

# SUMO-specific protease 2 (SEN2) functions as a tumor suppressor in osteosarcoma via SOX9 degradation

HONG PEI<sup>1\*</sup>, LIANG CHEN<sup>1\*</sup>, QUAN-MING LIAO<sup>1</sup>, KE-JUN WANG<sup>1</sup>,  
SHUN-GUANG CHEN<sup>1</sup>, ZHENG-JIE LIU<sup>1</sup> and ZHI-CAI ZHANG<sup>2</sup>

<sup>1</sup>Department of Orthopaedics, Jing Zhou Central Hospital, The Second Clinical Medical College, Yangtze University, Jingzhou, Hubei 434020; <sup>2</sup>Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, P.R. China

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**Abstract.** Osteosarcoma (OS) is the most common primary bone malignancy in children and adolescents, the pathogenesis of which remain largely unknown. Small ubiquitin-like modifier (SUMO)-Specific Protease 2 (SEN2) has been reported to serve as a tumor suppressor in hepatocellular carcinoma cells. The aim of the present study was to investigate the critical role of SEN2 in OS cells. Using reverse transcription-quantitative polymerase chain reaction and western blot assays, it was observed that SEN2 was significantly downregulated in clinical OS tissues compared with adjacent normal samples. Ectopic expression of SEN2 resulted in the suppression of proliferation, migration and invasion in OS cells, whereas SEN2 knockdown by CRISPR-Cas9-based gene editing had the opposite effect. SEN2 is associated with the proteasome-dependent ubiquitination and degradation of SRY-box-9 (SOX9). SOX9 silencing impaired SEN2-depletion-induced accelerated cell growth and migration. Together, these results suggest that SOX9 is a critical downstream effector of the tumor suppressor SEN2 in OS.

## Introduction

Osteosarcoma (OS) and the Ewing sarcoma family of tumors (ESFT) are the most common primary bone malignancies observed in children and adolescents worldwide (1). OS is an osteoid-producing malignancy of mesenchymal origin that accounts for <0.2% of all cancers (2). However,

the frequency of OS has been increasing by 0.3% per year over the last 10 years (2). OS occurs most frequently in patients between 5 years of age and early adulthood (2). Unfortunately, few improvements have been made in prognostic and therapeutic methods (3,4). As such, increasing our understanding of the molecular events associated with OS is of great importance.

Small ubiquitin-like modifier (SUMO)-ylation is a dynamic posttranslational modification that occurs on substrate proteins lysine residues to which a SUMO protein covalently attached (5,6). SUMO proteins are highly conserved and serve important roles in a number of cellular processes, including the pathogenesis of cancer (5,7). SUMOylation is a reversible process regulated by the SUMO-specific protease (SEN2) family, which exhibits isopeptidase activity to remove SUMO proteins from substrates (8). A total of six SEN2 members have been identified, including SEN1, SEN2, SEN3, SEN5, SEN6 and SEN7, each of which has various substrate specificities and subcellular localizations (9,10). SEN5 is reported to be overexpressed in OS cell lines and tissues (11). However, it remains unclear whether other SEN2 family members serve a role in OS.

SEN2 has a broad de-SUMOylation activity (12). SEN2 null mice exhibit developmental defects in trophoblast stem cell niches and lineages due to dysregulation of the Mdm2-p53 signaling pathway in the placenta (13). SEN2 also acts as a tumor suppressor in human hepatocellular carcinoma by modulating the stability of  $\beta$ -catenin (14,15).

The present study demonstrates that SEN2 negatively regulates the proliferation, migration and invasion of OS cells. SEN2 was significantly downregulated in OS tissues compared with adjacent normal samples. SEN2 was associated with and promoted proteasome-dependent SOX9 ubiquitination and degradation, exhibiting its tumor suppressor function.

## Materials and methods

**Samples, cell lines, cell culture and transfection.** A total of 18 paired primary OS tissues and adjacent non-tumor normal tissues were collected from patients (male, 11 and female, 7; age range, 9-20 years; mean age, 15.6 $\pm$ 4.8 years) with newly

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*Correspondence to:* Dr Zhi-Cai Zhang, Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Jiangnan District, Wuhan, Hubei 430022, P.R. China  
E-mail: zhicaizhang95@163.com

\*Contributed equally

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diagnosed with OS who had not received any previous surgical treatment from June 2014 to May 2016. The present study was approved by the Institutional Review Board of Jing Zhou Central Hospital, the Second Clinical Medical College, Yangtze University, Jing Zhou (China) and written consent was obtained from all participants or their families. Normal hFOB1.19 cells and OS cell lines including Saos2, U2OS, Hos and MG63 were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 IU/ml penicillin and 100 mg/ml streptomycin and maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All plasmids were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). For transfection, U2OS cells were cultured as described to 70-80% confluence in a 6-well plate. Flag-SENP2 (1 µg) and/or HA-SOX9 (1 µg) and pcDNA 3.1 control plasmids were co-transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. After 36 h, a total of 20 µM protease inhibitor MG132 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were added for additional 4 h.

**Immunoprecipitation (IP) with Flag M2 beads.** U2OS cells were cultured to 70-80% confluence in 10 cm dishes and co-transfected with Flag-SENP2 (4 µg) and/or HA-SOX9 (4 µg) and pcDNA 3.1 (4 µg) control plasmids as above described. Cells were lysed in lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) for 20 min with gentle rocking at 4°C. Lysates were cleared by centrifugation (12,000 x g for 30 min at 4°C) and then filtered through 0.45 µm spin filters (Beyotime Institute of Biotechnology) to further remove cell debris. The resulting material was subjected to IP with 20 µl anti-FLAG M2 affinity resin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) overnight at 4°C. Resin-containing immune complexes were washed with ice-cold lysis buffer (2x5 min) followed by TBS washes (2x5 min). Proteins were eluted using 100 µl 150 µg/ml Flag-peptide (Sigma-Aldrich; Merck KGaA) in TBS for 10 min at room temperature. Proteins in each fraction were precipitated with 0.5 ml cold acetone (Sigma-Aldrich; Merck KGaA) overnight at 4°C and collected by centrifugation (12,000 x g; 30 min; 4°C). Protein samples were mixed with SDS loading buffer (Beyotime Institute of Biotechnology, Shanghai, China) prior to denaturation in a boiling water bath for 10 min and subjected to western blot assays.

**CRISPR/Cas9 knock out (KO) cell lines.** KO plasmids, including SENP2 CRISPR/Cas9 KO Plasmid (h): sc-402148 and SENP2 homology directed repair (HDR) Plasmid (h): sc-402148-HDR, were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Hos cells at 70-80% confluence were transfected with 2 µg KO and 2 µg HDR plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions and cells were selected with 1 µg/ml puromycin over 1 week, as previously described (16). Cells were trypsin-digested and cultured as described in 96-well for 1-2 weeks. Single clones were selected from individual wells and cultured as described above in

10 cm dishes for 2 weeks. Western blotting was used to verify the knockout efficiency.

**Retroviral short hairpin (sh)RNA.** Retroviral shRNA for SOX9 (Shanghai Genechem Co., Ltd.) was cloned into plko.1-puro vector. SOX9 shRNA sequence was as follows: 5'-CCGGGC GGAGGAAGTCGGTGAAGAACTCGAGTTCTTCACCGA CTTCTCCGCTTTTGTG-3'. For transfection, SENP2 KO hos cells generated by CRISPR/Cas9 as mentioned were cultured to 70-80% confluence in a 6-well plate. plko.1 control vector (2 µg) or SOX9 shRNA plko.1 (2 µg) were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Following 24 h of transfection, cells were collected and subjected to migration and invasion assays.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was harvested from human tissue samples or cells using the RNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Then, cDNA was obtained by RT at 37°C for 1 h using a Reverse Transcription system (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using a SYBR Green Premix Ex Taq (Takara Bio, Inc., Otsu, Japan) with the following conditions: Denaturing DNA at 95°C for 5 min, followed by 45 cycles of amplification with 94°C for 30 sec, 60°C for 60 sec and 72°C for 60 sec. Differences in gene expression, expressed as fold-changes, were calculated using the 2<sup>-ΔΔC<sub>q</sub></sup> method (17). Results were normalized to β-actin. Primer sequences were as follows: Human SENP2, forward, 5'-CTCAGGAACAGGCTGTAACA-3' and reverse, 5'-CAG GACAGACAGAGTTTCCA-3'; and human β-actin, forward, 5'-ACGAGACCACCTTCAACTCGATC-3' and reverse, 5'-AGGTCCTTCCTGATGTCCACGT-3'.

**Western blotting.** Tissues and cells were lysed with cold 2X SDS lysis buffer (Beyotime Institute of Biotechnology) for 1 h at 4°C. Lysates were centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was collected and the protein concentration was determined by a bicinchoninic acid protein concentration determination kit (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples (100 µg) were mixed with SDS loading buffer (Beyotime Institute of Biotechnology) prior to denaturation in a boiling water bath for 10 min. Samples (10 µg) were separated using 10% SDS-PAGE gels and resolved proteins were transferred to nitrocellulose membranes on ice and blocked with 50 g/l skimmed milk at room temperature for 1 h. Then, the membranes were incubated with the following primary antibodies at 4°C overnight: Rabbit anti-human SENP2 (1:500; cat. no. sc-130871; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-human ubiquitin (1:1,000; cat. no. sc-166553; Santa Cruz Biotechnology, Inc.), mouse anti-SOX9 (1:500; cat. no. sc-166505; Santa Cruz Biotechnology, Inc.), mouse anti-human GAPDH (1:10,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.), mouse anti-Flag M2 (1:5,000; cat. no. F1804; Sigma-Aldrich; Merck KGaA) and mouse anti-HA (1:5,000; cat. no. H9658; Sigma-Aldrich; Merck KGaA). Following three washes with PBS plus Tween-20 (PBST), membranes were incubated with polyclonal goat anti-mouse horseradish peroxidase-conjugated

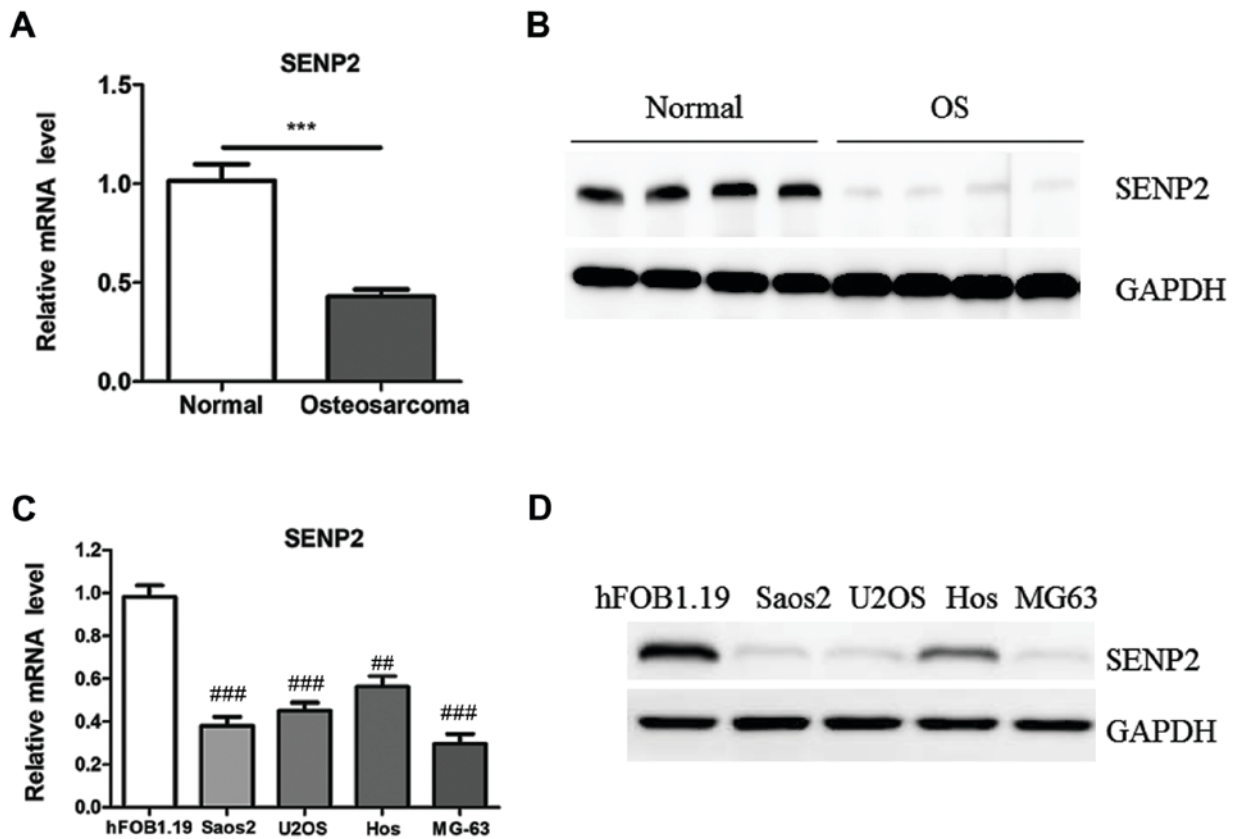


Figure 1. SENP2 was downregulated in OS samples and cell lines. SENP2 (A) mRNA and (B) protein expression in OS and adjacent normal tissues. SENP2. (C) mRNA and (D) protein expression in four OS cell lines and normal human osteoblastic hFOB1.19 cells. \*\*\* $P < 0.005$  vs. adjacent normal tissues; \*\* $P < 0.01$  and ### $P < 0.001$  vs. hFOB1.19. SENP2, small ubiquitin-like modifier-specific protease 2; OS, osteosarcoma.

secondary antibody (1:20,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) or polyclonal goat anti-rabbit horse-radish peroxidase-conjugated secondary antibody (1:20,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Membranes were washed three times with PBST and developed using an enhanced chemiluminescence detection kit (Sigma-Aldrich; Merck KGaA) for imaging. Image Lab v3.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to acquire and analyze imaging signals. GAPDH was used as controls for western blot assay.

**Migration and Matrigel invasion assays.** Migration assay and invasion assay were performed in 24-well plates with Boyden chambers (Corning Incorporated, Corning, NY, USA) with a filter of 6.5 mm diameter and 8  $\mu$ m pore size. All procedures were performed according to the manufacturer's protocol. Invasion assay was performed using the upper chambers coated with Matrigel (Collaborative Biomedical Products, Bedford, MA, USA). Matrigel was thawed at 4°C overnight and diluted with RPMI-1640 medium. The mixture (20  $\mu$ l) was then added into the upper chamber (BD Biosciences) and incubated at 37°C for 1 h. Cells ( $4 \times 10^4$ ) were seeded into the upper chamber containing 0.2 ml serum-free RPMI-1640 medium. In addition, 0.5 ml RPMI-1640 medium supplemented with 10% fetal bovine serum was added into the lower chamber. Following 48 h at 37°C, the chamber was removed and cells in the upper chamber were wiped off. Following fixing with 4% formaldehyde for 10 min at room temperature, samples were stained

at room temperature for 2 h using 0.2% crystal violet in 10% methanol. Migration and invasion activities were evaluated by counting the number of migrated cells on the lower surface of the filters using a light microscope (magnification,  $\times 100$ ; EclipseE200; Nikon Corporation, Tokyo, Japan).

**Cycloheximide inhibition test.** Control and SENP2 KO cells ( $5 \times 10^5$ ) were cultured to 70–80% confluence in a 6-well plate and treated with 20  $\mu$ g/ml cycloheximide (CHX; Sigma-Aldrich; Merck KGaA) for 0, 1, 2, 4 or 8 h at 37°C. SOX9 and SENP2 protein expression was measured by western blot as described, using GAPDH as loading control.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation of three independent experiments. Statistical significance was evaluated using one-way analysis of variance with the Newman-Keuls' post hoc test. Data were analyzed using GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA, USA) and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**SEN2 was downregulated in OS samples and cell lines.** To investigate the expression of SEN2 in clinic samples, SEN2 mRNA was measured in 18 paired primary OS samples and adjacent normal tissues using RT-qPCR. SEN2 expression was significantly decreased in OS samples compared with

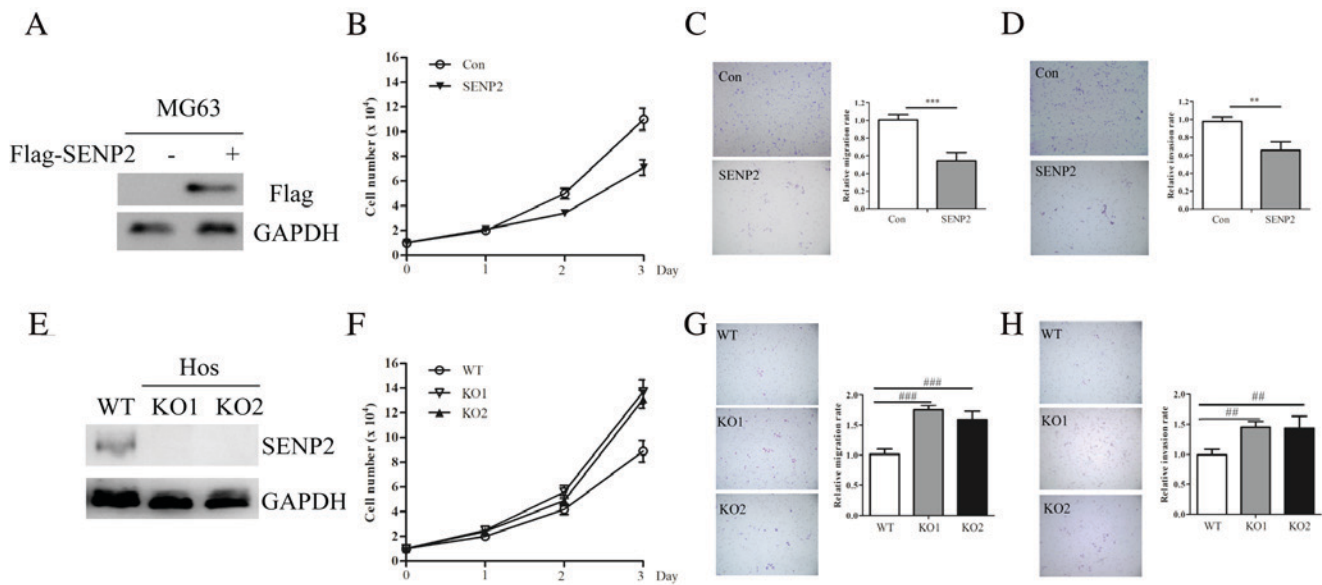


Figure 2. SENP2 regulates OS cell proliferation, migration and invasion. (A) SENP2 protein expression in MG63 cells with or without Flag-SENP2 overexpression. (B) Cell growth curve, (C) migration assay and (D) invasion assay for Con-MG63 and Flag-SENP2-MG63 cells. (E) SENP2 protein expression in control or SENP2 KO cells. (F) Cell growth curve, (G) migration assay and (H) invasion assay for control and SENP2 KO Hos cells. \*\* $P < 0.01$  and \*\*\* $P < 0.005$  vs. CON; ## $P < 0.01$  and ### $P < 0.005$  vs. WT. SENP2, small ubiquitin-like modifier-specific protease 2; OS, osteosarcoma; KO, knockout; WT, wild type; Con, control.

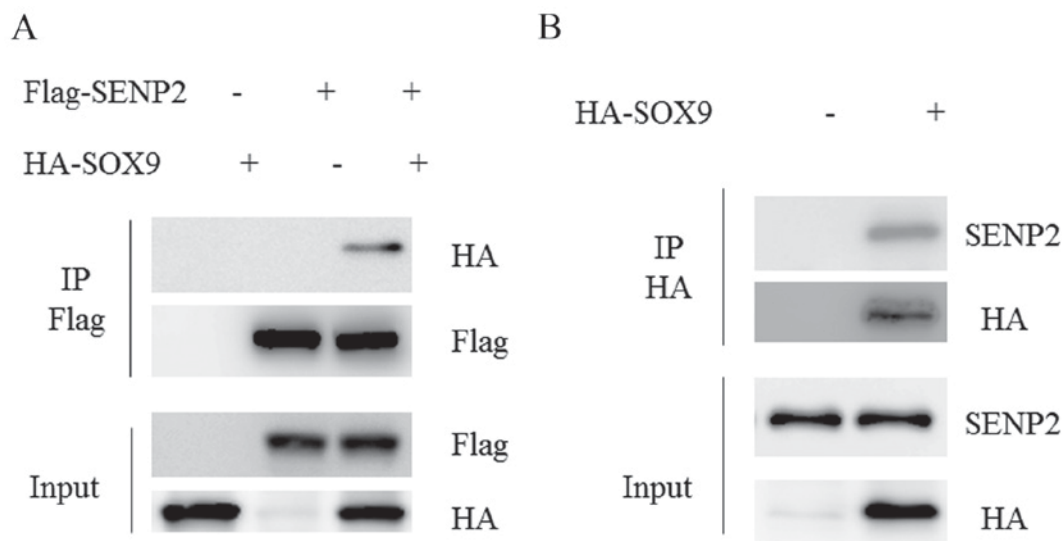


Figure 3. SENP2 is associated with SOX9. U2OS cells were transfected with (A) Flag-SENP2 and HA-SOX9 or (B) HA-SOX9 alone for 48 h and subjected to IP with Flag M2 beads as well as western blotting. SENP2, small ubiquitin-like modifier-specific protease 2; SOX9, Sry-box-9; HA, hemagglutinin; IP, immunoprecipitation.

adjacent normal tissues (Fig. 1A). SENP2 protein expression was compared between 4 paired OS samples using western blotting. The results demonstrated that SENP2 protein was also downregulated in OS samples compared with adjacent normal tissues (Fig. 1B). SENP2 mRNA and protein expression was also significantly downregulated in Saos2, U2OS, Hos and MG63 OS cell lines compared with normal hFOB1.19 cells (Fig. 1C and D). Taken together, these data demonstrate that SENP2 is downregulated in OS.

*SENP2 regulates proliferation, migration and invasion in OS cells.* SENP2 is downregulated in OS cells and it was observed

that MG63 cells had the lowest expression; as such, MG63 cells were selected for use in following experiments (Fig. 2A). SENP2 overexpression significantly inhibited the proliferation, migration and invasion of MG63 cells (Fig. 2B-D). SENP2 knockdown was achieved in Hos cells, which had the highest SENP2 expression of OS cells assessed in the present study, using a CRISPR/Cas9 system. Two clones were selected and SENP2 silencing was confirmed using western blotting (Fig. 2E). KO clones exhibited increased cell proliferation compared with control cells (Fig. 2F). Consistent with this, SENP2 depletion was demonstrated to enhance OS cell migration and invasion (Fig. 2G-H).



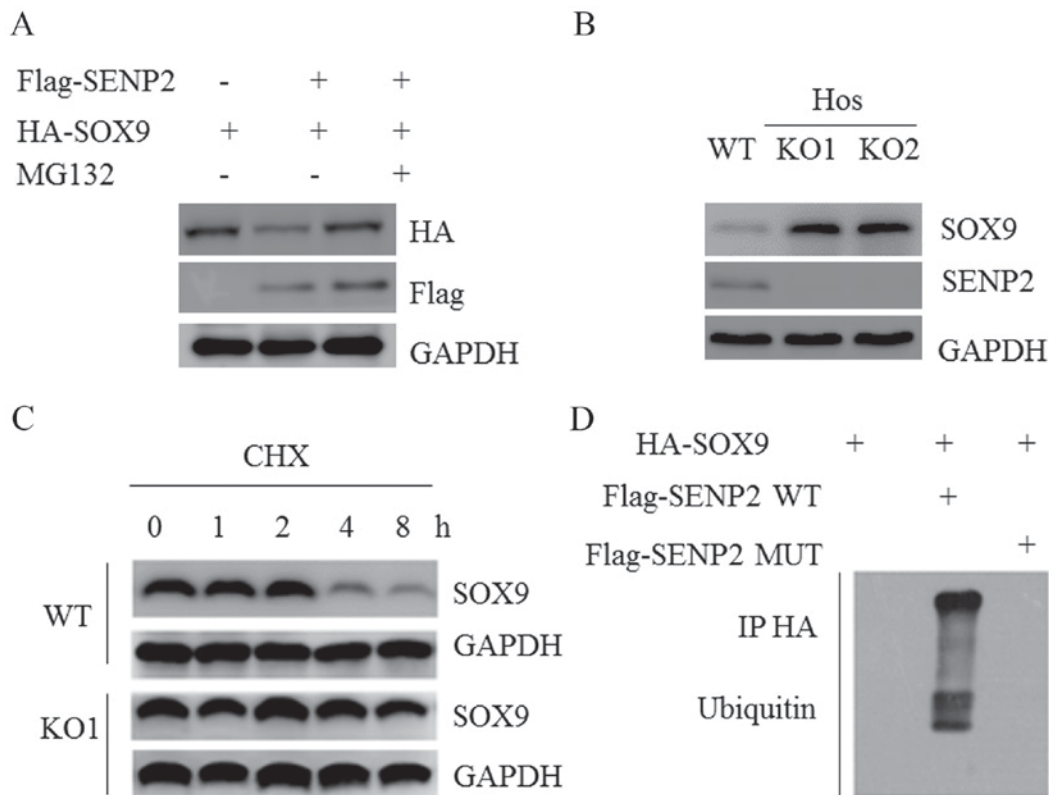


Figure 4. SENP2 promotes SOX9 ubiquitination and degradation. (A) U2OS cells were transfected with the indicated plasmids for 36 h and subsequently treated with 20  $\mu$ M MG132 for 4 h, following which western blotting was performed. (B) SOX9 protein expression was measured in control and SENP2 KO cells. (C) Control and SENP2 KO cells were treated with 20  $\mu$ g/ml CHX for 0, 1, 2, 4 or 8 h and the protein expression of SOX9 was measured. (D) U2OS cells were transfected with the indicated plasmids and subjected to immunoprecipitation with HA-antibody and western blotting. SENP2, small ubiquitin-like modifier-specific protease 2; SOX9, Sry-box-9; KO, knockout; MUT, mutant; WT, wild type; HA, hemagglutinin.

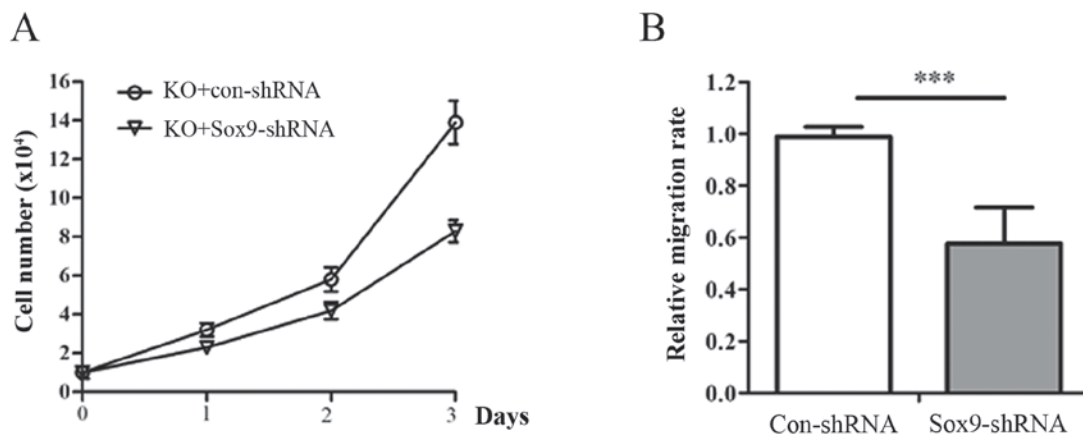


Figure 5. The SENP2-SOX9 axis regulates OS cell proliferation and invasion. (A) Cell growth curve and (B) migration assay for SENP2 KO cells transfected with control-shRNA or SOX9-shRNA. \*\*\* $P < 0.005$  vs. Con. SENP2, small ubiquitin-like modifier-specific protease 2; SOX9, Sry-box-9; OS, osteosarcoma; shRNA, short hairpin RNA; Con, control.

*SENP2 is associated with SOX9.* SOX9 is a transcription factor that serves crucial roles in tissue development and tumorigenesis (18). In a previous study, SOX9 was overexpressed in 120 out of 166 (72.3%) OS specimens and was demonstrated to be associated with advanced clinical stage, metastasis and poor response to chemotherapy (19). Given that SENP2 serves a tumor suppressing role in OS cells and SOX9 is a SUMOylated protein (20), the potential association between

SENP2 and SOX9 was investigated. To this end, U2OS cells were co-transfected with Flag-SENP2 and HA-SOX9 for 48 h, cells were then harvested and subjected to immunoprecipitation with Flag M2 beads. The results revealed that HA-SOX9 was readily detected in Flag-SENP2 immunoprecipitate (Fig. 3A). Furthermore, endogenous SENP2 was detected in HA-SOX9 immunoprecipitate (Fig. 3B). These results suggest that SENP2 interacts with SOX9 in OS.

*SENP2 promotes SOX9 ubiquitination and degradation.* In the presence of SENP2, the expression of exogenous SOX9 was decreased (Fig. 4A); however, this effect was reversed by treatment with the proteasome inhibitor MG132 (Fig. 4A), suggesting that SENP2 may regulate the protein stability of SOX9. SOX9 protein expression was upregulated in SENP2 KO cells (Fig. 4B). The effects of SENP2 on SOX9 protein stability were assessed using a cycloheximide (CHX) assay. CHX inhibits new protein synthesis and it was revealed that the half-life of SOX9 was significantly extended in OS cells in the absence of SENP2 (Fig. 4C). Furthermore, SENP2 WT was demonstrated to significantly promote the ubiquitination of SOX9. SENP2 Mut, a deSUMOylation catalytic inactive form of SENP2, was unable to induce SOX9 ubiquitination (Fig. 4D). Together, these data suggest that SENP2 promotes SOX9 ubiquitination and degradation dependent on its deSUMOylation activity.

*SENP2-SOX9 axis regulates OS cell proliferation and invasion.* To determine whether SOX9 is required for the biological function of SENP2 in OS, SENP2 KO cells were further transfected with shRNAs against SOX9. It was revealed that SOX9 knockdown largely inhibited the proliferation and invasion of SENP2 KO cells (Fig. 5A-B), suggesting that SOX9 is a downstream target of SENP2 in OS cells.

## Discussion

The transcription factor SOX9 is associated with the development of a variety of malignant tumors and increased aggressiveness in OS. SOX9 knockdown reduced the proliferation of OS cells by decreasing the expression of Wnt1 and its receptor Fzd1 (19). SOX9 protein levels are regulated by SUMOylation and the ubiquitin-proteasome pathway (21). SUMO1 and SUMOylation are able to suppress the transactivation of SOX9 (22). SOX9 has a HMG box and its transactivation serves critical roles in a number of developmental processes, including sex determination and chondrogenesis (23). However, it is not yet known whether SOX9 transactivation is required for the development of OS.

Unlike other SENP family members, SENP2 is regarded as a tumor suppressor in a number of malignant tumors (24-26). In the present study it was demonstrated that SENP2 exerted anti-tumor effects in OS cells. Firstly, SENP2 expression is downregulated in OS tissue samples and cultured cell lines. Secondly, SENP2 overexpression decreased the proliferation and invasion of OS cells, whereas SENP2 knockdown had the opposite effect. Thirdly, SOX9 was demonstrated to be a post-transcriptional target of SENP2 in OS cells. The results of the present study demonstrate that SENP2 is associated with SOX9 and promotes its proteasome-dependent degradation in a SUMOylation-dependent manner. Furthermore, SOX9 silencing was revealed to counteract the effects of SENP2 depletion.

In summary, the *in vitro* gain- and loss-of-function studies performed in the present study demonstrate that the SENP2-SOX9 regulatory axis serves a role in the regulation of OS cell proliferation and invasion. These results suggest that SENP2 dysregulation is an important feature of OS. As such, restoring the expression or activity of SENP2 may be a promising therapeutic treatment for patients with OS.

However, the present study is limited by the relative small number of clinical samples utilized. Future studies may include a larger number of clinical samples and animal experiments may further elucidate the biological function of SENP2-SOX9 regulatory axis in the development and treatment of OS.

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## Availability of data and materials

The datasets and materials used in the current study are available from the corresponding author upon reasonable request.

## Authors' contributions

HP and ZZ collaborated to design the study. HP, LC, QL and KW were responsible for experiments. HP, SC and ZL analyzed the data. HP and ZZ wrote the paper. All authors collaborated to interpret results and develop the manuscript. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Jing Zhou Central Hospital, the Second Clinical Medical College, Yangtze University, Jing Zhou (China) and written consent was obtained from all participants or their families.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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