Oleanolic acid inhibits osteosarcoma cell proliferation and invasion by suppressing the SOX9/Wnt1 signaling pathway

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Abstract. Osteosarcoma is the most common primary bone malignancy in children and adolescents. Inhibition of SOX9/Wnt1-mediated signaling might suppress osteosarcoma metastasis, and oleanolic acid (OA) might decrease the activity of the SOX9/Wnt1 signaling pathway. The aim of the present study was to determine the role of OA in osteosarcoma cell proliferation and invasion. Osteosarcoma cell lines (KHOS and U2OS) and an osteoblastic cell line (hFOB1.19) were used for cell viability, proliferation and invasion analysis. The data suggested that OA significantly inhibited cell viability on days 3, 4 and 5 compared with the control (Ctrl) group in both U2OS and KHOS cells. Cell proliferation in the OA-treated group was significantly decreased compared with the Ctrl group in the osteosarcoma cell lines. Analysis of the cell cycle indicated that OA significantly reduced the percentage of U2OS and KHOS cells in the S phase compared with the Ctrl group. The wound healing assay results indicated that the OA group displayed significantly decreased cell re-colonization of the wound at 48 h compared with the Ctrl group. The Transwell chamber assay results also indicated that cell invasion was significantly inhibited by OA compared with the Ctrl group. Furthermore, OA significantly increased osteosarcoma cell apoptosis compared with the Ctrl group. Similarly, the protein expression levels of SOX9 and Wnt1 were significantly decreased in OA-treated U2OS and KHOS cells compared with Ctrl cells. OA-mediated downregulation of Wnt1 expression was reversed following SOX9 small interfering RNA transfection. Collectively, the results indicated that OA inhibited SOX9/Wnt1-associated osteosarcoma cell proliferation, migration and invasion.

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Introduction

Osteosarcoma is the most common primary bone malignancy in children and adolescents worldwide (1). The majority of patients with osteosarcoma display invasion and metastasis, leading to a reduction in the efficiency of anticancer agents and the failure of therapy (2). Therefore, identification of potential therapeutic targets to suppress osteosarcoma cell proliferation and invasion is required.

Various mechanisms underlying osteosarcoma cell proliferation and invasion have been previously described (1,2). The Wnt signaling pathway, which regulates cell proliferation, differentiation and migration, serves important functions in osteosarcoma cell proliferation and invasion (3,4). Previous studies have reported that Wnt1 expression is increased in osteosarcoma tissues (3,4). Activation of Wnt signaling activates genes associated with cell proliferation, including c-Myc, matrix metallopeptidases and cyclin D1 (3-5), leading to osteosarcoma cell proliferation and invasion. Moreover, it has been reported that SOX9 regulates hyperexpression of Wnt1 in osteosarcoma tissues and cells (3). Therefore, it was hypothesized that inhibition of SOX9/Wnt1-mediated signaling might suppress osteosarcoma metastasis.

Oleanolic acid (OA), a naturally occurring triterpenoid, displays potential antitumor activity in several tumor cells, and has also has been reported to inhibit osteosarcoma cell proliferation and induce cell apoptosis (6-8). However, the mechanisms underlying OA during osteosarcoma are not completely understood. Previous studies have demonstrated that OA inhibits the Wnt signaling pathway in hepatoma cells (9) and derivatives of OA decrease the expression levels of SOX2 (10). The present study investigated the hypothesis that OA functions as an anticancer agent to protect against osteosarcoma.

Materials and methods

Osteosarcoma cell lines culture. Osteosarcoma cell lines (KHOS and U2OS) and an osteoblastic cell line (hFOB1.19) were purchased from American Type Culture Collection. Osteosarcoma cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco;

Thermo Fisher Scientific, Inc.), and osteoblastic cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS at 37˚C with 5% CO_2. Cells were incubated with 50 μM OA (cat. no. O5504; Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C.

Cell viability analysis. The Cell Counting Kit-8 (CCK-8) assay (cat. no. C0038; Beyotime Institute of Biotechnology) was performed to evaluate cell viability according to the manufacturer's protocol. Briefly, cells ($1x10^4$ cells/well) were seeded into a 96-well plate and incubated with 10 μl CCK-8 reagent for 2 h at 37˚C. The absorbance of each well was measured at a wavelength of 450 nm using a microplate spectrophotometer.

Colony formation assay. Cell proliferation was assessed by conducting a colony formation assay. Cells were seeded ($1x10^3$ cells/well) into a 6-well plate and cultured at 37˚C with 5% CO_2. Following culture for 21 days, cell colonies, which were defined as 0.5-1 mm in diameter cell assemblies, were fixed with 4% paraformaldehyde for 30 min at room temperature, stained using crystal violet for 10 min at room temperature and observed using a light microscope (magnification, x100).

Ki67 staining. Cell proliferation was also assessed by performing Ki67 staining. Briefly, cells were grown on coverslips and fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, cells were incubated with an Alexa Fluor 647-conjugated anti-Ki67 antibody (1:100; cat. no. ab196907; Abcam) for 30 min at 37˚C and the nuclei were stained with DAPI for 1 min at room temperature. Ki67-positive cells were counted in ten randomly selected fields of view using a fluorescence microscope (magnification, x100) and NIS-Elements Viewer software (version 4.2.0; Nikon Corporation).

Flow cytometry. The cell cycle distribution was analyzed by performing by flow cytometry. Cells were harvested and washed with PBS. Subsequently, cells were fixed with ice-cold 70% ethanol for 4 h at 4˚C. After permeabilization by 0.1% Triton X-100 for 30 min at room temperature, the cells were stained with PI containing RNase A (cat. no. C1052; Beyotime Institute of Biotechnology) at 37˚C for 1 h. The cell cycle distribution was analyzed via flow cytometry using a BD flow cytometry system (FACSVerse; BD Biosciences) and FlowJo software (version 10.0; BD Biosciences).

Wound healing assay. The wound healing assay was performed to assess cell migration. Cells were cultured in a 6-well plate. At 90% confluence, the cell layer was scratched using a 200-μl sterile pipette tip. Subsequently, cells were cultured in DMEM or RPMI-1640 without FBS at 37˚C with 5% CO_2. At 0 and 48 h, the wounds were observed using a phase contrast light microscope (magnification, x200). Taking the distance between the wound edges at 0 h as a baseline, the image of the same location after 48 h was captured. The percentage of the wound healing area was analyzed using ImageJ software (version 1.46r; National Institutes of Health).

Transwell assay. To assess cell invasion, cells ($5x10^4$) were seeded into the upper chambers of Transwell plates with 50 mg/l Matrigel (pore size, 8 μm; cat. no. CLS3374; Corning Inc.) and serum-free medium after Matrigel precoating at 37˚C for 1 h. DMEM or RPMI-1640 medium supplemented with 10% FBS was filled into the lower chambers. Following incubation for 8 h, cells on the upper surface of the membrane were removed and invading cells were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with crystal violet for 30 min at room temperature. Invading cells were visualized using an inverted light microscope (magnification, x100).

TUNEL assay. To detect osteosarcoma cell apoptosis, a TUNEL assay (cat. no. C1088; Beyotime Institute of Biotechnology) was performed. Briefly, 10^4 cells/well were cultured on coverslips in DMEM or RPMI-1640 supplemented with 10% FBS at 37˚C with 5% CO_2. Subsequently, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After rinsing with PBS, cells were stained using the TUNEL assay kit at 37˚C for 60 min and the nuclei were stained with DAPI (0.3 mM, Vector Laboratories, Inc.) at room temperature for 1 min. Following mounting with an anti-fluorescence quenching mounting medium (cat. no. P0126; Beyotime Institute of Biotechnology), the number of apoptotic nuclei per section was calculated using the following formula: The number of TUNEL-positive cell nuclei/the total number of DAPI-positive nuclei. The number of apoptotic nuclei were counted in ten randomly selected fields of view using a fluorescence microscope (magnification, x100).

Western blotting. After washing twice with ice-cold PBS, total protein was extracted from osteosarcoma cells using ice-cold lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology) and quantified using the bicinchoninic acid assay (cat. no. P0012; Beyotime Institute of Biotechnology). Protein homogenates (30 μg) were separated via 8-10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were washed with TBS and blocked with 5% milk powder in TBS for 1 h at room temperature. Subsequently, the membranes were incubated at 4˚C overnight with the following primary antibodies: Rabbit anti-caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), rabbit anti-cleaved- caspase-3 (1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.), rabbit anti-Bcl2 (1:1,000; cat. no. 3498; Cell Signaling Technology, Inc.), rabbit anti-SOX9 (1:1,000; cat. no. 82630; Cell Signaling Technology, Inc.), rabbit anti-β-catenin (1:1,000; cat. no. 9582; Cell Signaling Technology, Inc.), rabbit anti-Wnt1 (1:1,000; cat. no. 2915; Cell Signaling Technology, Inc.) and anti-GAPDH (1:1,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.). Following primary incubation, the membranes were washed with TBS and incubated with an HRP-conjugated goat anti-rabbit-IgG secondary antibody (1:4,000; cat. no. BA1039; Boster Biological Technology) for 1 h. Protein bands were visualized using enhanced chemiluminescence (EMD Millipore). Densitometry levels were analyzed using Quantity One software (version 4.6.6; Bio-Rad Laboratories, Inc.) with GAPDH as the loading control.

Small interfering (si)RNA) transfection. Cells in 6-well plates ($2x10^5$ cells/well) were transfected with 10 nM SOX9-specific

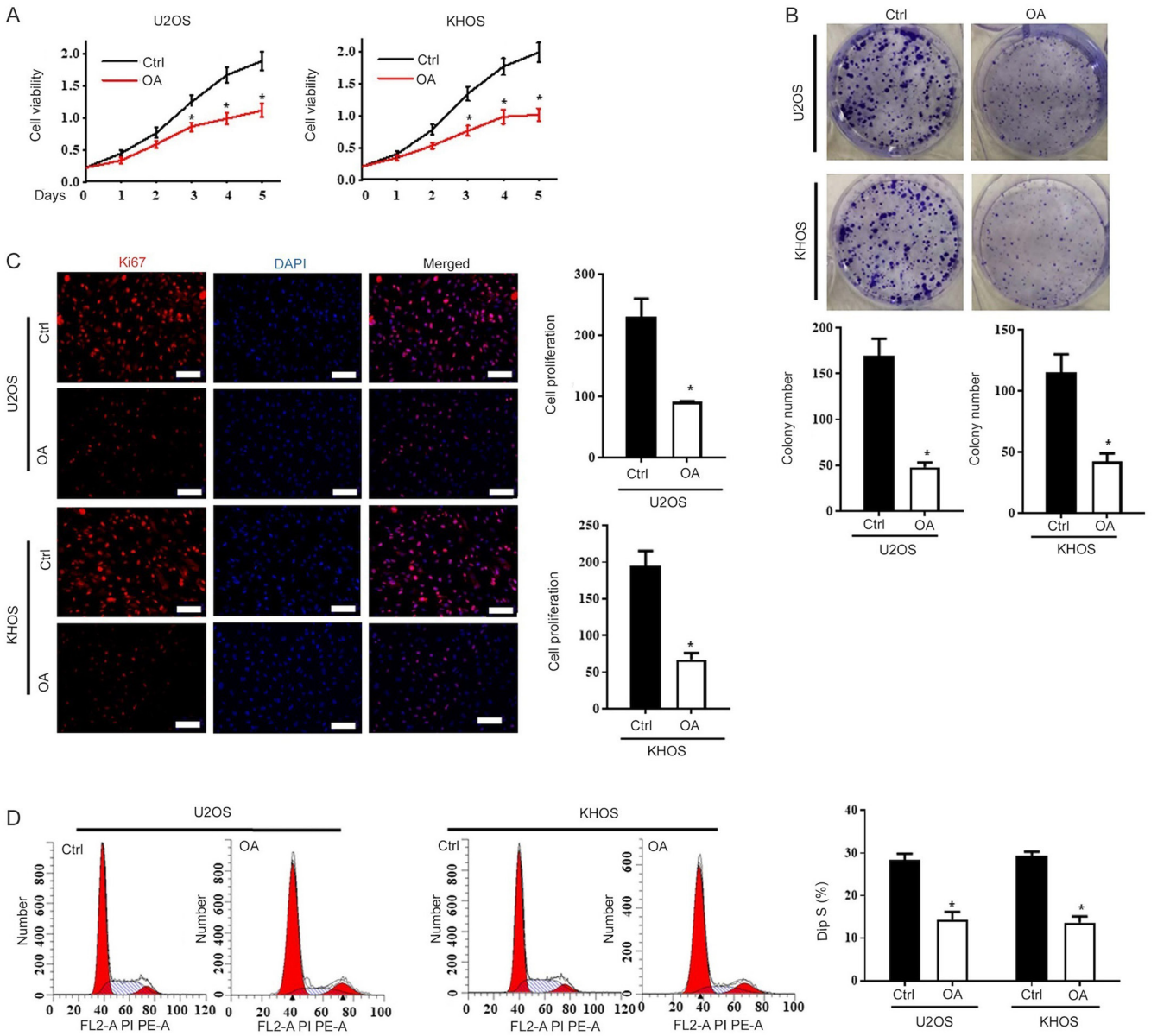

Figure 1. OA treatment inhibits osteosarcoma cell proliferation. (A) OA significantly inhibited osteosarcoma cell viability, as determined by performing the Cell Counting Kit-8 assay (n=8). (B) OA significantly inhibited osteosarcoma cell colony formation. Cell colonies were stained using crystal violet and observed under a microscope (magnification, x40; n=5). (C) Ki67 staining in osteosarcoma cells cultured with or without OA (scale bar, 40 μm; n=5). (D) Flow cytometry was performed to assess the cell cycle distribution of osteosarcoma cell lines (n=5). *P<0.05 vs. Ctrl. OA, oleanolic acid; Ctrl, control.

siRNA (5'-GCGACGUCAUCUCCAACAU-3') or scramble siRNA (5'-UAGAGCUAGAGCAAGGGUA-3') (both GE Healthcare Dharmacon, Inc.) using 6 μl Oligofectamine in Opti-MEM medium (Invitrogen; Thermo Fisher Scientific, Inc.). At 24 h post-transfection, cells were cultured in DMEM supplemented with 10% FBS for 24 h at 37˚C with 5% CO_2. Subsequently, transfection efficiency was determined via RT-qPCR and western blotting (Fig. S1).

Statistical analysis. Statistical analyses were performed using SPSS software (version 22.0; IBM Corp.). Comparisons among multiple groups were analyzed using one-way ANOVA followed by Holm-Sidak's post hoc test. Comparisons between two groups were analyzed using unpaired Student's t-test. Data are presented as the mean ± SEM. P<0.05 was considered to indicate a statistically significant difference.

Results

OA inhibits osteosarcoma cell proliferation and invasion. To investigate the protective effect of OA against osteosarcoma, the primary low-metastatic U2OS (11) and high-metastatic KHOS (12) cell lines were treated with OA. The CCK-8 assay was performed to assess cell viability, and the results indicated that OA significantly inhibited cell viability compared with the control (Ctrl) group on days 3, 4 and 5 in both U2OS and KHOS cells (Fig. 1A). Similar results were obtained in the colony formation assay (Fig. 1B). The Ki67 staining results indicated that cell proliferation in the OA-treated group was significantly lower compared with the Ctrl group in both osteosarcoma cell lines (Fig. 1C). Cell cycle analysis indicated that OA significantly reduced the percentage of U2OS and KHOS cells in the S phase compared with the Ctrl group (Fig. 1D).

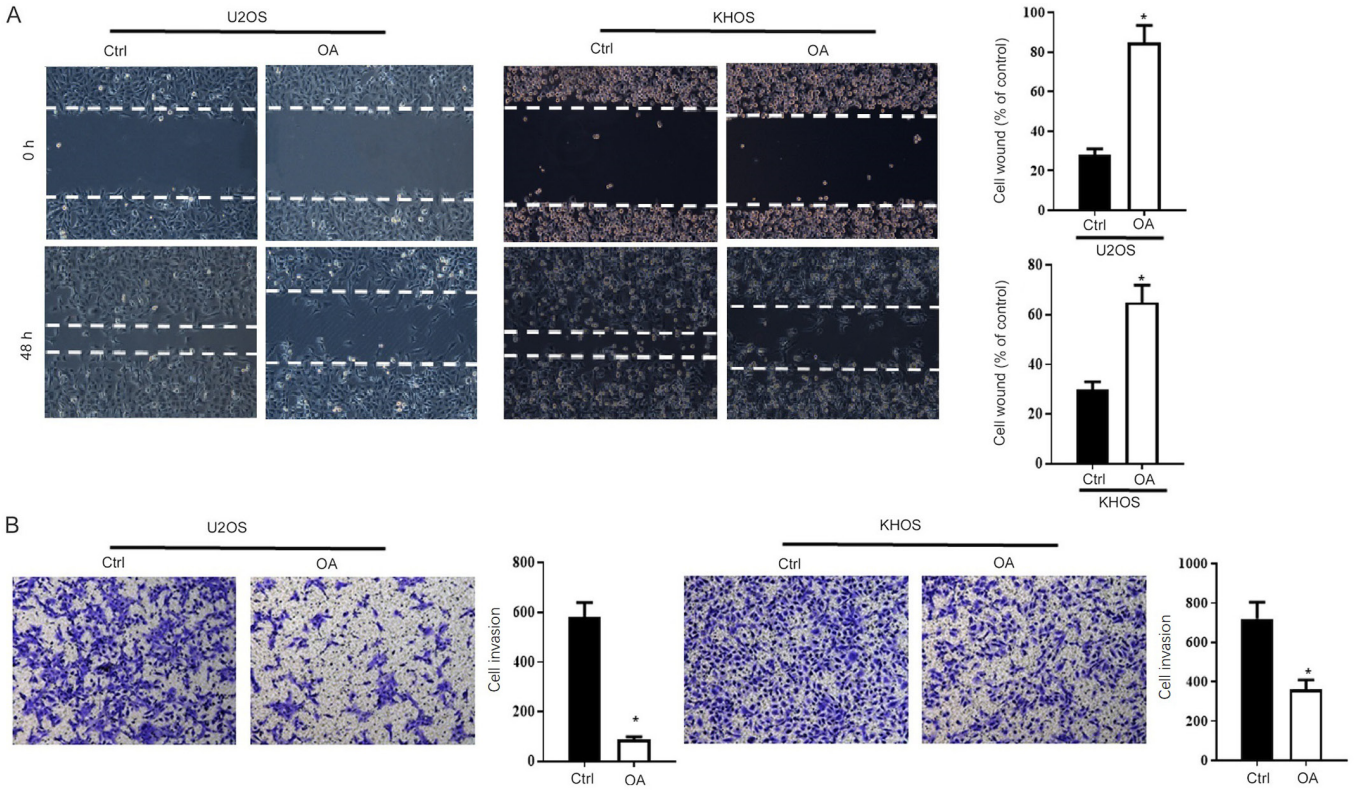

Figure 2. OA treatment inhibits osteosarcoma cell migration and invasion. Cell migration and invasion were assessed by performing (A) wound healing and (B) Transwell assays, respectively (magnification, x100; n=5). *P<0.05 vs. Ctrl. OA, oleanolic acid; Ctrl, control.

Further studies investigated the effects of OA on osteosarcoma cell migration and invasion. The wound healing assay results indicated that the OA group displayed significantly decreased cell re-colonization of the wound after 48 h compared with the Ctrl group (Fig. 2A). Moreover, the Transwell assay results indicated that OA significantly inhibited cell invasion compared with the Ctrl group (Fig. 2B). An additional osteoblastic cell line, hFOB1.19, was used to assess the effect of OA on non-tumor cells. Compared with the Ctrl group, OA did not significantly alter hFOB1.19 cell viability, colony formation, proliferation, migration or invasion, as determined by performing CCK-8, colony formation, Ki67 staining, wound healing and Transwell assays, respectively (Fig. S2).

OA enhances osteosarcoma cell apoptosis. Previous studies have reported that OA increased cell apoptosis in other tumors (6,13); therefore, the effects of OA on osteosarcoma cell apoptosis were assessed. The TUNEL staining results suggested that OA significantly increased osteosarcoma cell apoptosis compared with the Ctrl group (Fig. 3A). The western blotting results also suggested OA-mediated induction of cell apoptosis. Caspase-3, a key enzyme involved in apoptosis (14), was significantly activated in OA-treated osteosarcoma cells compared with control osteosarcoma cells. In addition, OA treatment markedly reduced Bcl-2 expression compared with the Ctrl group (Fig. 3B and C).

OA inactivates the SOX9/Wnt1 signaling pathway in osteosarcoma cells. Subsequently, whether OA downregulated the SOX9/Wnt1 signaling pathway was investigated. The protein expression levels of SOX9, β-catenin and Wnt1 were significantly decreased in OA-treated U2OS and KHOS cells compared with Ctrl cells (Fig. 4A and B). Moreover, OA-mediated downregulation of Wnt1 expression was significantly reversed by transfection with SOX9 siRNA (Fig. 4C).

Discussion

The results of the present study were consistent with the hypothesis that OA, the 3β-hydroxy-olean-12-en-28-oic acid that is widely distributed in the plant kingdom as free acid or as aglycone of triterpenoid saponins (13), reduced osteosarcoma cell proliferation and invasion, and promoted osteosarcoma cell apoptosis compared with the Ctrl group. Although OA displays protective effects against inflammation and oxidative stress-induced cell apoptosis (15), OA also displays a wide range of anticancer pharmacological activities (16,17). Similar to the results of the present study, previous studies also demonstrated that OA inhibits cell proliferation and invasion, and induces cell apoptosis in other tumor cells, such as thyroid, prostate and breast cancer cells, as well as hepatocellular carcinoma and glioblastoma cells (13,18-20).

OA serves an antioncogenic role during malignant tumor development by inhibiting cancer cell proliferation, migration and invasion, and triggering cell death via apoptosis, autophagy or mitophagy (6,17,21). Multiple signaling pathways are associated with OA-mediated anticancer activities. Previous studies have indicated that OA induces cancer cell apoptosis by inhibiting the Akt-mTOR signaling cascade (22-24), as well as ERK, STAT3 and NF-κB signaling pathways (21).

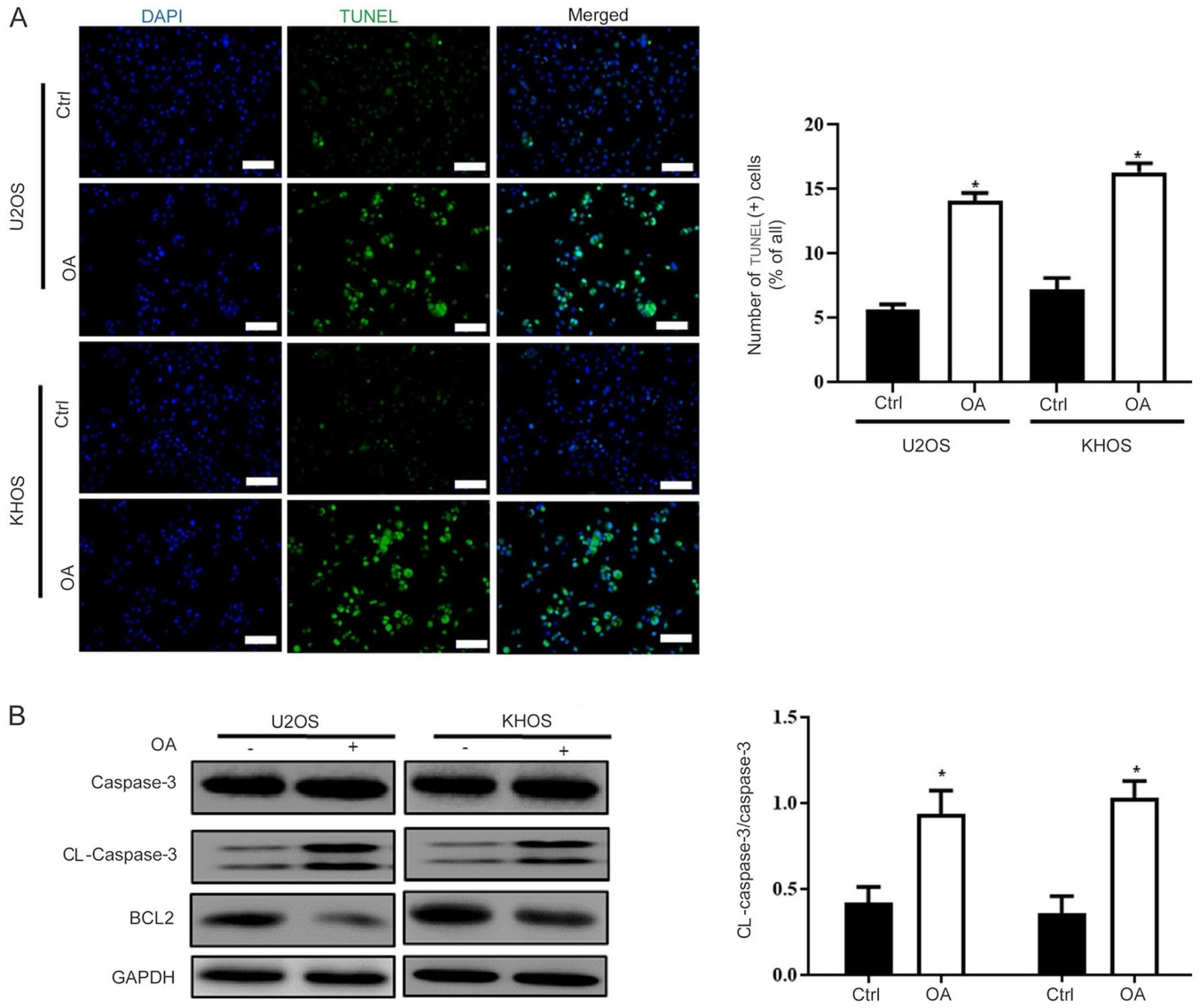

Figure 3. OA treatment enhances osteosarcoma cell apoptosis. (A) Cell apoptosis was assessed by performing a TUNEL assay (scale bar, 20 μm; n=5). (B) Caspase-3 activation and Bcl-2 expression in OA-treated osteosarcoma cells (n=4). *P<0.05 vs. Ctrl. OA, oleanolic acid; Ctrl, control; CL, cleaved.

Previous studies have also reported that OA induces osteosarcoma cell apoptosis and inhibits osteosarcoma cell proliferation; however, the underlying mechanism is not completely understood. Xu *et al* (6) reported that OA inhibits osteosarcoma cell proliferation and viability and interrupts the balance between proapoptotic and antiapoptotic factors by inhibiting the Notch signaling pathway. Moreover, the derivative of OA, N-formyl morpholine substituent of CDDO (CDDO-NFM), leads to degradation of c-Myc and decreases glucose uptake, lactate generation and adenosine triphosphate production to block glycolysis (7). In the present study, compared with the Ctrl group, OA inhibited osteosarcoma cell proliferation and invasion, and enhanced cell apoptosis by inactivating the SOX9/Wnt1 signaling pathway.

SOX9 is a member of the SOX family of transcription factors, which is closely associated with the development of a variety of malignant tumors (25). SOX9 enhances tumorigenesis by reactivating Wnt signaling (26,27). Moreover, the SOX9/Wnt1 signaling pathway also serves a key role in osteosarcoma (3). Suh *et al* (28) indicated that SOX9 regulates hyperexpression of Wnt1 in human osteosarcoma tissues and cells, and siRNA-mediated SOX9 knockdown inhibits human osteosarcoma cell proliferation by down-regulating Wnt1 expression. Furthermore, the synthetic oleanane triterpenoids, CDDO-Imidazolide (CDDO-Im) and CDDO-Ethyl amide (CDDO-EA), upregulate SOX9 expression in normal cartilage and induce chondrogenic differentiation. OA displays a contrary effect in normal and tumor cells (3,28-30); however, the results of the present study indicated that the expression levels of SOX9 and Wnt1 were significantly decreased in OA-treated osteosarcoma cells compared with Ctrl cells, which suggested that OA inhibited SOX9/Wnt1-associated osteosarcoma cell proliferation, migration and invasion.

The mechanisms underlying OA-mediated regulation of SOX9 expression have not been previously reported. Previous studies have indicated that SOX9 activity is regulated via multiple layers, including posttranslational modifications, such as Small Ubiquitin-like Modifier (SUMO)ylation. SUMOylation represses the gene transcriptional activity

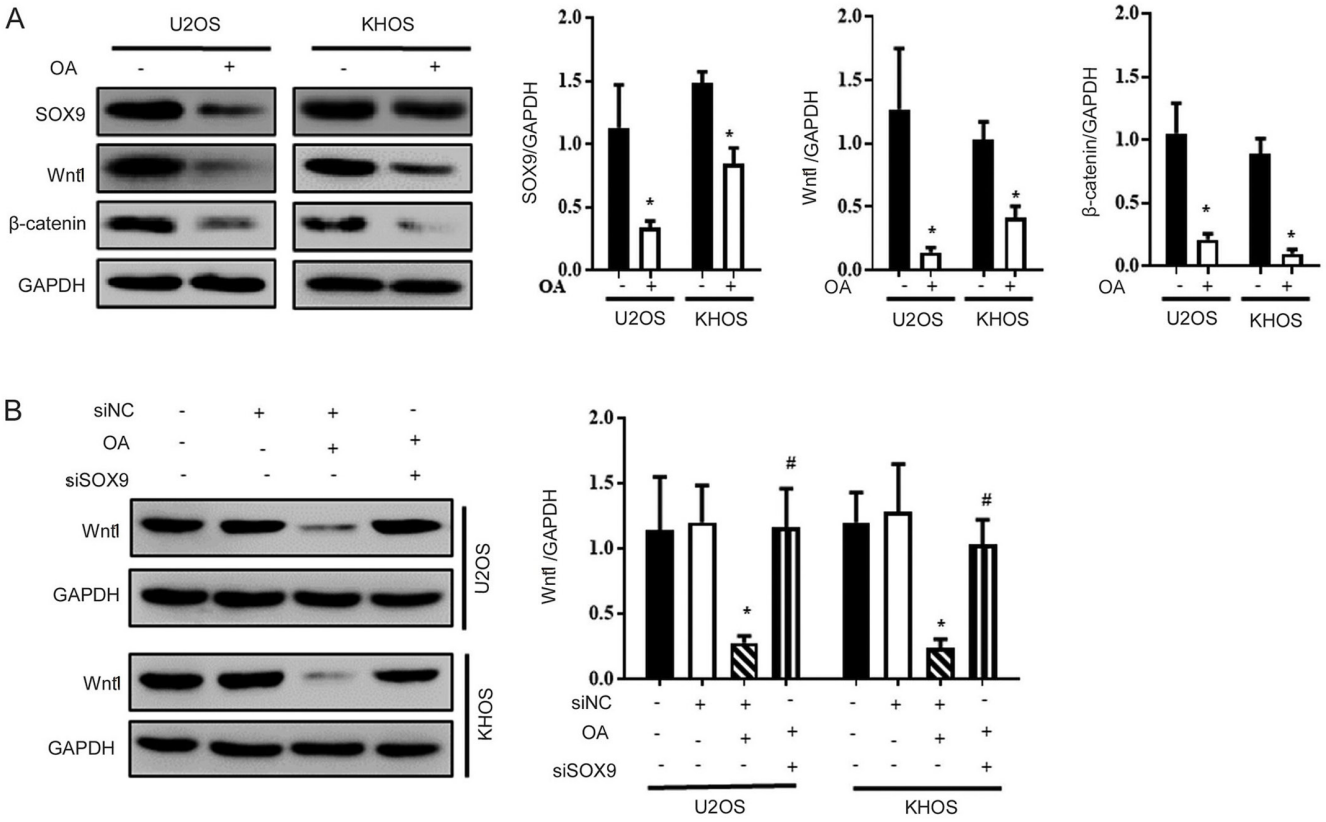

Figure 4. OA treatment inactivates the SOX9/Wnt1 signaling pathway. (A) SOX9, β-catenin and Wnt1 protein expression levels in OA-treated osteosarcoma cells. (B) SOX9 knockdown reversed OA-induced downregulation of Wnt1 expression (n=4). *P<0.05 vs. Ctrl; #P<0.05 vs. siNC + OA. OA, oleanolic acid; Ctrl, control; si, small interfering RNA; NC, negative control.

of SOX9, leading to decreased SOX9 expression (31-33). Meanwhile, Momordin Ic, an analog of OA, inhibits the activity of SUMO-specific protease 1, a member of the de-SUMOylation protease family (34), which might elevate the level of SOX9 SUMOylation and decease SOX9 expression. Future studies are required to identify the mechanisms underlying OA-mediated regulation of SOX9 expression. The current study has several limitations. Firstly, *in vivo* studies should be performed to identify the role of OA in tumor growth. Secondly, further research is required on the mechanisms that underlie the role of OA in the inhibition of SOX9 expression and the inactivation of the SOX9/Wnt1 signaling pathway.

In conclusion, the present study indicated that OA-mediated inactivation of the SOX9/Wnt1 signaling pathway may serve as a potential therapeutic strategy for osteosarcoma. The results of the present study indicated that OA significantly inhibited osteosarcoma cell proliferation, migration and invasion compared with the Ctrl group; therefore, OA may display potent antitumorigenic activities in osteosarcoma. For centuries, several medicinal plant extracts containing OA have been used in Asian countries for medical purposes (29) and have become registered drugs for the treatment of liver diseases in China (30). Chemically modified OA has been evaluated in several clinical trials for the treatment of various types of cancer such as breast, colorectal and lung cancer among others (8); however, further clinical trials are required to confirm the potential therapeutic application of OA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XC and ZW contributed to the conception or design of the work and drafting the article or revising it for important intellectual content. YZ, SZ, AW and QD performed the experiments and contributed to the acquisition of data, analysis and interpretation of data and drafting and revising the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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