Oncology Research, Vol. 25, pp. 743–751
Printed in the USA.

0965-0407/17 $90.00 + .00
DOI: https://doi.org/10.3727/096504016X14772395226335
E-ISSN 1555-3906
www.cognizantcommunication.com

Downregulation of Ubiquitin-Specific Protease 22 Inhibits Proliferation, Invasion, and Epithelial–Mesenchymal Transition in Osteosarcoma Cells

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Ubiquitin-specific protease 22 (USP22), a novel deubiquitinating enzyme, belongs to an extended family of proteins that have ubiquitin hydrolase activity. Recently, USP22 has attracted widespread attention because of its implication in carcinogenesis. However, there have been no studies, to our knowledge, investigating the expression of USP22 in osteosarcoma (OS) and its association with OS progression. In this study, we explored the role of USP22 in OS. We demonstrated that USP22 was highly expressed in OS tissue and cell lines. Downregulation of USP22 inhibited OS cell proliferation, invasion, and epithelial–mesenchymal transition (EMT) in vitro. In addition, downregulation of USP22 suppressed OS tumor growth and metastasis in vivo. We also found that the PI3K/Akt signaling pathway was involved in the tumor-promoting effect of USP22 on OS progression. Taken together, we suggest USP22 as a novel therapeutic target for OS.

Key words: Ubiquitin-specific protease 22 (USP22); Proliferation; Invasion; Epithelial–mesenchymal transition (EMT); Osteosarcoma (OS)

INTRODUCTION

Osteosarcoma (OS) is a common type of bone tumor and accounts for a not insignificant proportion of malignant tumors in children[1–3]. With the origin of primitive bone-forming mesenchymal stem cells, OS frequently starts from the metaphysis of long bones[4,5]. In the past, surgical resection dominated OS therapies, leading to poor prognosis of OS patients[6]. OS therapies have recently developed to a great degree and include neoadjuvant chemotherapies that are performed with cisplatin, ifosfamide, doxorubicin, and methotrexate[7,8]. The application of chemotherapies has increased the 5-year survival rate of OS patients to about 80% but has produced adverse effects such as renal and cardiac toxicity, suppression of bone marrow, and gastrointestinal problems[7–9]. Therefore, the development of a novel therapy for OS is urgently needed.

Ubiquitin-specific protease 22 (USP22), a novel deubiquitinating enzyme, belongs to an extended family of proteins having ubiquitin hydrolase activity[10]. First identified in a microarray-based study by Glinsky et al., USP22 is considered to be important in many physiological and pathological processes such as cell cycle, cell proliferation, and tumor invasion[11–14]. It has been found that USP22 is frequently overexpressed in different types of cancers[15–17]. Furthermore, the elevated expression of USP22 has proven to be associated with tumor recurrence, distant metastasis, poor prognosis, and therapeutic failure in various cancer patients[18–20]. Because of its significant role in cancer, USP22 has been given a lot of attention and has been extensively investigated. However, the expression pattern and biological significance of USP22 in OS remain largely unknown.

In this study, we investigated the role of USP22 in OS. We reported that USP22 was upregulated in OS tissues and cells lines. USP22 downregulation inhibited OS cell proliferation, invasion, and epithelial–mesenchymal transition (EMT) in vitro. It also suppressed OS tumor growth and metastasis in vivo. In addition, we demonstrated that USP22 functioned as a tumor promoter in OS by suppressing the PI3K/Akt signaling pathway.

MATERIALS AND METHODS

Patients and Tissue Samples

Twenty-eight pairs of OS tissues and matched noncancerous bone tissues were obtained from OS patients who were admitted to the Huaihe Hospital of Henan University (Kaifeng, P.R. China). Each patient taking part in the study provided written consent. All tissue samples

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were frozen in liquid nitrogen and stored at −80°C before use. The study was approved by the ethics committee of the Huaihe Hospital of Henan University.

Cell Lines and Cell Culture

Human OS cell lines (U2OS and MG-63) and osteoblastic cell line hFOB were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco).

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

Total RNA was extracted from tissues or cells with the TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA) and then reversely transcribed into cDNA using the Primer Script Kit (TaKaRa, Dalian, P.R. China). RT-PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 30 s, 35 cycles of 95°C for 15 s and 60°C for 30 s. The PCR primers were as follows: USP22, 5′-CCATTGATCTGATGTACGGAGG-3′ (forward) and 5′-TCCTTGGCGATTATTTCCATGTC-3′

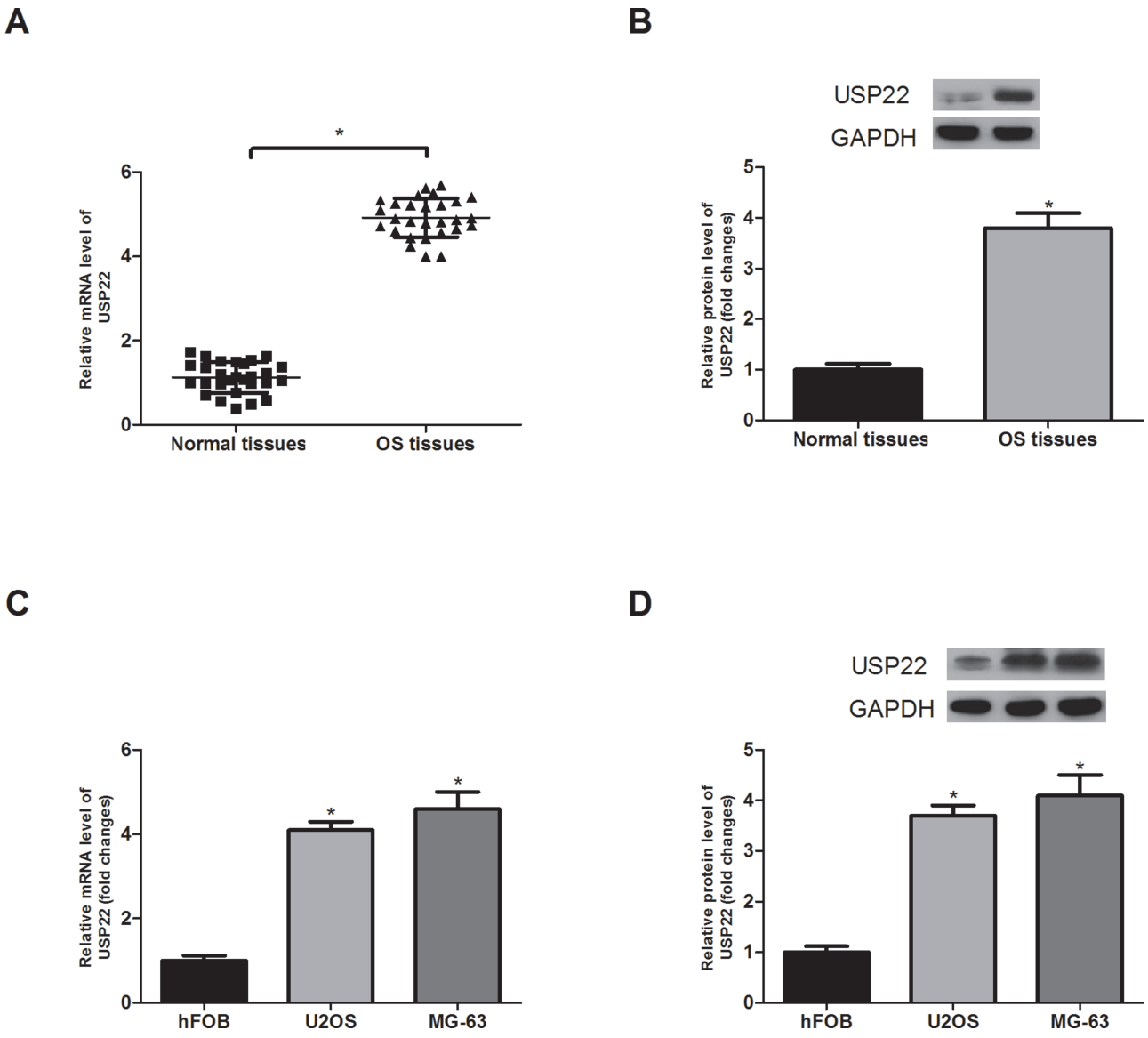

Figure 1. USP22 was upregulated in OS tissues and cell lines. (A, B) The mRNA and protein expression levels of USP22 were markedly increased in OS tissues compared with the corresponding normal tissues. (C, D) The expression of USP22 was much higher in the OS cell lines U2OS and MG-63 than in the osteoblastic cell line hFOB at both mRNA and protein levels. *$p<0.05$.

(reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-GACAAGCTTCCCGTTCTCAG-3′ (reverse). GAPDH was used as an internal control. Data analysis was performed through the $2^{-\Delta\Delta}$CT method[21].

Western Blot Analysis

Lysis buffer was used to extract total protein from tissues and cells. After centrifugation of lysates at 13,200 rpm for 10 min, the supernatants were collected for Western blot analysis. The protein was resolved by 12% SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Subsequent to blocking with nonfat milk at room temperature for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against USP22, E-cadherin, N-cadherin, vimentin, p-PI3K, PI3K, p-Akt, Akt, or GAPDH. After washing with TBST three times, the membranes were incubated with HRP-conjugated secondary antibody. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Protein bands were visualized by enhanced chemiluminescence. The protein expression was analyzed using an Odyssey infrared laser imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Small Interfering RNA (siRNA) and Transfection

The siRNA method was used for USP22 downregulation in U2OS and MG-63 cells. USP22 siRNA was purchased from RiboBio (Guangzhou, P.R. China), and the sequence was 5′-GGAGAAAGAUCACCUCGAA dTdT-3′. Ncontrol_05815 (NCsi; RiboBio) was used as a negative control. U2OS and MG-63 cells were transfected with USP22si or NCsi using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfection efficiency was determined via Western blot analysis.

MTT Assay

Cell proliferation was tested using an MTT assay. Transfected cells were seeded into 96-well plates at a density of 5×10^3 cells/well, followed by incubation for 24, 48, 72, and 96 h, respectively. After 25 µl of MTT (5 mg/ml; Sigma-Aldrich) was added to each well, cells were further incubated for 4 h. Subsequently, supernatants were removed, and 150 µl of DMSO (Sigma-Aldrich) was added to each well. The absorbance value (OD) was measured at a wavelength of 450 nm.

Transwell Assay

Transwell chambers (8-µm pore size; Costar, Cambridge, MA, USA) were used for the cell invasion assay. Briefly, 5×10^4 transfected cells were suspended in RPMI medium and seeded into the Matrigel-coated upper chamber. RPMI medium (500 µl) containing 10% FBS was added to the lower chamber. After incubation for 24 h with 5% CO_2 at 37°C, cells invading the lower surface of the filters were fixed with cold methanol and stained with 0.1% crystal violet. The number of invading cells was counted in four randomly selected fields under a microscope (400×).

In Vivo Xenograft Tumor Assay

Male BALB/c nude mice (4 to 6 weeks old) were purchased from the Experimental Animal Center of Henan University and maintained under specific pathogen-free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of Henan University. U2OS cells (1×10^5) transfected with USP22si or NCsi were suspended in 200 µl of PBS and subcutaneously injected into the left flank of nude mice ($n=6$ per group). Tumor volume was measured every 7 days and calculated by the following formula: tumor volume = (length × width^2)/2. After 35 days, mice were euthanatized, and the tumors were weighed.

To test tumor metastasis in vivo, 1×10^5 transfected U2OS cells were suspended in 100 µl of PBS and injected into the lateral tail vein of nude mice ($n=6$ per group). Thirty-five days later, mice were sacrificed to evaluate lung metastasis. The number of lung nodules was counted under a dissecting microscope.

Statistical Analysis

Data were shown as means ± standard deviation (SD). Differences were analyzed by the Student's *t*-tests. Statistical analysis was performed using SPSS 19.0 software (Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

RESULTS

USP22 Was Upregulated in OS Tissues and Cell Lines

To investigate the role of USP22 in OS, we first examined the expression levels of USP22 in OS tissues via RT-PCR and Western blot analysis. The results indicated that USP22 was significantly upregulated in OS tissues compared with their corresponding normal tissues (Fig. 1A and B). In addition, we detected the expression of USP22 in the OS cell lines U2OS and MG-63. The expression levels of USP22 were remarkably increased in OS cell lines compared with the osteoblastic cell line hFOB (Fig. 1C and D). These results showed that USP22 was upregulated in both OS tissues and cell lines.

Downregulation of USP22 Inhibited OS Cell Proliferation and Invasion In Vitro

To explore the effect of USP22 on OS cell proliferation and invasion, we decreased the expression of

USP22 in U2OS and MG-63 cells via siRNA transfection. USP22 downregulation in U2OS and MG-63 cells was confirmed by Western blot analysis (Fig. 2A and B). Cell proliferation and invasion were measured using the MTT and Transwell assays, respectively. The assay results showed that USP22 downregulation significantly inhibited the proliferation (Fig. 2C and D) and invasion (Fig. 2E and F) of U2OS and MG-63 cells.

Downregulation of USP22 Inhibited OS Tumor Growth and Metastasis In Vivo

To assess the effect of USP22 downregulation on OS tumor growth in vivo, U2OS cells transfected with USP22si or NCsi were subcutaneously injected into the left flank of nude mice ($n=6$). Tumor volume was measured every 7 days. After 35 days, mice were sacrificed, and tumors were weighed. The tumor growth assay showed that the tumor volume and weight of the U2OS/USP22si group were markedly reduced compared with that of the U2OS/NCsi group (Fig. 3A and B). We also investigated the effect of USP22 downregulation on OS tumor metastasis in vivo. Transfected U2OS cells were injected into the lateral tail vein of nude mice ($n=6$). Thirty-five days later, mice were sacrificed to check lung metastasis. The number of lung nodules was significantly decreased in the U2OS/USP22si group compared with the U2OS/NCsi group (Fig. 3C). These results suggest that USP22 downregulation inhibited OS tumor growth and metastasis in vivo.

Downregulation of USP22 Inhibited the EMT Process in OS Cells

Western blot analysis was used to detect the expression of EMT-related markers in U2OS cells after USP22 downregulation. USP22 downregulation significantly increased the expression of E-cadherin (epithelial marker) but decreased the expression of N-cadherin and vimentin (mesenchymal markers) (Fig. 4). The results suggested that USP22 downregulation obviously suppressed the EMT process in OS cells.

Downregulation of USP22 Inhibited the Activation of PI3K/Akt Signaling Pathway

Increasing evidence has proven the significant role of the PI3K/Akt signaling pathway in cancer progression[22]. Therefore, we explored the effect of USP22 downregulation on the PI3K/Akt signaling pathway. The protein expression of p-PI3K and p-Akt was remarkably decreased in USP22si-transfected U2OS cells compared with the control cells (Fig. 5A). The total protein levels of PI3K and Akt were unaffected. We also examined the effect of the Akt inhibitor (MK-2206) on OS cell invasion mediated by downregulation of USP22. First, we performed the MTT assay and obtained a dose–response curve of MK-2206 on the growth of U2OS and MG-63 cells (Fig. 5B). Subsequently, we carried out the Transwell assay and found that the USP22si-inhibited invasion of U2OS (Fig. 5C) and MG-63 (Fig. 5D) cells was dramatically potentiated after treatment with MK-2206.

DISCUSSION

OS, a common bone tumor, has a high potential for malignancy and metastasis[1]. With the development of more and more advanced treatment, the survival rate of OS patients has been significantly increasing but is still not satisfactory. Therefore, it will be of great help to explore a novel therapeutic target for OS.

Recently, USP22 has attracted widespread attention because of its identification as a key component of the 11-gene signature capable of predicting metastasis, recurrence, and therapy resistance in various cancers[11]. Several previous reports have demonstrated frequent overexpression of USP22 in some epithelial cancers such as breast cancer, colorectal cancer, and gastric cancer[15,19,20,23]. These findings suggest that USP22 may be implicated in carcinogenesis.

However, there have been no studies, to our knowledge, investigating the expression of USP22 in OS and its association with OS progression. We addressed these issues in the present study. We first detected the expression levels of USP22. The results showed that USP22 was upregulated in OS tissues and cell lines, which were consistent with previous studies. We also suggested that downregulation of USP22 inhibited OS cell proliferation and invasion in vitro. We performed in vivo experiments to verify the in vitro results. As expected, downregulation of USP22 suppressed OS tumor growth and metastasis in vivo. The findings in our study agreed with the fact that a growing body of evidence indicated the suppressive effect of USP22 knockdown on malignant behavior of cancer cells. For instance, Ding et al. demonstrated that USP22 silencing inhibited in vitro proliferation and in vivo tumor growth of non-small cell lung cancer cells[24]. Similarly, Zhao et al. reported that USP22 depletion suppressed cell survival and proliferation as well as tumor growth and lung metastasis of anaplastic thyroid carcinoma cells[25]. These observations support the notion that USP22 acts as an oncogene in cancer development.

It is well known that the PI3K/Akt signaling pathway is a player in the regulation of cell proliferation, migration, and invasion of diverse cancers[26–28]. PI3K is activated by oncogenes, and activation of PI3K contributes to cancer cell growth and survival[29]. Akt, a key molecule in the PI3K pathway, is frequently found to

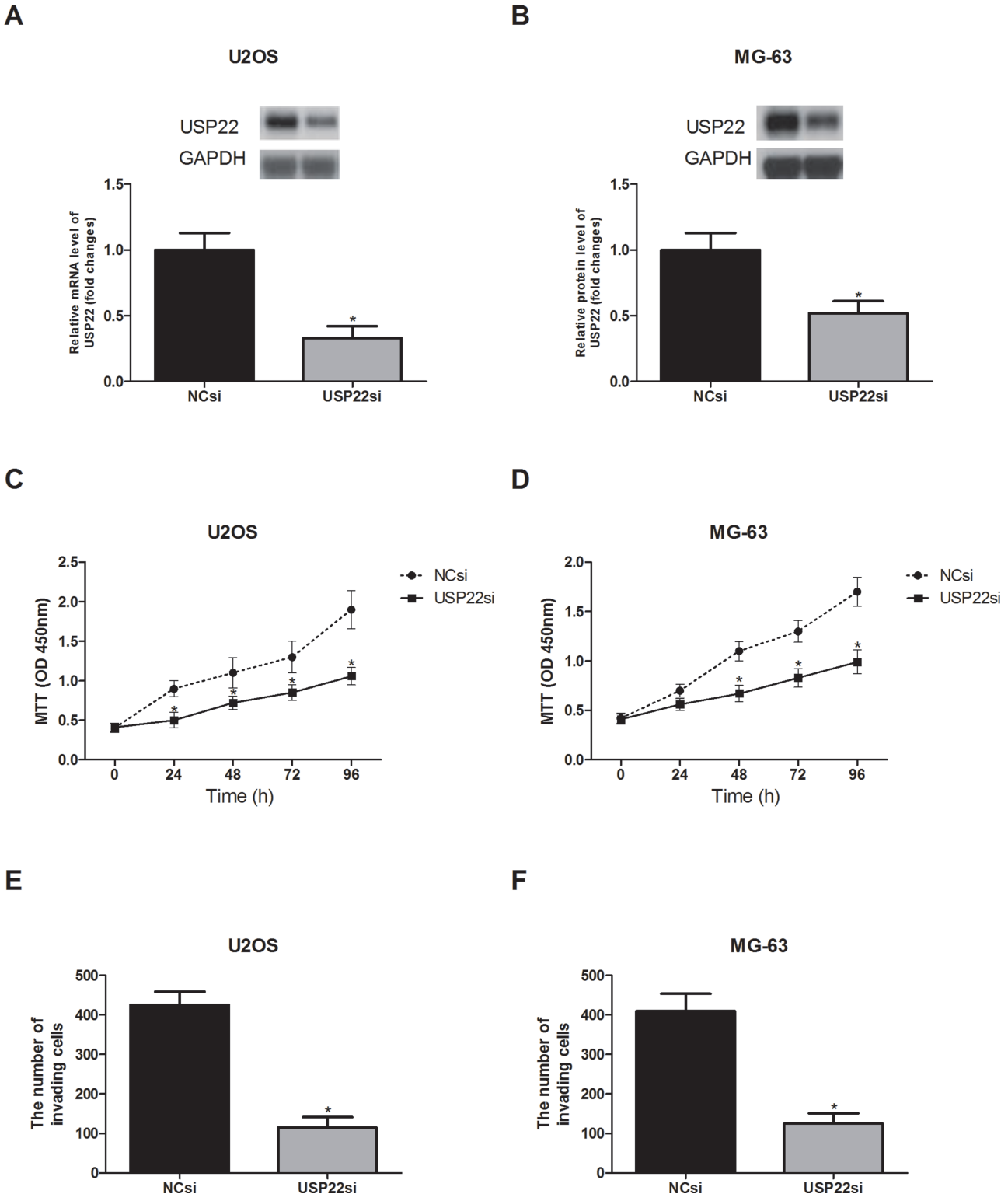

Figure 2. Downregulation of USP22 inhibited OS cell proliferation and invasion in vitro. (A, B) USP22 downregulation in U2OS and MG-63 cells was confirmed by Western blot analysis. (C, D) USP22 downregulation significantly inhibited the proliferative ability of U2OS and MG-63 cells. (E, F) USP22 downregulation obviously reduced the number of invading U2OS and MG-63 cells. *$p<0.05$.

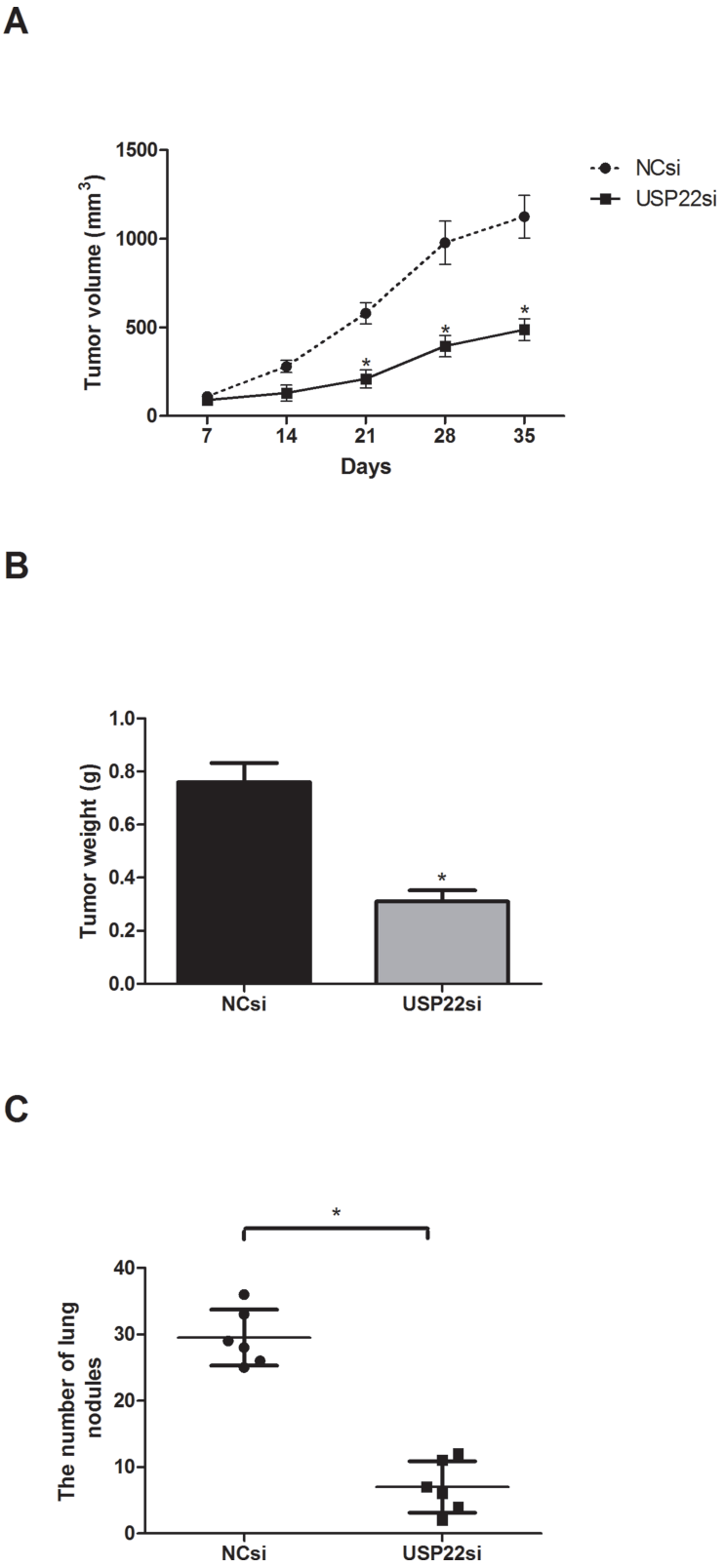

Figure 3. Downregulation of USP22 inhibited OS tumor growth and metastasis in vivo. (A, B) USP22 downregulation significantly decreased the tumor volume and weight of the U2OS/USP22si group compared with the U2OS/NCsi group. (C) The number of lung nodules was obviously reduced in the U2OS/USP22si group compared with the U2OS/NCsi group. *$p<0.05$.

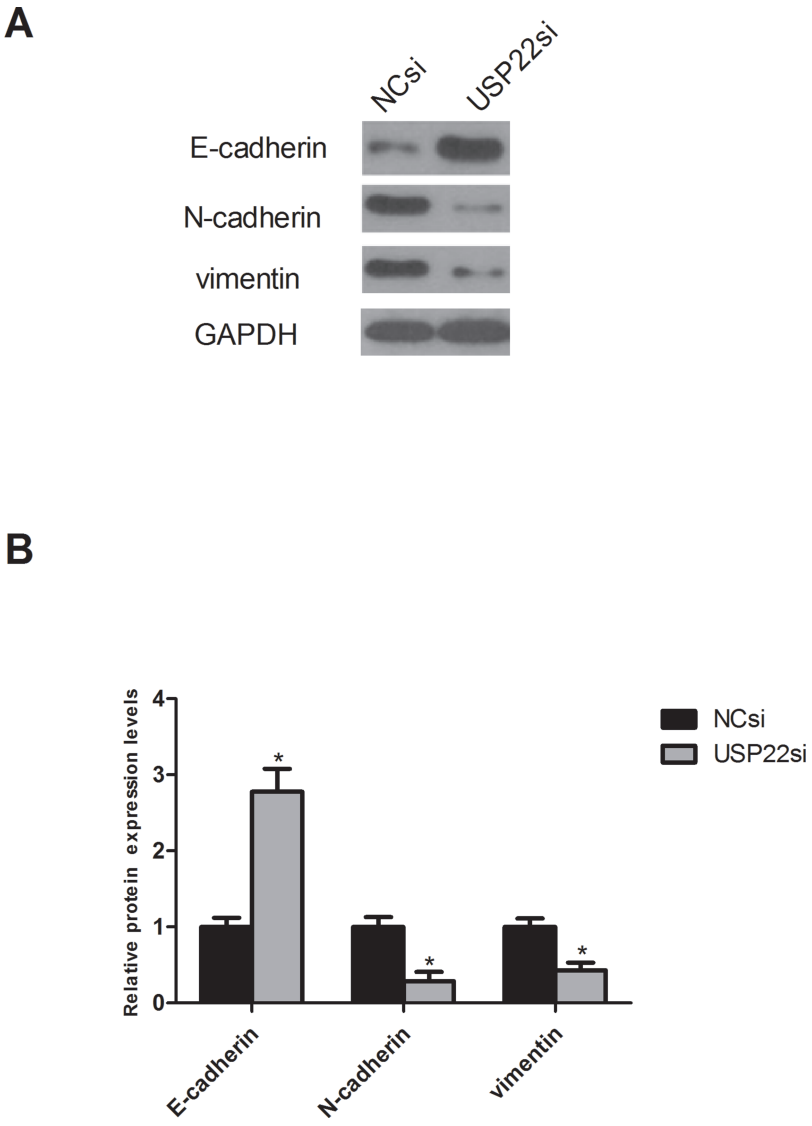

Figure 4. Downregulation of USP22 inhibited the EMT process in OS cells. (A) The protein expression levels of EMT-related markers in U2OS cells were detected by Western blot. (B) The protein expression of EMT-related markers in U2OS cells was quantified by an Odyssey infrared laser imaging system. *$p < 0.05$.

be activated in cancers, impacting various downstream targets[30,31]. Thus, the PI3K/Akt signaling pathway is considered to be a potential target for cancer therapies. More importantly, the PI3K/Akt signaling pathway is found to play a significant role in OS progression[32]. Therefore, we inferred that USP22 downregulation exerted the suppressive effect on OS cells via inactivating the PI3K/Akt signaling pathway. To prove our hypothesis, we detected the protein expression levels of p-PI3K, PI3K, p-Akt, and Akt after USP22 downregulation. The results showed that USP22 downregulation remarkably decreased the protein expression of p-PI3K and p-Akt without change in the total protein levels of PI3K and Akt. In addition, we tested the effect of an Akt inhibitor (MK-2206) on USP22si-mediated OS cell invasion, finding that MK-2206 significantly potentiated USP22si-inhibited OS cell invasion. These results suggest that USP22 downregulation inhibits OS cells by suppressing the PI3K/Akt pathway. Considering the complex relationship affecting cancer development, the mechanisms behind the oncogenic role of USP22 in OS need to be studied further.

In conclusion, we demonstrated that USP22 was highly expressed in OS tissues and cells lines. Downregulation of USP22 inhibited OS cell proliferation, invasion, and EMT in vitro. In addition, downregulation of USP22

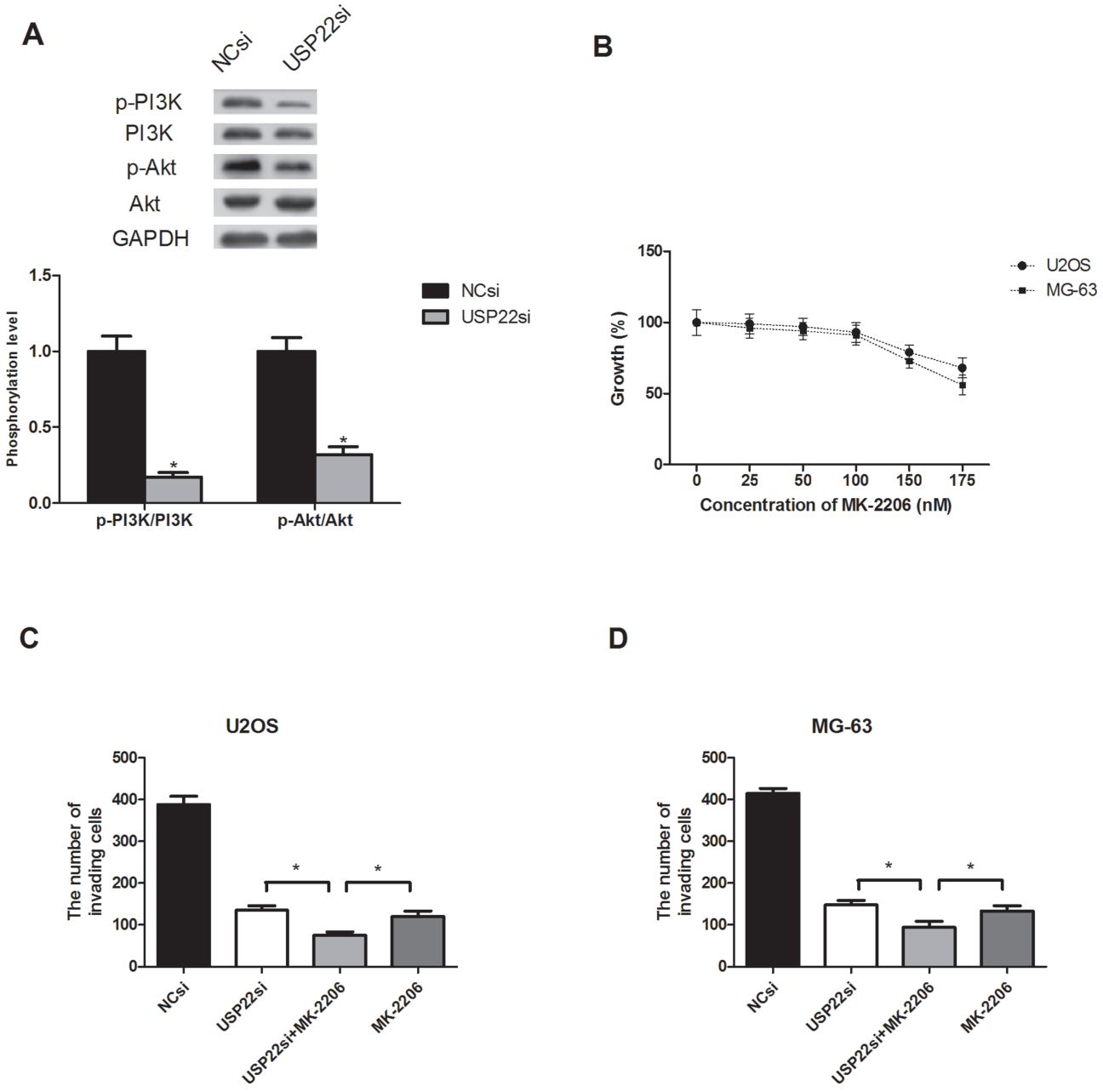

Figure 5. Downregulation of USP22 inhibited the activation of the PI3K/Akt signaling pathway. (A) The protein expression levels of p-PI3K, PI3K, p-Akt, and Akt in U2OS cells were detected by Western blot. (B) U2OS and MG-63 cells were incubated with different concentrations of MK-2206. Cell growth was determined after 24 h of using MTT assay. (C, D) U2OS and MG-63 cells were transfected with USP22si or NCsi in the presence or absence of MK-2206 (100 nM). The Transwell assay was carried out to measure cell invasion. *$p<0.05$.

suppressed OS tumor growth and metastasis in vivo. We also found that the PI3K/Akt signaling pathway was involved in the tumor-promoting effect of USP22 on OS progression.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

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