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Downregulation of Homeobox B7 Inhibits the Tumorigenesis and Progression of Osteosarcoma

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Homeobox B7 (HOXB7), a member of the HOX gene family, plays a role in tumorigenesis. However, until now the expression status and role of HOXB7 in osteosarcoma remain unclear. Therefore, the present study aimed to investigate the functional role and mechanism of HOXB7 in osteosarcoma. Our results demonstrated that HOXB7 was overexpressed in osteosarcoma cell lines. Downregulation of HOXB7 significantly inhibited osteosarcoma cell proliferation in vitro, as well as attenuated xenograft tumor growth in vivo. Downregulation of HOXB7 also inhibited the migration and invasion of osteosarcoma cells. Furthermore, downregulation of HOXB7 significantly suppressed the protein expression levels of p-PI3K and p-Akt in U2OS cells. In summary, our data demonstrated that downregulation of HOXB7 inhibited proliferation, invasion, and tumorigenesis, partly through suppressing the PI3K/Akt signaling pathway in osteosarcoma cells. Our findings provide new insights into the role of HOXB7 in osteosarcoma and new therapeutic targets for the treatment of osteosarcoma.

Key words: Homeobox B7 (HOXB7); Osteosarcoma; Proliferation; Invasion; PI3K/Akt pathway

INTRODUCTION

Osteosarcoma is the most common malignant bone tumor in children and young adults¹. Although the development of treatments has improved survival in patients with primary osteosarcoma²⁻⁴, the 5-year survival rate is less than 20% in those with recurrence or metastasis⁵. Thus, there is an urgent need to develop novel early molecular markers for diagnostic and therapeutic targets in osteosarcoma.

Homeobox (HOX) genes are the family of transcription factors that play an important role in regulating embryonic development, cellular proliferation, differentiation, and death⁶. HOX genes were originally identified in *Drosophila melanogaster*⁷. Aberrant expression of HOX genes has been reported in many human cancers, including colorectal, breast, oral, pancreatic, and prostate cancers⁸⁻¹⁰. HOXB7, a member of the HOX gene family, plays a role in tumorigenesis. Li et al. confirmed that the expression of HOXB7 was significantly upregulated in 23 samples of malignant esophageal squamous cell carcinoma (ESCC) tissues, and knockdown of HOXB7 remarkably suppressed proliferation, colony formation,

and tumorigenicity in ESCC cells¹¹. Overexpression of HOXB7 also increased proliferation and angiogenesis, as well as induced epithelial–mesenchymal transition (EMT) in breast cancer cells^{12,13}. However, until now the expression status and role of HOXB7 in osteosarcoma remain unclear. Therefore, the present study aimed to investigate the functional role and mechanism of HOXB7 in osteosarcoma. Our results demonstrated that downregulation of HOXB7 inhibited the tumorigenesis and progression of osteosarcoma.

MATERIALS AND METHODS

Cell Culture

Three human osteosarcoma cell lines (SaOS₂, MG-63, and U2OS) and a normal osteoblast cell line (hFOB1.19) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/ml; Sigma-Aldrich, St. Louis, MO, USA), and streptomycin (100 µg/ml; Sigma-Aldrich) at 37°C in a humidified 5% CO₂ atmosphere.

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RNA Interference and Transfection

The specific short hairpin RNA targeting HOXB7 (sh-HOXB7) and its negative control (sh-NC) were synthesized by Invitrogen (Carlsbad, CA, USA). U2OS cells (5×10^4 cells/ml) were seeded into 24-well plates and incubated at 37°C. The next day, when these cells were at about 80% confluence, cells were transfected with sh-HOXB7 or sh-NC using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested for assays 48 h after transfection.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from osteosarcoma cells using the TRIzol reagent (Invitrogen). Two micrograms of total RNA was employed as the template for synthesis of first-strand cDNA with an M-MLV RT kit (Fermentas, Burlington, Ontario, Canada). qPCRs were performed on a 7500 Real-Time PCR system (Applied Biosystems, Grand Island, NY, USA) with a SYBR Green Master Mix Kit (Takara Biotechnology, Dalian, P.R. China). The sequences of the primers used are as follows: HOXB7, 5'-TATGGGCTCGAGCCGAGTT-3' (forward) and 5'-GGCCTCGTTTGCAGT-3' (reverse); β -actin, 5'-AGAAAATCTGGCACCACACC-3' (forward) and 5'-TAGCACAGCCTGGATAGCAA-3' (reverse). Expression levels of the relative genes were calculated using control β -actin mRNA and the $2^{-\Delta\Delta CT}$ method.

Western Blot

The proteins were extracted from osteosarcoma cells with RIPA buffer consisting of 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk for 2 h and then incubated with primary antibodies (anti-HOXB7, anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt, anti-Akt, or anti-GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The blots were then washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK). The signals were quantified by densitometry using Sion Image software (Scion Corporation, Frederick, MD, USA).

Cell Proliferation Assay

Infected U2OS cells (1×10^5 cells per well) were seeded into 96-well flat-bottom microtiter plates (Pierce, Rockford, IL, USA) and then cultured at 24-h intervals for

4 days. Cell proliferation was then evaluated using the cell counting kit-8 (CCK-8; Sigma-Aldrich) assay according to the manufacturer's instructions. The absorbance was subsequently measured on a Bio-Rad Microplate Reader (Bio-Rad, Hercules, CA, USA) using a test wavelength of 490 nm.

Cell Migration and Invasion Assays

Cell migration assay was detected using Transwell chambers (Costar, Cambridge, MA, USA). The medium, including 1% FBS, was added into the lower compartment, and infected U2OS cells (5×10^4 cells/ml) suspended in DMEM were added to the upper chamber. Twenty-four hours later, cells on the upper surface of the filters were removed with a cotton swab. Cells that migrated to the lower surface of the filters were fixed in 3.7% paraformaldehyde in PBS at 4°C, stained with Giemsa, and counted under a microscope. The cell invasion assay was performed using a Boyden chamber coated with Matrigel (8- μ m pore; BD Biosciences, France).

In Vivo Xenograft Tumor Assay

Four-week-old male BALB/c nude mice were purchased from the Laboratory Animal Center of Hebei Province (P.R. China) and housed in a specific pathogen-free environment. U2OS cells (2×10^6 cells) transfected with sh-HOXB7 or sh-NC diluted in 200 μ l of PBS were inoculated subcutaneously into the right flank of nude mice ($n=6$ per group). Tumor volume was measured every week and calculated using the formula: volume = length \times width² $\times \pi/6$. About 4 weeks after inoculation, mice were anesthetized with sodium pentobarbital (40 mg/kg), and the tumors were weighed. All animal studies were approved by the Institutional Animal Care and Use Committee at Cangzhou Central Hospital.

Statistical Analysis

Data were processed as mean \pm SD. The differences were analyzed by the Student's *t*-test or one-way analysis of variance and Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Expression of HOXB7 in Human Osteosarcoma Cell Lines

We initially examined the expression of HOXB7 in three human osteosarcoma cell lines by qRT-PCR and Western blot assay. mRNA expression levels of HOXB7 were higher in all osteosarcoma cell lines than in the normal osteoblast cell line (hFOB1.19) (Fig. 1A). Similarly, Western blot analysis revealed that the mRNA expression levels of HOXB7 were significantly upregulated in all osteosarcoma cell lines (Fig. 1B).

Downregulation of HOXB7 Inhibited Osteosarcoma Cell Proliferation In Vitro

To examine the effect of HOXB7 on osteosarcoma cell proliferation, we generated the HOXB7-silencing osteosarcoma cell line U2OS. The results demonstrated that the expression levels of HOXB7 in both mRNA (Fig. 2A) and protein (Fig. 2B) were significantly reduced in U2OS cells, respectively. Then the effect of HOXB7 on osteosarcoma cell proliferation was detected using the CCK-8 assay. Compared with the sh-NC group, downregulation

of HOXB7 greatly suppressed the proliferation of U2OS cells (Fig. 2C).

Downregulation of HOXB7 Attenuated the Growth of Osteosarcoma In Vivo

To examine the effect of HOXB7 on osteosarcoma growth in vivo, U2OS cells transfected with sh-HOXB7

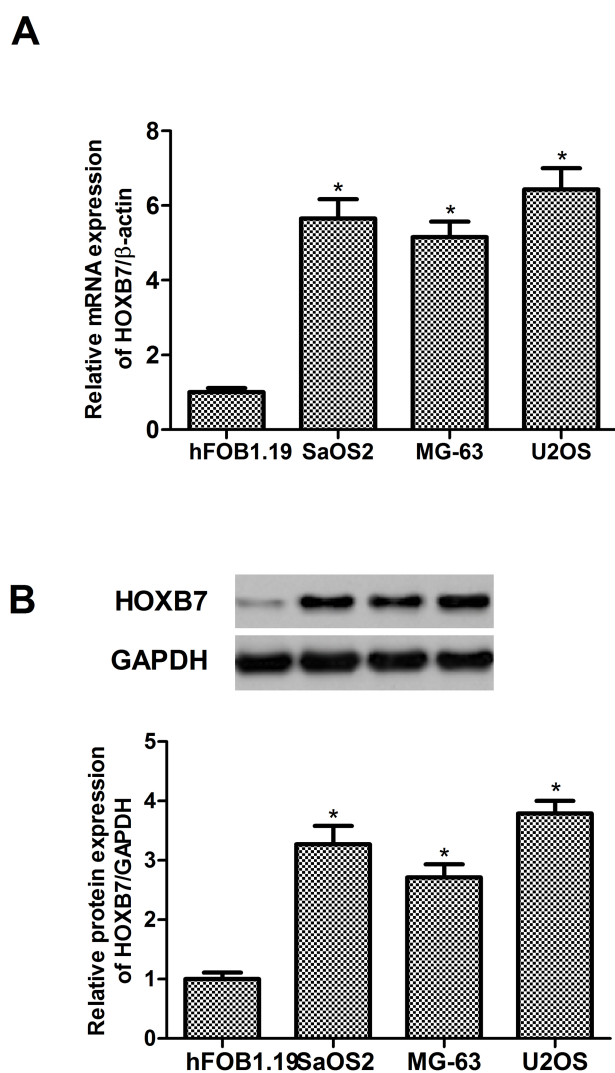


Figure 1. Expression of HOXB7 in human osteosarcoma cell lines. (A) The mRNA expression of HOXB7 in three human osteosarcoma cell lines was detected using the quantitative real-time polymerase chain reaction (qRT-PCR) assay. (B) The protein expression of HOXB7 in three human osteosarcoma cell lines was evaluated by Western blot assay. All experiments were repeated at least three times. * $p < 0.05$ compared with hFOB1.19 group.

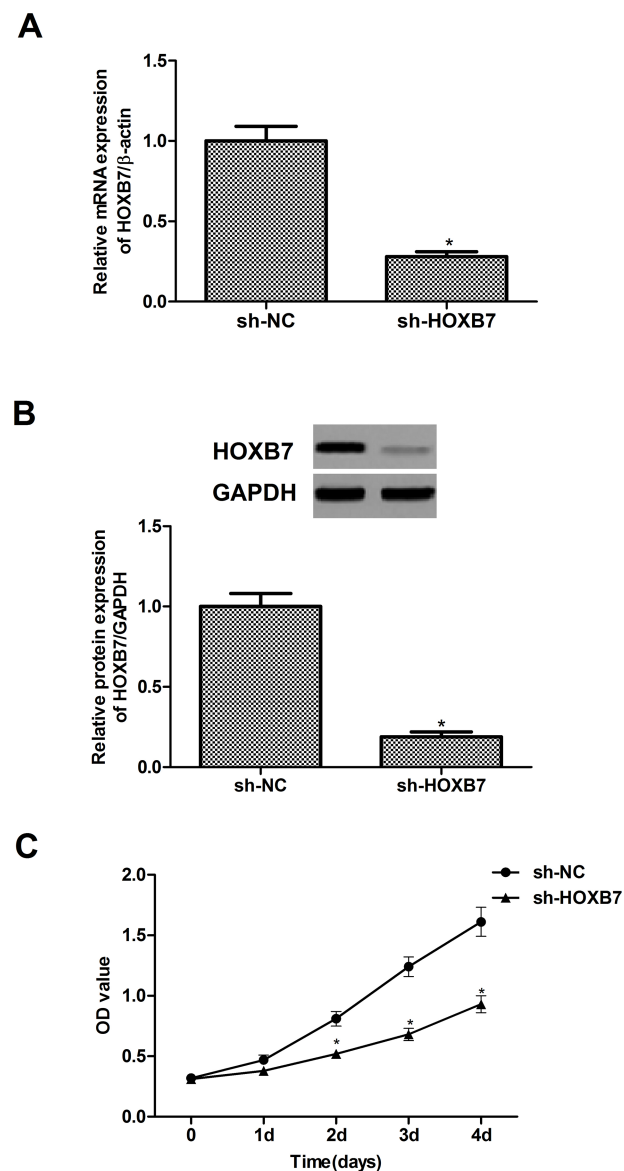


Figure 2. Downregulation of HOXB7 inhibited osteosarcoma cell proliferation in vitro. U2OS cells were transfected with sh-HOXB7 or sh-NC for 48 h, respectively. Transfection efficiency was confirmed by qRT-PCR (A) and Western blot (B) analysis. (C) The effect of HOXB7 on osteosarcoma cell proliferation was detected using the cell counting kit-8 (CCK-8) assay. All experiments were repeated at least three times. * $p < 0.05$ compared with sh-NC group.

or sh-NC diluted in 200 μ l of PBS were inoculated subcutaneously into the right flank of nude mice. The subcutaneous tumor volume in mice significantly declined in U2OS cells treated with sh-NC when compared with the controls (Fig. 3A). In addition, downregulation of HOXB7 sharply reduced the weight of tumors compared with control tumors (Fig. 3B).

Downregulation of HOXB7 Inhibited the Migration and Invasion via Blocking the EMT Process in Osteosarcoma Cells

Next, we analyzed the effect of HOXB7 on the migration and invasion of osteosarcoma cells. The number of cells that migrated into the lower chamber was significantly lower in sh-HOXB7-transfected U2OS cells than

in sh-NC-transfected cells (Fig. 4A). In addition, downregulation of HOXB7 obviously inhibited the invasion of U2OS cells (Fig. 4B). Furthermore, we examined the effect of HOXB7 on EMT-related marker expression in U2OS cells. The results of the Western blot analysis showed that

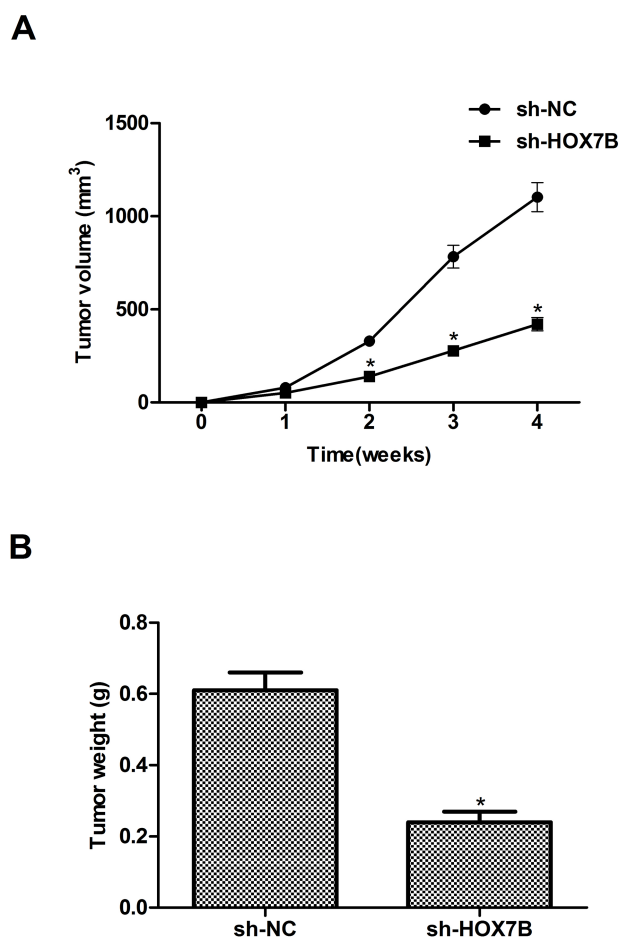


Figure 3. Downregulation of HOXB7 attenuated the growth of osteosarcoma in vivo. U2OS cells transfected with sh-HOXB7 or sh-NC diluted in 200 μ l of PBS were inoculated subcutaneously into the right flank of nude mice. (A) The tumor volumes were calculated in each group every week. (B) Downregulation of HOXB7 inhibited tumor weight of BALB/c nude mice. * $p < 0.05$ compared with sh-NC group.

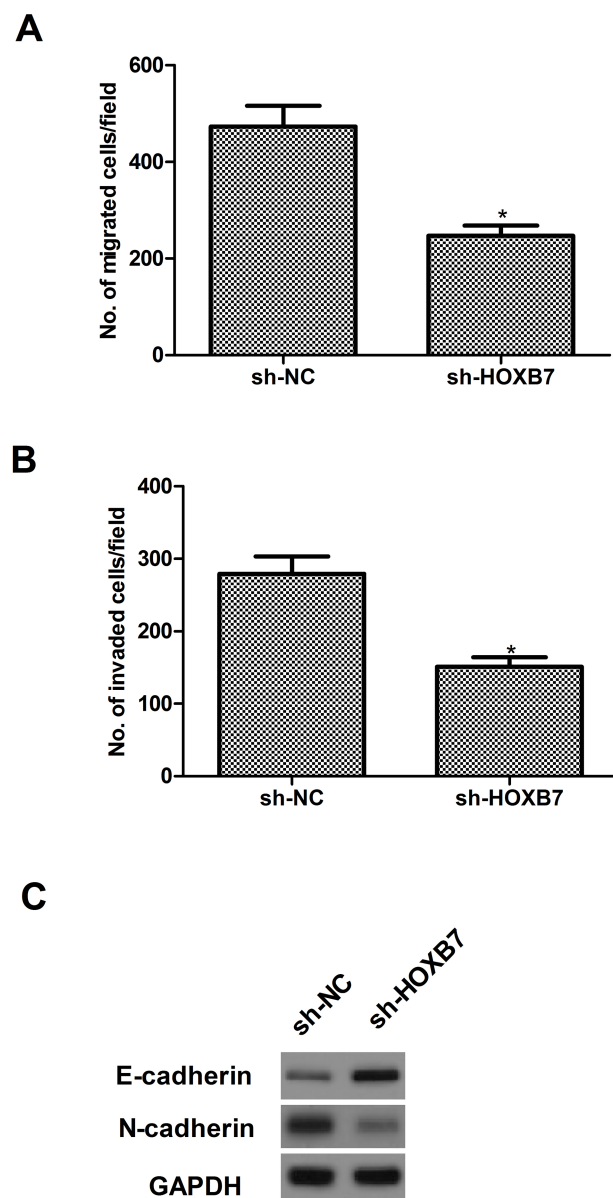


Figure 4. Downregulation of HOXB7 inhibited the migration and invasion via blocking the epithelial-mesenchymal transition (EMT) process in osteosarcoma cells. U2OS cells were transfected with sh-HOXB7 or sh-NC for 48 h, respectively. (A) Cell migration was assessed by the Transwell chamber. (B) Cell invasion was assessed by the Matrigel invasion chamber. (C) The protein levels of E-cadherin and N-cadherin were determined by Western blot. All experiments were repeated at least three times. * $p < 0.05$ compared with sh-NC group.

downregulation of HOXB7 induced an increase in the protein expression of E-cadherin, paralleling with a decrease in the protein expression of N-cadherin in U2OS cells (Fig. 4C).

Downregulation of HOXB7 Inhibited the Activation of the PI3K/Akt Pathway in Osteosarcoma Cells

In order to explore the underlying mechanism modulated by sh-HOXB7 in tumor inhibition, we examined PI3K and Akt phosphorylation levels in sh-HOXB7-transfected U2OS cells. The results of the Western blot analysis revealed that downregulation of HOXB7

significantly suppressed the protein expression levels of p-PI3K and p-Akt in U2OS cells, compared with the sh-NC group (Fig. 5A and B). Furthermore, we examined the effect of the PI3K/Akt inhibitor (LY294002) on the level of proteins, including p-Akt, E-cadherin, and N-cadherin, in U2OS cells. The results showed that treatment with LY294002 (20 μ M) significantly decreased the growth of U2OS cells; thus, 10 μ M LY294002 was chosen to be used for additional experiments (Fig. 5C). In addition, we observed that LY294002 notably upregulated the protein expression of E-cadherin, while it downregulated that of N-cadherin and p-Akt in U2OS cells (Fig. 5D).

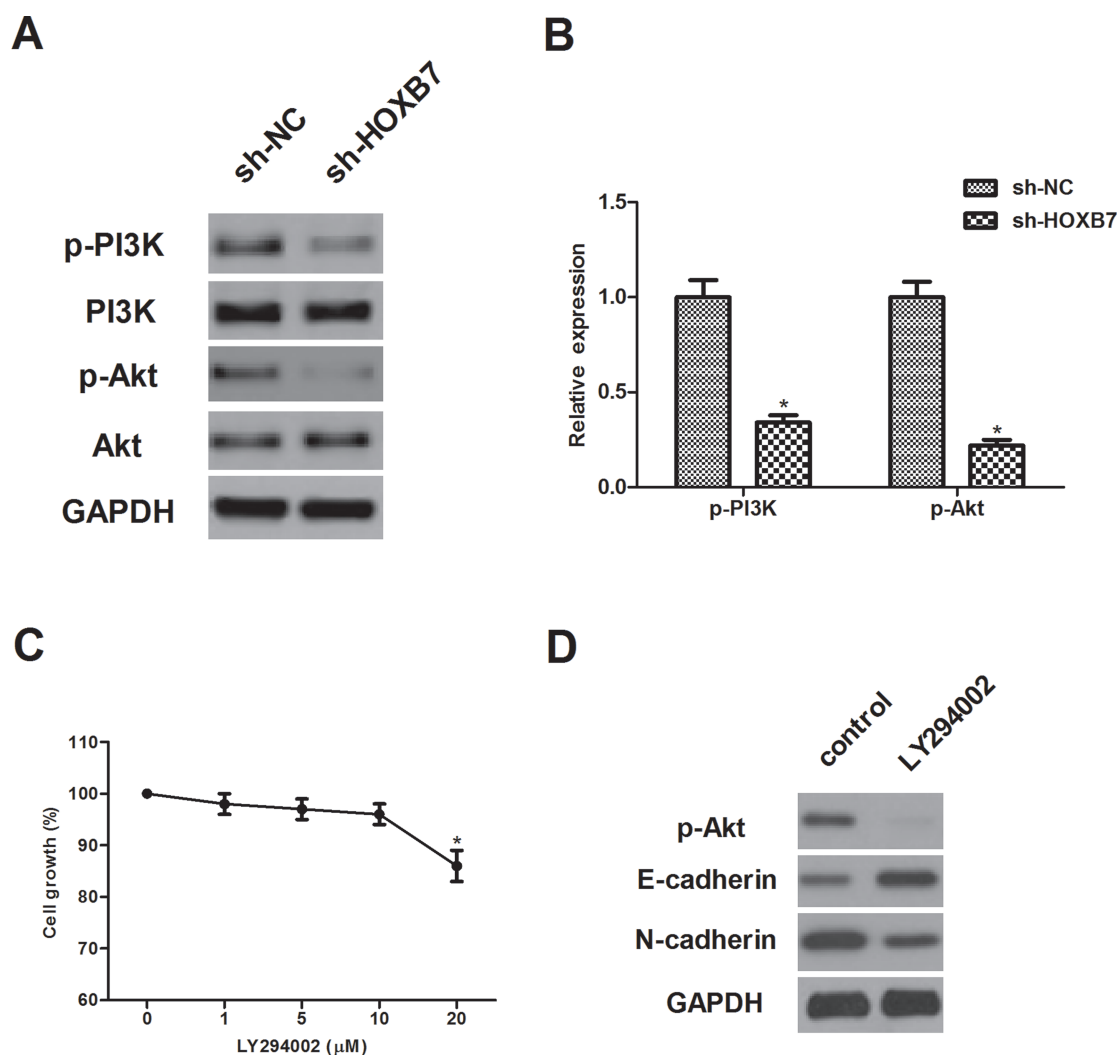


Figure 5. Downregulation of HOXB7 inhibited the activation of the PI3K/Akt pathway in osteosarcoma cells. U2OS cells were transfected with sh-HOXB7 or sh-NC for 48 h, respectively. (A) The protein levels of PI3K, p-PI3K, Akt, and p-Akt were determined by Western blot. (B) The relative protein expression levels of p-PI3K and p-Akt were quantified using the Sion Image software. All experiments were repeated at least three times. * $p < 0.05$ compared with sh-NC group. (C) U2OS cells were incubated with various concentrations of the PI3K/Akt inhibitor (LY294002), and cell growth was detected using the CCK-8 assay. (D) The protein levels of p-Akt, E-cadherin, and N-cadherin were determined by Western blot. * $p < 0.05$ compared with control group.

DISCUSSION

In the present study, we first elucidated the role of HOXB7 in osteosarcoma. Our results demonstrated that HOXB7 was overexpressed in osteosarcoma cell lines. Downregulation of HOXB7 significantly inhibited osteosarcoma cell proliferation *in vitro*, as well as attenuated xenograft tumor growth *in vivo*. Downregulation of HOXB7 also inhibited the migration and invasion of osteosarcoma cells. Furthermore, downregulation of HOXB7 significantly suppressed the protein expression levels of p-PI3K and p-Akt in U2OS cells.

Aberrant expression of HOXB7 has been associated with several types of cancers. A study by Komatsu et al. confirmed that HOXB7 expression was much higher in hepatocellular carcinoma tissues than in liver parenchyma and that HOXB7 expression was positively related to larger tumor size and a higher rate of biliary invasion¹⁴. Cai et al. reported that HOXB7 expression was highly expressed in gastric cancer cell lines and tissues and that upregulation of HOXB7 significantly promoted the tumorigenesis and progression of gastric cancer¹⁵. Consistent with the results of previous studies, we provided evidence that HOXB7 was overexpressed in osteosarcoma cell lines. Downregulation of HOXB7 significantly inhibited osteosarcoma cell proliferation *in vitro* and attenuated xenograft tumor growth *in vivo*. All of these results suggest that HOXB7 may be a novel oncogene in the development and progression of osteosarcoma.

EMT is considered to be one of the critical steps in cancer cell invasion and metastasis. In the process of EMT, tumor cells lose epithelial (E)-cadherin expression and cellular adhesion, and gain the higher ability of invasiveness and metastasis^{16,17}. Recently, Joo et al. reported that the expression of HOXB7 was obviously higher in primary or metastatic gastric cancer tissues than in chronic gastritis tissues and that knockdown of HOXB7 suppressed gastric cancer cell migration and invasion¹⁸. Similarly, the data presented here indicated that downregulation of HOXB7 inhibited the migration and invasion of osteosarcoma cells, upregulated the expression of E-cadherin, and downregulated the expression of N-cadherin in osteosarcoma cells. These results suggest that HOXB7 positively controls the EMT phenotype, consequently affecting the migration and invasion of osteosarcoma cells.

The PI3K/Akt signaling pathway is correlated with the development and progression of osteosarcoma^{19–21}. Studies have shown that the PI3K/Akt pathway is constitutively upregulated in osteosarcoma^{22–24}. In addition, activated Akt can trigger a series of downstream effects, such as activation of transcription factors p53 and NF- κ B, which have a major impact on cancer cell growth and survival²⁵, and induce EMT and invasion in tumor cells²⁶. Herein we found that downregulation of HOXB7

significantly suppressed the protein expression levels of p-PI3K and p-Akt in U2OS cells. These data suggest that downregulation of HOXB7 inhibits proliferation, invasion, and tumorigenesis, partly through suppressing the PI3K/Akt signaling pathway in osteosarcoma cells.

In summary, our data demonstrated that downregulation of HOXB7 inhibited proliferation, invasion, and tumorigenesis partly through suppressing the PI3K/Akt signaling pathway in osteosarcoma cells. Our findings provide new insight into the role of HOXB7 in osteosarcoma and new therapeutic targets for the treatment of osteosarcoma.

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