Inhibition of Skp2 suppresses the proliferation and invasion of osteosarcoma cells

LU DING1,2*, RONG LI3*, XIAOPING HAN2*, YUBO ZHOU4, HUA ZHANG2, YONG CU12, WU WANG2 and JINGPING BAI1

1Department of Orthopedics, Tumor Hospital Affiliated to Xinjiang Medical University;
2Department of Orthopedics, Fifth Affiliated Hospital, Xinjiang Medical University;
3Department of Maternal, Child and Adolescent Health, College of Public Health, Xinjiang Medical University Xinshi, Urumqi, Xinjiang 830000;
4Department of Orthopedics, Traditional Chinese Medicine Hospital Affiliated to Xinjiang Medical University, Xinshi, Urumqi, Xinjiang, P.R. China

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Abstract. Osteosarcoma (OS) is a common bone tumor that mainly affects children and young adults. S-phase kinase-associated protein 2 (Skp2) has been characterized to play a critical oncogenic role in a variety of human malignancies. However, the biological function of Skp2 in OS remains largely obscure. In the present study, we elucidated the role of Skp2 in cell growth, cell cycle, apoptosis and migration in OS cells. We found that depletion of Skp2 inhibited cell growth in both MG-63 and SW 1353 cells. Moreover, we observed that depletion of Skp2 triggered cell apoptosis in two OS cell lines. Furthermore, downregulation of Skp2 induced cell cycle arrest in the G0/G1 phase in OS cells. Notably, our wound healing assay results revealed that inhibition of Skp2 suppressed cell migration in OS cells. Invariably, our western blot results demonstrated that depletion of Skp2 in OS cells inhibited activation of pAkt and increased p27 expression in OS cells, suggesting that Skp2 exerted its oncogenic function partly through the regulation of Akt and p27. Our findings revealed that targeting Skp2 could be a promising therapeutic strategy for the treatment of OS.

Introduction

Osteosarcoma (OS) is a common bone cancer that mainly affects children and adolescents (1,2). Although multiple treatments including local control interventions and chemotherapy have improved the survival of patients with OS, the survival rate is still ~60-80% in OS patients (3). Moreover, the 5-year event-free survival for patients with high grade OS is still less than 50% (3). Therefore, it is imperative to develop more effective therapeutic strategies for the treatment of OS. It has been demonstrated that genetic and cytogenetic abnormalities are critically involved in the development and progression of OS (4). For example, mutations in tumor suppressors and activation of oncogenes are associated with OS (5). Therefore, it is essential to understand the molecular mechanism of OS tumorigenesis and to validate novel therapeutic targets for OS.

Emerging evidence has revealed that several biological molecules could be effective therapeutic targets for OS. For instance, multiple genes such as Notch, Wnt, nuclear factor-κB (NF-κB), p53, phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) are critical in the development of OS (4). Tao et al identified that Notch activation is a driver of osteogenic sarcoma (6). Recent studies have validated that S-phase kinase-associated protein 2 (Skp2) plays an essential role in the development of various human malignancies. Skp2 has been characterized to exhibit its oncogenic function via targeting of its substrates including p27 (7,8), p21 (9), p57 (10) and Forkhead box protein O1 (FOXO1) (11,12). Engineered mouse models revealed that conditional depletion of Skp2 in mice suppressed tumor growth in T cell lineage (13), B cell lineage (14), bone marrow (15), liver (16,17), breast (18), prostate (19) and skin cancer (20). Consistently, upregulation of Skp2 in mice enhanced tumor growth in lymphoma (21), prostate cancer (19) and breast tumor (18). Importantly, high expression of Skp2 has been observed in a wider spectrum of cancers including lymphomas (22,23), pancreatic cancer (24), breast...
carcinomas (25-29), prostate (30,31) and gastric cancer (32), melanoma (33-35), hepatocarcinoma (36) and nasopharyngeal carcinoma (37,38). Skp2 was also highly expressed and was correlated with relapse, metastasis and survival in OS patients (39). However, the role of Skp2 in OS has not been fully elucidated.

In the present study to explore the role of Skp2 in OS, we depleted Skp2 in MG-63 and SW 1353 cells. Then, we investigated the biological function of Skp2 in cell growth, apoptosis, and in the cell cycle of OS cells after knockdown of Skp2. Moreover, we assessed the effects of Skp2 depletion on OS cell migration. Methodically, we determined whether the downstream targets of Skp2 are involved in the progression of OS. We found that depletion of Skp2 inhibited cell growth, induced apoptosis, arrested the cell cycle and suppressed cell migration in OS cells. Moreover, depletion of Skp2 exerted its function partly through the regulation of Akt and p27 in OS cells. Our findings suggest that targeting Skp2 may be a promising therapeutic strategy for the treatment of OS.

Materials and methods

Cell culture, reagents and antibodies. The human OS MG-63 and SW 1353 cells (ATCC, Manassas, VA, USA) were cultured at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-Akt and anti-pAkt antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β-actin, Skp2 and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Transfection. Cells were seeded into 6-well plates and transfected with control siRNA or Skp2 siRNAs (GenePharma, Shanghai, China) using Lipofectamine 2000 according to the manufacturer’s protocol.

MTT assay. Cells were seeded at equal densities into 96-well culture plates. After 24 h, the cells were then treated with Skp2 siRNA for 48 h. An MTT assay was conducted to determine the absorbance at 560 nm using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA, USA). All values were normalized to those of the control.

Cell cycle analysis. The transfected cells were seeded into 100-mm dishes for 48 h. Then, the cells were fixed in ice-cold 70% ethanol in phosphate-buffered saline (PBS) for 12 h. The fixed cells were treated with 0.1 mg/ml RNase A for 20 min. The cells were re-suspended in propidium iodide (PI) (50 µg/ml). Cell cycle distribution was assessed using a FACScan flow cytometer and analyzed.

Apoptosis assay. The transfected cells were cultured into a 6-well plate for 48 h. Then, the cells were harvested and washed with PBS, resuspended in 500 µl of binding buffer with 5 µl of PI and 5 µl of FITC-conjugated anti-Annexin V antibody. Apoptosis was assessed by a FACSCalibur flow cytometer.

Real-time RT-PCR analysis. Total RNA from transfected cells was isolated using TRIzol and reversed-transcribed into cDNA by RevertAid First Strand cDNA Synthesis kit according to the manufacturer’s protocol. The primers used in the PCR reactions were: Skp2 forward, 5'-GCT GCT AAA CTT CTC TGG TGT-3' and reverse, 5'-AGG CTT AGA TCC TGC AAC TTG-3'; GAPDH forward, 5'-ACC CAG AAG ACT GTG GAT GG-3' and reverse, 5'-CAG TGA GTC CGT CGT TCA G-3'. The expression of GAPDH was used as an internal control.

Western blot analysis. Cells were lysed in lysis buffer and protein concentrations were detected by Brandford assay reagent. Equal amounts of proteins were resolved on SDS-PAGE, and then transferred to membranes. The membranes were immunoblotted with primary antibodies including Skp2, Akt, phosphor-Akt-Ser473, p27 and β-actin, followed by secondary antibodies conjugated with horse-radish peroxidase. Then, the bands were revealed using an enzyme-linked chemiluminescence (ECL) detection kit assay.

Wound healing assay. Cells were cultured in 6-well plates and grown to confluency. When cells converged to ~100%, monolayers of cells were scratched with 200 µ yellow pipette tips and washed with PBS. The scratched area was photographed with a microscope at 0 and 20 h, respectively.

Transwell invasion assay. An invasion assay was carried out using BD BioCoat Matrigel invasion chambers. Briefly, transfected cells were seeded in DMEM without serum in the upper chamber. DMEM containing 10% FBS was added to the lower chamber. After overnight incubation, the non-invading cells were removed. The cells that had invaded through the Matrigel matrix membrane were stained with 4 µg/ml calcein AM in Hanks’ buffered saline at 37°C for 1 h. The fluorescence of the invaded cells was read on Ultra Multifunctional Microplate Reader (Tecnal, Durham, NC, USA) at excitation/emission wavelengths of 530/590 nm. Invasiveness was assessed by calculating the invading cells with a microscope.

Statistical analysis. The data are expressed as the mean ± SD. A Student's t-test was performed to evaluate statistical significance between the Skp2-siRNA transfection group and the NC-siRNA treated group. The level of significance was considered as P<0.05.

Results

Skp2 expression is inhibited by its siRNAs in OS cells. To determine whether Skp2 is involved in the regulation of cell proliferation in OS cells, we depleted Skp2 using its specific siRNAs in both MG-63 and SW 1353 cells. Real-time reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis were applied for the detection of the efficacy of multiple Skp2 siRNAs on inhibition of Skp2 in OS cells. The results from RT-PCR revealed that Skp2 siRNAs significantly downregulated Skp2 mRNA levels in both MG-63 and SW 1353 cells (Fig. 1A). Consistently, our western
blot analysis demonstrated that Skp2 siRNAs markedly inhibited the expression of Skp2 in both OS cell lines (Fig. 1B). In the following experiments, Skp2 siRNA2 was used to deplete Skp2 expression in the two OS cell lines.

Depletion of Skp2 inhibits cell proliferation in OS cells. It has been demonstrated that downregulation of Skp2 suppressed cell proliferation in human cancer cells. To determine whether Skp2 regulates cell proliferation in OS cells, an MTT assay was performed to assess cell proliferation in OS cells transfected with Skp2 siRNA. We found that depletion of Skp2 significantly inhibited cell proliferation in MG-63 cells compared with the control group (Fig. 1C). Similarly, Skp2 siRNA treatment led to inhibition of cell proliferation in SW 1353 cells (Fig. 1C). Our findings revealed that depletion of Skp2 suppressed cell proliferation in OS cells.

Depletion of Skp2 triggers apoptosis in OS cells. Next, we used an Annexin V-FITC/PI apoptosis detection kit to assess apoptosis in OS cells after Skp2-siRNA transfection. We found that depletion of Skp2 induced apoptosis in both MG-63 and SW 1353 cells (Fig. 2A and B). The percentage of apoptotic cells increased from 13.43% in the control siRNA-treated group to 30.6% in the Skp2 siRNA-treated group in the SW 1353 cells (Fig. 2B). We also observed that downregulation of Skp2 enhanced apoptosis in the MG-63 cells (Fig. 2A). These data indicated that depletion of Skp2 led to increased apoptosis, which contributed to cell growth inhibition in both OS cell lines.

Depletion of Skp2 induces cell cycle arrest in OS cells. To explore whether Skp2 regulates cell cycle progression, PI staining and flow cytometry assays were conducted in OS cells treated with Skp2 siRNA. We found a typical
G0/G1 arrest pattern in both Skp2 siRNA-treated OS cell lines (Fig. 2C and D). The G0/G1 phase fraction increased from 50.72% in the control siRNA-treated cells to 79.58% in the Skp2 siRNA-treated MG-63 cells (Fig. 2C). Similarly, G0/G1 cell cycle arrest was found in the Skp2 siRNA-treated SW 1353 cells (Fig. 2D). These results demonstrated that depletion of Skp2 induced G0/G1 arrest in OS cells.

Depletion of Skp2 suppresses cell migration and invasion in OS cells. To explore whether depletion of Skp2 suppresses the motility of OS cells, wound healing assays were conducted to assess the migration of OS cells following Skp2-siRNA transfection. Our results from the wound healing assays demonstrated that inhibition of Skp2 significantly decreased cell migration in both OS cell lines (Fig. 3). To further validate this finding, migration and invasion assays were performed to determine the cell migratory and invasive activities of OS cells treated with Skp2 siRNA using Transwell chamber assays. We observed that downregulation of Skp2 markedly inhibited the migration and invasion (Fig. 4) in both OS cell
Figure 3. Depletion of Skp2 suppresses the motility activity in OS cells. (A and B) Wound healing assays were used to detect the migratory potential in (A) MG-63 and (B) SW 1353 cells after Skp2-siRNA treatments. Control, without any treatment group; NC siRNA, non-specific siRNA. (C) Quantitative results are illustrated for A and B; *P<0.05, **P<0.01 vs. the NC siRNA. Skp2, S-phase kinase-associated protein 2.

Figure 4. Depletion of Skp2 inhibits migration and invasion in OS cells. (A and B) Migration and invasion assays were used to assess the migratory capacity in (A) MG-63 and (B) SW 1353 cells treated with Skp2 siRNA. Control, without any treatment group; NC siRNA, non-specific siRNA. Skp2, S-phase kinase-associated protein 2.
Depletion of Skp2 inhibits tumorigenesis lines. Altogether, depletion of Skp2 may inhibit motility in OS cells.

Depletion of Skp2 increases the expression level of p27, but decreases the expression level of pAkt in OS cells. It has been demonstrated that Skp2 regulates the levels of p27 and pAkt, two key targets of Skp2, in several types of human cancers. To further determine whether depletion of Skp2 regulates the expression of p27 and pAkt in OS cells, we assessed the levels of these two genes in the Skp2 siRNA-treated OS cell lines. Our western blot analysis data revealed that downregulation of Skp2 increased p27 expression in both OS cell lines (Fig. 5). Furthermore, depletion of Skp2 decreased the expression of pAkt in OS cells, but not the total level of Akt (Fig. 5). These findings revealed that Skp2 suppressed the expression level of pAkt, but increased the expression of p27 in OS cells.

Discussion

Various studies have discovered that Skp2 plays an important role in tumorigenesis in a variety of human types of cancer (40,41). However, the function of Skp2 in OS remains largely obscure. Indirect evidence has indicated the function of Skp2 in OS cells. For example, the depletion of Forkhead box M1 (FoxM1) inhibited cell growth in human OS U2OS cells due to dysregulation of Skp2 and Cks1, leading to a mitotic block and accumulated levels of p21 and p27 (42). A previous study indicated that FoxM1 regulated the transcription of cell cycle genes including genes encoding the SCF (Skp2-Cks1) ubiquitin ligase complex (42). Another study demonstrated that inhibition of the Notch pathway by γ-secretase inhibitor suppressed the growth of OS in vitro and in vivo due to a decrease in the expression of accelerators of the cell cycle such as Skp2, cyclin D1, and cyclin E1 and E2 (43). Similarly, inhibition of smoothened, a key molecule in the Hedgehog pathway, slowed the growth of OS cells via the suppression of Skp2 and cyclin D1 and E1, but upregulation of p21 (44). In line with this, knockdown of Gli2, a key mediator of the Hedgehog pathway, prevented OS growth and anchorage-independent growth, and promoted G1 phase arrest through inhibition of Skp2 and cyclin D1 (45). In the present study, depletion of Skp2 in OS cells inhibited cell growth, arrested the G1 cell cycle, triggered cell apoptosis, and suppressed cell migration. This is direct evidence validating the biological function of Skp2 in OS cells. Our findings demonstrated that inactivation of Skp2 could be a useful approach for the treatment of OS patients.

Accumulated evidence has revealed that Akt directly binds to Skp2 and subsequently enhances the translocation of Skp2 from the nucleus to the cytoplasm, leading to activation of Skp2 function (46,47). Notably, one research...
group reported that Akt could promote Skp2 translocation to the cytoplasm (12,48). A recent study revealed that Skp2 stimulated Akt activation in human breast cancer cells (49). In line with this finding, our results revealed that depletion of Skp2 inhibited the activation of pAkt in OS cells. This result demonstrated that Skp2 exerted its oncogenic function partly through the Akt pathway in OS cells. Further detailed investigations are warranted to determine how Skp2 regulates Akt activation in OS cells.

Since inhibition of Skp2 represents an effective therapeutic approach for patients with OS, it is critical to discover and develop Skp2 inhibitors. To this end, several Skp2 inhibitors have been developed. For instance, 5-bromo-8-tolylsulfonyl-4-quinoline-1-inhibited the interaction of Skp2 with cyclin-dependent kinase regulatory subunit 1 (Cks1), leading to the inhibition of cell growth in lung cancer cells (50). Another compound M1 targeted the p300-binding site of Skp2 and disrupted the Skp2/p300 interaction, leading to apoptosis and cell death in cancer cells (51). Arsenic trioxide exhibited its antitumor activity via suppression of Skp2 in pancreatic cancer cells (52). Caffeic acid phenethyl ester suppressed cell proliferation and migration and arrested the cell cycle through inactivation of Skp2 in prostate cancer cells (53). Notably, multiple natural agents have been characterized as Skp2 inhibitors (53-56). Flavokawain A (FKA) selectively inhibited Skp2 in a proteasome-dependent manner in prostate cancer (57). Quercetin, curcumin and lycopene enhanced cell cycle arrest via the targeting of Skp2 expression in breast cancer cells (54). Moreover, curcumin was validated to regulate the PI3K/Akt-Skp2-Cip/Kips pathway in breast cancer cells (55,56). Furthermore, curcumin inhibited tumorigenesis via the downregulation of Skp2 in glioma cells (58). 15,16-Dihydrotanshinone I (DHTI) has been inhibited tumorigenesis via the downregulation of Skp2 in breast cancer cells (55,56). Furthermore, curcumin lycopene enhanced cell cycle arrest via the targeting of Skp2 expression in breast cancer cells (54). Moreover, curcumin suppression of Skp2, a p27Kip1

References


46. DING et al: DEPLETION OF Skp2 INHIBITS TUMORIGENESIS


