Eukaryotic translation initiation factor 3H suppression inhibits osteocarcinoma cell growth and tumorigenesis

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Abstract. Eukaryotic translation initiation factor 3H subunit (EIF3H) is a member of the EIF3 family and exhibits a central role in translation initiation in higher eukaryotes. Although EIF3H expression is upregulated in numerous tumour types, its potential role in human osteosarcoma (OS) has not yet been investigated. In the present study, it was demonstrated that *EIF3H* mRNA expression was upregulated in the human OS cell lines Saos-2 and U2OS. A recombinant lentivirus harbouring short hairpin RNA targeting *EIF3H* was constructed and successfully infected human OS Saos-2 and U2OS cells, resulting in 95% downregulated *EIF3H* expression compared with the respective control groups. Knockdown of *EIF3H* significantly inhibited the proliferation and colony formation of OS cells *in vitro*, and tumour growth in nude mice *in vivo*. Flow cytometry analysis revealed cell cycle arrest and promotion of apoptosis in OS cells with *EIF3H* knocked down. In conclusion, the results strongly suggested that EIF3H is a critical factor mediating the growth of OS cells and may represent a novel therapeutic target.

Introduction

Human osteosarcoma (OS) is the most common primary malignant bone tumour that mainly occurs in children and adolescents. Conventional therapeutic approaches include treatment of disseminated disease with multi-agent cytotoxic chemotherapy, and local control of the primary lesion by chemotherapy or surgery. However, the 5-year survival rate of OS patients is only 60-70%, and there have been no improvements in this rate in the last 30 years (1,2), especially for patients showing metastasis at diagnosis (3). Consequently, there is a critical need to identify novel diagnostic markers and effective therapeutic targets for OS.

Eukaryotic translation initiation factor 3 (EIF3) is the largest of the translation initiation factors, comprising 13 non-identical protein subunits with a mass that is approximately 50% less than that of the 40S ribosomal subunit. One member of this family, EIF3H, plays a central role in translation initiation in higher eukaryotes and is located on chromosome 8q23 (4), a region frequently amplified in many tumour types (5,6). Recently, expression of the *EIF3H* gene was shown to be significantly upregulated in many human cancers (7,8). Knockdown of *EIF3H* could decrease cell viability through both cell cycle arrest and apoptosis induction and inhibited the formation of colonies in anchorage-independent conditions in breast cancer cells (7). Cappuzzo *et al* examined 54 metastatic NSCLC patients treated with gefitinib and found 10 cases (18.5%) showed amplification of *EIF3H* (8). These results indicated that EIF3H could play an important role in the growth and malignant phenotypes of cancer cells. However, the participation of *EIF3H* in human OS development and progression has been scarcely studied, and therefore its function in human OS is poorly understood.

To fill this knowledge gap and evaluate *EIF3H* as a candidate therapeutic or diagnostic target, we synthesized a sequence-specific interfering short hairpin RNA (shRNA) lentivirus targeting the *EIF3H* gene in OS cell lines to evaluate whether *EIF3H* could affect OS cell proliferation *in vitro*. MTT, cell cycle and apoptosis assay were selected to assess cell proliferation. In order to further verified this effect.

We investigated the potential role of EIF3H in OS by knocking down its expression in two OS cell lines, and evaluated the effects *in vivo* using an animal model.

Materials and methods

Cell culture. 293T cells and the human OS cell lines Saos-2, U2OS and MG-63 were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). All cells were cultured in Dulbecco's minimum essential medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal

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Abbreviations: EIF3H, eukaryotic translation initiation factor 3H; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SDS, sodium dodecyl sulphate; shRNA, short hairpin RNA; OS, osteocarcinoma; GFP, green fluorescent protein

Key words: EIF3H, shRNA, osteosarcoma, knockdown, proliferation, tumorigenesis

bovine serum (Biowest, Riverside, MO, USA, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37˚C in a 5% CO_2 atmosphere incubator.

Construction of the shRNA interference lentiviral vector. Two RNA interference sequences targeting *EIF3H* mRNA were designed and synthesized according to the *EIF3H* mRNA sequence in GenBank (NCBI accession no. NM_003756): *EIF3H* siRNA s1, 5'-GCAACTCTTGGAAGAAATATA-3'; *EIF3H* siRNA s2, 5'-CCCAAGGATCTCTCTCACTAA-3'. A random sequence was also designed to serve as a negative control (shCon). Sequences in the form of shRNA were inserted to a shRNA cloning and expression lentivirus vector containing green fluorescent protein (GFP)-tagged (SBI, Palo Alto, CA, USA). The lentivirus particles were produced in 293T cells transfected with the shRNA vector (shEIF3H) or the control (shCon) vector together with pHelper plasmids SHP001 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Infection of cells by the lentivirus. Saos-2 and U2OS cells were seeded in 6-well plates at approximately $3x10^4$ cells/well and $2.5x10^4$ cells/well, respectively, and maintained in a 5% CO_2 incubator at 37˚C until reaching approximately 30% confluence. Saos-2 cells and U2OS cells were infected with the shEIF3H or shCon vectors at a multiplicity of infection of 40 and 20, respectively, according to the virus titre of the sequence. The medium was replaced after culturing for 16 h. Expression of the GFP reporter gene in lentivirus-infected cells was observed under a fluorescence microscope at 120 h post-infection.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was prepared from the human OS cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNA was synthesized from total RNA with M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. RT-qPCR was performed using the SYBR-Green mix (Thermo Fisher Scientific, Inc.) on the Bio-Rad CFX96 sequence detection system. The primer sequences for the human *EIF3H* gene were: Forward 5'-GTGCTTTTGGGTCTGGTTGT-3' and reverse 5'-ATACCAGCCCACGTGAAGAT-3'. The *EIF3H* gene expression levels obtained were normalized to the mRNA expression levels of actin, amplified with the following primers: Forward 5'-GTGGACATCCGCAAAGAC-3' and reverse 5'-AAAGGGTGTAACGCAACTA-3'. The reaction conditions were as follows: a predenaturation step of 1 min at 95˚C followed by 40 cycles at 95˚C for 5 sec and 60˚C for 20 sec. The melting curve was established under the following conditions: 95˚C for 15 sec, 55˚C for 30 sec, and 95˚C for 15 sec. Each reaction was repeated three times per sample. The relative expression level of *EIF3H* was calculated using the comparative quantification cycle (Cq) method $2^{-\Delta\Delta Cq}$ (9).

Western blot analysis. Cell lysates were prepared from Saos-2 and U2OS cells using 2X sodium dodecyl sulphate (SDS) Sample Buffer [100 mM Tris-HCl (pH 6.8), 10 mM ethylenediaminetetraacetic acid, 4% SDS, and 10% glycine]. Lysates were clarified by centrifugation at 13,000 x g for 5 min at 4˚C, and the total protein was quantified by the bicinchoninic acid method and read at 560 nm. Protein samples were fractionated on 12% SDS-polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with rabbit monoclonal anti-EIF3H antibody (1:500; Proteintech, Rosemont, IL, USA) followed by incubation with the secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Bands were detected using enhanced chemiluminescence (ECL-PLUS/kit; Amersham Pharmacia Biotech, Tokyo, Japan) reagents. Anti-GAPDH antibody (1:500,000; Santa Cruz Biotechnology, Inc.) was used as the loading control.

Cell proliferation assays. The effect of *EIF3H* knockdown on cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M2128; Sigma-Aldrich; Merck KGaA) plus acidic isopropanol. In brief, Saos-2 and U2OS cells were respectively seeded in 6-well plates at a density of $2x10^3$ cells/well. At 24, 48, 72, 96, and 120 h after viral infection, MTT plus acidic isopropanol solution was added to each well and the plates were incubated at 37˚C for 1 h. Absorbance values were determined at 450 nm on a microplate reader (Epoch; BioTek, Winooski, VT, USA).

Colony formation assays. For colony formation assays, Saos-2 and U2OS cells were plated on 6-well plates at 400 and 600 cells/well and cultured for 7 and 8 days, respectively, in a 5% CO_2 incubator at 37˚C (Thermo Fisher Scientific, Inc.). The colonies formed were washed with phosphate-buffered saline (PBS), fixed with methanol, and finally stained with 0.1% crystal violet solution (C0121; Beyotime Institute of Biotechnology, Haimen, China). The number of colonies containing 50 or more cells was counted under an inverted microscope (CKX41; Olympus, Tokyo, Japan). Each assay was repeated in triplicate.

Cell cycle analysis. For cell cycle analysis, Saos-2 and U2OS cells infected with shEIF3H or shCon were seeded in 6-cm dishes at $6x10^4$ cells/dish and $8x10^4$ cells/dish, respectively. After being cultured for 5 days, or until the cells reached approximately 80% confluence, the cells were stained with propidium iodide (Beyotime Institute of Biotechnology), and the cell cycle distribution was assayed on a Gallios flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). The percentages of cells infected with ShEFI3H or shCon at the G0/G1, S, and G2/M phases were determined and compared. The experiments were performed in triplicate.

Flow cytometric analysis of apoptosis. The quantification of apoptotic cells was determined by flow cytometry using the Annexin V/7-AAD double staining kit (KGA1026; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. In brief, Saos-2 cells and U2OS cells infected with ShRNA or shCon were seeded in 6-cm dishes at $6x10^4$ cells/dish and $1.5x10^5$ cells/dish, respectively. When the cells reached approximately 80% confluence, they

were harvested, washed twice with PBS, and suspended in 450 μl binding buffer. Annexin V was added at room temperature, let to stand for 15 min for staining without light, and then resuspended in 450 μl binding buffer. The cells were then stained with 7-AAD in the dark. Cell apoptosis was analysed on a Gallios flow cytometer (Beckman Coulter, Inc.).

Xenograft tumorigenicity assay. Male BALB/c-Nude mice, 6-9 weeks old, were purchased from SLRC Laboratory Animal Company (Shanghai, China), and were housed under pathogen-free conditions in the barrier animal facility. For *in vivo* tumorigenicity experiments, Saos-2 cells stably infected with shCon or shEIF3H were collected, resuspended in PBS, and injected subcutaneously into the right or left subaxillary region of each mouse ($2x10^4$ per mouse), respectively. From the 7th day on, tumour xenografts were measured with callipers every 3 days, and tumour volume was calculated using the following formula: (length x width^2) x 0.5. At the end of the experiments (day 28 post inoculation), the mice were anaesthetized with intraperitoneal injection of 100 mg/kg pentobarbital sodium, killed by cervical dislocation, and tumour xenografts were recovered and weighed. All animal experiments were approved by the Committee on the Ethics of Affiliated Hospital of Zunyi Medical College.

Statistical analysis. Student's t-test was performed using GraphPad Prism 5.0 software. Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Infection efficiency of Saos-2 and U2OS cells with lentiviral vectors. Expression of EIF3H in four human OS cell lines were detected and results showed higher expression in Saos-2 and U2OS (Fig. 1A), which were selected for the next experiment. At 120 h post-infection with shEIF3H and shCon, both Saos-2 and U2OS cells showed strong expression of GFP, with an infection efficiency of over 80% in both cell lines (Fig. 1B), indicating that the lentiviral vector was successfully constructed to establish two stable cell lines.

shRNA effectively knocked down EIF3H mRNA expression. RT-qPCR analysis showed a 95% reduction in the *EIF3H* mRNA levels in both OS cell lines infected with shEIF3H compared to those infected with shCon (P<0.001; Fig. 1C). Western blot analysis further confirmed the efficacy of gene silencing, given a significant decrease in the relative protein expression level of EIF3H in the shEIF3H groups for both cell types (Fig. 1D).

EIF3H knockdown inhibited OS cell proliferation and colony formation. As shown in Fig. 2A, knockdown of endogenous *EIF3H* significantly inhibited the proliferation of Saos-2 and U2OS cells at 24, 48, 72, 96 and 120 h after viral infection.

Moreover, the number cells forming colonies was visibly reduced in the EIF3H knockdown groups compared to that in the shCon-infected groups, with a significant difference (P<0.01; Fig. 2B and C).

Knockdown of EIF3H led to cell cycle arrest and promoted apoptosis in OS cells. To investigate the potential mechanism of the inhibition of proliferation in the two OS cell lines, we assessed the effect of *EIF3H* knockdown on the cell cycle of OS cells by flow cytometry analysis. Both Saos-2 and U2OS cells with suppressed EIF3H expression significantly accumulated in the G0/G1 phase, whereas the percentages of cells in the S phase were significantly decreased compared to controls (P<0.05; Fig. 3A and B). These results indicated that *EIF3H* knockdown contributed to induction of G0/G1 arrest in OS cells.

As shown in Fig. 4A and B, *EIF3H* knockdown also increased the rate of apoptosis in the two OS cell lines based on flow cytometry using Annexin V and 7-AAD double-staining at both the early and late phases of apoptosis (Saos2 cells: 14.13 and 3.83% vs. 4.97 and 0.67% in controls, respectively, P<0.05; U2OS cells: 7.96 and 12.53% vs. 6.89 and 7.14% in controls, respectively, P<0.01).

EIF3H knockdown inhibited tumour growth in nude mice. Saos-2 cells infected with shEIF3H developed significantly smaller and reduced weighted tumors in mice compared to those infected with shCon (Fig. 5A), indicating that *EIF3H* knockdown also inhibited the growth of OS cells *in vivo*.

Discussion

OS is a malignant tumour that has become a global health issue. Although advances have been made in OS diagnosis and treatment, patient prognosis remains poor. We have demonstrated a clear role of EIF3H in the growth of OS cells and tumour development, suggesting a new candidate therapeutic target.

Over the past decade, the contribution of EIF3 to malignant transformation and progression has been established, and a previous study demonstrated that *EIF3H* expression was up-regulated in 18% of breast cancers and 30% of prostate cancers (10). Earlier studies also indicated that *EIF3H* was essential for maintaining the malignant state in cells (11). Zhu *et al* (12) reported that knockdown of *EIF3H* expression in hepatocellular carcinoma cells promoted apoptosis, and inhibited cell growth, colony formation, migration, as well as tumour growth in nude mice. In another study, reduction of EIF3H levels reduced cell proliferation and anchorage-independent growth in soft agar in breast and prostate cancer cell lines (13). However, the roles of EIF3H in human OS cells have thus far remained unclear, and there has been minimal research conducted on the effects of *EIF3H* in OS initiation and progression.

ShRNA-mediated gene silencing has proven to be a powerful tool to investigate the roles of cancer-related genes. Mahmood *et al* (7) demonstrated that *EIF3H* knockdown with specific small interfering RNA induced cell cycle arrest and apoptosis in breast tumour cells. We confirmed that an RNA interference strategy could effectively reduce the protein and gene expression of EIF3H in both OS cell lines as confirmed by western blot and RT-qPCR.

Moreover, knockdown of *EIF3H* markedly inhibited the growth and colony formation, resulted in G1 arrest, and induced apoptosis in Saos-2 and U2OS cells. The *in vivo* tumorigenicity experiments showed that *EIF3H* knockdown

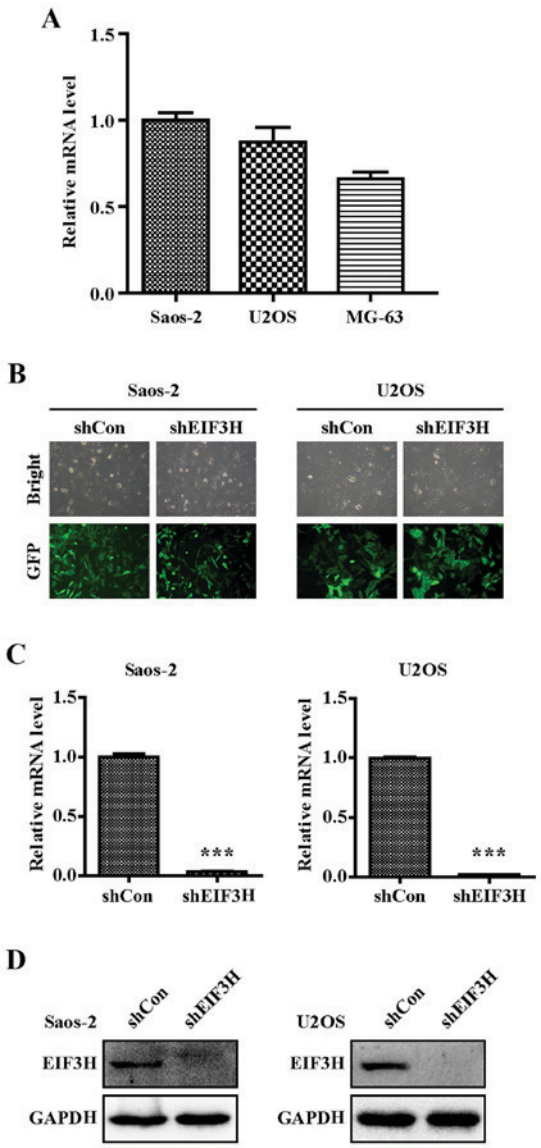

Figure 1. Validation of eukaryotic translation initiation factor 3H (*EIF3H*) knockdown in osteosarcoma (OS) cell lines. (A) Relative mRNA expression level of *EIF3H* in OS cell lines (Saos-2, U2OS, MG-63) quantified by RT-qPCR. (B) Infection efficiency of Saos-2 and U2OS cells observed under a fluorescence microscope. (C) Relative expression levels of *EIF3H* in Saos-2 and U2OS cells infected with shCon and shEIF3H detected by qPCR. (D) Western blotting of EIF3H protein expression in Saos-2 and U2OS cells after infection with shEIF3H and shCon. Student's t-test was performed using GraphPad Prism 5.0 software to calculated P-value. ***P<0.001 vs. shCon.

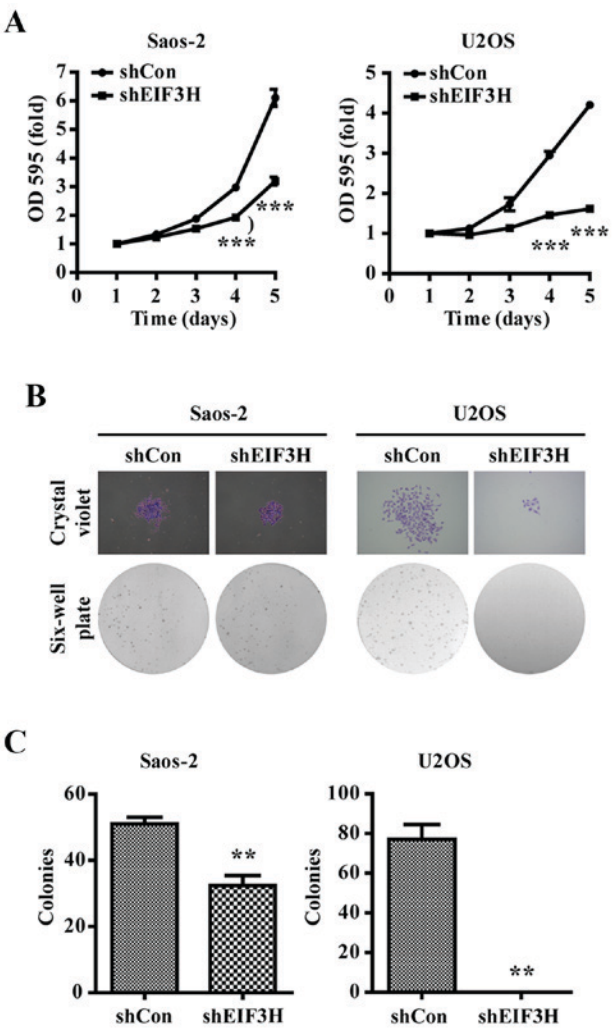

Figure 2. Eukaryotic translation initiation factor 3H (*EIF3H*) knockdown on inhibited cell proliferation and colony formation in osteosarcoma (OS) cell lines. (A) Line chart of MTT assay was to determine the proliferation rate after shEIF3H infection. (B) Representative microscopic images of colonies were stained by crystal violet. The full-sized vision of six-well plate under microscope showed significant colony formation inhibition in *EIF3H* knockdown group. Single clone was stained with crystal violet reagent and photographed (upper panel). Colonies were photographed (lower panel). (C) Quantitative analysis of colony numbers in (B). Student's t-test was performed using GraphPad Prism 5.0 software to calculated P-value. ***P<0.001, **P<0.01 vs. shCon.

further inhibited the growth of xenograft OS tumours *in vivo*. Collectively, these results suggest that EIF3H may play an important role in OS, and that an *EIF3H* knockdown approach may be a potential therapy for the treatment of OS. Therefore, targeting EIF3H may provide a new tool for the clinical prevention and treatment of human OS.

Although we did not determine the mechanism by which high levels of EIF3H influence cell growth, previous studies have shown that dysregulation of protein synthesis is implicated in oncogenesis through influencing the mRNA levels of proteins involved in cell proliferation, which are translated with activation of the protein synthesis apparatus (14,15). Therefore, changing the translational apparatus elements or activity, particularly the initiation factors, may be an efficient strategy to modify protein synthesis (16,17), because the initiation phase is the rate-limiting step for the translation of most mRNAs (18). When EIF3H is overactivated, the translation of mRNAs related to malignancy would be disproportionately enhanced to contribute to malignant activity (19-21). Zhu and colleagues (12) identified that the transforming growth factor-beta and mitogen-activated protein kinase pathways are potentially targeted by EIF3H using microarray analysis. This mechanism along with others are worthy of further detailed investigation to establish a new therapeutic strategy for cancer.

In conclusion, our study provides the first demonstration that knockdown of *EIF3H* using shRNA technology could inhibit the growth and colony formation of two OS cell lines and further suppress the development of xenograft tumours. These findings suggest that knocking down EIF3H expression

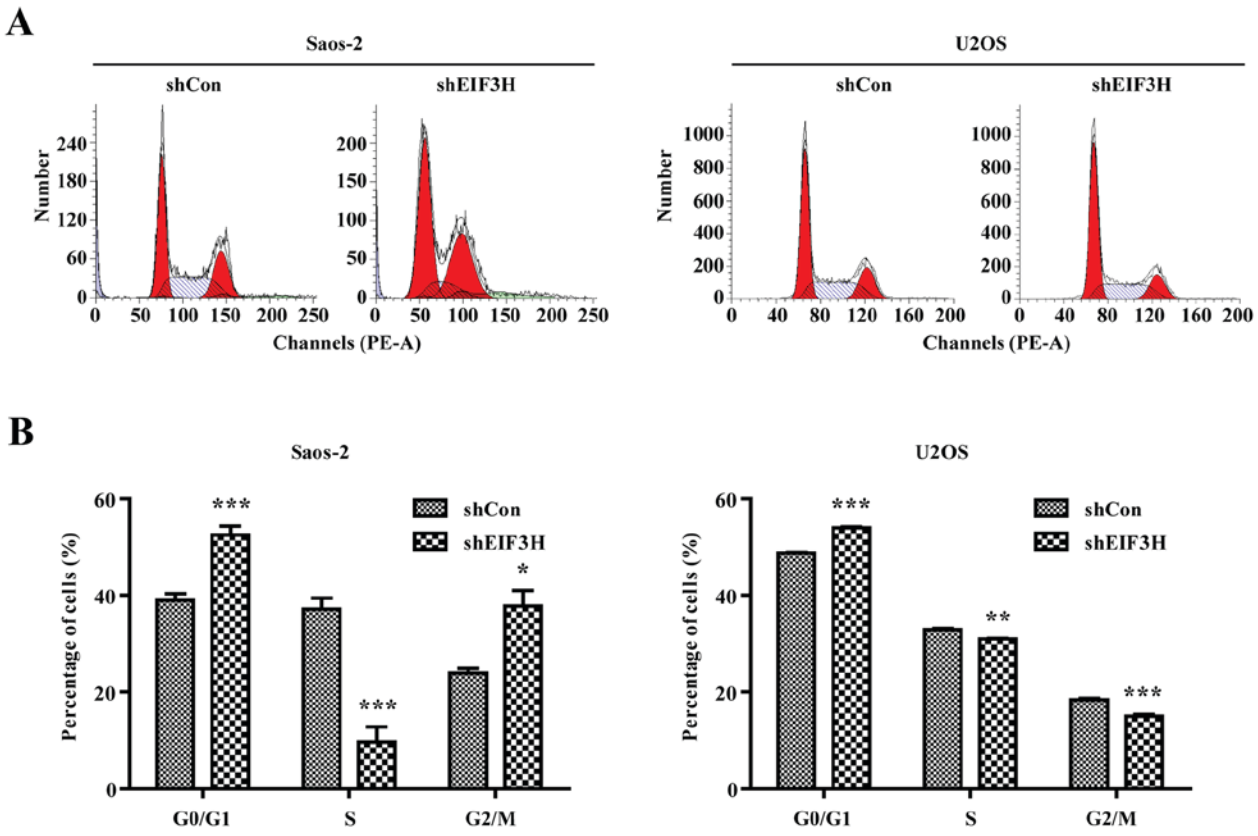

Figure 3. Cell cycle distribution after eukaryotic translation initiation factor 3H (*EIF3H*) knockdown in osteosarcoma (OS) cell lines. (A) Representative images of cell cycle distribution of Saos-2 cells (left panel) and U2OS cells (right panel) were measured by flow cytometry after infection with shEIF3H or shCon. (B) Saos-2 and U2OS cells at 48 h after infection with shEIF3H or shCon and the data was analysed (B). Student's t-test was performed using GraphPad Prism 5.0 software to calculated P-value. *P<0.05, **P<0.01, ***P<0.001 vs. shCon.

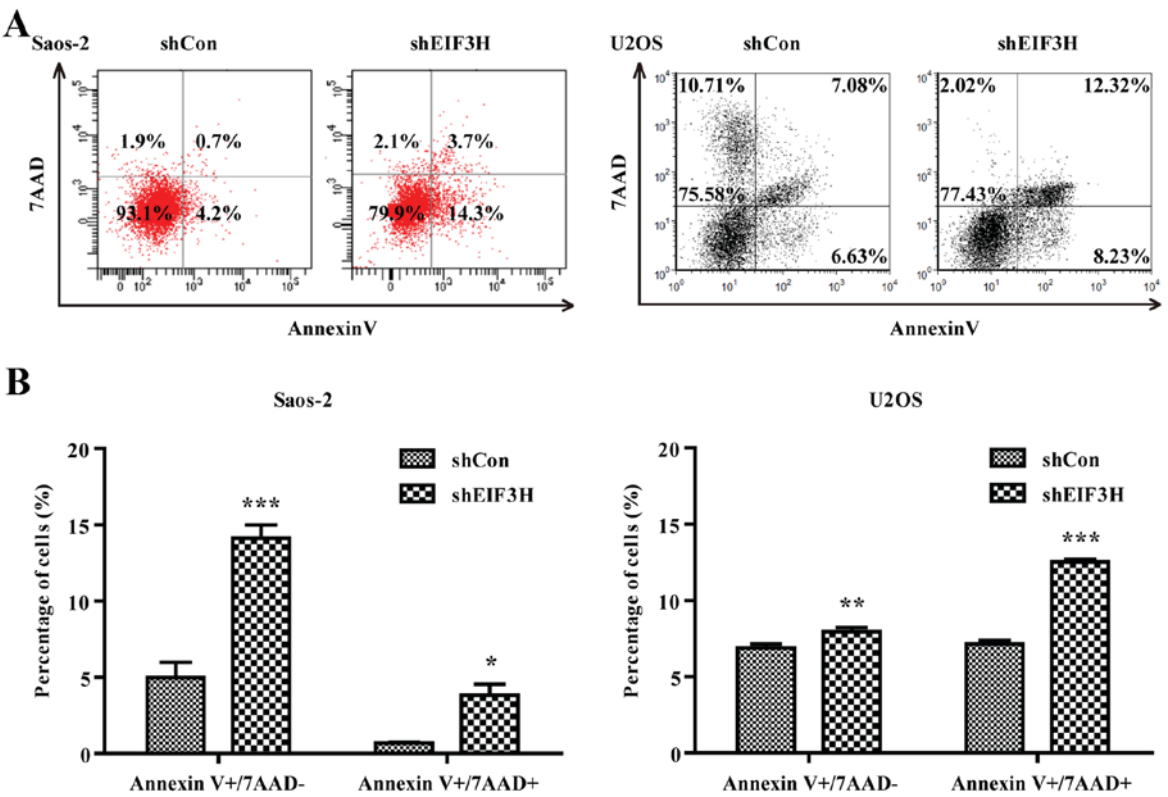

Figure 4. (A) Representative flow cytometry results for apoptosis detected at 36 h after infection with shEIF3H or shCon. (B) Percentages of the Annexin V/7-AAD positive apoptotic cells. Data represent the means ± SD from three independent experiments. Student's t-test was performed using GraphPad Prism 5.0 software to calculated P-value. *P<0.05; **P<0.01; ***P<0.001 vs. shCon. EIF3H, eukaryotic translation initiation factor 3H.

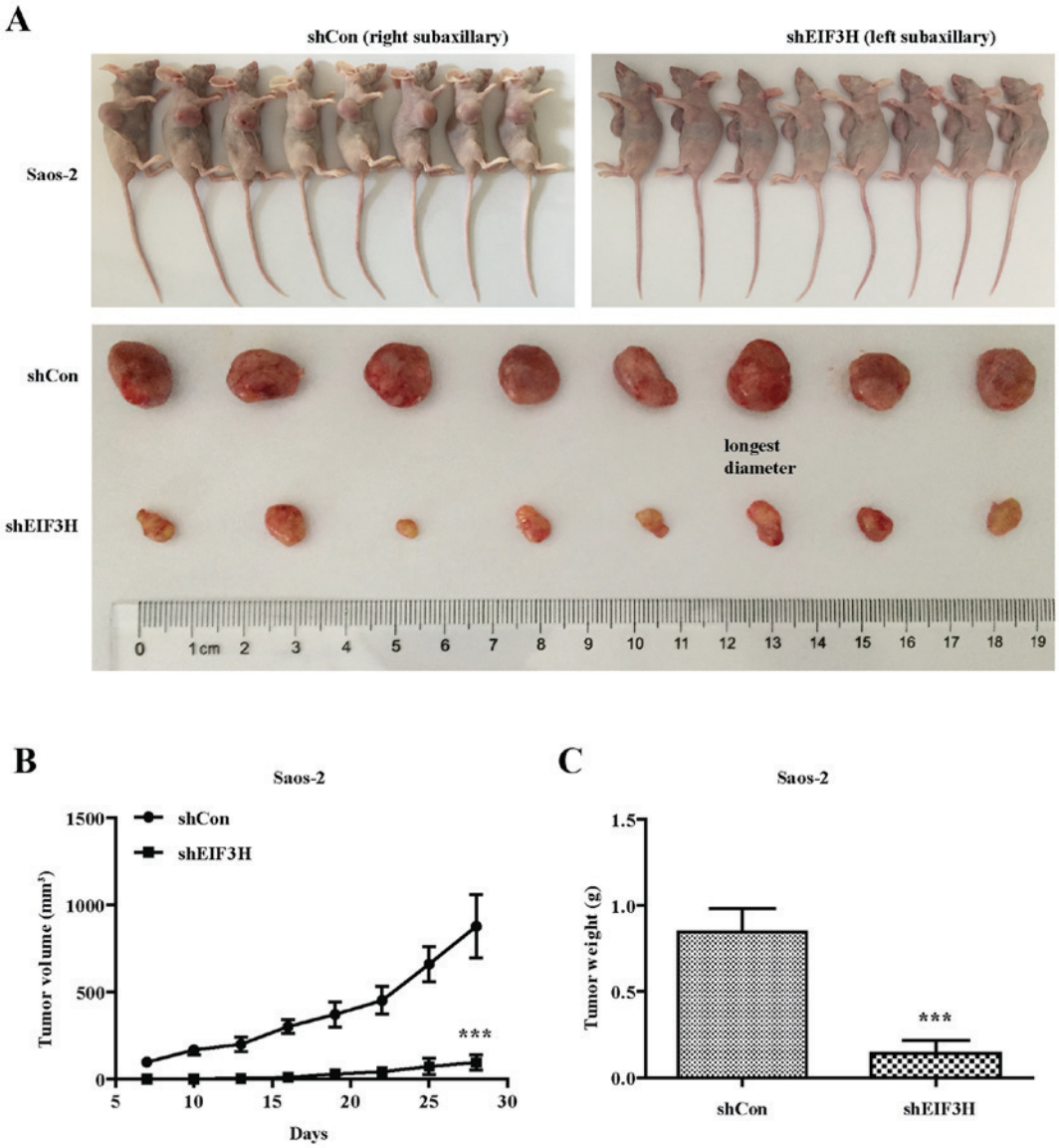

Figure 5. (A) Representative images of nude mice bearing osteosarcoma (OS) tumours and photographs of xenograft tumours at week 5 after inoculation. (B) Tumour volumes measured at the indicated time points after the OS cell inoculation. (***P<0.001 vs. shCon.). (C) Mean tumour weight of each group. Similar results were observed in two independent experiments. ***P<0.001 vs. scrambled control (shCon) groups. Student's t-test was performed using GraphPad Prism 5.0 software to calculated P-value.

could become a novel therapeutic strategy for OS prevention and treatment.

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