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Knockdown of UBE2T Inhibits Osteosarcoma Cell Proliferation, Migration, and Invasion by Suppressing the PI3K/Akt Signaling Pathway

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Ubiquitin-conjugating enzyme E2T (UBE2T), a member of the E2 family, was found to be overexpressed in a great many cancers such as bladder cancer, lung cancer, and prostate cancer. However, there have been no reports on the role of UBE2T in osteosarcoma. In this study, we tried to make the effects of UBE2T on osteosarcoma clear. The study results showed that UBE2T was overexpressed in osteosarcoma tissues and cell lines. Moreover, UBE2T knockdown inhibited osteosarcoma cell proliferation, migration, and invasion. We also observed that UBE2T downregulation could suppress the activity of the PI3K/Akt signaling pathway. Therefore, we concluded that UBE2T exerted its inhibitory effects on osteosarcoma cells via suppressing the PI3K/Akt signaling pathway. These findings indicated that UBE2T may be a potential therapeutic target for osteosarcoma treatment.

Key words: Ubiquitin-conjugating enzyme E2T (UBE2T); Osteosarcoma; Proliferation; Invasion; PI3K/Akt signaling pathway

INTRODUCTION

Osteosarcoma, a common malignant bone tumor often occurring in children and adolescents, possesses a highly metastatic potential (1–3). About 20% of osteosarcoma patients, at initial diagnosis, suffer from clinically detectable metastatic disease; about 40% of the patients, at the advanced stage, suffer from metastases mainly developing in the lungs (4). With more and more advanced treatment, the survival rate for osteosarcoma patients without metastasis has been greatly increased to 60–70% (5). However, the situation is still not optimistic for patients with metastatic tumors, who have a survival rate of only 30% (6,7). Therefore, it is desirable to discover a novel strategy for a better therapeutic outcome for osteosarcoma patients.

Ubiquitin-conjugating enzyme E2T (UBE2T), a member of the E2 family, was first discovered in a case of Fanconi anemia (FA) (8–10). It is part of the ubiquitin–proteasome degradation system and plays its role in combination with specific E3 ubiquitin ligase to achieve degradation or functional changes of relevant substrates

(11,12). This process governs the turnover of regulatory proteins involving primary cellular processes such as differentiation, angiogenesis, apoptosis, cell cycle progression, and cellular signaling pathways (11). For example, UBE2T has been reported to be independently employed in regulating FANCD2 monoubiquitination and to take part in the FA pathway together with FANCD2 (9,13). Recent studies have indicated that disrupted UBE2T expression, by affecting the DNA damage–repair response, could directly result in FA and increase the sensitivity of tumor cells to crosslink agents (10,14). What is interesting is the UBE2T location, 1q32.1, which is amplified in a variety of cancers (15). In addition, UBE2T overexpression has been found in a number of cancers such as bladder cancer, lung cancer, and prostate cancer (16–19). However, there have been no reports on the role of UBE2T in osteosarcoma.

In this study, we concentrated on making the role of UBE2T in osteosarcoma clear. The results showed that UBE2T was overexpressed in osteosarcoma tissues and cell lines. Moreover, UBE2T downregulation inhibited

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osteosarcoma cell proliferation, migration, and invasion via suppressing the PI3K/Akt signaling pathway. These findings indicated that UBE2T may be a potential therapeutic target for osteosarcoma.

MATERIALS AND METHODS

Patients and Osteosarcoma Samples

Osteosarcoma tissues and matching noncancerous bone tissues were obtained from osteosarcoma patients at Nanfang Hospital (Guangzhou, China). All the tissue samples were collected and frozen in liquid nitrogen for future experiments. All the patients agreed to participate in the study and gave written consent. The use of tissue samples in the study was approved by the Ethics Committee of Nanfang Hospital.

Cell Lines and Cell Culture

Human osteosarcoma cell lines (HOS, MG63, and SaOS2) and human osteoblast cell line (HCO) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Tauranga, New Zealand) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 100 U/ml penicillin (Sigma-Aldrich), followed by incubation in a humidified atmosphere with 5% CO₂ at room temperature.

Quantitative Real-Time PCR

Total RNA was isolated from the tissues or the cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was conducted with the PrimeScript RT Reagent kit (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's instructions. The PCR was performed under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The following primers were used: UBE2T, 5'-CAAATATTAGGTGGAGCCAA CAC-3' (forward) and 5'-TAGATCACCTTGGCAAAGA ACC-3' (reverse); β-actin, 5'-AGAAAATCTGGCACCA CACC-3' (forward) and 5'-TAGCACAGCCTGGATAGC AA-3' (reverse). β-Actin was used as an internal control. The relative mRNA expression levels were calculated using the comparative CT method ($2^{-\Delta\Delta C_t}$) (20).

Western Blot

The proteins were extracted from the tissues or the cells in RIPA lysis buffer (Beyotime, Jiangsu, China). After centrifugation at 12,000×g for 10 min at 4°C, the supernatants were separated using 10% SDS-PAGE (Bioworld Technology, St. Louis Park, MN, USA), and then the separated proteins were transferred to a PVDF membrane (Life Technologies, Gaithersburg, MD, USA). The membrane

was blocked in PBS and 5% skim milk for 1 h and then incubated overnight at 4°C with the primary antibodies against UBE2T, PI3K, p-PI3K, Akt, p-Akt, or β-actin (Cell Signaling Technology, San Jose, CA, USA). The membrane was incubated at room temperature for 1 h with secondary HRP-conjugated antibodies (Cell Signaling Technology). The relative protein expression levels were evaluated using the Quantity One software (Life Technologies).

Plasmids and Transfection Assay

UBE2T siRNA (siUBE2T) and scrambled siRNA (siNC) plasmids were purchased from Life Technologies. The sequences of siUBE2T and siNC were GCUGACAUU CCUCAGAAUTT and UUCUCCGAACGUGUCACGU TT, respectively. HOS and MG63 cells were transfected with siUBE2T or siNC plasmid using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instruction. After 24 h of transfection, the cells were collected for the following studies. Western blot assays were performed to determine the transfection efficiency.

Proliferation Assay In Vitro

MTT assays were performed to measure the rate of cell proliferation in vitro. Briefly, the cells transfected with siUBE2T or siNC were planted into 96-well plates at a density of 1×10^5 cells/well and then cultured for 24, 48, or 72 h, respectively. The transfected cells were incubated with 25 µl of MTT (Sigma-Aldrich) for 4 h at 37°C, followed by removing of supernatants and adding of 150 µl of DMSO (Sigma-Aldrich). The absorbance value was measured at 450 nm with a microplate reader (BioTek Instruments, Winooski, VT, USA).

Transwell Assay

To measure the effects of UBE2T on invasion capacities of osteosarcoma cells, the matrix-coated Transwell assay was conducted. In brief, transfected cells (1×10^5 cells/well) were suspended in serum-free medium and then added to the upper chamber coated with matrix. The lower chamber was filled with normal cell culture medium. After 24 h of incubation at 37°C, cells on the membrane were clipped off, and those below the membrane were fixed and stained. The number of invaded cells was counted under a microscope (200×). For migration assay, common Transwell chambers were applied instead of the matrix-coated ones.

Tumor Xenograft Growth Assay In Vivo

Male BALB/c nude mice that were 4 to 6 weeks old were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). All animal studies were approved by the Institutional Animal Care and Use Committee of Nanfang Hospital. For the tumor growth assay, 5×10^3 cells transfected with siUBE2T or siNC were resuspended in PBS medium and then inoculated

subcutaneously into nude mice. The treatment time was 35 days. The tumors were measured every 7 days since their appearance, and the tumor volume (cm^3) was calculated by the following formula: $\text{length} \times \text{width}^2/2$.

Statistical Analysis

Statistical analysis was performed using the SPSS 13.0 software (IBM, Armonk, NY, USA). Student's *t*-tests were carried out to make a comparison between different groups. Data were expressed as mean \pm SD. A value of $p < 0.05$ was considered statistically significant.

RESULTS

UBE2T Was Overexpressed in Osteosarcoma Tissues and Cell Lines

UBE2T expression in human osteosarcoma tissues and corresponding normal tissues was measured by RT-PCR and Western blot assays. As shown in Figure 1A and B, UBE2T was overexpressed in osteosarcoma tissues but was hardly detected in the matching normal tissues. Additionally, as shown in Figure 1C and D, a higher expression level of UBE2T was observed in three osteosarcoma cell lines (HOS, MG63, and SaOS2) when compared to the osteoblast cell line HCO.

UBE2T Knockdown Inhibited Osteosarcoma Cell Proliferation In Vitro and Tumor Growth In Vivo

UBE2T knockdown was achieved by transfection of siRNA plasmids. The transfection efficiency was verified by the Western blot assay. As shown in Figure 2A, the protein expression levels of UBE2T were significantly decreased in HOS-siUBE2T cells in comparison with the HOS-siNC group. Similar results were found in MG63-siUBE2T cells (Fig. 2B).

MTT assays were conducted to investigate the effects of UBE2T knockdown on proliferation of osteosarcoma cells. The results revealed that UBE2T knockdown dramatically inhibited the growth of HOS-siUBE2T (Fig. 2C) and MG63-siUBE2T (Fig. 2D) cells. To confirm whether the growth-inhibiting effect of UBE2T knockdown on osteosarcoma cells was associated with the tumor growth in vivo, MG63 cells transfected with siUBE2T or siNC were subcutaneously inoculated into mice. The results showed that knockdown of UBE2T remarkably decreased the tumor volume (Fig. 2E) and the tumor weight (Fig. 2F).

UBE2T Knockdown Inhibited Osteosarcoma Cell Migration and Invasion In Vitro

We performed Transwell assays to evaluate the effects of UBE2T knockdown on migration and invasion of osteosarcoma cells. We used matrix-coated Transwell chambers to measure the invasive capability and matrix-free ones to measure the migratory capability. As shown in

Figure 3A and B, UBE2T knockdown greatly reduced the number of migrated and invaded HOS-siUBE2T cells, compared to the HOS-siNC group. In addition, we found that UBE2T knockdown similarly affected migration and invasion of MG63-siUBE2T cells (Fig. 3C and D).

UBE2T Knockdown Inhibited the Activity of PI3K/Akt Signaling Pathway

We investigated the effects of UBE2T knockdown on the activity of the PI3K/Akt signaling pathway. As indicated by the Western blot assay, a significant decrease in the protein levels of p-PI3K and p-Akt was observed in MG63-siUBE2T cells (Fig. 4A–C) in comparison with corresponding control cells. However, the total protein levels of PI3K and Akt were little affected. We also tested the effects of an Akt inhibitor (wortmannin) on osteosarcoma cell proliferation and invasion mediated by knockdown of UBE2T. As assessed by the cell proliferation assay, wortmannin dramatically potentiated siUBE2T-inhibited MG63 cell proliferation (Fig. 4D). In addition, wortmannin similarly enhanced the suppressive effect of siUBE2T on MG63 cell invasion (Fig. 4E).

DISCUSSION

Osteosarcoma is a kind of malignant bone tumor mainly occurring in children and adolescents. Although the survival rate of osteosarcoma patients has been greatly increased by chemotherapy, the overall outcome of osteosarcoma patients is still poor because of drug resistance, especially multidrug resistance. Therefore, it is desperately necessary to find a new therapeutic strategy for a more effective osteosarcoma treatment.

UBE2T, a novel therapeutic target, has been found to be overexpressed in various cancers such as bladder, lung, and prostate cancers (10,17–19). Furthermore, recent studies have shown that UBE2T downregulation inhibited proliferation, migration, and invasion of nasopharyngeal carcinoma cells, and its depletion significantly suppressed tumor formation and metastasis of prostate cancer cells (19,21). Consistent with all these findings, the results of our in vitro and in vivo studies indicated that UBE2T was overexpressed in osteosarcoma tissues and cell lines and that UBE2T knockdown inhibited osteosarcoma cell proliferation, migration, and invasion.

Proliferation, migration, and invasion are all essential cellular processes and important for tumor formation and development. In the present study, we performed corresponding experiments to investigate the effects of UBE2T knockdown on these processes. First, we carried out RT-PCR and Western blot assays to detect the mRNA and protein expression levels of UBE2T in osteosarcoma tissues and cell lines. As revealed by the study results, UBE2T was upregulated in osteosarcoma

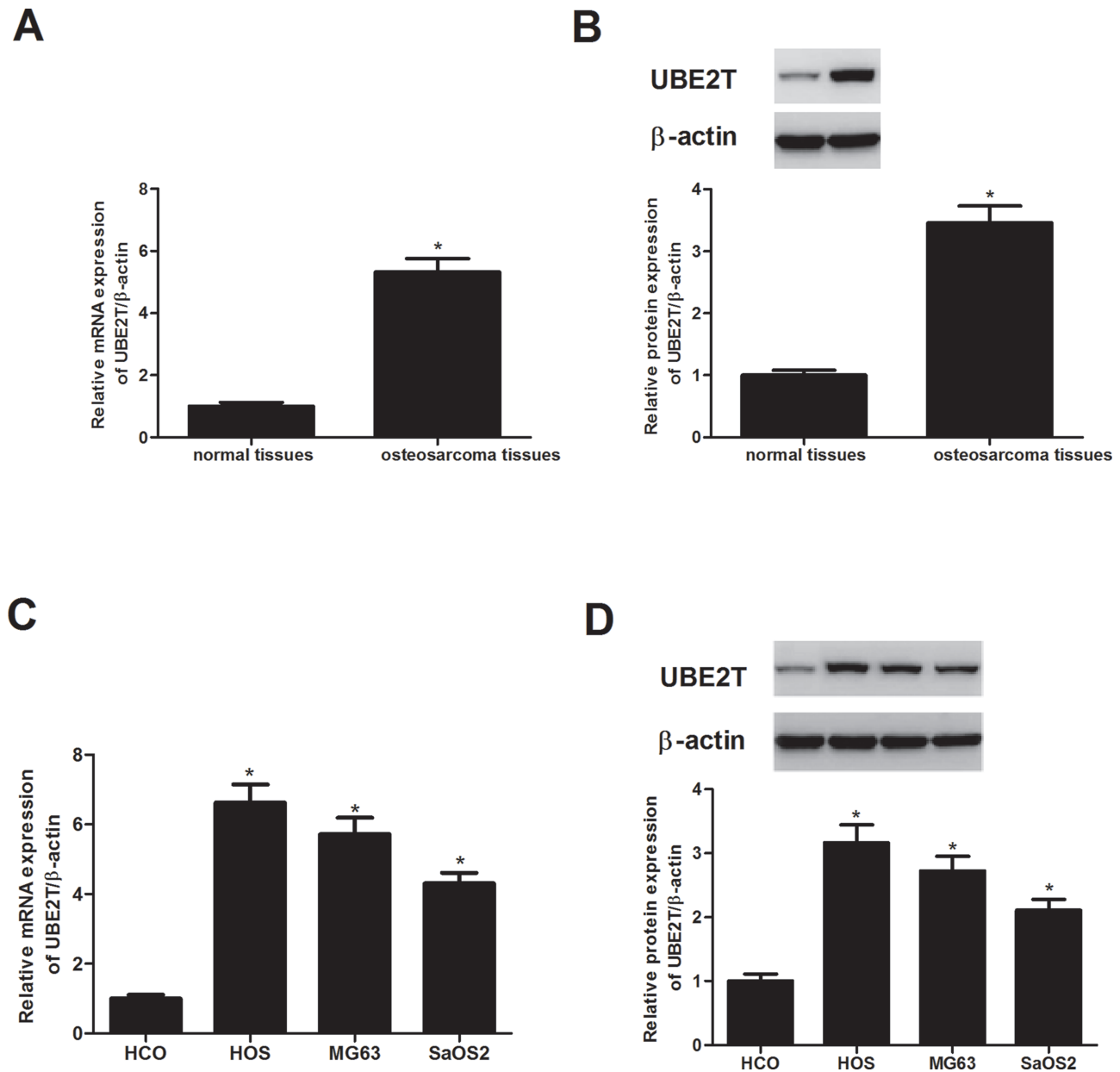


Figure 1. Ubiquitin-conjugating enzyme E2T (UBE2T) was overexpressed in osteosarcoma tissues and cell lines. (A, B) RT-PCR and Western blot analysis were conducted to evaluate the expression levels of UBE2T in osteosarcoma tissues and matching normal tissues. (C, D) mRNA and protein expression levels of UBE2T in three osteosarcoma cell lines (HOS, MG63, and SaOS2) and the osteoblast cell line HCO. * $p < 0.05$.

tissues and cell lines. After we downregulated UBE2T in two osteosarcoma cell lines, HOS and MG63, via transfection of siRNA plasmids, we performed both in vitro and in vivo assays to evaluate the role of UBE2T knockdown in proliferation, migration, and invasion of osteosarcoma cell lines. As expected, we observed that UBE2T knockdown exerted a significant inhibitory effect on these processes. However, the underlying mechanisms remained unclear.

The PI3K/Akt signaling pathway is of great importance in the malignant development of various tumors (22,23). This signaling pathway acts as a mediator of proliferation, migration, and invasion of cancer cells (24,25). It is reported that PI3K is often activated by oncogenes, and its activation can facilitate cancer cell proliferation (26). In carcinogenesis of PI3K, Akt plays a significant role as a major downstream effector of PI3K (27). Akt is capable of regulating many cellular

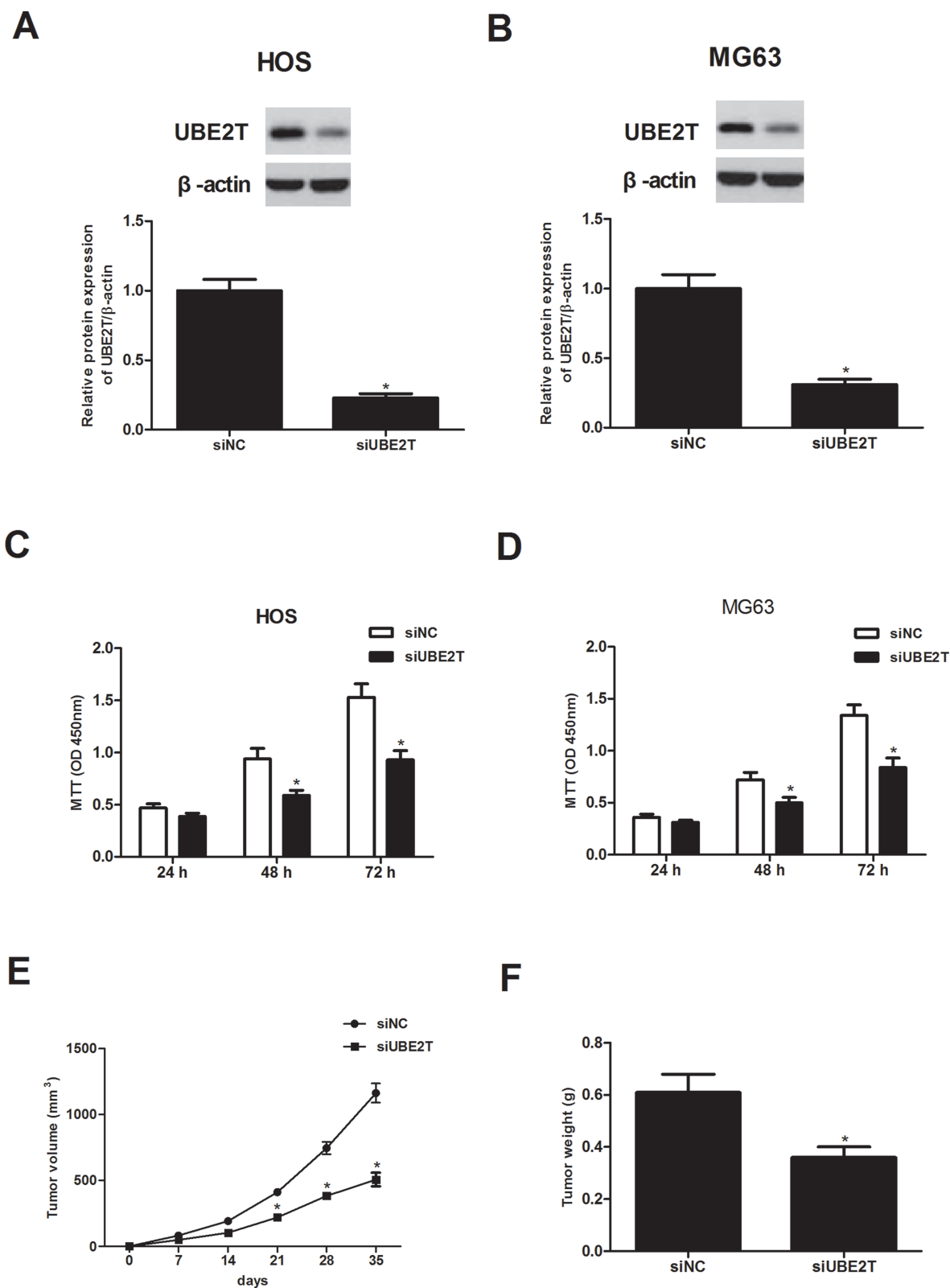


Figure 2. UBE2T knockdown inhibited osteosarcoma cell proliferation in vitro and tumor growth in vivo. (A, B) The Western blot assay was performed to detect the transfection efficiency of HOS-siUBE2T and MG63-siUBE2T cells. (C, D) A lower proliferation rate was found in HOS-siUBE2T and MG63-siUBE2T cells, compared to corresponding control cells. (E, F) A significant decrease in tumor volume and tumor weight was observed in MG63-siUBE2T cells, compared to the MG63-siNC group ($n=5$). * $p<0.05$.

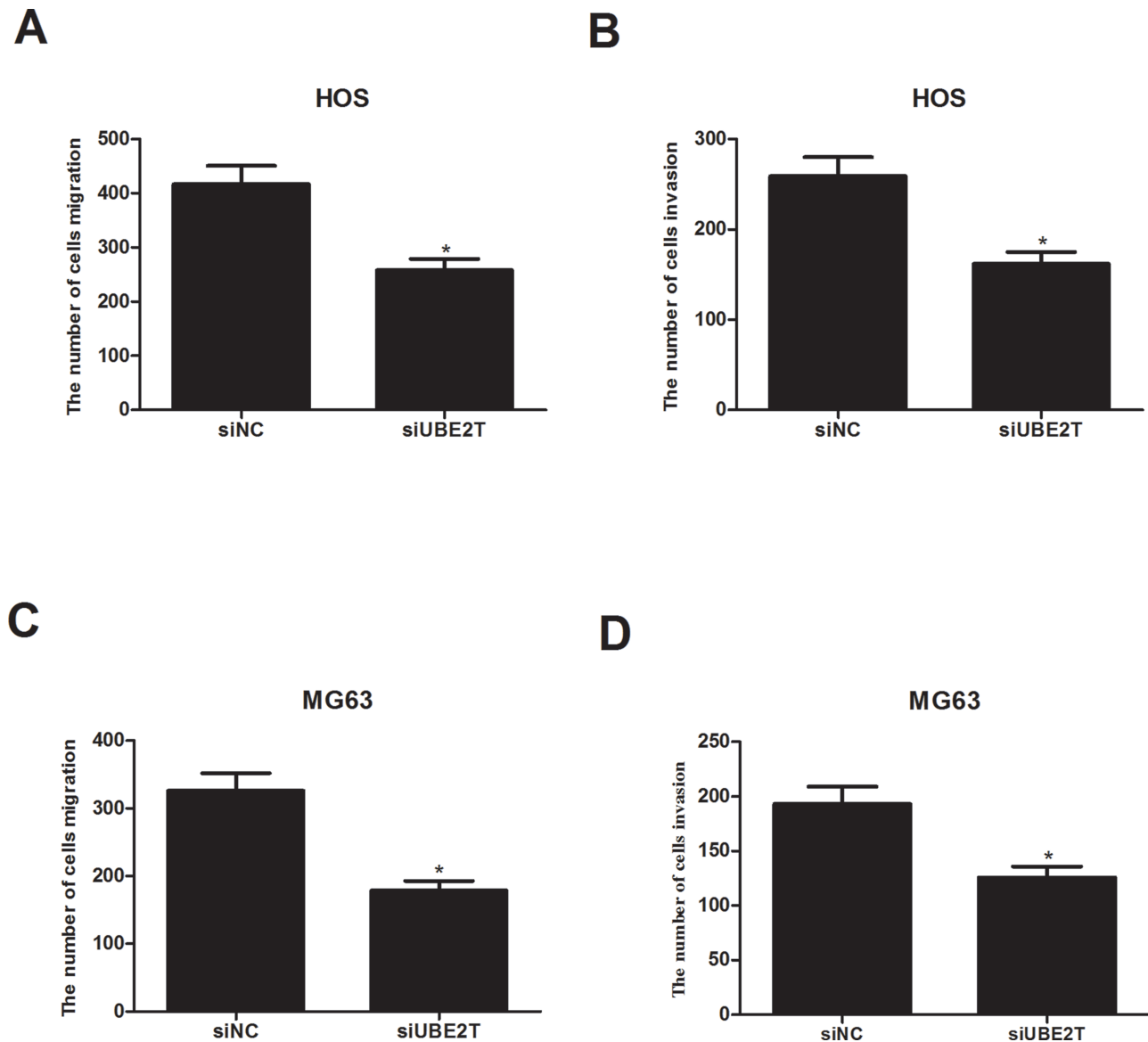


Figure 3. UBE2T knockdown inhibited osteosarcoma cell migration and invasion in vitro. (A, B) As indicated by Transwell assays, UBE2T knockdown obviously weakened the migratory and invasive capabilities of HOS-siUBE2T cells, in comparison with corresponding control cells. (C, D) UBE2T knockdown exerted a similar effect on the migration and invasion of MG63-siUBE2T cells. * $p < 0.05$.

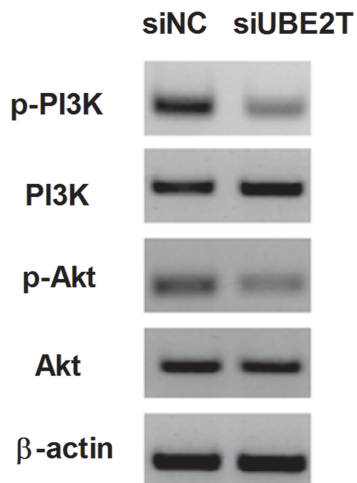
processes, and its deregulation is essential in activating tumorigenic characteristics such as unbounded cell proliferation, invasion, and metastasis (28). In addition, Akt is found to be downregulated in a variety of cancers including osteosarcoma (29). In view of the crucial role

of the PI3K/Akt signaling pathway in tumor progression, in our study we investigated whether UBE2T exerted any influence on this signaling pathway in osteosarcoma. We performed Western blot to examine the effects of UBE2T knockdown on the protein expression levels of

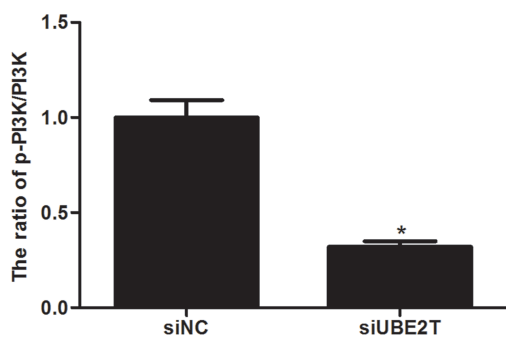
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Figure 4. UBE2T knockdown inhibited the activity of the PI3K/Akt signaling pathway. (A) Western blot assays were performed to measure the protein levels of p-PI3K, PI3K, p-Akt, and Akt, with β -actin as the control. A significant decrease in the protein levels of p-PI3K (B) and p-Akt (C) was observed in MG63-siUBE2T cells, compared to the MG63-siNC group. However, the total protein levels of PI3K and Akt were unaffected. (D) MG63 cells were transfected with siUBE2T or siNC in the presence or absence of wortmannin (100 nM) for 48 h. Cell proliferation was measured by the MTT assay. (E) Cell invasion was detected by the Transwell assay. * $p < 0.05$.

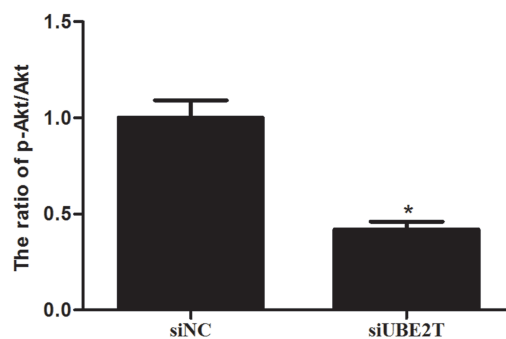
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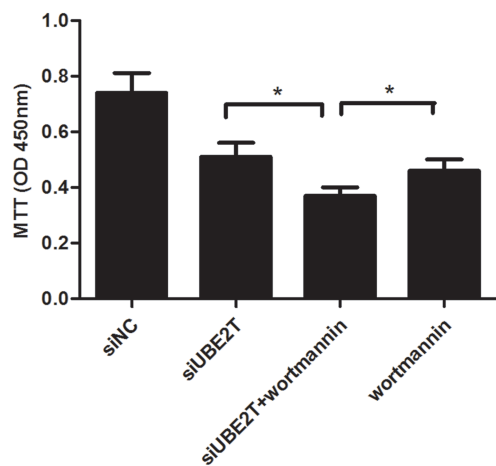
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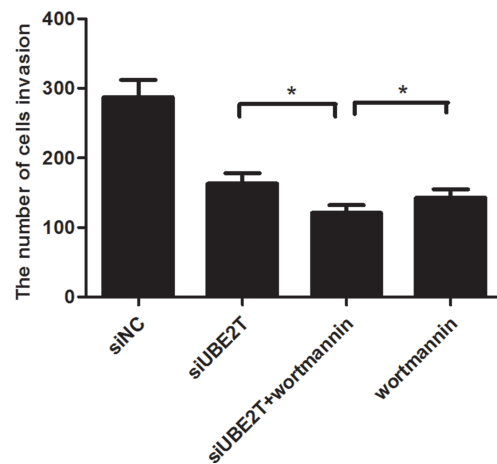
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E



PI3K, p-PI3K, Akt, and p-Akt. The assay results showed that knockdown of UBE2T remarkably decreased the protein levels of pPI3K and p-Akt without affecting the total protein levels of PI3K and Akt. We also examined the effects of an Akt inhibitor (wortmannin) on siUBE2T-mediated osteosarcoma cell proliferation and invasion. The results showed that wortmannin remarkably potentiated siUBE2T-inhibited MG63 cell proliferation and invasion. Taken together, we concluded that UBE2T knockdown inhibited osteosarcoma cell proliferation, migration, and invasion via suppressing the PI3K/Akt signaling pathway. Considering that the development of osteosarcoma is a multistep process involving diverse cellular pathways, more studies will be needed to make clear the molecular mechanisms involved in osteosarcoma initiation and progression (30).

In conclusion, we demonstrated that UBE2T was overexpressed in osteosarcoma tissues and cell lines. Furthermore, UBE2T knockdown inhibited osteosarcoma cell proliferation, migration, and invasion via suppressing the PI3K/Akt signaling pathway. These findings may be used as evidence supporting the role of UBE2T, a potential therapeutic target for osteosarcoma treatment.

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