

# S-phase kinase-associated protein 2 is involved in epithelial-mesenchymal transition in methotrexate-resistant osteosarcoma cells

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**Abstract.** Osteosarcoma (OS), a common worldwide primary aggressive bone malignancy, arises from primitive transformed cells of mesenchymal origin and usually attacks adolescents and young adults. Methotrexate (MTX) is the anti-folate drug used as a pivotal chemotherapeutic agent in the treatment of OS. However, patients with OS often develop drug resistance, leading to poor treatment outcomes. In the present study, in order to explore the underlying mechanisms responsible for MTX resistance, we established MTX-resistant OS cells using the U2OS and MG63 cell lines and examined whether MTX-resistant OS cells underwent epithelial-mesenchymal transition (EMT) by Transwell assay, wound healing assay, MTT assay, RT-PCR and western blot analysis. We found that the viability of the MTX-resistant cells remained relatively unaltered following further treatment with MTX compared to the parental cells. The resistant cells appeared to possess a mesenchymal phenotype, with an elongated and more spindle-like shape, and acquired enhanced invasive, migratory and attachment abilities. The measurement of EMT markers also supported EMT transition in the MTX-resistant

OS cells. Our result further demonstrated that the overexpression of S-phase kinase-associated protein 2 (Skp2) was closely involved in the resistance of OS cells to MTX and in the acquirement of EMT properties. Thus, the pharmacological inhibition of Skp2 may prove to be a novel therapeutic strategy with which to overcome drug resistance in OS.

## Introduction

Osteosarcoma (OS) is a common primary aggressive tumor affecting the bones, which arises from primitive transformed cells of mesenchymal origin (1). It is locally aggressive and is inclined to generate early systemic metastases (2). Comprehensive data regarding the epidemiology of OS have revealed that OS is most prevalent in teenagers and young adults (3,4). Current therapeutic strategies include pre-operative (neoadjuvant) chemotherapy followed by the surgical removal of all detectable cancerous lesions (including metastases) and post-operative (adjuvant) chemotherapeutic management (1). Patients with high-grade OS have acquired favorable long-term outcomes in the conjunction of systemic chemotherapy with surgery (5). A 5-year event-free survival of 60-70% is achieved in extremity localized, non-metastatic disease following the introduction of chemotherapy (6,7). Chemotherapeutic agents that have shown toxicity against OS include cisplatin, doxorubicin, oxazaphosphorines and high-dose methotrexate (HDMTX) (8-10).

Methotrexate (MTX), originally known as 4-aminopteroyl-glutamic acid, is a pivotal chemotherapeutic agent that was discovered in the 1940s and was originally used in the treatment of childhood leukemia and lymphoma (11). MTX has been used as a chemotherapeutic agent in the treatment of various types of human cancer, including breast cancer (12,13), leukemia (14,15), lung cancer (16), gastric choriocarcinoma (17), lymphoma (18) and OS (19), either alone or in combination with other agents. However, 35-45% of patients with OS acquire drug resistance due to the inherent resistance to chemotherapeutic agents or due to the fact that they become

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unresponsive to these drugs during chemotherapy (20,21). There is evidence to suggest that epithelial-mesenchymal transition (EMT) is associated with acquired resistance to chemotherapeutic drugs in human malignancies (22). EMT is a biological process during which the phenotype of polarized epithelial cells transforms into one of mesenchymal cells (23). At the molecular level, during the transition, a decrease in the expression of epithelial cell markers [such as Zonula occludens-1 (ZO-1) and E-cadherin] is observed, as well as an increase in the expression of stromal cell markers [such as N-cadherin, Slug, Snail, Twist, Vimentin, and zinc finger E-box binding homeobox (ZEB)1 and ZEB2] (24,25). Chemotherapy has been reported to induce EMT in tumor cells. Fang *et al* found that Snail inhibition by transfection with specific small interfering RNA (siRNA) promoted cisplatin sensitivity, and cisplatin-induced EMT was significantly blocked (26). In addition, baicalin has been shown to inhibit human OS cell invasion, metastasis and anoikis resistance by suppressing transforming growth factor (TGF)- $\beta$ 1-induced EMT (27). Recently, it was reported that catalpol suppresses OS cell proliferation by blocking EMT and inducing apoptosis (28). Ohbayashi *et al* found that lung cancer cells treated with MTX exhibited an EMT-like phenotype accompanied by the elevation of the expression of interleukin-6 (IL)-6 and TGF- $\beta$ 1, as well as an enhancement of migration (29). However, whether MTX triggers EMT in OS remains to be fully determined.

F-box E3 ubiquitin ligase S-phase kinase-associated protein 2 (Skp2) belongs to the ubiquitin proteasome system (UPS). The deregulation of Skp2-mediated ubiquitination and the proteolysis of its substrates is involved in tumorigenesis in various types of human cancer (30). A previous study revealed that Skp2 was overexpressed and was associated with a poor prognosis in prostate cancer (31), lymphomas (32), gastric cancer (33), breast cancer (34), liver cancer (35) and nasopharyngeal carcinoma (NPC) (36), thereby functioning as a proto-oncogene. Skp2 has been reported to modulate the cell cycle, cell proliferation, apoptosis and metastasis in a variety of human cancers by regulating numerous substrates (30,37,38). Targeting Skp2 suppresses tumorigenesis by Arf-p53-independent cellular senescence (39). Skp2 has been shown to be highly expressed in NPC specimens and to be associated with a poor prognosis, and Skp2 inactivation has been shown to promote cellular senescence in NPC cell lines through p21<sup>cip</sup>/WAF and p27<sup>Kip</sup> (40). Furthermore, Skp2 has been reported to function as a critical component in the PTEN/PI3-kinase pathway for the regulation of p27 and cell proliferation in carcinomas (41). Skp2 has also been shown to promote the ubiquitin-mediated proteolysis of forkhead box O1 (Foxo1) and to play a key role in tumorigenesis (42). Inuzuka *et al* found that Skp2 enhanced cellular migration through ubiquitination and the destruction of E-cadherin (43). Recently, it was reported that the depletion of Skp2 inhibited cell growth and triggered the apoptosis of the OS cell lines, MG63 and SW 1353 cells (44). Therefore, Skp2 may be an effective therapeutic target in the coming age of cancer therapy.

In this study, we examined whether Skp2 was associated with MTX-induced EMT in OS cells. We established MTX-resistant OS cell lines using the U2OS and MG63 cells. We then examined whether the MTX-resistant OS cells underwent the transition from an epithelial into a mesenchymal

phenotype. Finally, we provide evidence that Skp2 is involved in the resistance of OS cells to MTX and is closely associated with the acquirement of mesenchymal characteristics.

## Materials and methods

**Cell culture and reagents.** The human osteosarcoma cell lines, U2OS and MG63, were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) medium supplemented with penicillin (100 U/ml), and streptomycin (100 U/ml) and 10% fetal bovine serum (FBS). MTX, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and anti- $\alpha$ -tubulin (T9028) primary antibody were purchased from Sigma (St. Louis, MO, USA). Matrigel was purchased from BD Biosciences (San Jose, CA, USA). Primary antibodies against ZO-1 (#5406), N-cadherin (#4061), E-cadherin (#3195), Slug #9585), Vimentin (#5741), Nanog (#4903), octamer-binding transcription factor 4 (Oct4, #2750), ATP-binding cassette sub-family B member 1 (ABCB1, #12683), FoxO1 (#2880) and p21 (#2946) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-Skp2 (sc-7164) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). To establish MTX-resistant cell lines, the U2OS and MG63 cells were cultured at 37°C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) in increasing concentrations of MTX (10-40  $\mu$ M) for >6 months. The MTX-resistant OS cells developed a resistance to 40  $\mu$ M MTX.

**Cell viability assay.** The parental and MTX-resistant OS cells (4x10<sup>3</sup> cells/well) were seeded in 96-well plates and incubated at 37°C overnight. Various concentrations of MTX (10, 20, 30 and 40  $\mu$ M) were added and cell culture was continued for 48 and 72 h. MTT assay was then performed to measure cell viability using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 570 nm.

**Cell attachment and detachment.** For attachment assay, 5x10<sup>4</sup>/well cells were seeded in a 24-well plate and incubated at 37°C. One hour later, the unattached cells were removed and the attached cells were counted using the Countess II FL Automated Cell Counter (Thermo Fisher Scientific). For cell detachment assay, the cells were seeded and incubated at 37°C for 24 h. The cells were treated with 0.05% trypsin for 3 min, and then counted as detached cells using the Countess II FL Automated Cell Counter.

**Viral infection.** Skp2 knockdown was performed using Skp2 short hairpin (Skp2-RNAi; shRNA1, shRNA2, shRNA3, Genechem, Shanghai, China) or scrambled shRNA (CON054) lentiviral particles (Genechem). 293T cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin at 37°C with 5% CO<sub>2</sub>. The 293T cells were co-transfected with the packaging plasmids, pVSV-G, p $\Delta$ R-rev (Jiran Co., Shanghai, China) and shRNA or scrambled shRNA expression plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to produce lentivirus particles according to the manufacturer's

instructions (Invitrogen). Supernatant from the 293T cells was collected at 48 h following transfection. The MTX-resistant OS cells were grown to 40-50% confluency in DMEM with 10% FBS and exposed to the collected lentivirus particles for 48 h. Subsequently, the cells were selected by 4  $\mu\text{g}/\text{ml}$  puromycin. The puromycin-contained medium was displaced every 3 days for approximately 2 weeks until the non-transduced cells disappeared. Single clones were selected, and inoculated onto a new plate to grow in the presence of puromycin. The single clone with the stable knockdown of the *Skp2* gene was expanded and passaged for use in subsequent experiments.

**Invasion assay.** The MTX-resistant and parental OS cells were observed and photographed under a microscope (Olympus IX71; Olympus, Tokyo, Japan) to observe any morphological changes. Subsequently, the invasive capacity of the MTX-resistant cells was determined by placing the cells into 24-well Transwell inserts pre-coated with Matrigel following the manufacturer's instructions. Briefly, the OS cells, MTX-resistant cells with control shRNA-transfected or *Skp2* shRNA-transfected cells were cultured in the upper chamber of the inserts with 200  $\mu\text{l}$  FBS-free DMEM. The bottom chamber contained 500  $\mu\text{l}$  medium with 10% FBS. Following incubation at 37°C for approximately 24 h, the non-invading cells on the upper surface of the filter membrane were removed carefully using a cotton swab, while the invading cells on the bottom surface of the membrane were stained with Calcein-AM (C3099, Invitrogen) for 10 min. Subsequently, the membrane was rinsed with water and photographed and the invading cells were counted under a fluorescent microscope (Olympus, IX71; Olympus).

**Wound healing assay.** The parental, MTX-resistant cells with control shRNA transfection and MTX-resistant cells in which *Skp2* was knocked down were seeded ( $1 \times 10^5$  cells/ml) in a 6-well plate. The supernatant was absorbed after the cells grew to approximately 90% confluence. The scratch wound was generated by scratching the monolayers with a 10  $\mu\text{l}$  sterile pipette tip. The cells were washed carefully with PBS to remove floating cell debris and then supplemented with DMEM. Following cultured for approximately 20 h, the cells that had migrated into the wound area were photographed under an inverted microscope (Olympus, IX71; Olympus).

**Reverse transcription-quantitative RT-PCR (RT-qPCR).** Total RNA was isolated from the parental and MTX-resistant OS cells using the RNeasy Plus Mini kit (Qiagen China Co., Ltd, Shanghai, China). The concentrations and purities of the RNA were determined by an ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). First-strand cDNA was reverse transcribed using the TaqMan Reverse Transcription Reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mRNA levels of EMT markers, including Vimentin, Slug, N-cadherin, ZO-1 and E-cadherin were detected by RT-qPCR assay using the SYBR-Green assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI 7900 HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were as follows: *Skp2* forward, 5'-GCT GCT AAA GGT CTC TGG TGT-3' and reverse, 5'-AGG CTT AGA TTC

TGC AAC TTG-3'; E-cadherin forward, 5'-GAA GTG TCC GAG GAC TTT GG-3' and reverse, 5'-CAG TGT CTC TCC AAA TCC GAT A-3'; N-cadherin forward, 5'-CCT GCG CGT GAA GGT TTG CC-3' and reverse, 5'-CCA AGC CCC GCA CCC ACA AT-3'; Vimentin forward, 5'-TGT CCA AAT CGA TGT GGA TGT TTC-3' and reverse, 5'-TTG TAC CAT TCT TCT GCC TCC TG-3'; Slug forward, 5'-CAT GCC TGT CAT ACC ACA AC-3' and reverse, 5'-GGT GTC AGA TGG AGG AGG G-3'; ZO-1 forward, 5'-AGA AGA TAG CCC TGC AGC-3' and reverse, 5'-AGT CCA TAG GGA GAT TCC-3'; and GAPDH forward, 5'-ACC CAG AAG ACT GTG GAT GG-3' and reverse, (5'-CAG TGA GCT TCC CGT TCA G-3'). The expression of GAPDH was used as an internal control.

Target sequences were amplified at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.  $\beta$ -actin was amplified as an endogenous normalization control. The fold change in the mRNA level was calculated according to  $2^{-\Delta\Delta C_q}$  method (45).

**Western blot analysis.** Total proteins were isolated from the cells with protein lysis buffer. The concentrations of the protein samples were determined by bicinchoninic acid (BCA; Thermo Scientific) protein assay. Proteins samples (40  $\mu\text{g}$ ) were run and separated on a 10% of SDS-polyacrylamide gel (SDS-PAGE), and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking in blocking buffer (1X TBST with 5% w/v de-fatted milk powder), the membranes were incubated with specific primary antibodies at 4°C overnight. Primary antibodies against *Skp2* (1:1,000), ZO-1 (1:1,000), N-cadherin (1:1,000), E-cadherin (1:2,000), Slug (1:1,500), Vimentin (1:1,000), Nanog (1:1,000), Oct4 (1:1,500), ABCB1 (1:1,500), Foxo1 (1:1,500) and p21 (1:1,000) were used. The membranes were then washed with TBST and probed with anti-mouse (Cat. no. #A3682, 1:4,000, Sigma-Aldrich, St. Louis, MO, USA) or anti-rabbit secondary antibodies (cat. no. A16110 1:3,000, Thermo Fisher Scientific) at room temperature for 1 h. Finally, the membranes were washed again and detected using enhanced chemiluminescence substrate (ECL) (Sigma-Aldrich; EMD Millipore). Quantitative analysis was carried out using QuantiOne imaging software with gel imaging equipment (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Statistical analysis was carried out using GraphPad Prism 4.0 (Graph pad Software, La Jolla, CA, USA). The mean, standard error and P-values were analyzed using the two-tailed Student's t-test. Data are presented as the means  $\pm$  SEM.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Establishment of MTX-resistant human osteosarcoma cell lines.** MTX-resistant human osteosarcoma cell lines were established by continuous stepwise selection with increasing concentrations of MTX in the parental OS cell lines for >6 months. Briefly, the cells were cultured at the exponential phase and exposed to a low concentration of MTX for 3-4 days. The dead cells were removed and increasing concentrations of MTX were then added to the culture medium. After the OS

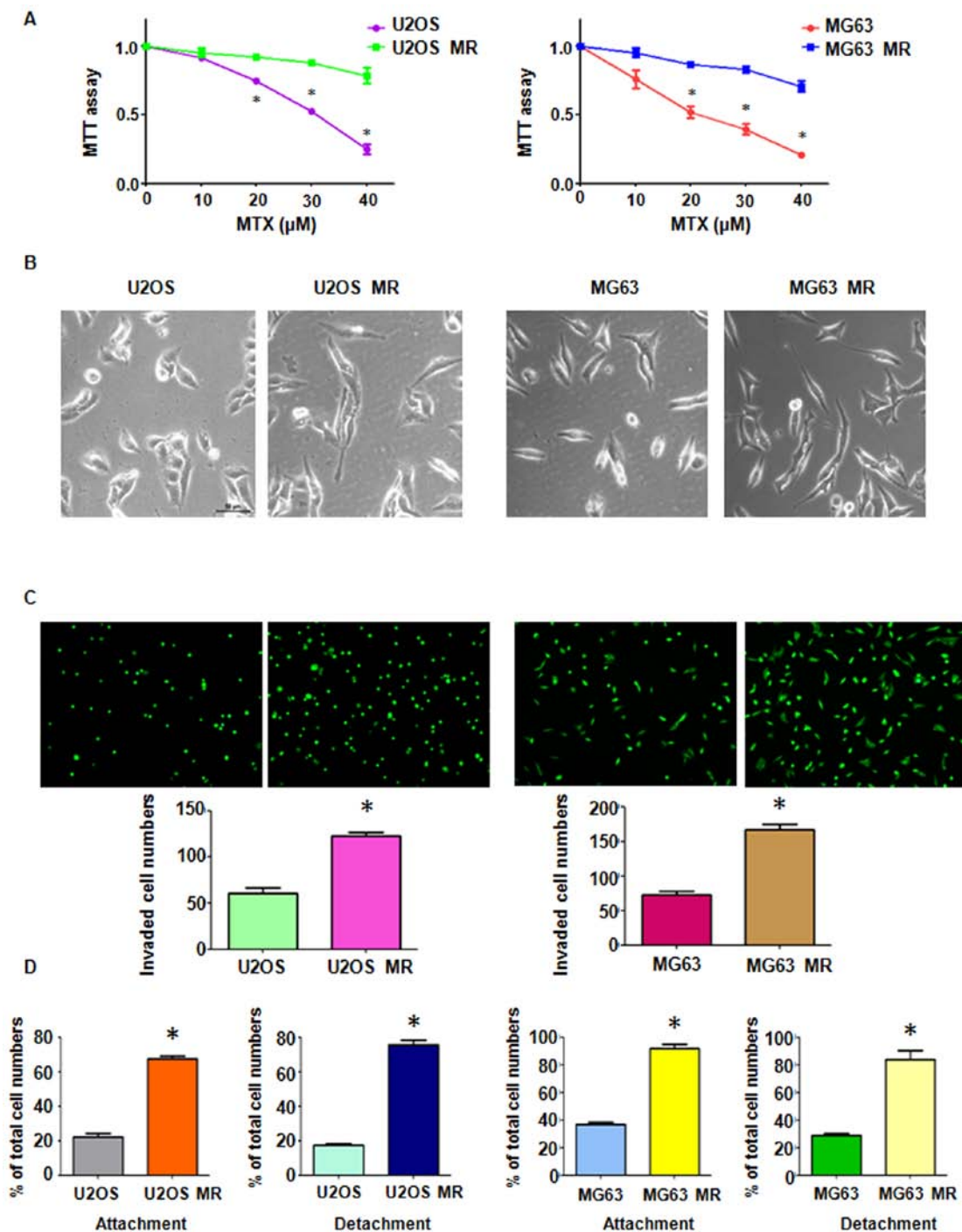


Figure 1. Methotrexate (MTX)-resistant cells acquire the epithelial-mesenchymal transition (EMT) phenotype. (A) MTX-resistant osteosarcoma cells were observed to have an increased cell proliferation (thus great resistance to MTX), as shown by MTT assay. (B) Cell morphology was observed by phase contrast microscopy in parental and MTX-resistant osteosarcoma (OS) cells. (C) Upper panel: Cell invasion assay was performed to measure the invasive capacity of the parental and MTX-resistant OS cells. Lower panel: Results of quantitative analysis are shown for the findings in the upper panel. \* $P < 0.05$  vs. control (parental cells). (D) Cell attachment and detachment assays were carried out in the parental and MTX-resistant cells. \* $P < 0.05$  vs control (parental cells).

cells were cultured for more than half a year with increasing concentrations of MTX, MTX-resistant cells were established. The surviving cells were observed to exhibit an enhanced resistance to MTX. MTT assay revealed that the U2OS and MG63 MTX-resistant cell lines were successfully established, as the MTX-resistant cells had an increased viability compared to the parental cells (Fig. 1A). The MTX-resistant OS cells developed a resistance to 40  $\mu\text{M}$  MTX. During the maintenance of MTX-resistant OS cells in drug-free medium,

the stable resistance to MTX was guaranteed by continuously measuring the  $\text{IC}_{50}$  value monthly.

*MTX treatment promotes mesenchymal-like properties in MTX-resistant OS cells.* Drug-resistant cells always exhibited the EMT phenotype (46). Cell morphological changes in the MTX-resistant OS cells were observed under a light microscope. We found that both the U2OS and MG63 MTX-resistant cell lines appeared to possess the mesenchymal phenotype, as

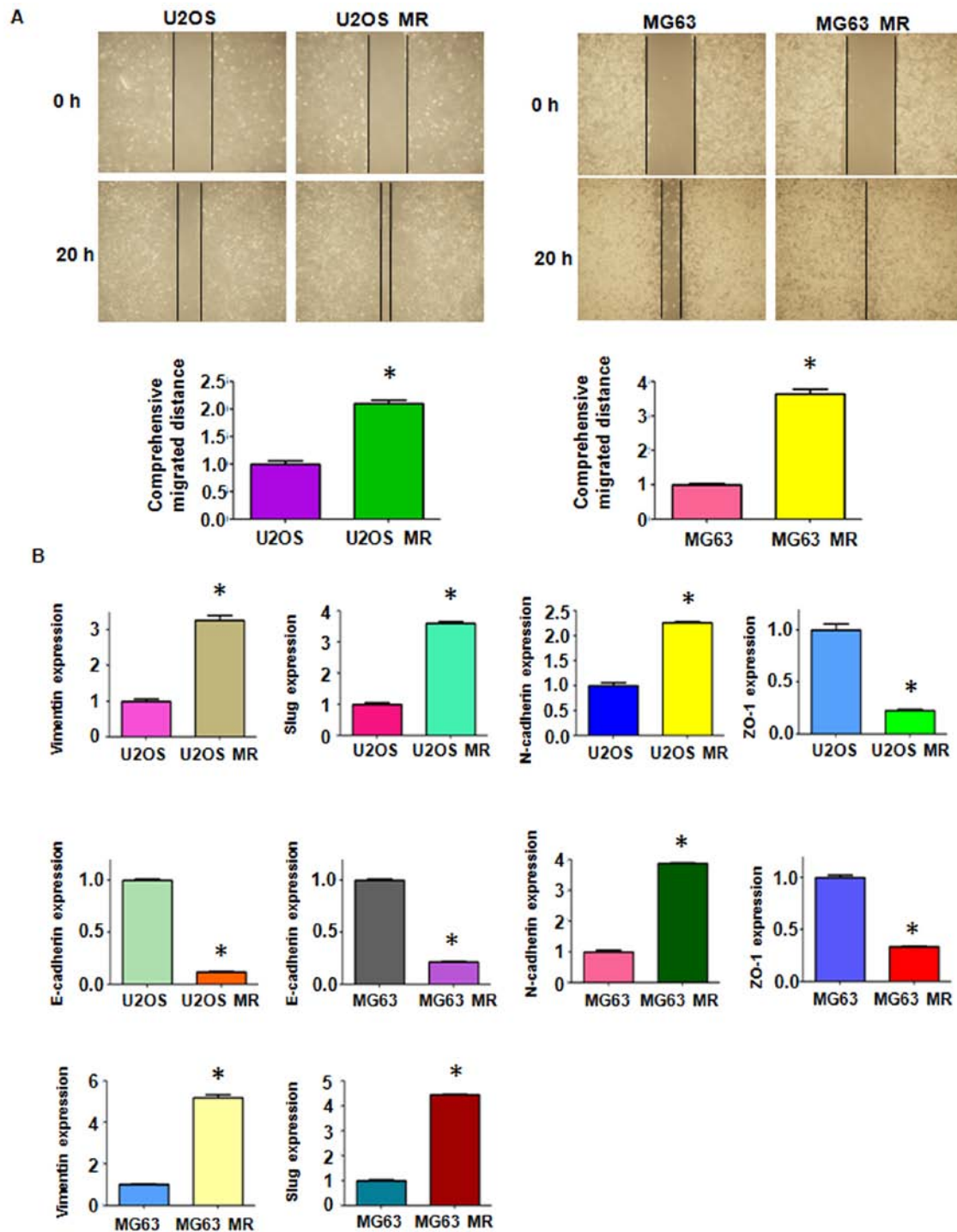


Figure 2. Methotrexate (MTX)-resistant cells exhibit an enhanced migration and acquire epithelial-mesenchymal transition (EMT)-like characteristics. (A) Upper panel: Wound healing assays were performed using cell monolayers to detect the motility of MTX-resistant osteosarcoma (OS) cells. Cells that migrated into the wounded region were photographed. Lower panel: Results of quantitative of the findings shown in the upper panel by comprehensive migrated distance. \* $P < 0.05$  vs. control (parental cells). (B) RT-qPCR revealed that the mRNA expression levels of the mesenchymal markers, Vimentin, Slug and N-cadherin, were upregulated, whereas those of the epithelial molecules, ZO-1 and E-cadherin, were markedly decreased in the MTX-resistant OS cells. \* $P < 0.05$  vs. control (parental cells).

the cells had developed into elongated and more spindle-like shapes (Fig. 1B).

**EMT characteristics of MTX-resistant OS cells.** Multiple biological changes were examined in the MTX-resistant OS cells. The results of Transwell assay revealed a significant increase in the invasive ability of both MTX-resistant OS

cell lines (Fig. 1C). Moreover, the MTX-resistant OS cells developed intensive attachment and detachment capacities, compared with their parental cell lines (Fig. 1D). The cell motility activity was further detected by wound healing assay. We observed an increased amount of MTX-resistant cells which had migrated into the wound area, indicating an enhanced motility activity of the drug-resistant cells (Fig. 2A).

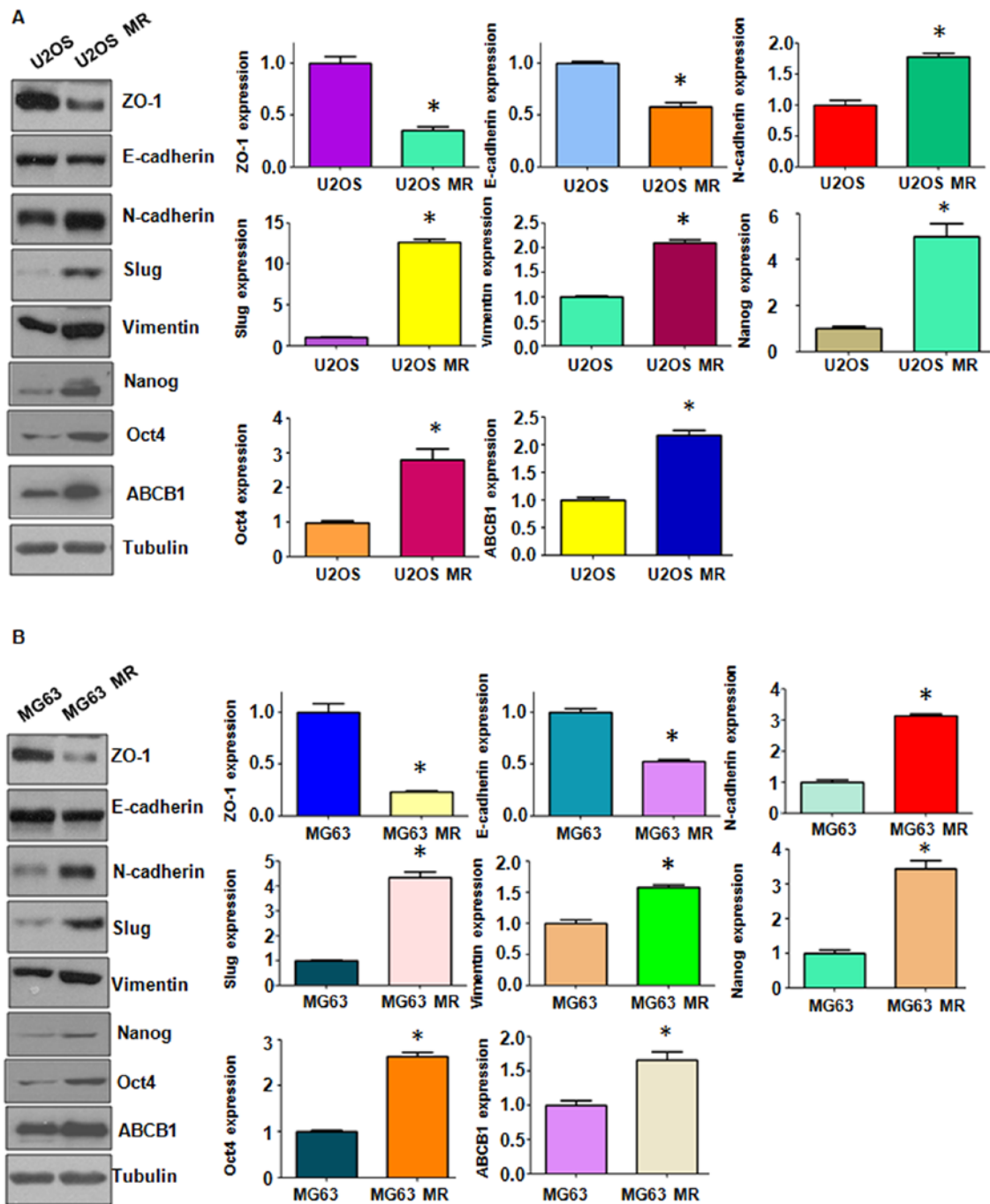


Figure 3. Methotrexate (MTX)-resistant osteosarcoma (OS) cells undergo epithelial-mesenchymal transition (EMT)-like changes. (A) Left panel: The expression levels of E-cadherin, N-cadherin, Slug, Vimentin, Nanog, Oct4, ABCB1 and ZO-1 were detected by western blot analysis in the U2OS and U2OS MTX-resistant cells. Right panel: Results of quantitative of the blots shown in the left panel. \* $P < 0.05$  vs. control (parental cells). (B) Left panel: The expression levels of the above-mentioned EMT-related and stem cell markers were measured by western blot analysis in the MG63 and MG63 MTX-resistant cells. Right panel: Results of quantitative of the blots shown in the left panel. \* $P < 0.05$  vs. control (parental cells).

*MTX-resistant OS cells undergo EMT and acquire stem cell molecular markers.* To investigate whether drug-resistant cells undergo EMT-related molecular marker changes, the mRNA and protein levels of several EMT markers were measured between the resistant cells and their paired parental cells. RT-qPCR analysis was performed to detect the expression of mRNAs. The results revealed a significant increase in the mRNA levels of mesenchymal markers, such as Vimentin, Slug and N-cadherin in the MTX-resistant OS cells (Fig. 2B). By contrast, the expression levels of the epithelial molecules,

ZO-1 and E-cadherin, were markedly decreased in the MTX-resistant OS cells (Fig. 2B). We further confirmed the changes in the protein expression levels of EMT markers by western blot analysis. We observed changes in the protein levels of EMT markers in the MTX-resistant OS cells (Fig. 3). We also found that the stem cell markers, Nanog and Oct4, were highly expressed in the MTX-resistant cells (Fig. 3). Importantly, we found that ABCB1 expression was increased in the MTX-resistant cells (Fig. 3). Thus the MTX-resistant OS cells acquired EMT-like and stem cell-like characteristics;

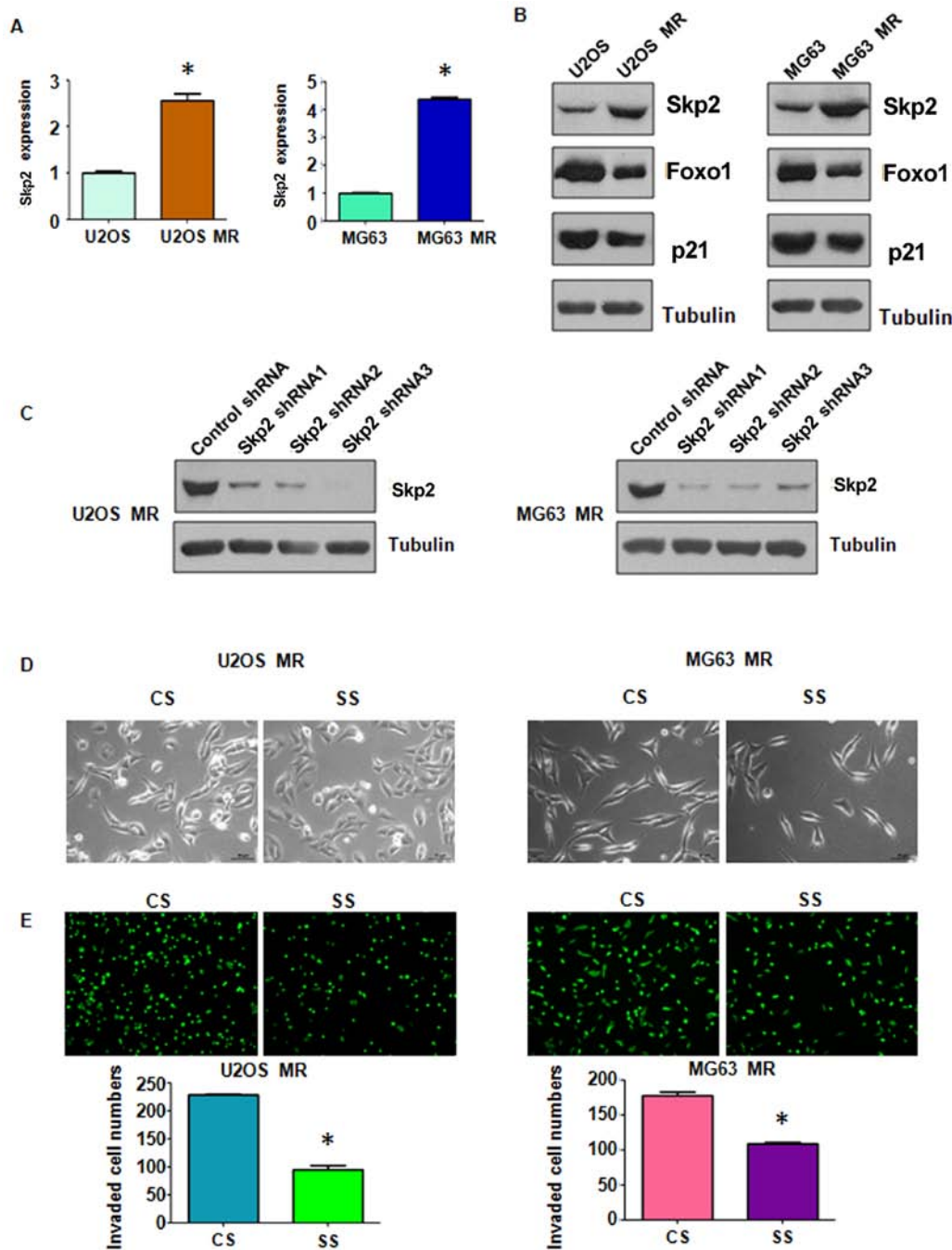


Figure 4. Skp2 is overexpressed in methotrexate (MTX)-resistant osteosarcoma (OS) cells and the downregulation of Skp2 suppresses cell invasion. (A) The mRNA expression of Skp2 was detected by RT-qPCR assay in the parental and MTX-resistant OS cells. \* $P < 0.05$  vs. control (parental cells). (B) The protein expression levels of Skp2 and its targets, Foxo1 and p21, were detected by western blot analysis. (C) RNAi efficiency was determined by western blot analysis in the MTX-resistant OS cells transfected with Skp2 shRNA. (D) Cell morphology was observed under a phase contrast microscope. (E) Upper panel: Cell invasion assay were performed in the MTX-resistant OS cells in which Skp2 was knocked down. Lower panel: Results of quantitative of the findings shown in the upper panel. \* $P < 0.05$  vs. control. CS, control shRNA-transfected MTX-resistant OS cells; SS, Skp2 shRNA-transfected MTX-resistant OS cells.

their drug-resistant capabilities may be attributed to mesenchymal transition.

*Skp2 expression is elevated in MTX-resistant OS cells.* Skp2 enhances tumor metastasis by modulating molecular markers of EMT (47,48). In accordance with this finding, in this study, we observed that Skp2 expression was significantly elevated in the MTX-resistant OS cells at both the mRNA and protein level (Fig. 4A). Moreover, we found that the levels of downstream molecules of Skp2, Foxo1 and p21, were markedly

downregulated in the MTX-resistant cells compared with the parental cells (Fig. 4A and B). These findings suggest that Skp2 is closely involved in EMT induced by MTX resistance and may thus play a critical role in human OS.

*Stable downregulation of Skp2 reverses EMT to mesenchymal-epithelial transition (MET) in MTX-resistant OS cells.* Stable Skp2 knockdown in the MTX-resistant OS cells was established by using Skp2 shRNA lentiviral particles infection. The efficiency of RNAi was confirmed by western blot analysis. As

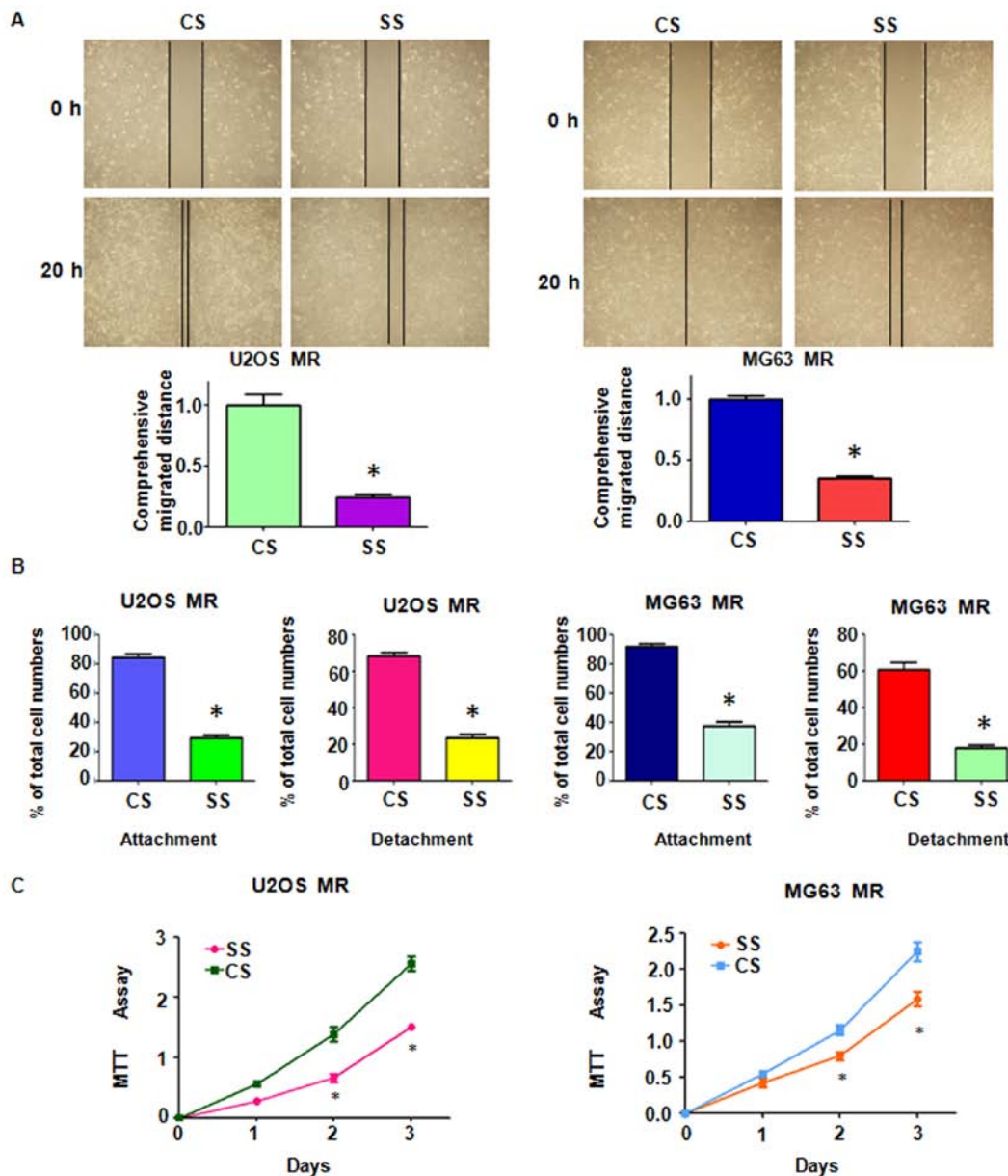


Figure 5. Stable knockdown of Skp2 inhibits motility and restores sensitivity to methotrexate (MTX) in MTX-resistant osteosarcoma (OS) cells. (A) Upper panel: Cell monolayers were scratched with a clean tip. Cells that had migrated into the wounded region were photographed. Lower panel: Results of quantitative analysis of the findings shown in the upper panel. \* $P < 0.05$  vs. control. (B) Cell attachment and detachment ability were measured in the MTX-resistant OS cells following the knockdown of Skp2. \* $P < 0.05$  vs. control. (C) MTT assay revealed that the stable knockdown of Skp2 restored the sensitivity of the MTX-resistant cells to MTX, as evidenced by the decrease proliferation of the cells in which MTX was knocked down. CS, control shRNA-transfected MTX-resistant OS cells; SS, Skp2 shRNA-transfected MTX-resistant OS cells. \* $P < 0.05$  vs. control.

shown in Fig. 4C, Skp2 expression was effectively suppressed in both the U2OS and MG63 MTX-resistant cells. We selected Skp2 shRNA2 lentiviral particles to infect the MTX-resistant OS cells in the subsequent experiments. We observed that following the exposure of MTX-resistant OS cells in which Skp2 was knocked down (SS group in Fig. 4C) to MTX, they exhibited a less spindle-like shape (Fig. 4D). Thus, Skp2 knockdown partially reversed EMT to MET. Moreover, the results of Transwell assay revealed that the invasive ability of the MTX-resistant OS cells was markedly inhibited following Skp2 knockdown (Fig. 4E). The effects of Skp2 knockdown on mobility of the MTX-resistant cells were further determined by wound healing assay. Skp2 knockdown suppressed the migratory ability of the MTX-resistant OS cells (Fig. 5A).

Furthermore, Skp2 knockdown markedly abrogated the attachment and detachment capacity of the MTX-resistant OS cells (Fig. 5B). It is important to note that Skp2 shRNA did not inhibit cell growth at 24 h (Fig. 5C). However, Skp2 shRNA suppressed cell migration (Fig. 5A) and invasion (Fig. 4E) at 20 and 24 h, respectively, suggesting that the suppression of cell migration and invasion by Skp2 shRNA was not due to cell growth inhibition by Skp2 downregulation. Taken together, these results demonstrate that Skp2 may play an important role in the regulation of EMT in MTX-resistant OS cells.

*Stable downregulation of Skp2 enhances the sensitivity of resistant OS cells to MTX treatment.* MTT assay was carried out to further examine the effects of stable the downregulation



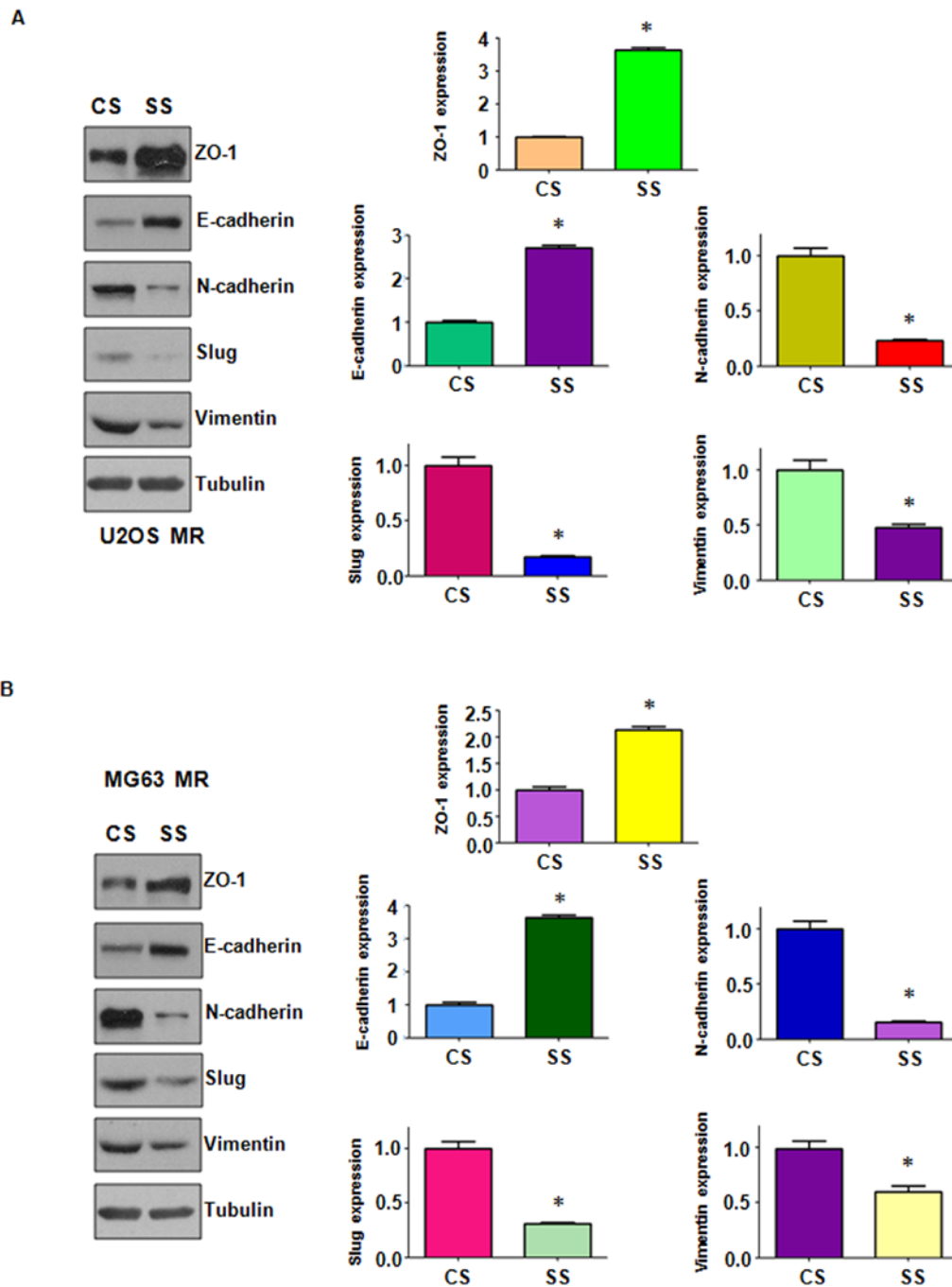


Figure 6. Stable knockdown of Skp2 abrogates epithelial-mesenchymal transition (EMT) induced by methotrexate (MTX) resistance in osteosarcoma (OS) cells. (A) Left panel: Stable knockdown of Skp2 in MTX-resistant U2OS cells promoted the expression of epithelial markers, such as ZO-1 and E-cadherin, whereas it suppressed the expression of mesenchymal markers, including N-cadherin, Slug and Vimentin. Right panel: Results of quantitative analysis of the findings shown in the left panel. \* $P < 0.05$  vs. control. (B) Left panel: Western blot analysis was performed to measure the expression of EMT markers in the MTX-resistant MG63 cells after Skp2 knockdown. Right panel: Results of quantitative analysis of the findings shown in the left panel. \* $P < 0.05$  vs. control. CS, control shRNA-transfected MTX-resistant OS cells; SS, Skp2 shRNA-transfected MTX-resistant OS cells.

of Skp2 on sensitivity of OS cells to the treatment drug. As shown in Fig. 5C, the increased viability of the MTX-resistant OS cells was antagonized by Skp2 knockdown, indicating that the sensitivity of the OS cells to MTX was enhanced following the downregulation of Skp2. This finding suggested that Skp2 may be used as a credible therapeutic target with which to combat drug resistance in OS.

*Stable downregulation of Skp2 regulates the expression of EMT markers.* We observed changes in EMT-related characteristics

in the MTX-resistant OS cells following Skp2 knockdown and further performed western blot analysis to detect whether the knockdown of Skp2 could modulate the expression of EMT-related molecules. The results revealed that Skp2 knockdown promoted the expression of the epithelial markers, ZO-1 and E-cadherin, whereas it suppressed the expression of the mesenchymal markers, N-cadherin, Slug and Vimentin (Fig. 6). These results indicated that the EMT-like characteristics of MTX-resistant OS cells can be abrogated by Skp2 knockdown.

## Discussion

Osteosarcoma is the most common aggressive bone malignancy affecting adolescents and young adults (49). The treatment outcomes have been greatly improved since the introduction of chemotherapy. MTX is one of the most widely used and effective anti-neoplastic drugs in the treatment of various types of solid tumors. Pre-operative high-dose methotrexate (HD-MTX) with folinic acid (leucovorin) is still a mainstay in the treatment of patients with OS (19,50,51). However, multidrug resistance often develops during the late stages of treatment. The detailed mechanisms responsible for drug resistance remain to be determined, and novel therapeutic strategies are required in order to overcome drug resistance in tumor cells and to prevent tumor progression.

In this study, we established MTX-resistant cells using U2OS and MG63 cells. These two MTX-resistant cell lines exhibited a much higher resistant ability to MTX than their parental cells (Fig. 1A). It may be of great importance to explore new molecular mechanisms responsible for MTX resistance in OS cells. Recently, chemotherapeutic agent-resistant tumor cells, were found to acquire an EMT-like morphology and molecular markers (26,27,52,53). Tumor metastasis is a complex process involving vessel formation, cell attachment, invasion, migration and cell proliferation, leading to tumor cell growth in other sites of the body (54). EMT has been reported to contribute to tumor cell invasion and distant metastases in human cancers (22). In this study, OS cells were treated with a sublethal dose of MTX, and any surviving cells presented with enhanced mesenchymal-like characteristics (Fig. 1B). MTX-resistant OS cells also acquired enhanced invasive, migratory and attachment abilities (Figs. 1C and D, and 2A). Moreover, alterations in the levels of EMT markers were observed. The MTX-resistant MR OS cells exhibited a significantly decreased expression of ZO-1 and E-cadherin, and a simultaneously increased expression of mesenchymal markers (Figs. 2B and 3). These findings suggest that EMT may be essential for the development of MTX resistance in OS cells and may thus play an important role in tumor metastasis in OSs.

The effectiveness of chemotherapeutic drugs, such as MTX in cancer is limited due to drug resistance. Thus, the further elucidation of the molecular mechanisms responsible for drug resistance in OS is of utmost importance. To this end, in this study, we detected cell signaling molecular changes associated with EMT in the MTX-resistant OS cells. We also found that Skp2 expression was increased in the MTX-resistant OS cells. Skp2 targets cell cycle-negative regulators, such as p27<sup>Kip1</sup>, p21<sup>Cip1</sup>, p130<sup>Cas</sup> and Foxo1, for ubiquitination and proteasomal degradation, ultimately positively maintaining and preserving cell cycle progression (30,55). In this series of experiments, the expression of Foxo1 and p21 was also downregulated in the resistant cells (Fig. 4A and B) in which Skp2 was upregulated. It has been reported that rapamycin resistance is linked to the defective regulation of Skp2, and that the RNAi-mediated silencing of Skp2 in human tumor cells enhances their sensitivity to rapamycin *in vitro* and inhibits the growth of tumor xenografts *in vivo* (56). Skp2 has also been shown to regulate salinomycin-induced cell cycle arrest and the apoptosis of drug-resistant cancer cells (57). The mitotic arrest deficient protein (MAD2B), a well-defined anaphase-promoting

complex/cyclosome (APC/C) inhibitor, promotes tubular EMT and renal tubulointerstitial fibrosis by inducing Skp2 expression (58). It has been recently reported that the acquisition of EMT-like characteristics is associated with Skp2 expression in paclitaxel-resistant breast cancer cells (48). Skp2 is associated with prostate cancer cell resistance to paclitaxel (59) and the pharmacological inhibition of Skp2 has been shown to sensitize lung cancer cells to paclitaxel (60).

Consistent with the findings of these above-mentioned previous studies, in this study, the use of targeted shRNA against Skp2 resulted in an enhancement of the sensitivity of the resistant OS cells to MTX, evidenced by a decrease in cell proliferation in the MTX-resistant cells in which Skp2 was knocked down (Fig. 5). Importantly, the stable knockdown of Skp2 abrogated the EMT-like characteristics, and decreased the migratory and attachment abilities of the MTX-resistant cells (Figs. 4-6). These results indicated that Skp2 overexpression is closely associated with the MTX resistance of OS cells and EMT properties. The silencing of Skp2 probably prevents EMT and metastasis, and restores the sensitivity of OS cells to MTX. Thus, the pharmacological inhibition of Skp2 may be used as a novel therapeutic strategy with which to overcome drug resistance in OS. Recently, compound 25, a novel Skp2 inhibitor, was shown to exhibit potent antitumor activities and to cooperate with chemotherapeutic agents to suppress cancer cell survival (61). Several natural compounds have been reported to exert their antitumor activities via the inhibition of Skp2 expression in human cancers (62-64). It is important to note that natural compounds do not specifically inhibit Skp2. The current study implied that targeting Skp2 may prove to be helpful for overcoming MTX resistance in OS. However, future studies are warranted to investigate other types of cancer cell lines in order to increase credibility. In addition, the use of animal models and clinical trials are required to fully assess the effects of Skp2 targeting on the prevention of cancer relapse, metastasis and chemoresistance.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

LD, JB and RL were involved in the conceptualization of the study; LD, CW and YC were involved in data curation; XH and YZ were involved in formal analysis; LD, CW, YC, XH and YZ were involved in the investigative aspects of the study; JB and RL were involved in project administration; JB and RL supervised

the study; LD, JB and RL wrote and edited the manuscript. All authors have read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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