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SPOP suppresses osteosarcoma invasion via PI3K/AKT/NF-κB signaling pathway

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Abstract. – OBJECTIVE: Speckle-type POZ protein (SPOP), is an E3 ubiquitin ligase adaptor that is frequently mutated in prostate and endometrial cancers. SPOP has been shown to be responsible for oncogene SRC-3 ubiquitination and proteolysis in prostate cancers. However, whether SPOP plays a role in osteosarcoma (OS) is unknown. In this study, we investigated the inhibitory effect of SPOP on invasion and migration of OS cells.

PATIENTS AND METHODS: Real-time PCR and Western blot were used to detect the expression of SPOP in human OS samples and cell lines. Short hairpin RNA (shRNA) was used to silencing the expression of SPOP. Small scale Real-time PCR screen was used to identify the matrix metalloproteases (MMP) family members responsible for the phenotype caused by SPOP depletion. Matrigel-coated invasion chambers were used to detect the invasion ability of SPOP in OS cells.

RESULTS: We found that SPOP was down-regulated in clinic OS samples and cultured OS cells. Furthermore, we showed that silencing of SPOP promoted cell migratory and invasive ability of OS cells *in vitro*, whereas restored the expression of SPOP achieved the opposite effects. At the molecular level, we found that SPOP regulated the activity of "PI3K/Akt/NF-κB" signaling pathway in OS cells.

CONCLUSIONS: Our results suggested that down-regulation of SPOP promoted OS cells migratory and invasive ability via modulating the "PI3K/Akt/NF-κB" signaling pathway. Thus, SPOP could be a promising drug target for the treatment of OS invasion.

Key Words:
SPOP, Osteosarcoma, Invasion, PI3K/Akt/NF-κB.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor among children and adolescents[1]. The five-year survival rate for OS patients has been reported at 55-80% due to the advent of effective chemotherapy. However, the 5-year survival rate for osteosarcoma patients with metastases is less than 20%[2]. The pulmonary metastasis is the leading cause of death for the patients with extremities OS. The current treatment strategies have limited efficacy in the treatment of metastatic and recurrent osteosarcoma[3]. Therefore, the better understanding of the clear molecular mechanism of metastasis of OS and identify novel drug targets are necessary for the treatment of OS.

SPOP has been identified as a Cul3-based E3 ubiquitin ligase adaptor. It contains three domains: an N-terminal MATH domain that recruits substrates, an internal BTB domain that binds Cul3, and a C-terminal nuclear localization sequence[4]. SPOP is mostly studied in ubiquitin-dependent proteolysis and substrates identification, but its role in tumorigenesis is largely unknown and even contradictory[5]. Wild-type SPOP has been identified as a critical tumor suppressor in prostate cancer cells by promoting the turnover of SRC-3 protein and suppressing androgen receptor transcription activity[6]. However, another study showed that hypoxia derived SPOP accumulation in clear cell renal cell carcinoma cell cytoplasm which targeted PTEN, DUSP7, DAXX, and Gli2, thus leading to tumorigenesis[7]. The limited studies indicate the importance of SPOP in tumorigenesis, which toke us to use osteosarcoma cells to investigate its role in tumorigenesis.

PI3K/Akt plays a crucial role in the cell-extracellular matrix (ECM) and cell-cell adhesion[8]. The phosphorylation and activation of Akt have been recognized as an important regulatory factor in the NF-κB signaling pathway, which acts as the upstream regulator of Matrix metalloproteinases (MMPs), promoting the tumor cell invasion and migration. MMPs are involved in the degradation of the basement membrane and epimatrix,

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among which MMP-2 and 9 markedly correlated with tumor invasion[9]. MMP-2 and 9 have been found to be over-expressed in OS cells and able to promote OS cells migration and invasion by degrading components of the basement membrane and epimatrix[10-11]. Thus, blocking the PI3K/Akt/NF-κB pathway might be contributed to the OS treatment.

In this study, we investigated the effect of inhibition SPOP on the activity of PI3K/Akt/NF-κB signaling pathway, invasion, and migration in OS cells.

Patients and Methods

Patient Specimens

A total of 20 paired osteosarcoma tissues and adjacent normal tissues were obtained from patients who underwent surgery in our Department from 2015-08 to 2016-06. All the patients have no history of prior therapies with anticancer drugs or radiotherapy. The study had the approval from the Ethics Committee of Jing Zhou Central Hospital.

Cell Culture

The human OS cell line Saos2, U2OS, Hos, and MG63 as well as normal osteoblast hFOB1.19 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator containing 5% CO_2 at 37°C.

Real-time PCR

The total RNA from OS cells or tissues was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The mRNA levels of SPOP were examined by using SYBR green Real-time quantitative reverse transcription-PCR and normalized with β-actin. The amplification reaction was performed by on Light Cycler 480 (Roche, Basel, Switzerland) for 40 cycles. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used here are available upon request.

Western Blot

The total protein from the cells or tissues was extracted by 2x SDS loading buffer, separated by 10-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% milk for 45 min, and probed with the following primary antibodies: rabbit anti-SPOP, rabbit anti-PI3K, anti-p-PI3K (Tyr199), anti-NF-κB (p65) and anti-p-Akt (Ser473) IgG, rabbit anti-AKT IgG, mouse anti-Flag and mouse anti-GAPDH overnight at 4°C. After incubation with the appropriate secondary antibody for 1 hour at room temperature, the chemiluminescence dissolvent (Thermo Scientific, Waltham, MA, USA) was used to visualize immunoreactive bands, which were then exposed to the X-ray film (Kodak, Rochester, NY, USA).

Cell Migration and Invasion Assays

Cell migration was assessed by using six-well culture dishes. The cell monolayer was scraped with a pipet tip to make a straight line. Photographs of the scratch were taken at 0 h and 48 h, respectively. For invasion assay, Matrigel-coated invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) pre-hydrated in serum-free medium were utilized, according to the manufacturer's protocol. After 24 h incubation, the cells in the upper chamber were removed, and the cells were then fixed in ice-cold methanol, stained with Wright-Giemsa solution. The positively stained cells were counted in ten random fields under a microscope, and the average value was expressed.

Statistical Analysis

Data were summarized as mean ± SEM. Statistical significance was analyzed by Student's *t*-test using GraphPad prism7.0 software (La Jolla, CA, USA). Statistical significance has been shown as $^{*}(p < 0.05)$, $^{**}(p < 0.01)$ or $^{***}(p < 0.001)$.

Results

SPOP was Down-Regulated in Clinic Osteosarcoma Samples and Cell Lines

To investigate the potential role of SPOP in OS, we firstly determined the mRNA level of SPOP in 20 fresh osteosarcoma tissues and adjacent normal ones. As shown in Figure 1A, we found that SPOP is commonly down-regulated in osteosarcoma tissues when compared with adjacent normal ones. Moreover, our Western blot data revealed that SPOP protein was indeed down-regulated in OS tissues (Figure 1B). Furthermore, we detected both the mRNA and protein levels of SPOP in several OS cell lines Saos2, U2OS, Hos, and MG63,

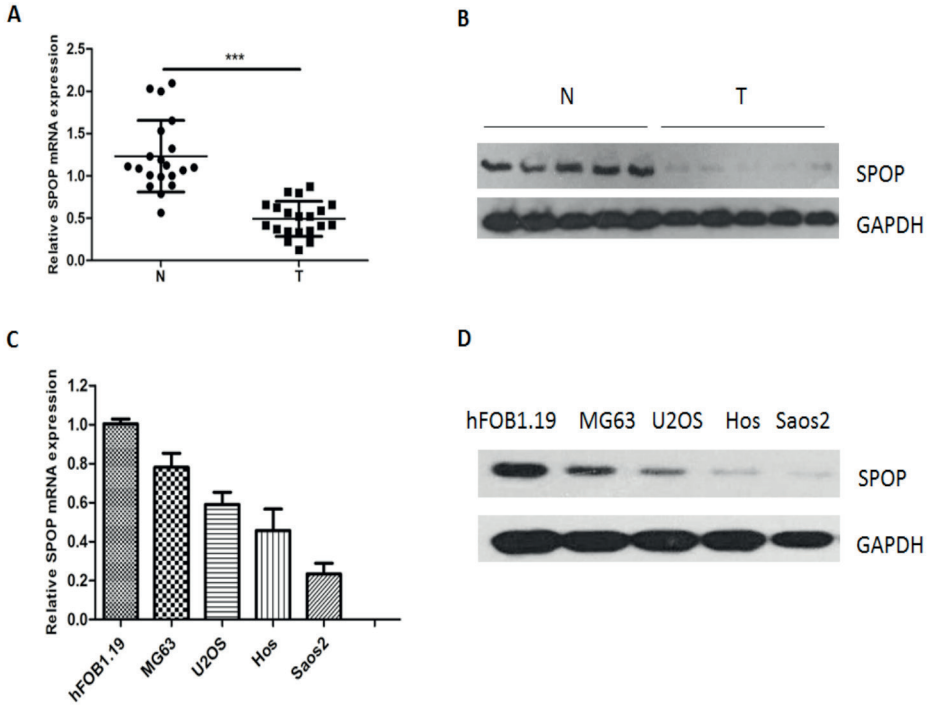

Figure 1. SPOP was down-regulated in clinic osteosarcoma samples and cell lines. ***A,*** Comparison of SPOP expression levels between 20 fresh pairs OS tissues and adjacent normal ones by Real-time PCR analysis. ***B,*** The protein levels of SPOP in 20 fresh pairs OS tissues and adjacent normal ones were determined by Western blot with indicated antibodies. ***C,*** Comparison of SPOP expression levels between 4 OS cell lines and normal hFOB1.19 cells by Real-time PCR analysis. ***D,*** The protein levels of SPOP in 4 OS cell lines and normal hFOB1.19 cells were determined by Western blot with indicated antibodies.

as well as in normal osteoblast hFOB1.19 cells. Inconsistent with the clinic OS tissues expression data, we found that both mRNA and protein levels of SPOP were down-regulated in OS cell lines when compared with normal hFOB1.19 cells (Figure 1C-D). These results suggest that SPOP is significantly down-regulated in the OS samples and cells.

Restoring of the Expression of SPOP Promoted OS Cells Migration and Invasion without Cell Proliferation Inhibition

As SPOP is down-regulated in OS tissues and cell lines with the relatively lowest expression level in Saos2 cells, we selected Saos2 to restore SPOP expression (Figure 2A). To our surprise, restoring the expression of SPOP exhibited an undetectable effect on Saos2 cells proliferation as determined by both cell counting and CCK-8 cell viability assays (Figure 2B-C). Considering that migration and invasion are critical steps for carcinogenesis, we then asked whether SPOP affected the migration and invasion of OS cells. We found that restored the expression of SPOP significantly promoted OS cells migration and invasion (Figure 2D-E), suggesting that SPOP played a critical role in OS cells migration and invasion instead of cell proliferation.

Silencing of SPOP Blocked OS Cells Invasion and Migration

Next, we silenced the expression of SPOP in MG63 cells, which possessed the relatively highest level of SPOP when compared with other OS cells we selected. Two different shRNAs against SPOP were used to knock-down SPOP expression in OS cells and achieved the similar inhibition efficacy (Figure 3A). In consistent with the rescue data, we found that SPOP depletion largely decreased OS cells migration and invasion (Figure 3B-C).

SPOP Regulated the Expression of MMP2 and MMP9 in OS Cells

To investigate the molecular mechanism of SPOP-mediated modulation of OS cells migration and invasion, we evaluated the genes' expression

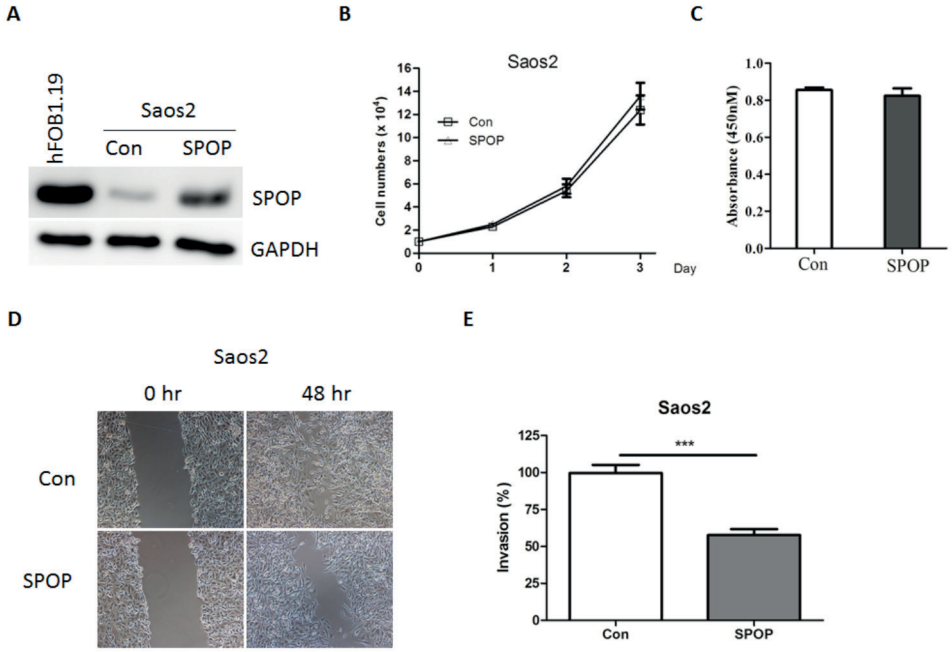

Figure 2. Restoring of the expression of SPOP promoted OS cells migration and invasion without proliferation inhibition. ***A,*** The protein levels of SPOP in normal hFOB1.19 cells and Saos2 cells with or without SPOP over-expression were determined by Western blot with indicated antibodies. ***B,*** The cell growth curve of Con-Saos2 and SPOP-Saos2 cells. ***C,*** The CCK-8 cell viability assays for Con-Saos2 and SPOP-Saos2 cells. ***D,*** A representative image of the wound healing assays is shown for Con-Saos2 and SPOP-Saos2 cells, indicating the migration of Saos2 cells were inhibited by SPOP over-expression. ***E,*** The cell invasion assays for Con-Saos2 and SPOP-Saos2 cells.

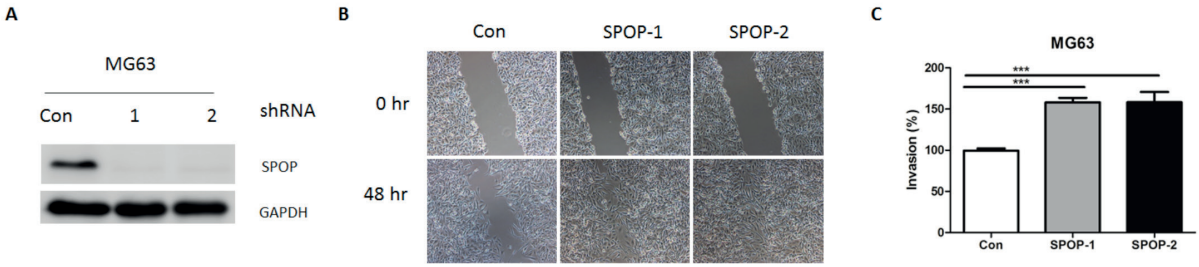

Figure 3. Silencing of SPOP blocked OS cells invasion and migration. ***A,*** MG63 cells stable expression of indicated shRNAs were subjected to Western blot with indicated antibodies. ***B,*** A representative image of the wound healing assays is shown for control and SPOP knockdown cells, indicating the migration of MG63 cells were increased by SPOP knockdown. ***C,*** The cell invasion assays for control and SPOP knockdown MG63 cells.

of matrix metalloproteases (MMPs), which were involved in cell migration and invasion in tumors by Real-time PCR. Of the twelve genes we tested, only two genes, MMP2 and MMP9, were found to be significantly down-regulated in Saos2 with SPOP over-expression (Figure 4A). In consistent with the mRNA data, we found the protein levels of MMP2 and MMP9 were also down-regulated in SPOP over-expression Saos2 cells (Figure 4B). In contrast, MMP2 and MMP9 were up-regulated in MG63 cells with SPOP knockdown (Figure 4C), suggesting SPOP regulated the expression of MMP2 and MMP9 in OS cells.

SPOP Suppressed the Expression of p-PI3K, p-Akt and NF-κB Proteins in OS Cells

To investigate how SPOP regulates the expression of MMP2 and MMP9, we evaluated the effects of SPOP on the activity of the PI3K/Akt/NF-κB signaling pathway in OS cells. The proteins of p-PI3K (Tyr199), PI3K, AKT, p-Akt (Ser473)

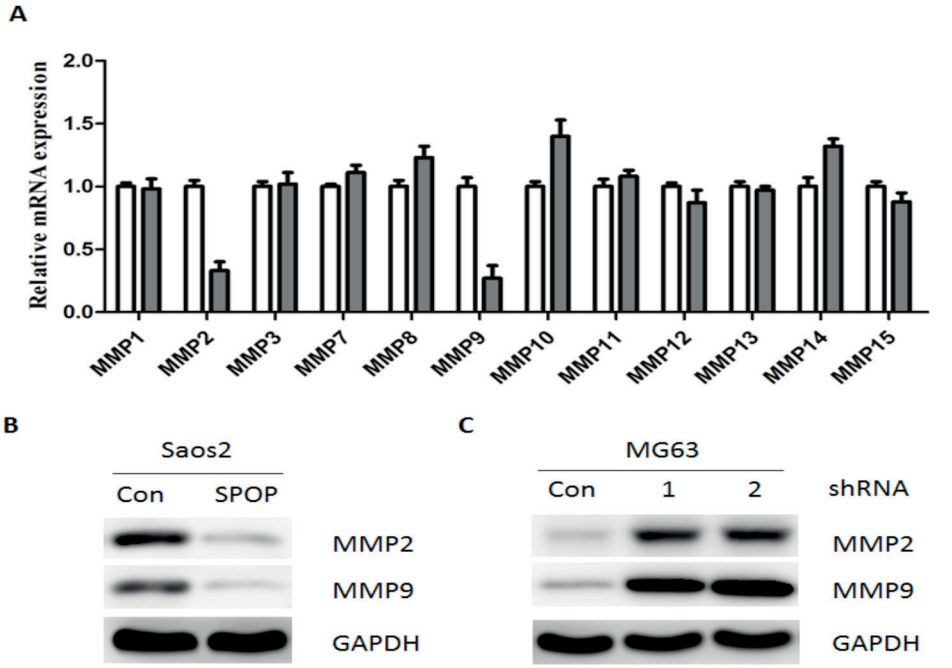

Figure 4. SPOP regulated the expression of MMP2 and MMP9 in OS cells. ***A,*** The mRNA levels of 12 MMP family members were detected by Real-time PCR in Con-Saos2 or SPOP-Saos2 cells. ***B,*** The protein levels of MMP2 and MMP9 in Con-Saos2 or SPOP-Saos2 cells were detected by Western blot with indicated antibodies. ***C,*** The protein levels of MMP2 and MMP9 in MG63 cells with or without SPOP knockdown were detected by Western blot with indicated antibodies.

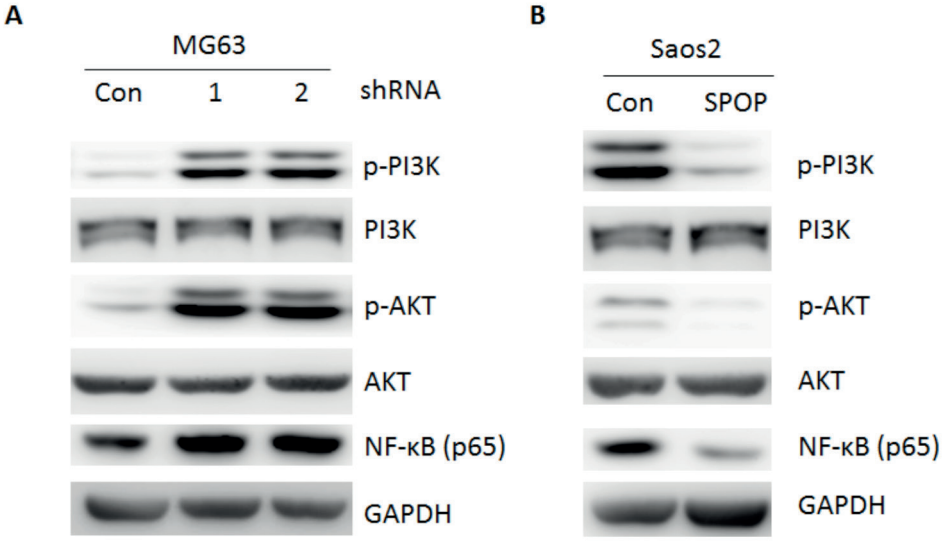

Figure 5. SPOP suppressed the expression of p-PI3K, p-Akt and NF-κB proteins in OS cells. ***A,*** The expression of p-PI3K, p-Akt and NF-κB proteins were determined in Con-Saos2 or SPOP-Saos2 cells by Western blot with indicated antibodies. ***B,*** The expression of p-PI3K, p-Akt and NF-κB proteins were determined in Con-MG63 or SPOP knockdown Saos2 cells by Western blot with indicated antibodies.

and NF-κB (p65) were measured by Western blot. We found that p-PI3K and p-Akt, NF-κB proteins expressions in SPOP knockdown cells were significantly higher than control cells (Figure 5A). On the contrary, restored the expression of SPOP in Saos2 cells decreased the expression of p-PI3K and p-Akt, NF-κB proteins (Figure 5B). Taken together, these data suggested that SPOP suppressed

the expression of p-PI3K, p-Akt and NF-κB proteins in OS cells.

Discussion

NF-κB is composed of DNA-binding subunits (p50 and p52) and subunits with transcriptional activity (p65 and RelB or c-Rel)[12]. The primary form of NF-κB is a heterodimer of the p50 and p65 subunits and mainly localized to the cytoplasm in an inactive form bound to an inhibitor of κB (IκB)[13]. The phosphorylation of Akt has been recognized as an important regulatory factor to activate NF-κB by promoting the proteasome degradation of IκB[14]. Activated NF-κB played an important role in the regulation of MMPs, including MMP-2 and MMP-9, to promote OS cells metastasis by degrading components of the basement membrane and epimatrix[15,16].

The tumor suppressor gene SPOP has been identified as a significantly mutated gene in human prostate cancers by cancer genomic analyses and confirmed via subsequent larger prostate cancer patient cohorts analyses[17]. SPOP is the substrate adaptor of a Cul3-based ubiquitin ligase by utilizing its MATH domain to mediate substrates binding. Interestingly, most SPOP mutations were occurred in its MATH domain, indicating impaired substrate-binding ability[18, 19]. Recently, in prostate cancer, several SPOP substrates have been identified including androgen receptor (AR), steroid receptor coactivator (SRC)-3, and SENP7 [20-22]. However, the cancer-associated SPOP mutants in other cancer types were barely reported, suggesting an alternative regulation of SPOP might exist.

In this study, we found that SPOP was significantly downregulated in both clinic OS samples and OS cell lines. Notably, we provided evidence to show that SPOP was dispensable for OS cells proliferation but indispensable for OS cells migration and invasion. By utilizing a small scale mRNAs expression screen, we found that SPOP could specially regulate the expression of both MMP-2 and MMP-9 among twelve MMPs. Importantly, we revealed the SPOP could modulate the activity of the PI3K/Akt/NF-κB signaling pathway to regulate MMP-2 and MMP-9 expression, leading to enhanced OS cells migration and invasion.

Conclusions

Given the implications of our findings about SPOP, further studies are warranted to characterize the mechanism of SPOP-mediated effects on OS metastasis. Moreover, how SPOP is down-regulated in OS cells remains an important question to be answered in the future. Nevertheless, we demonstrated here that down-regulation of SPOP promoted the migratory and invasive ability OS cells via modulating the "PI3K/Akt/NF-κB" signaling pathway. Thus, SPOP could be a promising drug target for the treatment of OS invasion.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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