10^3 cells/well and cultured at different times. Subsequently, MTT (Sigma-Aldrich) was added to each well, and cell culturing was continued for 4 h. After removal of supernatants, DMSO (Sigma-Aldrich) was added. The absorbance was measured with a microplate reader at 490 nm.

Colony Formation Assay

Cells were placed into a six-well plate at a density of 500 cells/well. After culturing for 10 days at 37°C in a humidified incubator with 5% CO₂, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Stained colonies were photographed, and the number of colonies (>50 cells per colony) was counted.

Transwell Assay

The migratory and invasive capabilities of osteosarcoma cells were tested by Transwell assays. To measure the migratory capability, 2×10^4 cells were seeded in the upper chamber with serum-free DMEM. FBS (10%) was added to the bottom chamber. After incubation for 24 h, cells that did not migrate were removed with cotton swabs, and the cells that migrated were stained with Giemsa. The number of migrating cells was counted under a microscope (400 \times). The invasive capability was tested according to the same procedure as mentioned above, except that the Matrigel-coated upper chamber was used.

In Vivo Tumorigenesis and Metastasis

Female nude mice (5 weeks old) were obtained from the Experimental Animal Center of Henan University. All mice were used for in vivo experiments with the approval of the Institutional Animal Care and Use Committee of Henan University. Transfected osteosarcoma cells (5×10^6) in 100 μ l of PBS were subcutaneously injected into nude mice (eight mice per group). Tumor volume was measured every 5 days after injection. Thirty days later, mice were sacrificed, and tumors were dissected and weighed.

Tumor metastasis was also tested. Briefly, 5×10^6 transfected osteosarcoma cells were intravenously injected into the tail vein of nude mice (eight mice per group). Thirty days later, mice were sacrificed, and lung metastasis was evaluated by counting the number of tumor nodules under a microscope.

Statistical Analysis

All experiments were conducted in triplicate. Data were presented as means \pm standard deviation (SD). Student's *t*-tests were performed to determine statistical significance. All analysis was performed with the SPSS 13.0 software. A value of $p < 0.05$ was considered statistically significant.

RESULTS

SALL4 Is Overexpressed in Osteosarcoma Tissues and Cell Lines

We surveyed the expression of SALL4 in osteosarcoma tissues and normal bone tissues. Thirty pairs of tissue specimens from osteosarcoma patients were evaluated by RT-PCR and Western blot analysis. The results showed that osteosarcoma tissues presented a higher expression level of SALL4 compared with the matching normal tissues (Fig. 1A and B). We also examined the expression of SALL4 in osteosarcoma cell lines and obtained similar results; SALL4 was highly expressed at both mRNA (Fig. 1C) and protein (Fig. 1D) levels in SAOS2 and 143B cells compared to the normal osteoblast hFOB1.19.

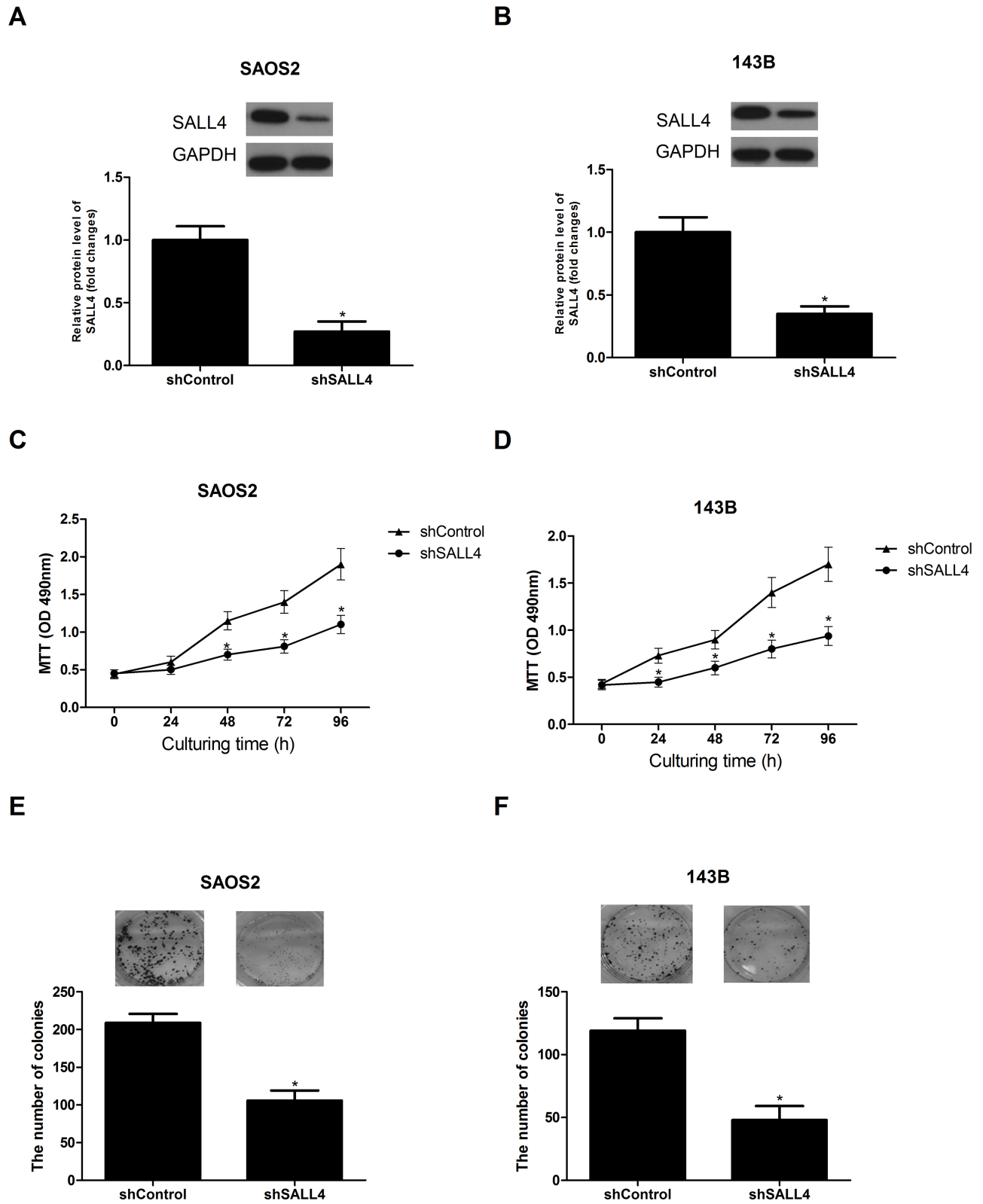


Figure 2. Knockdown of SALL4 inhibits the proliferation of osteosarcoma cells. (A, B) Western blot was carried out to determine the protein expression of SALL4 in SAOS2 and 143B cells after transfection. (C–F) The effect of SALL4 knockdown on the proliferation of SAOS2 and 143B cells was evaluated by MTT and colony formation assays. * $p < 0.05$.

Knockdown of SALL4 Inhibits the Proliferation of Osteosarcoma Cells

Osteosarcoma cell lines SAOS2 and 143B were transfected with shSALL4. Compared to cells transfected with shControl, the protein expression of SALL4 was distinctly reduced in the shSALL4 group (Fig. 2A and B). We performed an MTT assay to analyze the role of SALL4 knockdown in the proliferative ability of osteosarcoma cells. The assay demonstrated that SALL4 knockdown greatly reduced the proliferation of SAOS2 (Fig. 2C) and 143B (Fig. 2D) cells compared to the hFOB1.19 cells. Consistent with the MTT assay, the colony formation assay showed that SALL4 knockdown remarkably decreased the growth of SAOS2 (Fig. 2E) and 143B (Fig. 2F) cells compared to the control cells.

Knockdown of SALL4 Inhibits the Migration and Invasion of Osteosarcoma Cells

We performed Transwell assays to investigate the effect of SALL4 knockdown on the migratory and invasive potential of osteosarcoma cells. SALL4 knockdown markedly reduced the migratory and invasive capabilities of SAOS2 cells compared to the control group (Fig. 3A and B). A similar effect was achieved in 143B cells (Fig. 3C and D).

Knockdown of SALL4 Inhibits Osteosarcoma Growth and Metastasis In Vivo

We used xenograft models to investigate the effect of SALL4 knockdown on osteosarcoma growth in vivo. SAOS2 cells treated with shSALL4 or shControl

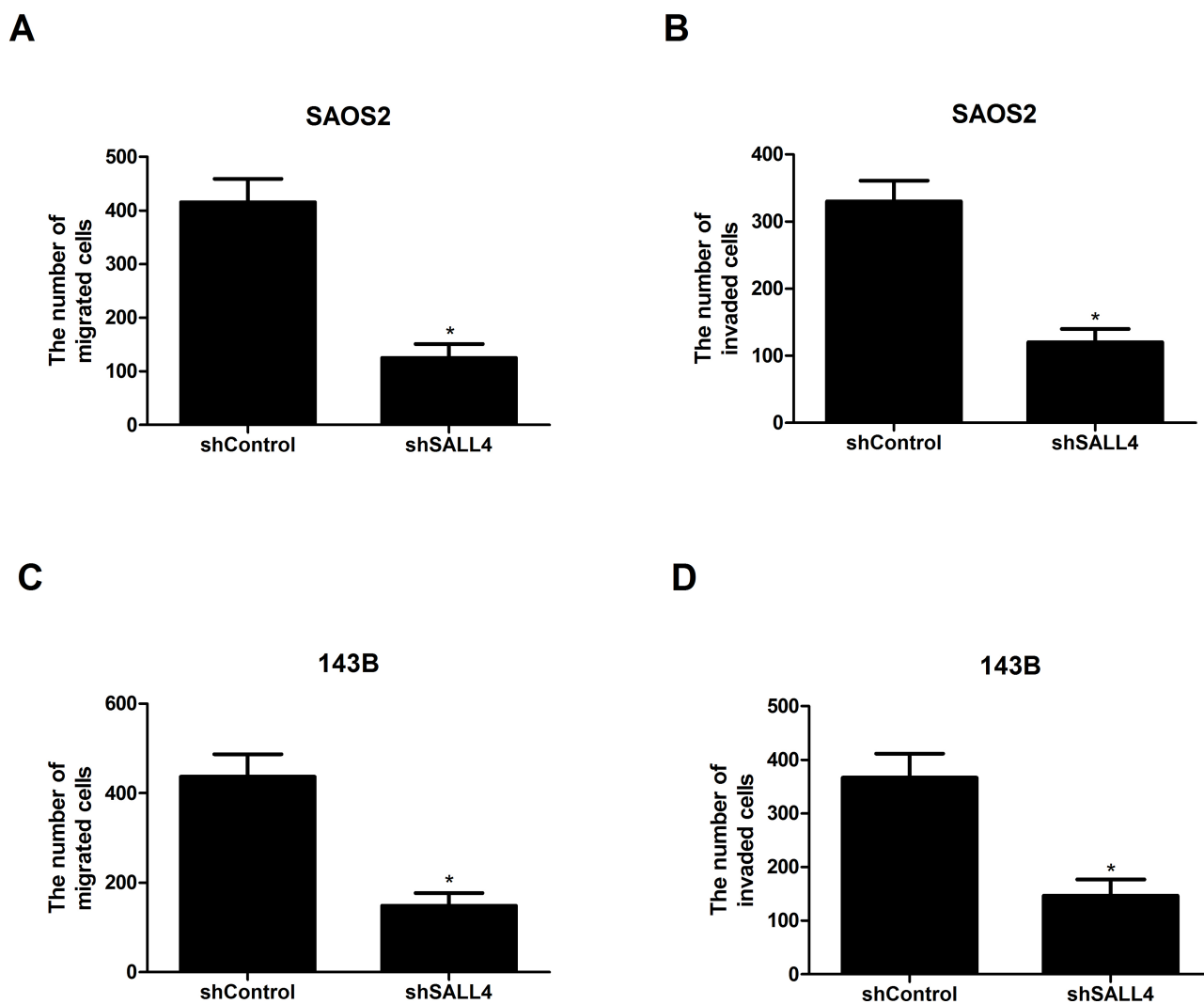


Figure 3. Knockdown of SALL4 inhibits the migration and invasion of osteosarcoma cells. (A, B) The migratory and invasive abilities of SAOS2 cells were significantly weakened after knockdown of SALL4, compared to the control group. (C, D) The migratory and invasive abilities of 143B cells were obviously suppressed after knockdown of SALL4, compared to the control group. * $p < 0.05$.

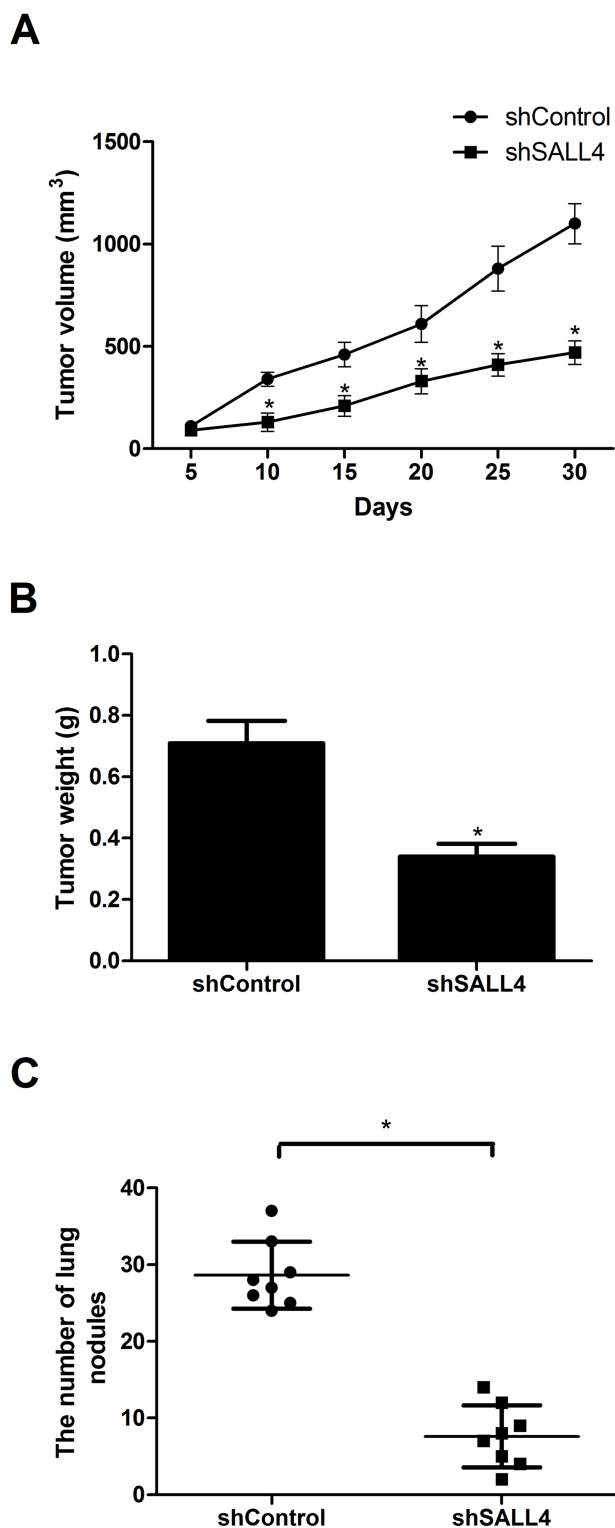


Figure 4. Knockdown of SALL4 inhibits osteosarcoma growth and metastasis in vivo. (A) The volume of tumors formed by SAOS2 cells was measured every 5 days after injection, and a tumor growth curve was plotted. (B) The weight of tumors formed by SAOS2 cells was measured 30 days after injection. (C) The number of lung nodules was counted 30 days after injection ($n=8$). $*p<0.05$.

were subcutaneously injected into nude mice ($n=8$). The in vivo tumorigenesis assay lasted for 30 days, and the results showed that both volume (Fig. 4A) and weight (Fig. 4B) of tumors formed by SAOS2 cells were decreased by SALL4 knockdown, compared with the control group.

We also examined osteosarcoma metastasis in vivo. Thirty days after intravenous injection of SAOS2 cells into nude mice ($n=8$), we sacrificed mice to check for lung metastasis, finding that the lung metastasis was markedly reduced in the SAOS2-shSALL4 group compared to the SAOS2-shControl group (Fig. 4C).

Knockdown of SALL4 Downregulates Wnt/ β -Catenin Signaling in Osteosarcoma Cells

The Wnt/ β -catenin signaling pathway has been widely reported to be involved in the development of various cancers including osteosarcoma^{23–26}. SALL4 was also found to interact with the Wnt/ β -catenin signaling pathway to promote progression of some kinds of cancers^{27–29}. Therefore, we detected the effect of SALL4 knockdown on the expression of Wnt3a and β -catenin in SAOS2 cells using Western blot analysis. SALL4 knockdown remarkably decreased the protein expression of Wnt3a and β -catenin in SAOS2 cells compared to the control cells (Fig. 5), suggesting the inhibitory effect of SALL4 knockdown on the Wnt/ β -catenin signaling pathway.

DISCUSSION

Osteosarcoma, a kind of bone tumor, often occurs in children and adolescents. Characterized by a high potential for malignancy, osteosarcoma poses a great threat to public health. Patients with osteosarcoma usually suffer from unsuccessful treatment because of metastatic lesions and resistance to chemotherapy. Thus, finding new therapeutic targets may help improve the poor outcome of osteosarcoma patients.

SALL4 is a zinc finger transcription factor that has been identified as an oncogene in many cancers. Its expression is found in fetal organs, gradually reduced during embryo development and silenced in tissues of most adults. Many reports have demonstrated that SALL4 is aberrantly expressed in several human malignancies. For example, He et al. reported upregulated SALL4 in esophageal squamous cell carcinoma tissues²⁹. Similarly, Chen et al. found overexpression of SALL4 in breast cancer cells³⁰. Consistent with their findings, we observed that SALL4 was elevated in osteosarcoma tissues and cell lines compared to corresponding normal tissues and cells. In addition, we found that knockdown of SALL4 inhibited the proliferation, migration, and invasion of osteosarcoma cells. These in vitro results were further verified by our in vivo experiments, which showed the suppressive effect of SALL4 knockdown on osteosarcoma growth and

metastasis. The results of our study were similar to those obtained by Cheng et al., who suggested the promoting effect of SALL4 on oncogenesis of colorectal cancer²⁰. Taken together, our study supported the notion that SALL4 played an oncogenic role in osteosarcoma progression.

Several mechanisms such as the NF- κ B pathway, the Slug/MMP-9 pathway, and the Wnt signaling have been reported to be involved in osteosarcoma development³¹⁻³³. Here we focused on the Wnt signaling pathway since increasing evidence has demonstrated the interaction of SALL4 with this pathway in several types of cancers. The Wnt signaling pathway plays an essential role in the determination of cell fate and proliferation³⁴. Aberrant changes in the pathway often cause cancer³⁵. As an important player of the Wnt signaling pathway, β -catenin exerts a promoting effect on tumor development via accumulation in the nucleus and cytoplasm³⁶. More importantly, the Wnt/ β -catenin signaling pathway is frequently found to be deregulated in various cancers and thus contributes to cancer progression^{35,37,38}. In this

study, we tested the effect of SALL4 knockdown on the Wnt/ β -catenin signaling pathway by examining the protein expression of Wnt3a and β -catenin in osteosarcoma cells. The Western blot assay showed that SALL4 knockdown significantly reduced the protein expression levels of Wnt3a and β -catenin in osteosarcoma cells. In combination with the previous results, we may infer that SALL4 knockdown exerted an inhibitory effect on osteosarcoma cells, at least in part, via suppressing the Wnt/ β -catenin signaling pathway. However, the mechanisms underlying the tumor-promoting role of SALL4 in osteosarcoma progression involve multiple molecular levels and therefore require further investigation.

In summary, our study, for the first time, showed that SALL4 was overexpressed in osteosarcoma tissues and cell lines. We demonstrated that knockdown of SALL4 inhibited osteosarcoma cell proliferation, migration, and invasion in vitro. Moreover, SALL4 knockdown suppressed osteosarcoma growth and metastasis in vivo. We also found that SALL4 knockdown downregulated the

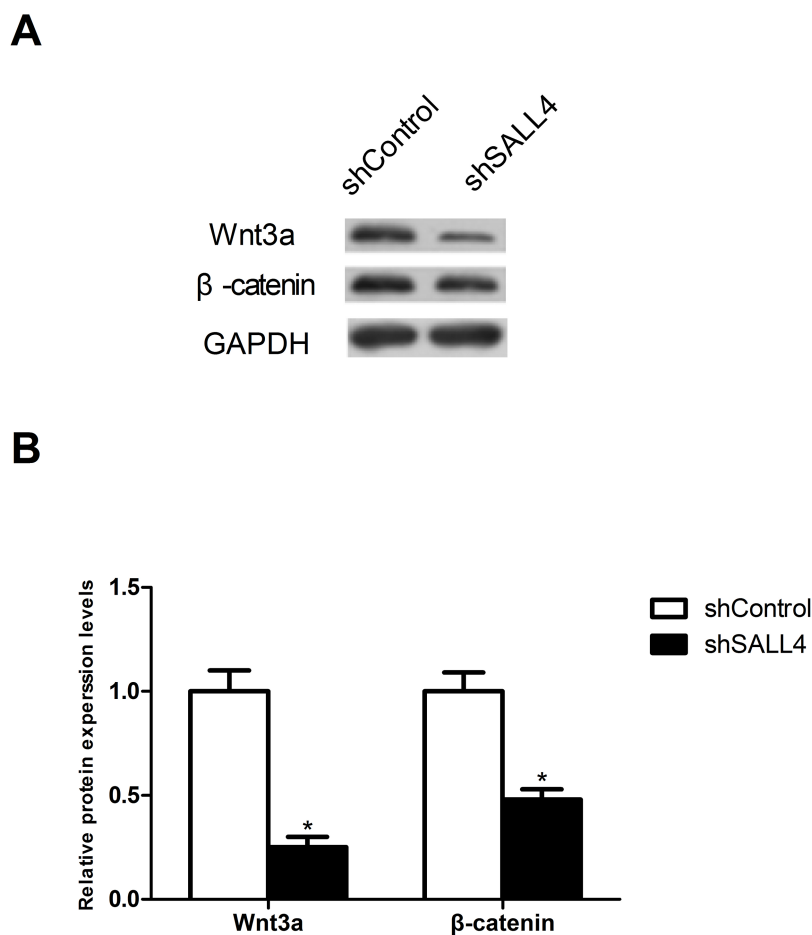


Figure 5. Knockdown of SALL4 downregulates the Wnt/ β -catenin signaling in osteosarcoma cells. (A) The Western blot assay showed a significant decrease in the protein expression of Wnt3a and β -catenin in SAOS2 cells. (B) The relative protein expression levels of Wnt3a and β -catenin in SAOS2 cells were quantified with the Quantity One 4.6 software. * $p < 0.05$.

Wnt/ β -catenin signaling pathway in osteosarcoma cells. Overall, our findings indicated that targeting SALL4 may be helpful for therapeutic intervention in osteosarcoma.

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