

# Knockdown of Ski decreases osteosarcoma cell proliferation and migration by suppressing the PI3K/Akt signaling pathway

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**Abstract.** Ski, an evolutionary conserved protein, is involved in the development of a number of tumors, such as Barrett's esophagus, leukemia, colorectal cancer, gastric cancer, pancreatic cancer, hemangiomas and melanoma. However, studies on the functions of Ski in osteosarcoma (OS) are limited. In this study, firstly the differential expression of Ski in OS tissues and osteochondroma tissues was detected, and the expression of Ski in both human OS cell lines (MG63 and U2OS) and normal osteoblasts (hFoB1.19) was then detected. The results demonstrated that Ski expression was significantly upregulated in both human OS tissues and cell lines. The results led us to hypothesize that Ski may play an essential role in the pathological process of OS. Thus, Ski specific small interfere RNA (Ski-siRNA) was used. The results revealed that OS cell proliferation was markedly inhibited following the knockdown of Ski, which was identified by CCK8 assay, EdU staining and cell cycle analysis. In addition, OS cell migration was significantly suppressed following Ski knockdown, which was identified by wound healing assay. Moreover, the protein levels of p-PI3K and p-Akt in OS cells declined prominently following Ski knockdown. On the whole, the findings of this study revealed that Ski expression was significantly upregulated in OS tissue and OS cells. The knockdown of Ski decreased OS cell proliferation and migration, which was

mediated by blocking the PI3K/Akt signaling pathway. Thus, Ski may act as a tumor promoter gene in tumorigenesis, and Ski may prove to be a potential therapeutic target for the treatment of OS.

## Introduction

Osteosarcoma (OS), a malignant primary tumor, is one of the most common primary bone tumors and mainly generates in teenagers (1). OS originates from primitive bone-forming mesenchymal stem cells and is usually associated with an aggressive behavior and growth speed, a high local recurrence rate and a high metastatic potential (2,3). With the improvement of medical technology, the 5-year survival rate for patients with local OS remains at 70%, while for patients with metastasis it remains at merely 20% (4). Over the past decades, no marked improvements have been made in OS therapy (at least to the best of our knowledge) and this is mainly due to local recurrence or distant metastasis (5). Thus, the study of the molecular mechanisms of OS tumorigenesis is imperative, as well as the exploration of novel biomarkers for the early diagnosis of OS.

The Ski oncogene was first isolated from the avian Sloan-Kettering viruses, and its expression can induce the transformation of chicken embryo fibroblasts, and its oncogenic activity is achieved through the inhibition of the transforming growth factor- $\beta$ (TGF- $\beta$ )/Smad signaling pathway (6-8). A number of studies have proven that Ski expression is notably upregulated in some human cancer cell lines and is associated with a number of advanced stages tumors, including Barrett's esophagus (9), colorectal cancer (10), gastric cancer (11), pancreatic cancer (12) and melanoma (13,14). However, the exact function of Ski in human OS and the underlying mechanisms in tumorigenesis remain unclear.

In this study, we concentrated on revealing the roles of Ski in OS and the underlying mechanisms. We found that Ski was over expression in OS tissues and cell lines. Moreover, the knockdown of Ski decreased OS cell proliferative and migratory abilities, and these effects may be mediated via the inhibition of the PI3K/Akt signaling pathway. These findings indicate that Ski may prove to be a potential therapeutic target in the treatment of OS.

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## Materials and methods

**OS samples.** The study protocol was specifically approved by the Ethics Committee of West China Hospital of Sichuan University (Chengdu, China) and complied with the Declaration of Helsinki. A total of 6 OS tissue samples and 6 osteochondroma tissue samples were collected during surgery from patients between October 1, 2017 and December 31, 2018, and the patient information is presented in Table I. No patients had received radiotherapy or chemotherapy prior to surgery. All patients provided written informed consent and agreed to participate in this research. All collected tissues were frozen instantly in liquid nitrogen following resection and were preserved in an ultralow temperature refrigerator prior to use in the experiments.

**Immunohistochemistry (IHC) analysis.** The expression of Ski in both OS tissues and osteochondroma tissues was detected by IHC under the standard immunoperoxidase staining procedure. In brief, the tissues were embedded with paraffin and conventionally sliced into 4- $\mu$ m-thick sections. The sections were baked for 2 h at 60°C before being dewaxed in xylene, and were then rehydrated through graded ethanol, and subsequently placed in sodium citrate buffer (pH 6.0) and heated up to 100°C for 5 min for antigen repair. After cooling down, the sections were incubated with 5% goat serum (Solarbio) at 37°C for 30 min, and the sections were then incubated with rabbit anti-human Ski monoclonal antibodies (1:200; sc-33693, Santa Cruz Biotechnology) at 4°C overnight, and then incubated with the secondary antibody (1:1,000; biotinylated goat anti-mouse IgG, ZB-2305; ZSBIO) at 37°C for 1 h. The sections were developed with diaminobenzidine (DAB; Beyotime Biotechnology) to detect the bound antibody. After staining, the sections were sealed up with balsam before observation using an optical microscope (Olympus Optical). The brownish-yellow presented in cytoplasm or cytomembrane indicated positive results, or otherwise indicated negative results. Five fields were randomly selected for detection to calculate the positive expression. The experiment was performed 3 times.

**Cell lines and cell culture.** All the cell lines were purchased from the Cellular Center of Institute of Basic Medical Sciences. Human OS cell lines, including MG63 and U2OS were cultured in Eagle's minimum essential medium containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific). The human normal osteoblasts (hFOB1.19) were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; HyClone) containing 10% FBS (Gibco; Thermo Fisher Scientific) and 1% streptomycin and penicillin (Gibco; Thermo Fisher Scientific). The cells were cultured in a humidified atmosphere in CO<sub>2</sub> cell incubator (37°C, 5% CO<sub>2</sub>).

**Western blot analysis.** The protein samples were extracted from the tissues or cells using RIPA lysis buffer (990  $\mu$ l RIPA and 10  $\mu$ l phenylmethylsulfonyl fluoride; Beyotime) on the ice. The supernatants were then transferred into a new 1.5 ml centrifuge tube and centrifuged in a low temperature environment for 15 min at 17,900 x g, and insoluble material was discarded. The sample concentration was detected using

the BCA kit (Beyotime). The protein samples (20  $\mu$ g) were separated by 10 or 12% SDS-PAGE, and the proteins were then transferred onto PVDF membranes (0.45  $\mu$ m; Millipore) using a transfer unit (Bio-Rad). Thereafter, the membranes were blocked in TBS solution containing 5% BSA for 2 h at room temperature. The membranes were then incubated with primary antibodies including anti-Ski (mouse, 1:200, sc-33693; Santa Cruz Biotechnology), proliferating cell nuclear antigen (PCNA; 1:1,000, 10205-2-AP, rabbit; Proteintech), CDK4 (sc-23896), cyclin D1 (sc-450) (both mouse, 1:500; Santa Cruz Biotechnology), matrix metalloproteinase (MMP)2 (4022S), MMP9 (3852S) (both rabbit, 1:1,000; Cell Signaling Technology), PI3K (4292S), p-PI3K (4228S), Akt (9272S) (all rabbit, 1:1,000; Cell Signaling Technology), p-Akt (4060S, rabbit, 1:2,000; Cell Signaling Technology) and  $\beta$ -actin (TA09, mouse, 1:1,000; ZSBIO) overnight at 4°C. Following washing with TBST, the membranes were then incubated with secondary antibodies (ZB-2301 and ZB2305, 1:10,000; ZSBIO) for 1 h. After washing with TBST 3 times, the proteins on the membranes were visualized by ECL, and the results were scanned by Quantity One software (Bio-Rad).

**Immunofluorescence staining.** The cells were cultured on small coverslips and allowed to adhere. Thereafter, the cells were washed with PBS solution for 5 min each, then fixed with 4% paraformaldehyde for 30 min, and washed with PBS for 5 min each. The cells were then permeabilized with 0.3% Triton X-100 for 20 min, and washed in PBS for 5 min each. The cells were blocked for 30 min with 10% goat serum at 37°C, and thereafter, anti-Ski (sc-33693, anti-human 1:300; Santa Cruz Biotechnology); antibodies were added followed by incubation overnight at 4°C. The following day, the cells were washed in PBS 3 times, and then incubated with secondary antibodies (Alexa Fluor 488-conjugated anti-mouse, 1:300; A21202; Invitrogen; Thermo Fisher Scientific) for 2 h at room temperature. Finally, the samples were incubated with DAPI for 10 min at room temperature. The images were acquired with a fluorescence microscope (Zeiss; Carl Zeiss).

**Transfection assay.** Ski specific siRNA (Ski-siRNA) and scrambled sequence siRNA (negative control siRNA, NC-siRNA) were purchased from Thermo Fisher Scientific. The target sequence of Ski-siRNA was 5'-CGGACCTTGGCTGGTTCC TCCAATA-3', and the sequence of 5'-TTCTCCGAACGTGTC ACGT-3' was considered to be NC-siRNA. The MG-63 and U2OS cells were transfected with Ski-siRNA or NC-siRNA using Lipofectamine<sup>®</sup> RNAiMAX Reagent (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Following 48 h of transfection, the cells were collected for the used in the following experiments. Western blot analyses were used to assess the silencing effect of Ski-siRNA. The cells were pre-treated with basic culture medium DMEM/F12 with or without 50  $\mu$ M LY294002 (a specific PI3K inhibitor, S1737, Beyotime) for 6 h prior to transfection.

**Proliferation assay in vitro.** Cell Counting kit-8 (CCK-8) assays (Dojindo) were used to assess cell proliferation. In brief, at 48 h following transfection with Ski-siRNA and NC-siRNA, the cells were trypsinized, and then replanted into

Table I. Clinical profiles of the 12 patients with osteosarcoma and osteochondroma.

Clinicopathological parameters	No. of osteosarcoma patients (n=6)	No. of osteochondroma patients (n=6)
Sex		
Male	3	4
Female	3	2
Age, years		
Median	38	17
Range	10-49	13-52
Tumor location		
Femur	3	4
Tibia and fibula	2	2
Others	1	0
Enneking stage		
I/II A	2	-
IIB/III	4	-
Metastasis		
Yes	4	-
No	2	-

96-well plates with  $3 \times 10^3$  cells/well for the cell proliferation assay. This was followed by the addition of DMEM (100  $\mu$ l) containing 10  $\mu$ l of CCK-8 working solution to each well at corresponding time-points and incubation at 37°C for 3 h in an incubator. Following incubation, cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Bio-Rad).

The EdU reagent kit (Ribobio) was also used to evaluate cell proliferation based on our previous study (15). Briefly, the OS cells were transfected with siRNA for 48 h and the medium was then changed with culture medium to continue culture for 2 and 4 days. The cells were then incubated with complete medium solution containing 50 M EdU for 1 h at corresponding time-points. The following procedure was carried out in accordance with the manufacturer's protocol. Finally, images were acquired with a fluorescence microscope (Zeiss; Carl Zeiss).

**Cell cycle analysis.** The OS cells were planted in a cell culture plate (35 mm) with  $3 \times 10^5$  cells and then incubated overnight until 50-70% confluent. Prior to transfection, the cells were synchronized for 6 h in serum-free DMEM culture medium. At 48 h following transfection with siRNA, the cells were trypsinized and transferred to a 10 ml centrifuge tube (centrifugation at 200 x g for 5 min at 4°C, washed with cold PBS solution twice, fixed with 70% pre-cold ethanol overnight at -20°C. Subsequently, 190  $\mu$ l EDTA and 10  $\mu$ l Rnase A (1 mg/ml) were added to tubes for 5 min at room temperature, and 18  $\mu$ l propidium iodide and 97  $\mu$ l PBS were then added for 10 min at 4°C in the dark, respectively. The volume was adjusted to 1 ml with PBS. Subsequently, the percentage of cells cycle was analyzed using a BD FACScan (BD Biosciences).

**Wound healing assay.** Wound healing assay was performed to estimate the OS cell migratory activities. The transfected OS cells were plated into a 6-well plate at a density of  $5 \times 10^5$  cells/well and two horizontal lines were marked on the back of the well plate. When the confluence of the cells reached 100%, the cells were scratched with a sterile 100  $\mu$ l pipet tip, and the cells debris was then removed using PBS solution. Following the addition of serum-free medium containing 2% FBS, the cells were placed back into the incubator (37°C, 5% CO<sub>2</sub>) for 48 h. The migration of the cells was photographed under a microscope with Zen Imaging software at 0, 12, 24 and 48 h after scratching. The percentage of wound healing was determined as described in our previous study (16).

**Statistical analysis.** Statistical analysis was performed using SPSS 21.0 software (IBM). All data are expressed as the means  $\pm$  SD. Statistical significance between two groups were analyzed with the Student's t-test. The significance among multiple groups was using one-way ANOVA followed by Tukey's post hoc test. P-values <0.05 were considered to indicate statistically significant differences. Each experiment was carried out in triplicate.

## Results

**Ski is highly expressed in OS tissues and cell lines.** Compared with the osteochondroma tissues, Ski was more highly expressed in the OS tissues. As shown in Fig. 1A and B, Ski was overexpressed in OS tissues, whereas its expression was hardly detected in the osteochondroma tissues by immunohistochemistry. The results of western blot analysis also demonstrated that Ski (the molecular weight of Ski varies between 90 and 132 kDa) increased significantly in the OS sample tissues compared with the osteochondroma samples (Fig. 1C and D). These results indicated that Ski may play an essential role in the tumorigenesis of OS. Thus, we compared Ski expression level in OS cell lines (MG63 and U2OS) with hFOB1.19 normal osteoblast cells. The results of immunofluorescence staining revealed that Ski expression was more abundant in the OS cell lines compared with the osteoblasts (Fig. 2A). Furthermore, western blot analysis confirmed the results again (Fig. 2B).

**Ski knockdown decreases the proliferation of OS cells.** In order to examine the role of Ski in OS cells, a specific siRNA (Ski-siRNA) was applied in the OS cell lines, MG63 and U2OS cells. The results indicated that Ski expression was inhibited efficiently in the MG63 and U2OS cells (Fig. 3A and D). Subsequently, CCK-8 assay was performed to investigate the effects of Ski-siRNA on OS cell proliferation. The results revealed that OS cell proliferation was markedly suppressed following transfection with Ski-siRNA at 48 and 72 h in the MG63 cells (P<0.001) compared with the control group and NC-siRNA group (Fig. 3E), and similar results were obtained with the U2-OS cells (Fig. 3F). In addition, EdU kit assay was applied to investigate the OS cell proliferative ability, and the results indicated that the MG63 and U2-OS cell proliferative ability was inhibited significantly following the knockdown of Ski (Fig. 4A-D). Moreover, to further confirm the results



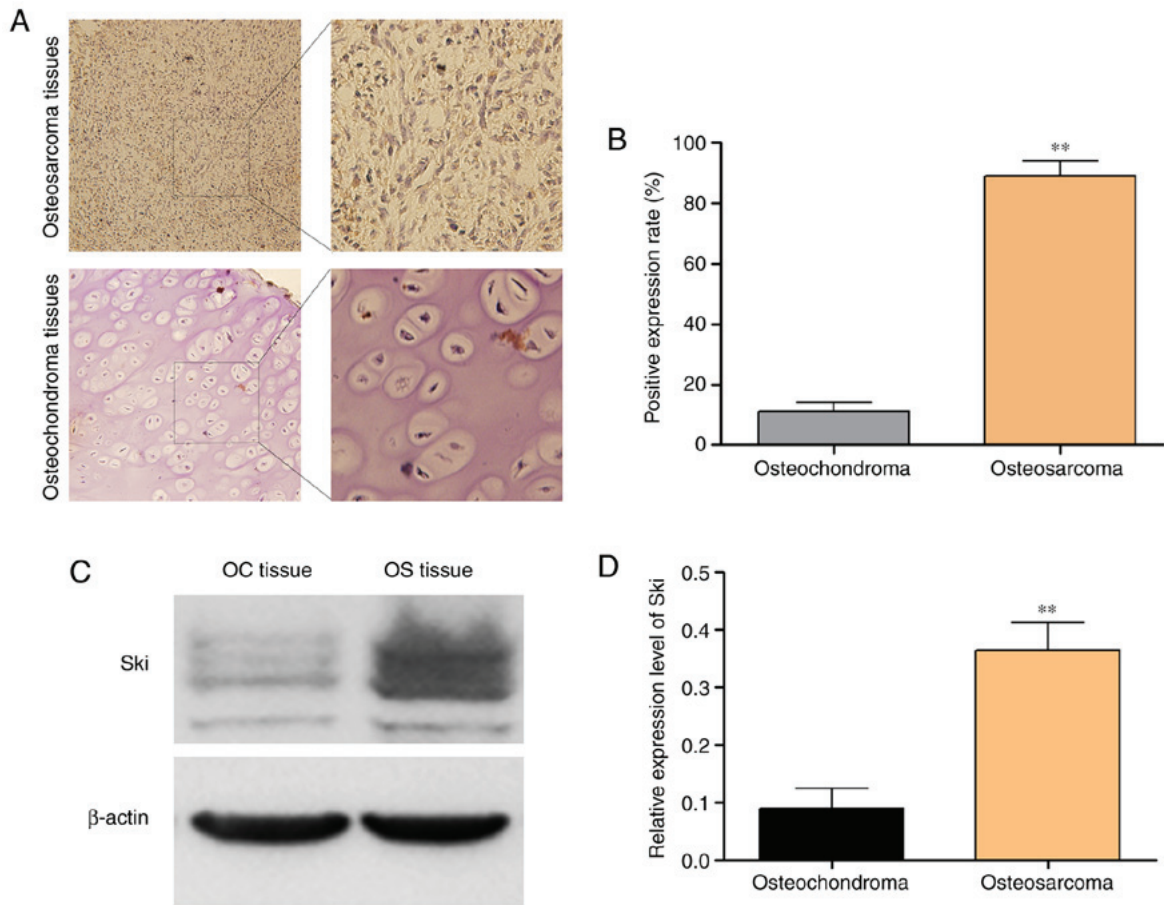


Figure 1. Ski expression in OS and OC tissue samples. (A) Immunohistochemical Ski protein staining results in OS and OC tissue samples. (B) Positive Ski expression rate in OS and OC tissue samples. (C) The protein expression of Ski in OS and OC tissue samples detected by western blot analysis. (D) Statistical analysis of Ski expression in OS and OC tissue samples. Values are expressed as the means  $\pm$  SD (n=3). \*\*P<0.001 compared with OC tissue samples. OS, osteosarcoma; OC, osteochondroma.

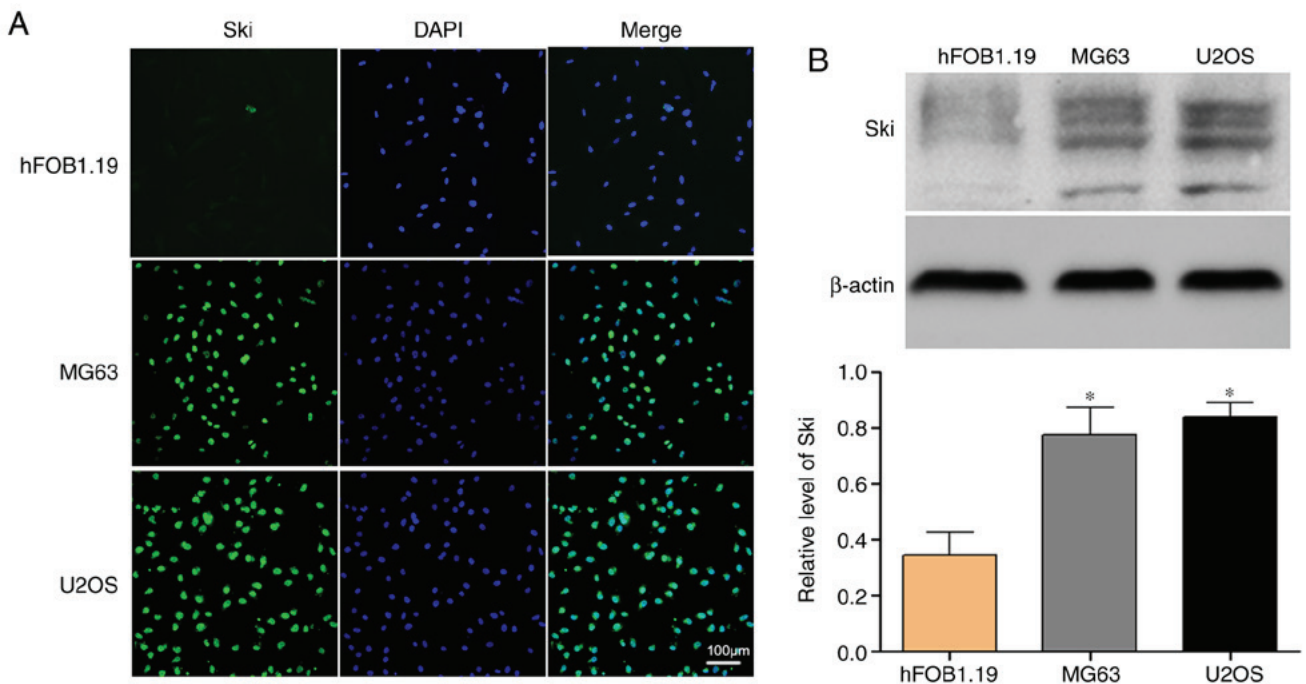


Figure 2. Ski is upregulated in OS cell lines. (A) Immunofluorescence staining showing that the fluorescence intensity of Ski was highly increased in OS cell lines (MG63 and U2OS) compared with the normal osteoblasts (hFOB1.19). Scale bars, 100  $\mu$ m. (B) Western blot analysis was performed to determine the expression Ski in the OS cell lines (MG63 and U2OS) and in normal osteoblasts (hFOB1.19); the expression of Ski was significantly upregulated in OS cell lines. No significant differences were observed between MG63 and U2OS cells (n=3). \*P<0.05 compared with hFOB1.19 normal osteoblasts. OS, osteosarcoma.

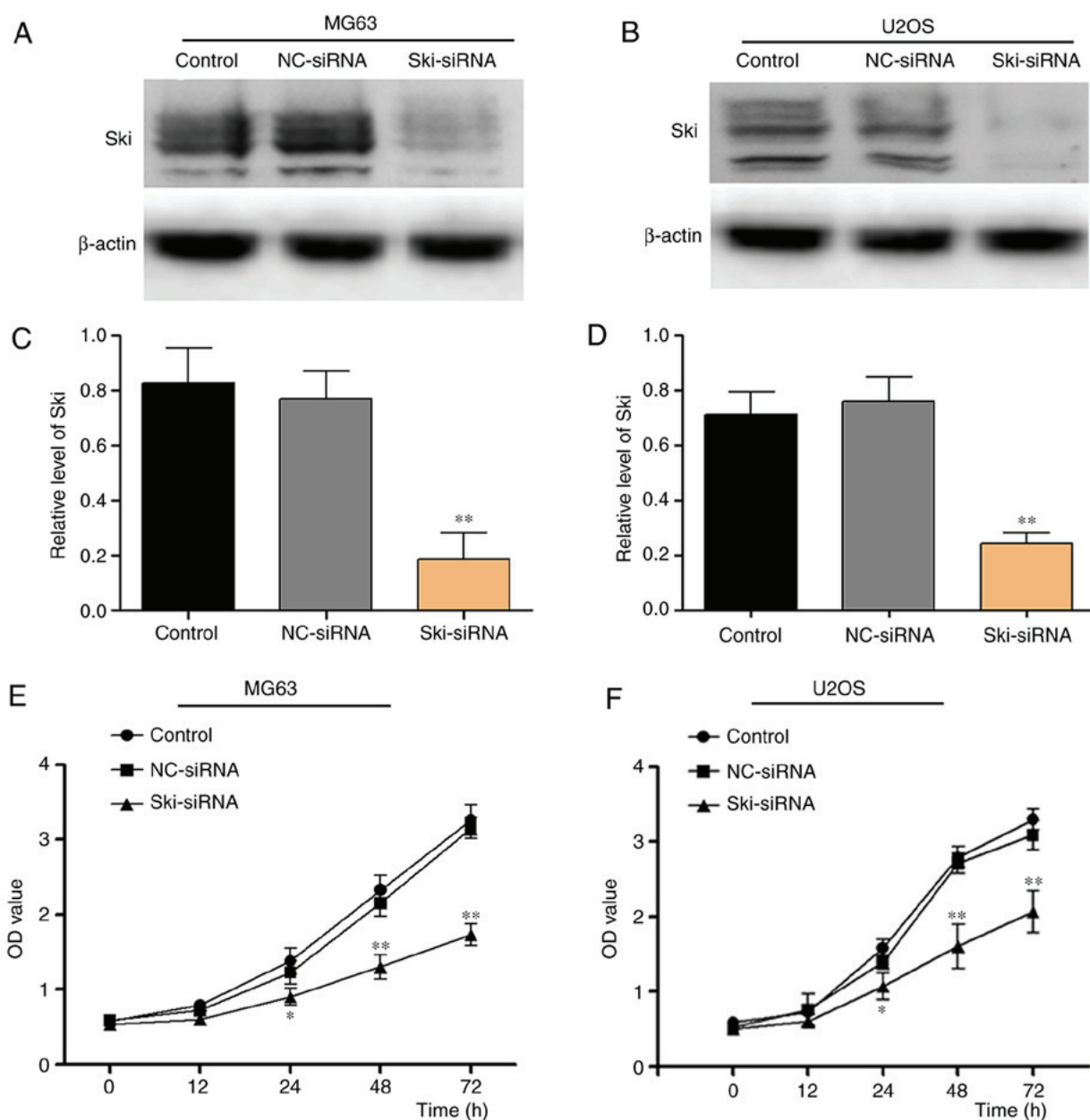


Figure 3. OS cells were transfected with Ski-siRNA for 48 h, and western blot analysis was used to detect Ski expression following transfected with Ski-siRNA. (A) The expression of Ski in MG63 was significantly decreased following transfection with Ski-siRNA. (B) The expression of Ski in U2OS was significantly decreased following transfection with Ski-siRNA. (C) Statistical analysis of Ski/ $\beta$ -actin following the knockdown Ski in MG63 cells. (D) Statistical analysis of Ski/ $\beta$ -actin following the knockdown Ski in U2OS cells. (E) MG63 cell proliferation was assessed by CCK8 assay at 12, 24, 48 and 72 h following transfection. (F) U2OS cell proliferation was assessed by CCK8 assay at 12, 24, 48 and 72 h following transfection. Values are expressed as the means  $\pm$  SD (n=3). \*P<0.05, \*\*P<0.001 vs. the control or NC-siRNA group). OS, osteosarcoma.

mentioned above, the proportions of cell cycle profiles were investigated following transfection with Ski-siRNA by flow cytometry. The results revealed that, the percentage of MG63 cells at the G1 phase increased significantly, while the percentage at the G2/M phase decreased in the Ski-siRNA group compared with the NC-siRNA group (Fig. 4E and G); however, no significant difference was observed in the cells in the S phase. Similar results were obtained for the U2OS cells (Fig. 4F and H). Furthermore, the results of western blot analysis revealed that the expression of proliferation-related proteins, including PCNA, CDK4 and cyclin D1 decreased significantly following transfection with Ski-siRNA in OS cells (Fig. 5). All the above-mentioned results strongly

indicate that Ski plays a crucial role in the proliferation of OS cells.

*Ski knockdown decreases the migration of OS cells.* In addition to proliferation, OS cell migration also plays a critical role in tumor metastasis. Thus, in this study, a wound healing assay was performed to assess the effects of Ski knockdown on MG63 and U2OS cell migration. Following the knockdown of Ski, the percentages of MG63 cells after wound healing at 48 h in the NC-siRNA group and Ski-siRNA group were  $94.47 \pm 5.36$  and  $52.53 \pm 6.57\%$ , respectively, and the rate of wound healing in the Ski-siRNA group was much lower than that of the NC-siRNA group (Fig. 6A and B); similar results

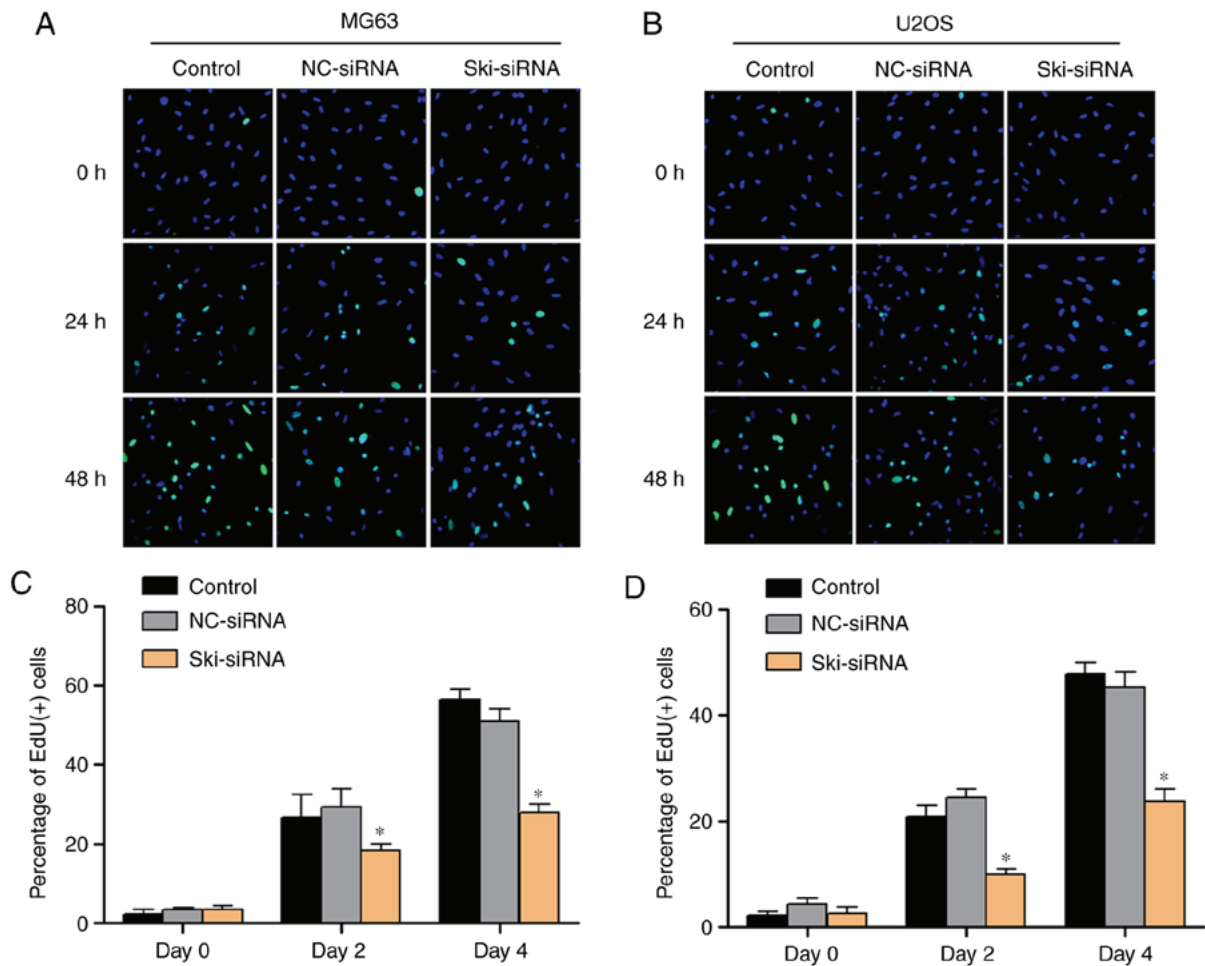


Figure 4. EdU staining assay and cell cycle distribution were employed to detect the cell proliferation rates following transfection. (A) Proliferation rates of MG63 cells among the control, NC-siRNA and Ski-siRNA groups at 24 and 48 h following transfection. (B) Proliferation rate of U2OS cells among the control, NC-siRNA and Ski-siRNA groups at 24 and 48 h following transfection. (C) Statistical analysis of percentages of EdU-positive MG63 cells in each group for corresponding time-points. (D) Statistical analysis of percentages of EdU-positive U2OS cells in each group for corresponding time-points. Data are shown as the means  $\pm$  SD. \* $P < 0.05$  compared with control or NC-siRNA groups.

were obtained for the U2OS cells as well (Fig. 6A and C). In addition, the results of western blot analysis revealed that the expression of migration-related proteins, including MMP2 and MMP9 markedly decreased following the knockdown of Ski (Fig. 7).

*Ski knockdown suppresses the activation of the PI3K/Akt signaling pathway in OS cells.* The PI3K/Akt signaling pathway plays an essential role in tumorigenesis, which has been well expounded as an essential pathway for the proliferation and migration of OS cells. Thus, in this study, the effects of Ski on the certain molecules which are involved in the PI3K/Akt signaling pathway in MG63 and U2-OS cells were examined. The results revealed that the knockdown of Ski decreased the PI3K and Akt phosphorylation levels significantly in the MG63 cells compared with the control and NC-siRNA groups (Fig. 8A). Similar results were obtained in the U2OS cells (Fig. 8B). Furthermore, to verify the function of the PI3K/Akt pathway, we examined the effects of the LY294002 on the Ski-mediated proliferation and migration of OS cells. The results revealed that LY294002 significantly enhanced the inhibitory effects of Ski-siRNA on OS cell proliferation and

migration, and these results were further verified by CCK8 assay (Fig. 8C and D) and wound healing assay (Fig. 8E and F).

## Discussion

OS, as a malignant bone tumor, mainly occurs in children and young individuals (17,18). With the development of new therapeutic technologies, the survival rate of patients with OS has markedly increased. However, the overall outcome of patients with OS remains poor due to drug or multidrug resistance (19,20). Thus, the study of the mechanisms responsible for the development of OS and the identification of novel effective treatable methods for patients with OS is crucial.

Ski, as an evolutionary conserved protein, has been reported to exist in various tissues and species, and participates in diverse cellular processes, such as cell proliferation, metastasis, transformation and tumor progressions (6). Previous studies have demonstrated that Ski plays an essential role in certain pathophysiological processes, such as wound healing and astrocyte proliferation (15,21), vascular smooth muscle cell proliferation (22,23), muscle differentiation (24,25) and liver regeneration (26). Notably, Ski, as a novel therapeutic

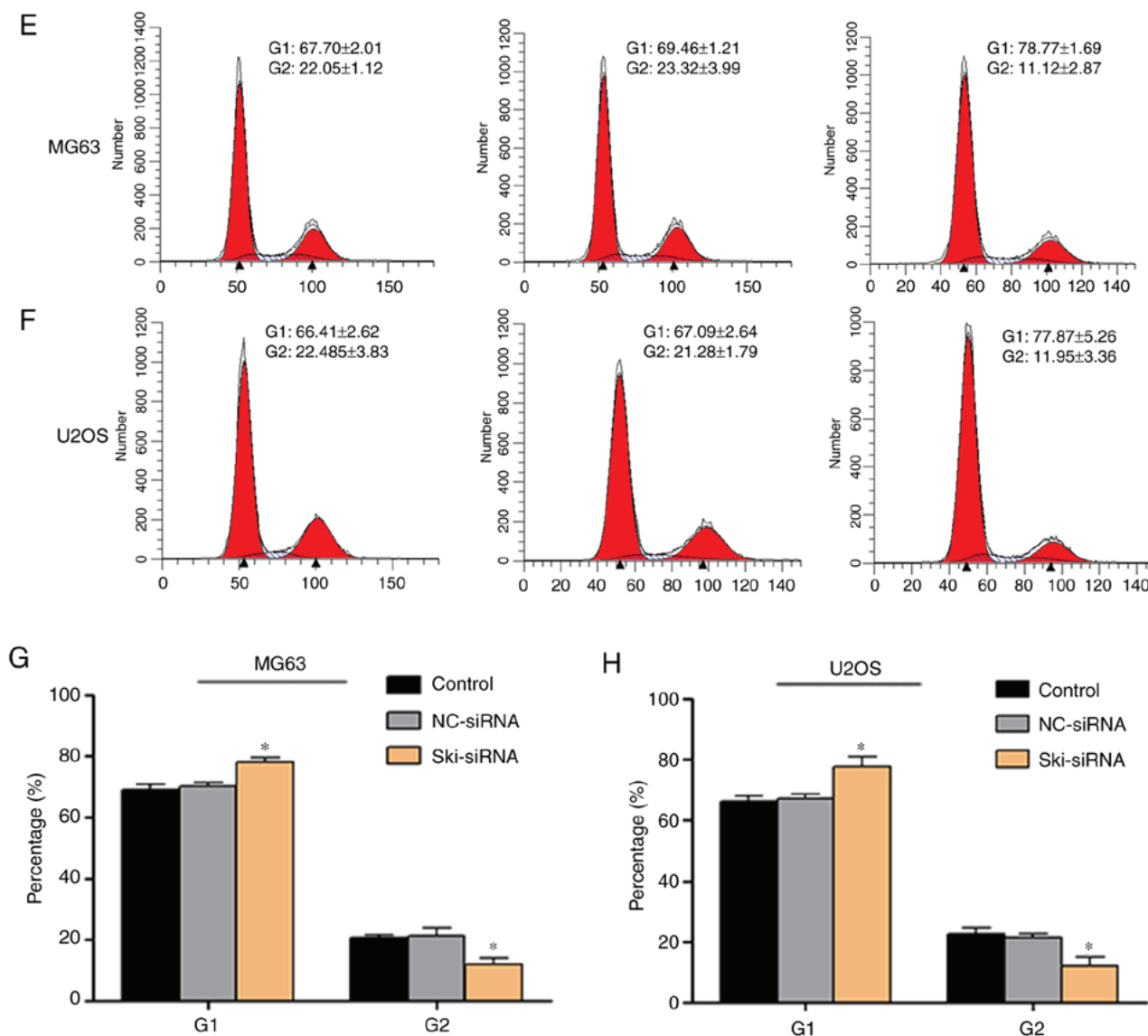


Figure 4. Continued. (E) The cell cycle distribution of MG63 was examined by flow cytometry. (F) The cell cycle distribution of U2OS was examined by flow cytometry. (G) Bar chart shows the average data of cell cycle distribution of MG63. (H) Bar chart shows the average data of cell cycle distribution of U2OS. Data are shown as the means  $\pm$  SD. \*P<0.05 compared with control or NC-siRNA groups.

target, has been found over expression in the development of solid tumors (27). However, the function and roles of Ski in human OS remain largely unknown. The present study focused on investigating Ski expression and its roles in OS cell lines.

In this study, we have found that Ski expressed much more in OS tissues sample compared with osteochondroma samples. Similarly, Ski expression was also more abundant in MG63 and U2OS cell lines compared with normal osteoblast cell line. However, the low sample size was a limitation to the present study. A previous study demonstrated that the Ski expression level was markedly increased during human melanoma tumor progression (13,28). Wang *et al* demonstrated that Ski was overexpressed in pancreatic cancer cell lines and that Ski may act as a tumor proliferation-promoting factor in pancreatic cancer (29). Combined with our results, therefore, we highly suspected that Ski may play a vital role in the pathological process of OS.

To the best of our knowledge, the definite role of Ski in OS has not yet been extensively studied. This study provides

the first evidence that Ski definitely plays an important role in OS. In the present study, we demonstrated that the knockdown of Ski decreased OS cell line proliferation verified by CCK8 assay and EdU staining assay. Moreover, the expression of proliferation-association proteins, including PCNA, CDK4 and cyclin D1 was downregulated in OS cells following transfection with Ski-siRNA. Furthermore, OS cell cycle arrest in the G1/G0 phase occurred following the knockdown of Ski. Atanasoski *et al* demonstrated that Ski controls the proliferation of Schwann cell and myelination process (30). Zhao *et al* also revealed that Ski plays a vital role in the proliferation of astrocyte and astrogliosis process (16). Both of these studies demonstrated that Ski participates in several types of cell proliferation biological properties. Combined with the findings of the present study, it is thus proven that Ski is positively associated with the proliferation of OS cell lines.

OS cell proliferation plays a vital role in tumor metastasis, while migration is also a critical step for tumor metastasis (31). In the present study, OS cell migration markedly decreased



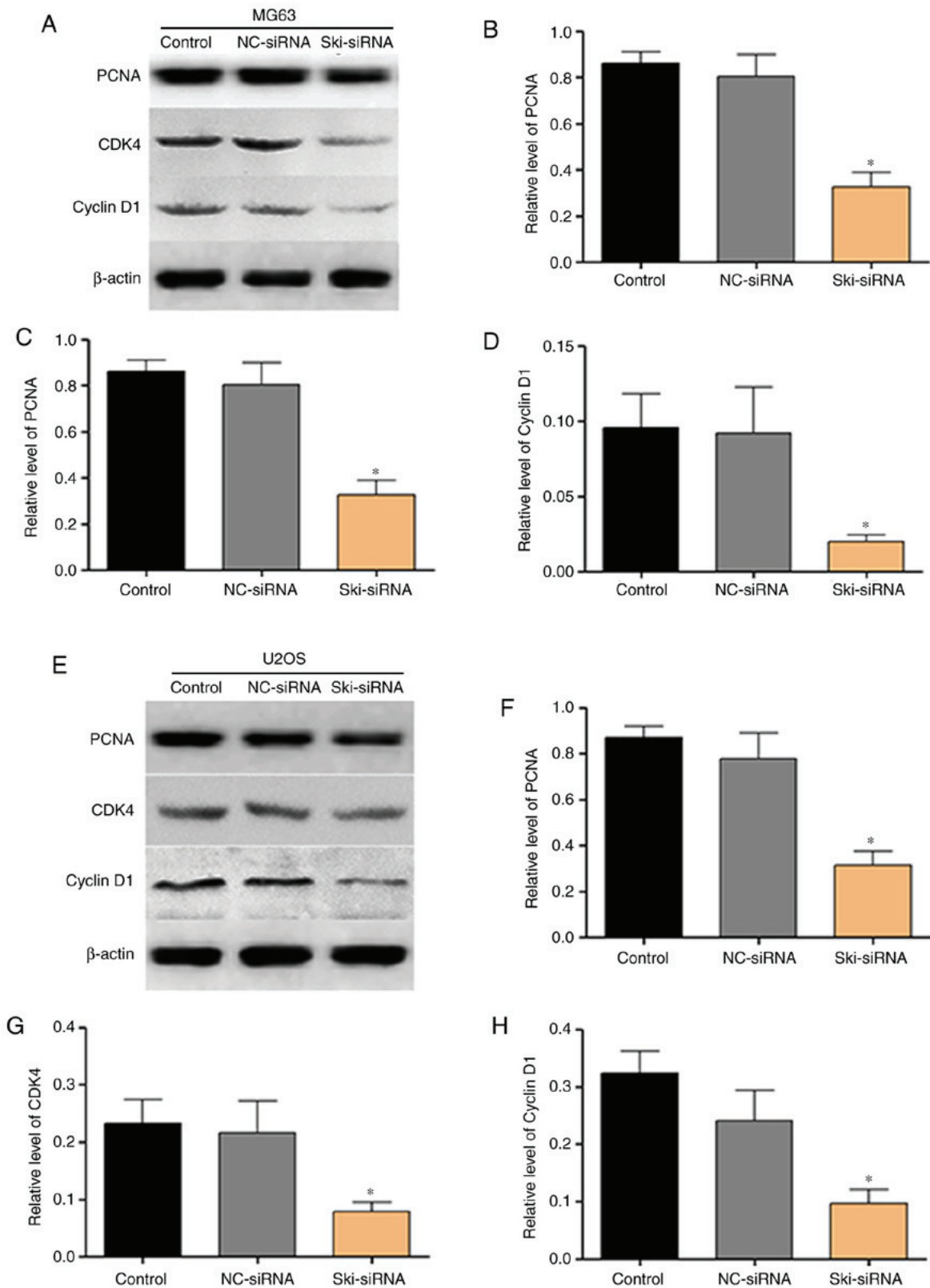


Figure 5. Protein expression of PCNA, CDK4 and cyclin D1 in OS cell lines following transfection with Ski-siRNA. (A) Western blot analysis revealed that the knockdown of Ski significantly decreased the PCNA, CDK4 and cyclin D1 protein levels in MG63 cells. (B-D) Statistical analysis of PCNA, CDK4 and cyclin D1 protein levels in MG63 cell following the knockdown of Ski at 48 h. (E) Western blot analysis revealed that the knockdown of Ski significantly decreased PCNA, CDK4 and cyclin D1 protein levels in U2OS cells. (F-H) Statistical analysis of PCNA, CDK4 and cyclin D1 protein levels in U2OS cell following the knockdown of Ski at 48 h. Values are expressed as the means  $\pm$  SD (n=3). \*P<0.05 compared with control or NC-siRNA groups. OS, osteosarcoma.

following the knockdown of the Ski gene, as shown by wound healing assays. Therefore, the results revealed that Ski knockdown suppressed OS cell metastasis. In addition, MMPs are

considered to play an essential role in collagen degradation, and can promote the migration and invasion of cancer cells (32,33), thereby exerting a profound effect on tumor metastasis. In



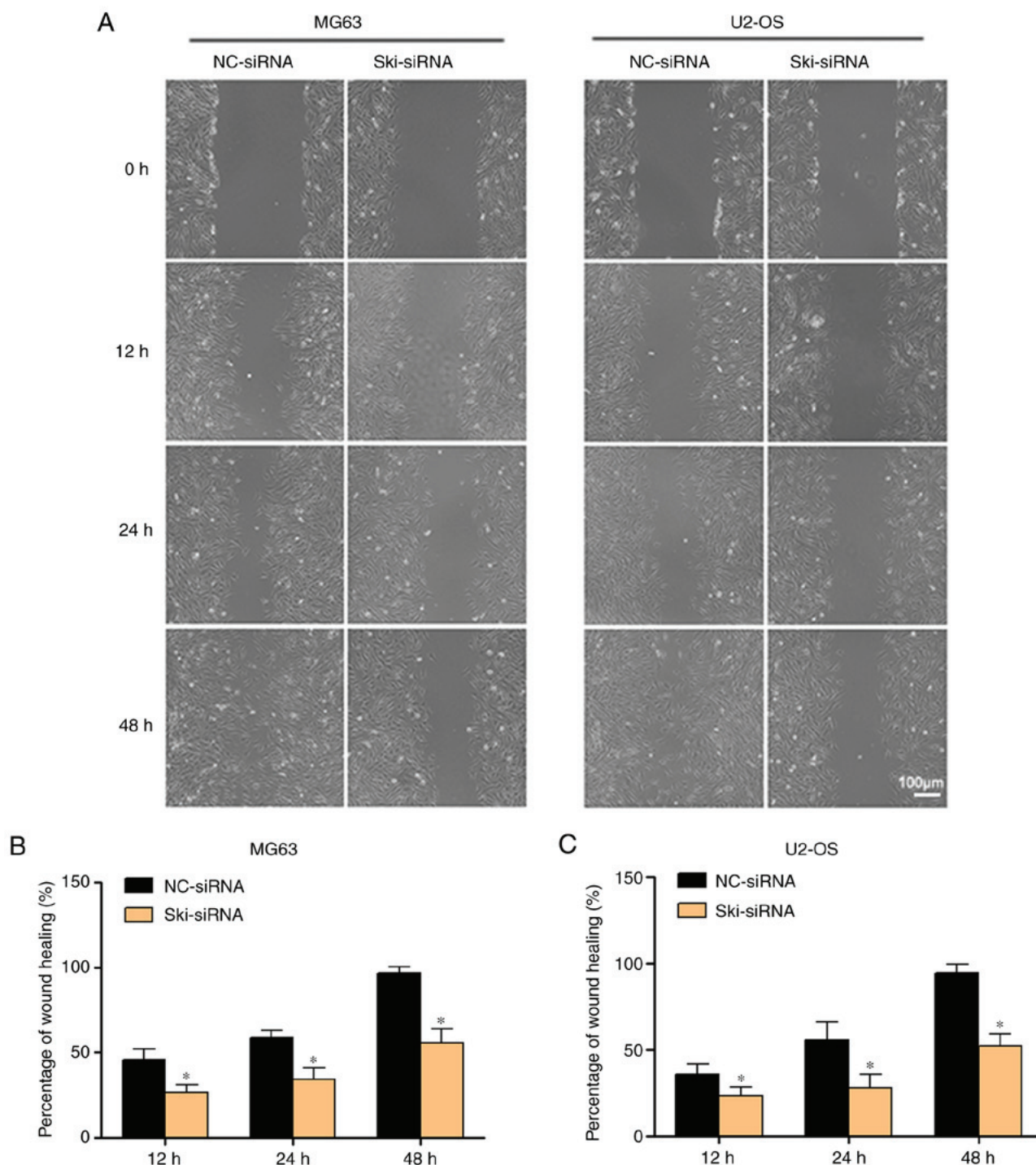


Figure 6. Knockdown of Ski decreased the migration of OS cells. (A) The wound healing assay revealed that the knockdown of Ski significantly decreased the migration of MG63 cells and U2OS cells. (B) Statistical analysis of the migration rate of MG63 cells. (C) Statistical analysis of migration rate of U2OS cells. Values are expressed as the means  $\pm$  SD (n=3). \*P<0.05 compared with NC-siRNA groups). OS, osteosarcoma.

this study, it was found that MMP2 and MMP9 expression levels were significantly decreased following the knockdown of Ski. Qin *et al* reported that PADI1 promotes breast cancer cell metastasis by regulating the ERK1/2/MMP2 signaling pathway (34). Li *et al* found that the knockdown of TKTL1 decreased ESCC cell metastasis by downregulating MMP2 and MMP9 expression (35). Moreover, Arndt *et al* reported that Fussel-15, a new member of the Ski family, plays a vital role in fibroblast migration (36). In this study, it was found that the knockdown of the Ski gene markedly suppressed the

migration in OS cells, and that the expression of MMP2 and MMP9 decreased significantly. The above-mentioned data demonstrated that Ski knockdown significantly decreased OS cell migration by suppressing MMP2 and MMP9 expression.

The PI3K/Akt pathway plays a critical regulatory role in tumorigenesis by regulating cell proliferation and metastasis (37,38). There is evidence to indicate that the PI3K/Akt pathway is activated in the pathological process of OS (39,40). The activation of Akt further phosphorylates multiple proteins that regulate cellular proliferation and migration (41). Therefore,

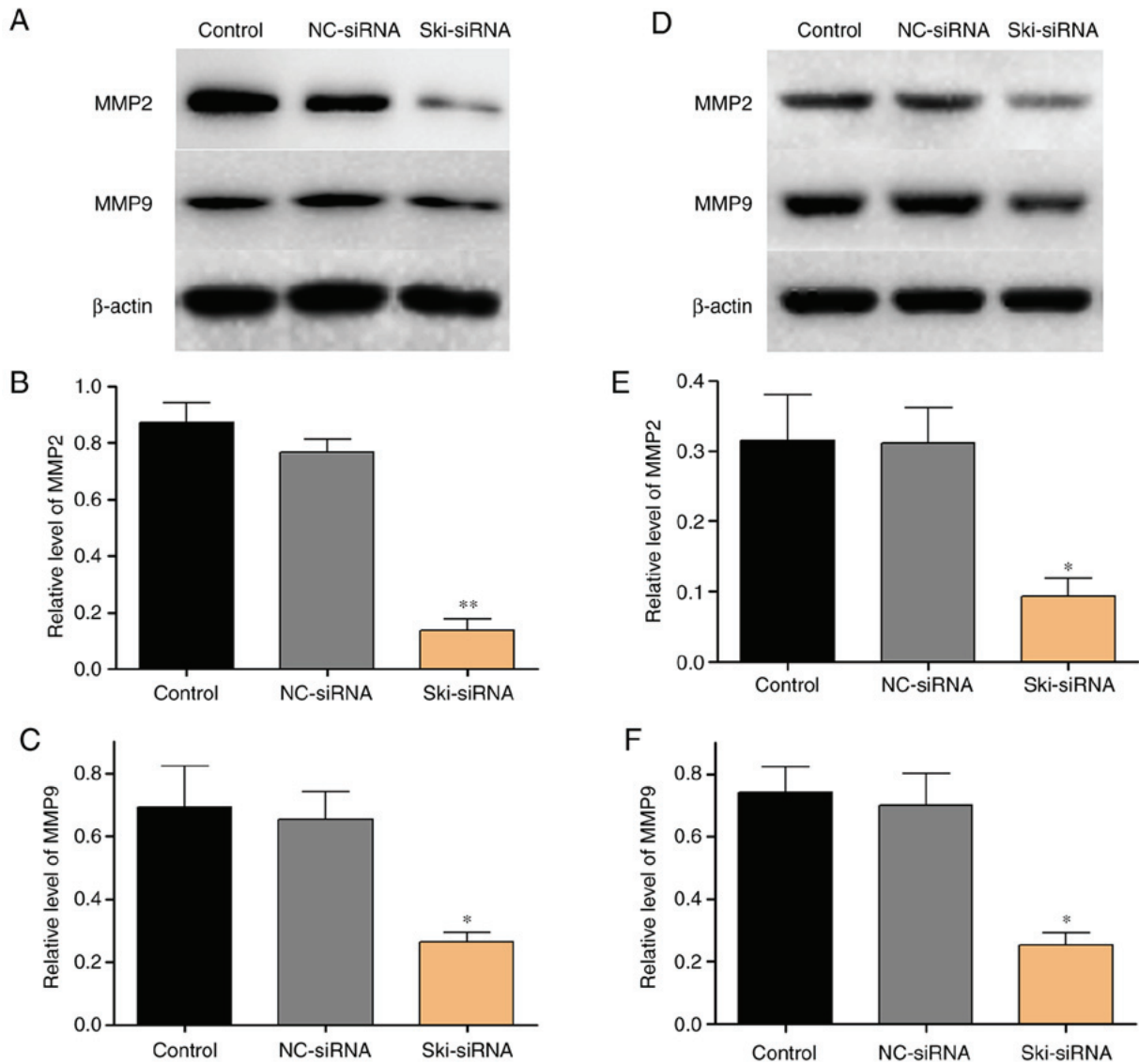


Figure 7. Protein expression of MMP2 and MMP9 in OS cell lines following transfection with Ski-siRNA. (A) Knockdown of Ski significantly decreased MMP2 and MMP9 protein levels in MG63 cells. (B and C) Statistical analysis of MMP2 and MMP9 expression in MG63 cells following the knockdown of Ski at 48 h. Values are expressed as the means  $\pm$  SD (n=3). \*P<0.05, vs. the control or NC-siRNA group). (D) Knockdown of Ski significantly decreased MMP2 and MMP9 protein levels in U2OS cells. (E and F) Statistical analysis of MMP2 and MMP9 expression in U2OS cells following the knockdown of Ski at 48 h. Values are expressed as the means  $\pm$  SD (n=3). \*P<0.05, vs. the control or NC-siRNA group). OS, osteosarcoma.

inhibiting the phosphorylation of the PI3K/Akt pathway represents a potential treatment method for OS (42,43). Thus, inhibiting the phosphorylation of the PI3K/Akt pathway, by various means, disrupts OS progression. It has been reported that the PI3K-specific inhibitor, LY294002, markedly suppresses OS cell proliferation and migration by downregulating the activity of the PI3K/Akt pathway (44-46). Jiang *et al* found that the knockdown of the DDX46 gene inhibited the tumorigenesis of OS cells by suppressing the phosphorylation of the PI3K/Akt signaling pathway (47). Chen *et al* found that isoliquiritigenin suppressed OS cell proliferation by downregulating the PI3K/Akt pathway (48). Similarly, it has been revealed that TROP promotes OS cell proliferation and migration by activating the PI3K/Akt signaling pathway (40). Of note, a previous study demonstrated that there may be a potential connection between Ski and the PI3K/Akt signaling pathway; for example, Ski can be phosphorylated by Akt and this

phosphorylation is elevated by the activation of the PI3K/Akt pathway (49). However, whether the roles of Ski in OS cells are regulated by the PI3K/Akt pathway remain unclear.

In the present study, the underlying mechanisms of the biological functions of Ski, including the proliferation and migration of OS cell lines were investigated. It was found that Ski knockdown significantly inhibited the phosphorylation levels of both PI3K and Akt in OS cell lines. In order to confirm the association between Ski and the PI3K/Akt signaling pathway in OS cell lines, an inhibitor of PI3K (LY 294002) was used. In this study, it was found that LY 294002 significantly enhanced the inhibitory effects of Ski-siRNA on MG63 cell proliferation and migration, and similar results were obtained with the U2OS cells. The above-mentioned results strongly demonstrated that Ski knockdown notably inhibited OS proliferation and migration by blocking the PI3K/Akt signaling pathway. To further confirm the function of Ski in

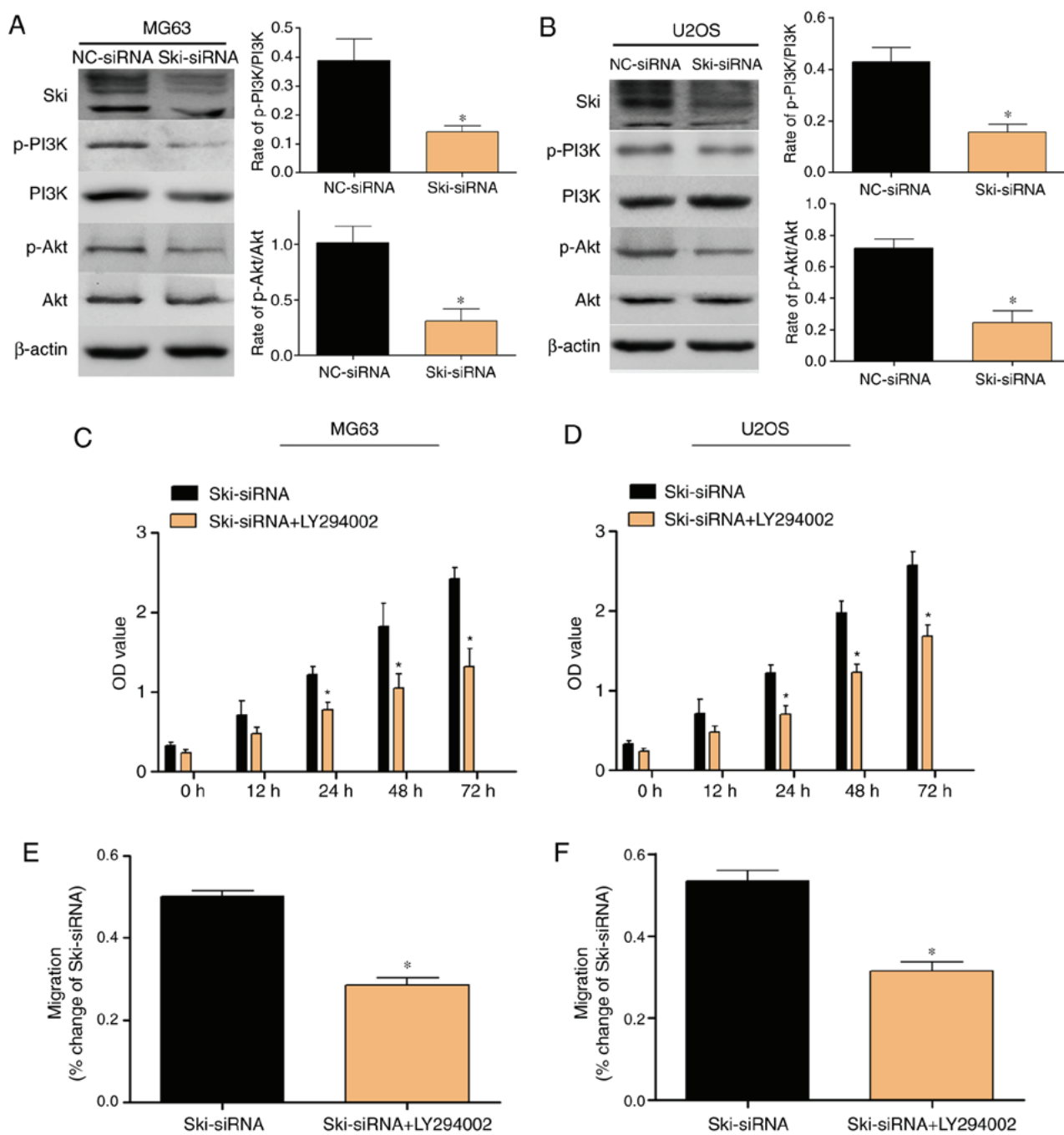


Figure 8. Knockdown of Ski reduces the levels of p-PI3K and p-Akt in OS cell lines, and the effect of Ski-siRNA in the presence or absence of the LY294002 on OS cells proliferation and migration. (A) Knockdown of Ski significantly decreased p-PI3K and p-Akt protein levels in MG63 cells. (B) Knockdown of Ski significantly decreased p-PI3K and p-Akt protein levels in U2OS cells. Values are expressed as the mean  $\pm$  SD (n=3). \*P<0.05, vs. the NC-siRNA group). (C and D) Proliferation of MG63 and U2OS cells examined by CCK8 assay; LY294002 markedly enhanced the inhibitory effects of Ski-siRNA on OS cell proliferation. (E and F) Statistical analysis the effect of LY294002 on the migration rate of OS cells following transfection with Ski-siRNA. Values are expressed as the means  $\pm$  SD (n=3). \*P<0.05, vs. the Ski-siRNA group).

OS, further studies on cell proliferation and migration trends following Ski gene transfection are warranted and our team aims to continue to study its role in OS cells.

In conclusion, this study demonstrates for the first time, to the best of our knowledge, that the expression of Ski was markedly increased in OS tissues and cell lines, and that Ski knockdown decreased OS cell proliferation and migration, which was performed by blocking the PI3K/Akt signaling pathway. It can thus be concluded that Ski may become a potential therapeutic target molecule in the treatment of OS.

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#### Availability of data and materials

The datasets used and analyzed in the present study are available from the corresponding author on reasonable request.

#### Authors' contributions

XZ and YF were involved in the design of the study, performed the experiments and drafted the manuscript. XW performed the experiments. ZY and DL designed and analyzed the immunohistochemistry data. PK and MT were involved in the design of the study, and in the critical appraisal of the manuscript. All authors have read and approved the final version of the manuscript to be published.

#### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of West China Hospital of Sichuan University (Chengdu, China). Written informed consent was obtained from all patients.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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