Gene silencing of USP1 by lentivirus effectively inhibits proliferation and invasion of human osteosarcoma cells

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Abstract. Osteosarcoma is the most frequent malignant bone tumor, affecting the extremities of adolescents and young adults. Ubiquitin-specific protease 1 (USP1) plays a critical role in many cellular processes including proteasome degradation, chromatin remodeling and cell cycle regulation. In the present study, we discovered that USP1 was overexpressed in 26 out of 30 osteosarcoma tissues compared to cartilage tumor tissues and normal bone tissues. We then constructed a lentiviral vector mediating RNA interference (RNAi) targeting USP1 and demonstrated that it significantly suppressed the mRNA and protein expression of the USP1 gene in U2OS cells. Knockdown of USP1 inhibited the growth and colony-forming, as well as significantly reduced the invasiveness of U2OS cells. Western blot analysis indicated that suppression of USP1 downregulated the expression of many proteins including SIK2, MMP-2, GSK-3β, Bcl-2, Stat3, cyclin E1, Notch1, Wnt-1 and cyclin A1. Most of these proteins are associated with tumor genesis and development. RNAi of SIK2 significantly decreased SIK2 protein expression and inhibited the ability of forming colonies, as well as induced apoptosis and reduced the invasiveness of U2OS cells. Collectively, our results suggest that silencing USP1 inhibits cell proliferation and invasion in U2OS cells. Therefore, USP1 may provide a novel therapeutic target for the treatment of osteosarcoma.

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Introduction

Osteosarcoma is the most common bone malignancy in the pediatric age group, with a very high propensity for local invasion and distant metastasis (1,2). In recent years, despite the development of new multimodal therapeutics, the prognosis for patients with this disease is generally poor (3,4). Therefore, effective therapeutic methods for osteosarcoma are urgently needed.

Ubiquitin (Ub), a kind of important signaling protein, is involved in many cellular processes, including cell cycle regulation, DNA damage response, chromatin remodeling and proteasome degradation. Similar to phosphorylation, ubiquitin modification is reversible, and deubiquitinases or DUBs are known to mediate ubiquitin removal (5). DUBs can remove Ub moieties from Ub-protein conjugates, resulting in reduced ubiquitination signaling (6).

The misexpression of DUBs has been demonstrated to be associated with a number of human diseases, especially cancers (7). Among them, USP1 is one of the best-characterized human DUBs, which is involved in the control of cell differentiation and plays an important role in the regulation of the cellular response to DNA damage (8).

USP1 has been reported to be overexpressed in several human malignant tumors (9,10). Moreover, USP1 has been identified as the DUB responsible for deubiquitinating Fanconi anemia complementation group I (FANCI), Fanconi anemia group D2 (FANCD2) and proliferating cell nuclear antigen (PCNA) in the DNA damage response. PCNA is monoubiquitinated in response to DNA damage that stalls progression of the replication fork and then initiates recruitment of translesion DNA synthesis (TLS) DNA polymerases in the DNA

damage tolerance pathway (11,12). USP1 plays as a regulator of PCNA ubiquitination in the DNA damage response. It may deubiquitinate the DNA replication processivity factor, PCNA, thus, contributing to preventing unscheduled recruitment of error-prone TLS DNA polymerases, as a safeguard against error-prone TLS of DNA (13).

USP1-associated factor 1 (UAF1) stabilizes USP1 and allosterically increases its catalytic activity, which is very low in the absence of the cofactor (14,15). In addition, UAF1 contributes to targeting USP1 to its nuclear substrates. USP1 performs its functions in the context of a heterodimeric complex with UAF1 (16).

RNAi is a powerful approach that silences the expression of endogenous genes via distinct messenger RNA degradation pathways. It may be a potential therapeutic agent for many kinds of diseases, including HIV, cerebrovascular and cardiovascular diseases, viral hepatitis, metabolic diseases, neurodegenerative disorders and cancers (17,18).

In the present study, specific-shRNA with lentivirus vector was employed to knock down USP1 in osteosarcoma U2OS cells, and the effects of USP1 silencing on cell growth and invasion were explored.

Materials and methods

Main reagents. Mouse anti-human USP1 polyclonal antibody was purchased from the ProteinTech Group (Wuhan, China); mouse anti-human monoclonal antibody to Bcl-2 and mouse anti-human polyclonal antibodies to MMP-2, GSK-3β, Stat3, cyclin E1, Notch1, Wnt-1 and cyclin A1 were purchased from ImmunoWay Biotechnology Co. (Plano, TX, USA); MG132 and mouse anti-human monoclonal antibody to USP1 were obtained from Merck Millipore (Darmstadt, Germany); mouse anti-human monoclonal antibody to SIK2 were obtained from BioLegend (San Diego, CA, USA); TRIzol reagent, Lipofectamine™ 2000, Opti-MEM and the SuperScript III reverse transcriptase (RT) kit were obtained from Invitrogen Corp. (Carlsbad, CA, USA); Taq DNA polymerase was purchased from Fermentas, Inc. (Waltham, MA, USA); cell culture media and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA).

Tissue samples, cell lines and cell culture conditions. Tissue samples from 30 osteosarcomas were obtained from patients who underwent surgery at the Third Affiliated Hospital of Soochow University. All participants who had undergone surgical resection signed an informed consent form for this study. All osteosarcoma cases had been confirmed both clinically and pathologically. The experimental protocols for the present study were approved by the Hospital's Protection of Human Subjects Committee.

The human osteosarcoma U2OS cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) in 2014 and were identified by short tandem repeat (STR) method in 2015. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Immunohistochemistry. Surgical specimens were fixed in 10% formalin solution and embedded in paraffin. Four-μm thick sections were deparaffinized, rehydrated and immersed in 3% hydrogen peroxide solution for 15 min to block endogenous peroxidase activity. For antigen retrieval, the sections were boiled in EDTA buffer (pH 9.0) for 10 min. Histologic sections were pre-treated in 10% normal goat serum in TBS for 30 min at room temperature in order to block non-specific binding sites. Then sections were incubated with polyclonal antibody against USP1 at a dilution of 1:100 in phosphate-buffered saline (PBS) at 4˚C for 1 h. After being rinsed five times with PBS, sections were incubated with goat anti-mouse IgG conjugated with horseradish peroxidase at room temperature for 1 h. Sections were developed with diaminobenzidene (DAB) as a chromogen and counterstained with haematoxylin. The sections were analyzed under light microscopy.

USP1 gene silence mediated by lentivirus-delivered shRNA. The human USP1-specific small interfering RNA (siRNA) sequence is 5'-GAAAGCTCCACATCAATAA-3', designed with an online Invitrogen software using the USP1 sequence (GenBank code: NM_003600) as a reference. The non-silencing sequence (5'-TTCTCCGAACGTGTCACGT-3') was used as a scrambled control. The recombinant lentiviral particles were regenerated in 293T cells by co-transfection of the modified pGCSIL-GFP viral vector together with the pHelper 1.0 and pHelper 2.0 plasmids using the Lipofectamine™ 2000 reagent. The lentiviral particles were referred to as LV-USP1 siRNA or USP1-siRNA. We generated lentiviruses that express non-silencing-shRNA as controls and named them scramble siRNA or scr-siRNA. The osteosarcoma cells transfected with the USP1-siRNA or scrambled siRNA were designated USP1-siRNA and scr-siRNA, respectively. For lentiviral transduction, U2OS cells were cultured in 6-well plates at 70% confluence and infected with control lentivirus or USP1-specific siRNA lentivirus at MOI of 20. After 5 days of infection, U2OS cells expressing GFP protein were observed by fluorescence microscopy to determine the infection efficiency.

SIK2 siRNA preparation. The siRNA sequences were designed by commercial software (Applied Biosystems/Ambion, Austin, TX, USA). For SIK2, the siRNA sense sequence is 5-CUA CCGAGAAGUACAAAUADTDT-3' and the antisense sequence is 5'-UAUUUGUACUUCUCGGUAGdTdT-3'. The siRNA sequences employed as negative controls were 5'-GG CCUCAGCUGCGCGACGCdTdT-3' (sense) and 5'-GCGUC GCGCAGCUGGGCCAdTdT-3' (antisense). The selected sequences were confirmed by NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/) to make sure that the selected genes were specifically targeted. Chemically synthesized siRNAs were designed and sequenced by Guangzhou RiboBio, Co., Ltd. (Guangzhou, China).

SIK2 siRNA transfection. U2OS cells were transiently transfected with synthetic siRNA using Lipofectamine 2000 reagent according to the manufacturer's instructions. Briefly, U2OS cells were seeded onto 6-well plates in the maintenance medium at a density of $2x10^5$ cells/well and allowed to grow for 12 h. Then, 5 μl Lipofectamine 2,000 and 200 pmol siRNA were diluted in serum-free medium to a total volume of 250 μl and incubated for 20 min at room temperature. After the cells were washed with DMEM medium without FBS, the diluted

siRNA-Lipofectamine mix was added to each well and incubated for 24 h. Then the mix was replaced with fresh DMEM medium containing 10% FBS. Forty-eight hours after the transfection, the U2OS cells were collected for protein and RNA isolation. The osteosarcoma cells transfected with the SIK2-siRNA or scrambled siRNA were designated SIK2-siRNA and scr-siRNA, respectively.

Quantitative real-time PCR analysis. Total RNA was extracted from osteosarcoma cells using TRIzol reagent following the manufacturer's instructions. RNA samples (1 μg/reaction) were reverse-transcribed with the RevertAid First Strand cDNA Synthesis kit according to the manufacturer's recommended protocol. The RT reaction was used for amplification with Taq polymerase and a SYBR-Green RT-PCR kit (Takara, Kyoto, Japan) was used to detect specific real-time RT-PCR products. The resulting cDNA was amplified using specific primers, which were designed by using the GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). The primer sequences were: For USP1, sense: 5'-AGGTTG CTAGTACAGCGTTTGC-3' and antisense: 5'-CACTGGATT CCTTGTTTCTATCAGA-3'; for SIK2, sense: 5'-AGTCTGA AGCCAGGGAAAA-3' and antisense: 5'-CATGTTGCCAGC AGTTCACC-3'; for β-actin, sense: 5'-GGCACTCTTCCAGC CTTCC-3' and antisense: 5'-GAGCCGCCGATCCACAC-3'. Relative gene-expression levels were calculated using the comparative Ct method, also called the $2^{-\Delta\Delta CT}$ analysis method.

Cell proliferation assay. U2OS cells were seeded onto 96-well plates and incubated in corresponding media supplemented with 10% FBS. After 24, 48, 72 and 96 h of incubation, 10 μl of Cell Counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added into each well, followed by 4 h of incubation. The optical density values were measured at 450 nm using a Bio-Rad microplate reader to determine cell viability. The results were based on three independent experiments and are presented as mean ± SD.

Colony-forming assay. Soft agar colony-formation assay was used to determine the effect of USP1 knockdown on the transformation capability of U2OS cells. In brief, U2OS cells were counted and inoculated in 6-well plates at a density of 3,000 cells/well. After being cultured at 37˚C in 5% CO_2 in a humidified incubator for 2 weeks, U2OS cells were immobilized by 4% paraformaldehyde and stained using Giemsa dye for 20 min. The cells were rinsed with distilled water. Plates were then scanned and photographed under an inverted microscope. All experiments were performed three times.

Flow cytometric analysis. The U2OS cells were harvested and washed with ice-cold PBS twice. Cells were then resuspended with 100 μl of Annexin V binding buffer, and incubated with APC-labeled Annexin V at room temperature for 15 min. Fluorescence-activated cell sorting (FACS) analysis for Annexin V staining was performed to determine cell apoptosis. Each experiment was performed in triplicate.

In vitro invasion assay. Twenty-four hours after the infection, the invasion ability of U2OS cells was determined using a Transwell chamber. A total of 40 μl Matrigel (10 mg/ml) (BD Biosciences, San Jose, CA, USA) was coated to 48-well plates and polymerized at 37˚C for 30 min. U2OS cells were collected into DMEM medium, supplemented with 1% FBS. In brief, a 100 μl cell suspension containing $2.0x10^5$ cells was added to the upper chamber of each insert. For each Transwell chamber, 500 μl of DMEM supplemented with 20% FBS was added in the lower chamber. After 24 h of incubation at 37˚C, cells and Matrigel on the upper surface of the well were removed. Cells invaded to the lower chamber were fixed with methanol, stained with 0.5% crystal violet for 20 min and then were counted under the light microscope. In addition, invaded cells were segregated, lysed and quantified at 570 nm using spectrophotometer to evaluate the amount of cells.

Western blot analysis. The U2OS cell pellets were lysed with protein extraction solution and incubated at 20˚C for 20 min. After protein quantification, the samples were separated by electrophoresis in SDS-PAGE and then transferred to a PVDF (polyvinylidene fluoride) membrane. After blocking in Tris-buffered saline Tween-20 (TBST) containing 5% non-fat milk for 1 h at room temperature, membranes were subsequently incubated with primary antibodies against USP1, SIK2, matrix metalloproteinase-2 (MMP-2), glycogen synthase kinase-3β (GSK-3β), B-cell lymphoma 2 (Bcl-2), signal transducer and activator of transcription 3 (Stat3), cyclin E1, Notch homolog 1 (Notch1), Wingless-type protein-1 (Wnt-1) and cyclin A1 at 4˚C overnight. After incubation with secondary antibodies (1:1,000; peroxidase-conjugated anti-mouse IgG) for another 2 h, the membranes were washed in TBST buffer and protein were detected using enhanced chemiluminescence. GAPDH and β-actin bands were used as loading controls.

Effect of MG132 on SIK2 protein levels. The U2OS cells were cultured in 6-well plates at a density of $4x10^4$ cells/well. After 24 h of incubation, cells were treated with various concentrations of MG132 (2, 5 and 10 μmol/l) and cultured for 0, 4, 8 and 16 h. Level of SIK2 protein was determined using western blot analysis.

Statistical analysis. Data are reported as the mean ± SEM of three independent experiments. Statistical analysis between comparable groups was performed using a one-way ANOVA by using GraphPad Prism 5.0 software. In each case, $P<0.05$ was considered statistically significant.

Results

USP1 is overexpressed in osteosarcoma samples. We examined the expression of USP1 in 30 samples from patients with osteosarcoma. Of the 30 patients, 26 (86.67%) were classified as positive expression of USP1. USP1 protein with positive staining was shown in the nuclei and cytoplasm of osteosarcoma tissues (Fig. 1A-a), while rare visible USP1 staining was detected in cartilage tumor tissues (Fig. 1A-b) and normal bone tissues (Fig. 1A-c). The USP1 expression in osteosarcoma tissues was significantly higher than that in cartilage tumor tissues and normal bone tissues.

Construction of a lentiviral vector mediating RNAi targeting of USP1 (LV-USP1 siRNA) and its effects on USP1 expres-

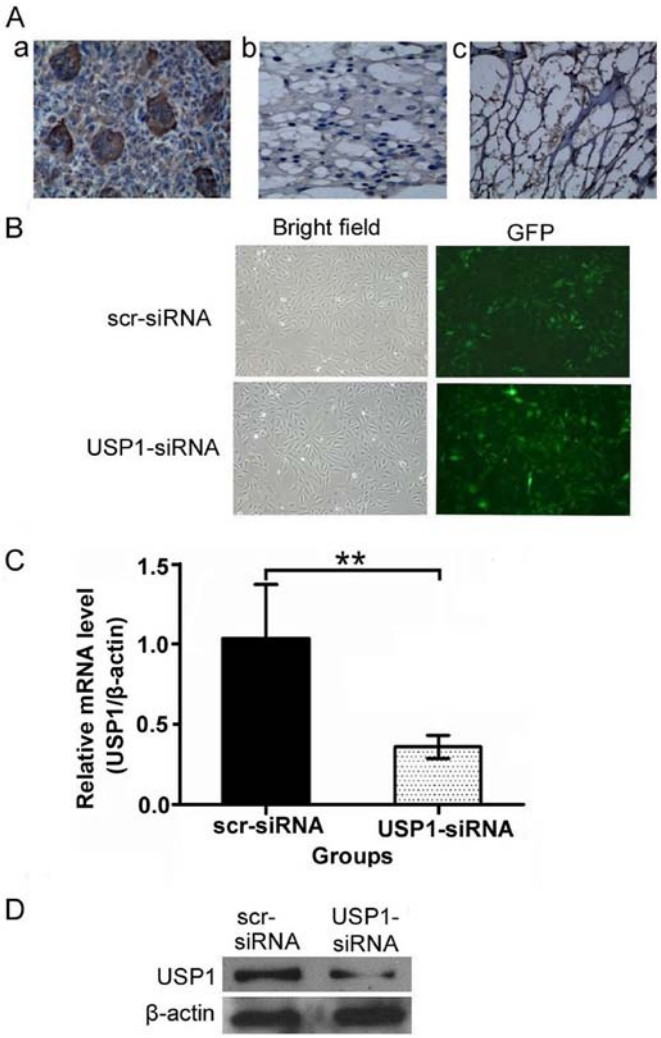

Figure 1. Analysis for expression of USP1 in osteosarcoma tissues and lentivirus infection in U2OS cells. (A) Immunohistochemistry analysis. Immunohistochemistry analysis of USP1 protein expression in osteosarcoma tissues (a), cartilage tumor tissues (b) and normal bone tissues (c) (magnification, x400). (B) Lentivirus infection in osteosarcoma cells. Green fluorescence indicates the successful delivery of siRNA into U2OS cells (magnification, x400). (C) The USP1 mRNA expression level was quantified by real-time RT-PCR. USP1 siRNA significantly decreased the level of USP1 mRNA in U2OS cells. (D) The USP1 protein expression level was quantified by western blot analysis. USP1 siRNA significantly decreased the level of USP1 protein in U2OS cells. *P<0.05, **P<0.01, in comparison to the scr-siRNA group.

sion. To further illuminate the relationship between USP1 and osteosarcoma, we constructed a lentivirus-based USP1-specific siRNA vector and a scramble-siRNA vector. The two vectors were infected into U2OS cells for 3 days and >90% of the cells showed a green fluorescence indicating successful infection (Fig. 1B). To confirm whether the USP1-siRNA had silenced the expression of USP1 mRNA and protein, real-time PCR and western blot analysis were performed on lentivirus-infected cells. The results showed that both mRNA (Fig. 1C) and protein (Fig. 1D) expression levels of USP1 were significantly reduced by infection of USP1-siRNA, compared to scr-siRNA. These data suggest that the infection with USP1-siRNA effectively downregulates USP1 expression in U2OS cells.

Effects of USP1-siRNA on cell viability. CCK-8 assay was performed to study the effect of USP1-siRNA on U2OS cell growth. As shown in Fig. 2A, the cell growth of USP1-siRNA groups showed a significant (P<0.01) reduction in cell viability in comparison to the scr-siRNA groups. The result of a colony formation assay showed that the number of colonies of the USP1-siRNA group (2249.33±156.31) was significantly less than that of the scr-siRNA group (2751.00±83.07) in U2OS cells (P<0.01) (Fig. 2B). These results demonstrate that the reduction in USP1 expression decreased the ability of U2OS cells to form colonies. Furthermore, these results suggest that cell proliferation is reduced due to the silencing of the USP1 gene.

USP1 silencing induces apoptosis in U2OS cells. To determine whether USP1 depletion induced cell apoptosis, flow cytometry was used after the silencing of USP1. The flow cytometric analysis shows that the percentage of apoptotic U2OS cells was 3.88±0.22% in the scr-siRNA group cells, and the percentage of apoptotic cells increased to 20.57±0.64% in the USP1-siRNA group cells (P<0.01) (Fig. 2C). These data suggest that the depletion of USP1 specifically induces apoptosis of the U2OS cells.

Depletion of USP1 inhibits the invasion of U2OS cells. Next, we performed an *in vitro* invasion assay, the results showed that OD value of 570 nm was 0.27±0.04 in the scr-siRNA group and 0.17±0.01 in the USP1-siRNA group (P<0.01) (Fig. 2D). These results demonstrate that USP1-siRNA could reduce the invasion of U2OS cells.

Western blot analysis. The protein levels of related molecules were also examined. The results showed that USP1 knockdown downregulated SIK2, MMP-2, GSK-3β, Bcl-2, cyclin E1, Notch1, Stat3, cyclin A1 and Wnt-1 in U2OS cells (Fig. 3A). These results suggest that USP1 downregulation inhibits growth and invasion of U2OS cells through these proteins.

MG132 affects SIK2 protein level. We further examined whether proteasome inhibitor MG132 can increase the level of SIK2 protein in U2OS cells. The result showed that cells treated with MG132 (5 μmol/l) for 4 or 8 h exhibited an elevation of SIK2 protein (Fig. 3B). It suggests that MG132 upregulates SIK2 expression by decreasing its protein degradation, and therefore, inhibition of proteasome function increases SIK2 protein level in U2OS cells.

SIK2 siRNA significantly inhibits the mRNA and protein expression of SIK2. Real-time PCR and western blot analysis were performed to confirm whether the SIK2 siRNA inhibited the expression of SIK2 mRNA and protein. The results showed that SIK2 mRNA and protein expression in SIK2-siRNA group were significantly inhibited compared to scr-siRNA group as shown in Fig. 4A and B. It indicates that the targeted SIK2 siRNA inhibited significantly the expression of SIK2 gene.

Downregulation of SIK2 suppresses colony formation in U2OS cells. Colony-forming assays were performed to determine the effect of SIK2 deletion on colony formation. The

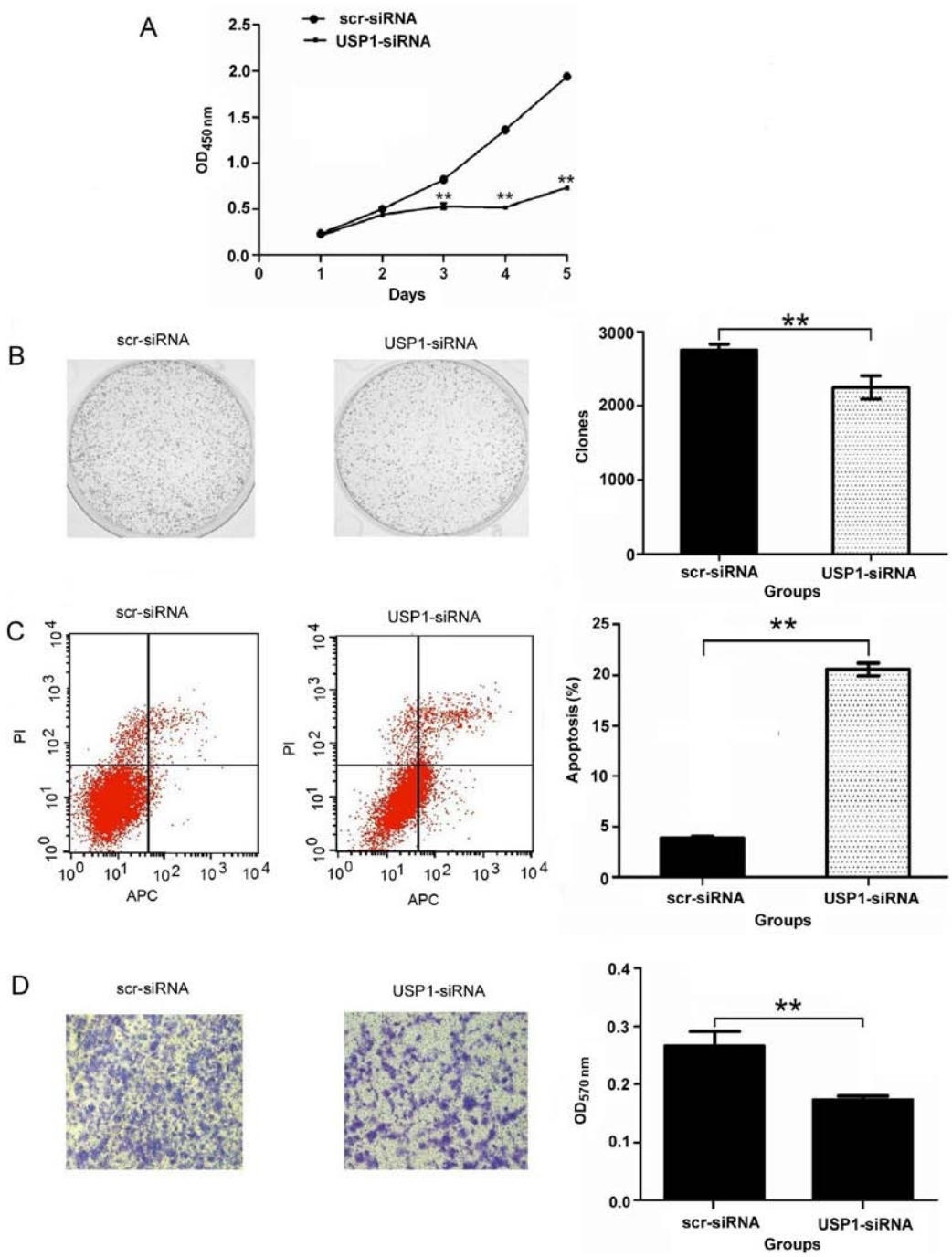

Figure 2. Silencing effect of USP1 siRNA in U2OS cells. (A) Effect of USP1 silencing on the proliferation of osteosarcoma cells. The impact of USP1 suppression on proliferation of U2OS cells was assessed by CCK-8 assay, USP1 siRNA depressed the growth curve of U2OS cells as compared with the scr-siRNA group. (B) Inhibition of USP1 decreased colony formation of U2OS cells. Colony formation assay showed that USP1 siRNA decreased the clone numbers of U2OS cells as compared with scr-siRNA group. (C) The effects of USP1 siRNA on apoptosis of U2OS cells was determined by flow cytometric analysis. Flow cytometric analysis showed that knockdown of USP1 expression increased cell apoptosis in U2OS cells. (D) Effects of USP1 siRNA on invasion of U2OS cells. Depletion of USP1 inhibits the invasion of U2OS cells. *P<0.05, **P<0.01 in comparison to the scr-siRNA group.

results showed that the number of colonies of the SIK2-siRNA group (1492.00±74.65) was significantly less than that of the scr-siRNA group (1713.00±159.46) in U2OS cells (P<0.01) (Fig. 4C). This clearly demonstrates that the number of the colonies decreased significantly with the SIK2 deletion.

SIK2 silencing induces apoptosis in U2OS cells. The flow cytometric analysis shows that the percentage of apoptotic U2OS cells was 8.05±0.20% in the scr-siRNA group cells, and the percentage of apoptotic cells increased to 27.09±3.07% in the SIK2-siRNA group cells (P<0.01) (Fig. 4D). It indicates that the percentage of apoptotic cells increased significantly with the SIK2 deletion.

SIK2 is involved in U2OS cell invasion. The images of cells stained with crystal violet suggests that knocking down the expression of SIK2 resulted in the decrease of the amount of invaded cells. Additional observation of absorption assay also

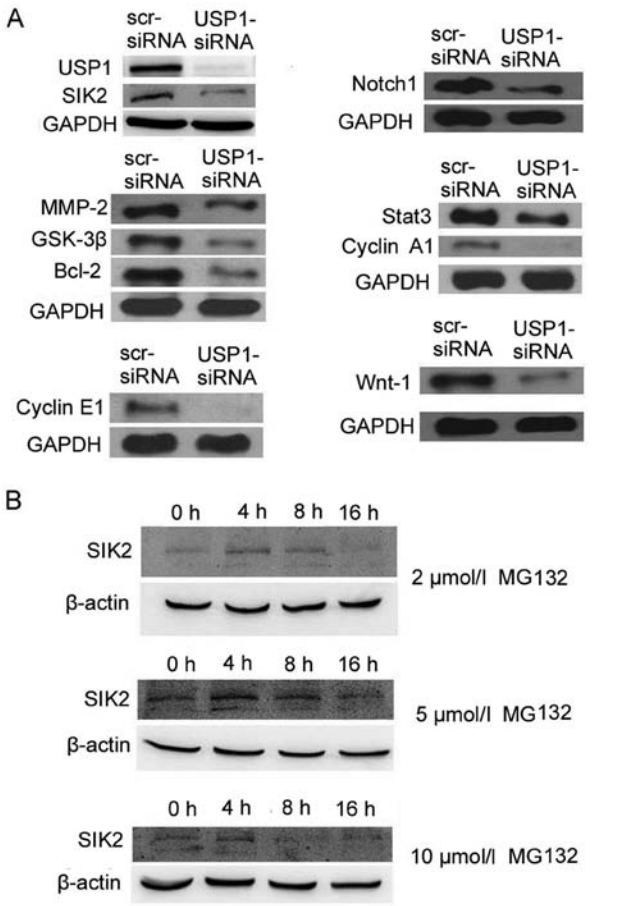

Figure 3. Western blot analysis. (A) The protein levels of related molecules. Protein expression levels of USP1, SIK2, MMP-2, GSK-3β, Bcl-2, cyclin E1, Notch1, Stat3, cyclin A1 and Wnt-1 were significantly decreased after USP1 silencing. (B) MG132 affects SIK2 protein level in U2OS cells. U2OS cells treated with MG132 (5 μmol/l) for 4 or 8 h exhibited an elevation of SIK2 protein.

proved that the migration ability of U2OS cells reduced after knocking down the expression of SIK2. The results showed that OD value of 570 nm was 0.13±0.02 in the SIK2-siRNA group and is significantly less than the reading in the scr-siRNA group (0.19±0.01, P<0.01) (Fig. 4E), all of these data suggest that SIK2 was involved in the invasion of U2OS cells.

Discussion

Osteosarcoma occurs most frequently at the metaphysis of long bones during longitudinal growth spurts in children and young adults (19). The treatment and prognosis for this disease still need to be improved.

USP1 belongs to the cysteine protease family, the USP1 gene encodes a 785 amino acid protein with a predicted molecular weight of 88.2 kDa (20). USP1 contains the conserved USP domain that characterizes this DUB family, with an amino-terminal Cys box motif and a carboxy-terminal, His box motif that contain the catalytic residues (Cys90, His593 and Asp751) (15,21). Notably, it has been uncovered that the catalytic activity of USP1 is stimulated through the formation of a tight complex with a WD40 repeat protein UAF1 (22). UAF1 plays a critical role as a cofactor and UAF1 binding induces conformational changes in USP1 active site increasing the enzyme activity dramatically by stabilizing it. Importantly, studies show that several inhibitors of the USP1/UAF1 complex act synergistically with cisplatin in cancer-derived cell lines, indicating that this complex may represent a potential therapeutic target in cancer (23-25).

DUBs play crucial roles in cancer development, progression and metastasis (26). Targeting DUBs is a new strategy for anti-tumorigenic therapeutics (27). USP1-null mice were hypersensitive to DNA damage, it is likely that targeting USP1 could increase the sensitivity of cancer cells to DNA damaging agents (28).

In the present study, we found that silencing of USP1 could inhibit osteosarcoma cell proliferation and invasion, which provides direct evidence that USP1 may serve as a target for such tumor treatment. The detailed mechanisms underlying how USP1 functions in osteosarcoma still need to be elucidated in the future.

Salt-inducible kinase 2 (SIK2) is a multifunctional serine/threonine kinase of the AMP-activated protein kinase (AMPK) sub-family, and it plays a role in cAMP response element-binding protein (CREB)-mediated gene transcription. Previous studies have demonstrated that increased SIK2 expression is significantly correlated with poor prognosis in overall survival in patients with high-grade serous ovarian cancer (HGSC). Recent reports have shown that SIK2 is required for bipolar mitotic spindle formation and is a potential target for ovarian cancer therapy. Examination of SIK2 found that it regulates mitotic progression and transcription in prostate cancer cells. Knockdown of SIK2 expression inhibits growth and induces cell cycle arrest and apoptosis (29,30). The results showed that knockdown of USP1 could downregulate SIK2.

MG132 is a specific proteasome inhibitor and reduces the degradation of ubiquitin-conjugated proteins in mammalian cells (31). In order to explore the relationship between USP1 and SIK2, the U2OS cells were treated with MG132. The study found that MG132 could upregulate the expression of SIK2, it suggests that USP1 may stabilize the expression of SIK2 through protein ubiquitination. In the present study, we also found that knockdown of SIK2 could inhibit the ability of forming colonies, induce apoptosis and reduce the invasiveness of osteosarcoma cells, which indicates the essential roles of SIK2 in such tumors.

MMP-2, an important member of the MMP family, is able to degrade extracellular matrix components to promote cancer cell migration and invasion in multiple tumor types (32-34). GSK-3β is a ubiquitous serine/threonine kinase that plays different roles in different types of cancers (35). The GSK-3β gene may function as an oncogene in various types of human cancer, including colon cancer and osteosarcoma (36,37). Bcl-2, a kind of classic proto-oncogene, plays an important regulatory role in many kinds of malignant tumor cells apoptosis and distant metastasis, such as liver, lung, kidney, bladder and gastric cancer (38). Experimental and pathological evidence shows that cyclin E1 deregulation is oncogenic (39,40). Elevated cyclin E1 is associated with aggressive disease in a variety of human tumors (41). The Notch signaling pathway is shown to be an important driver of tumor growth and metastasis and dysregulated in many cancers (42,43). Notch1 is required for multiple

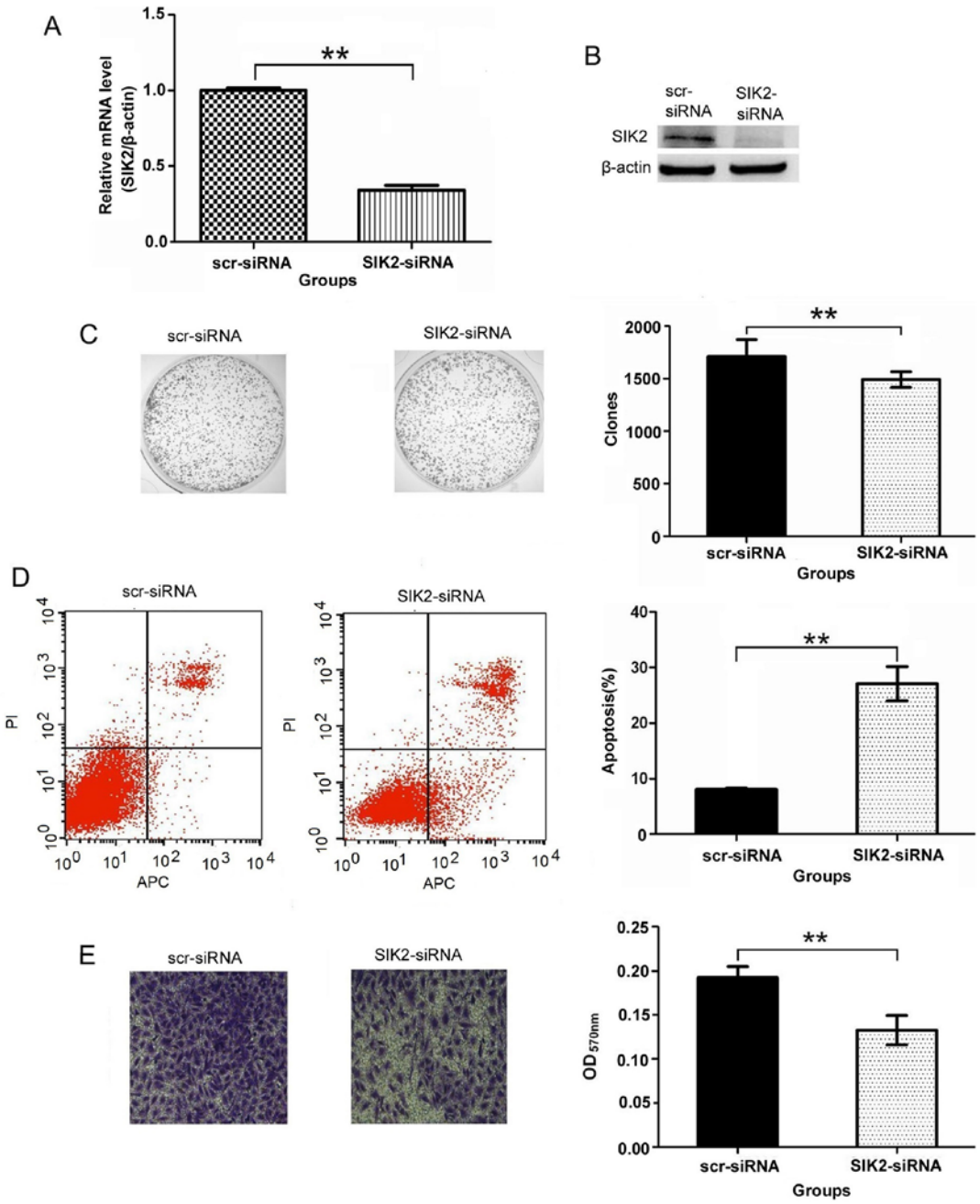

Figure 4. Silencing effect of SIK2 siRNA in U2OS cells. (A) The SIK2 mRNA expression level was quantified by real-time RT-PCR. SIK2 siRNA significantly decreased the level of SIK2 mRNA in U2OS cells. (B) The SIK2 protein expression level was quantified by western blot analysis. SIK2 siRNA significantly decreased the level of SIK2 protein in U2OS cells. (C) Inhibition of SIK2 decreased colony formation of U2OS cells. Colony formation assay showed that SIK2 siRNA decreased the clone numbers of U2OS cells as compared with scr-siRNA group. (D) The effects of SIK2 siRNA on apoptosis of U2OS cells was determined by flow cytometric analysis. Flow cytometric analysis showed that knockdown of SIK2 expression increased cell apoptosis in U2OS cells. (E) Effects of SIK2 siRNA on invasion of U2OS cells. Depletion of SIK2 inhibits the invasion of U2OS cells. *P<0.05, **P<0.01 in comparison to the scr-siRNA group.

cell fate determination processes, the deregulation of the Notch1 signaling cascade plays a crucial role in some solid tumors (44). Stat3, a central cytoplasmic transcription factor, is an important regulator of many biological processes (45). It regulates a number of genes that are critical to tumor cell proliferation, invasion, metastasis, angiogenesis and immune evasion (46,47). Previous studies revealed that cyclin A1 is highly expressed in many types of cancer, such as cancers of breast, lung and prostate (48-50). As a member of Wingless family and a secreted glycoprotein, Wnt-1 binds and activates the frizzled receptor, triggering a signaling cascade through GSK-3β and adenomatous polyposis coli protein (APC). Some cancer cells show upregulation of Wnt-1 signaling and continue to metastasize (51-53).

Notably, in the present study, all the above factors were reduced after knowdown of USP1 expression, which indicated that the inhibition of proliferation and invasion of human osteosarcoma cells after knockdown of USP1 expression is through reducing expression of these factors. Further experiments are required.

It is important to note that USP1 expression is increased in osteosarcoma tissues compared to cartilage tumor tissues and

normal bone tissues. This suggests an association between the overexpression of USP1 and osteosarcoma genesis.

In summary, our results suggest that silencing of USP1 inhibits cell proliferation and invasion in osteosarcoma cells *in vitro*, and reduce some tumor-related gene expression, which in turn provides the potential mechanisms of how UPS1 functions in osteosarcoma cells, and therapeutic targets for such tumors in the future.

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