

# Inhibition of focal adhesion kinase induces apoptosis in human osteosarcoma SAOS-2 cells

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**Abstract** Focal adhesion kinase (FAK), a non-receptor tyrosine kinase protein, acts as an early modulator of integrin signaling cascade, regulating basic cellular functions. In transformed cells, unopposed FAK signaling has been considered to promote tumor growth, progression, and metastasis. The aim of this study was to assess the role of focal adhesion kinase in human osteosarcoma SAOS-2 cells. SAOS-2 cells were transfected with PGPU6/GFP/shNC, and PGPU6/GFP/FAK-334 (shRNA-334), respectively. Expression of FAK was detected by real-time PCR and western blots. MTT assay was used to examine changes in cell proliferation. Cell apoptosis was analyzed by flow cytometry. The expression of caspase-3,-7,-9 was measured by Western blots. The expression of FAK in SAOS-2 cells significantly decreased in shRNA-334 group contrast to the control group ( $P < 0.01$ ). Cells proliferation was inhibited by shRNA-334 and shRNA-334 + cisplatin, and the effects were clearly enhanced when cells treated with the anticancer agents. The level of cell apoptosis in shRNA-334 and shRNA-334 + cisplatin group was higher than in the control group ( $P < 0.01$ ). The current data support evidence that down-regulation of FAK could induce SAOS-2 apoptosis through the caspase-dependent cell death pathway. Inhibition of the kinases may be important for therapies designed to enhance the apoptosis in osteosarcoma.

**Keywords** Human osteosarcoma cells · Apoptosis · Focal adhesion kinase · SAOS-2 cells

## Introduction

Focal adhesion kinase (FAK) is a 125-kDa cytoplasmic non-receptor tyrosine kinase enzyme initially described as a putative substrate for the Rous sarcoma virus-encoded oncoprotein pp60v-src [1, 2]. FAK was reported to be tyrosine-phosphorylated in response to integrin-mediated cell adhesion, integrin clustering, cell motility, and migration [3, 4]. It was also shown that FAK, forming a signaling complex with Src, a member of the Src family of cytoplasmic tyrosine kinases, activates downstream enzymes such as mitogen-activated protein kinases [1, 4, 5], resulting in the activation of tumor cells. Structural and functional analysis of FAK led to the identification of multiple binding interacting sites of this molecule with other proteins important for signaling. Activation and subsequent autophosphorylation of FAK, in response to cell adhesion, leads to its association with several signaling molecules triggering signal transduction [6]. Tyrosine kinase inhibitors have also been reported to reduce tyrosine phosphorylation of FAK and subsequently, decreased cellular migration of tumor cells in vitro [7]. Recently, it was shown that increased FAK expression correlated with increased cell motility, invasiveness, and proliferation [8–10]. FAK expression also correlates with tumor recurrence [11].

Osteosarcoma (OS) is a high-grade malignant bone tumor, and is the most frequent malignant bone tumor found in children and adolescents. It affects distal long bones through the formation of neoplastic bone tissue [12, 13]. The therapeutic modality that is currently favored involves neoadjuvant chemotherapy, followed by surgical resection, and the 5-year survival of patients with osteosarcoma has increased to 60 % through the use of such protocols. Induction of apoptosis is one goal in treating neoplasms, and recent studies have focused on the testing of novel methods for their ability to induce and enhance apoptosis [14–16]. Unfortunately, very few studies have been carried on inhibitory effect of FAK on

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osteosarcoma and the mechanisms of the anticancer capacity remain poorly understood.

The present study aimed to assess the decreased cell proliferation and apoptosis of SAOS-2 cells as a result of the down-regulation of FAK expression.

## Materials and methods

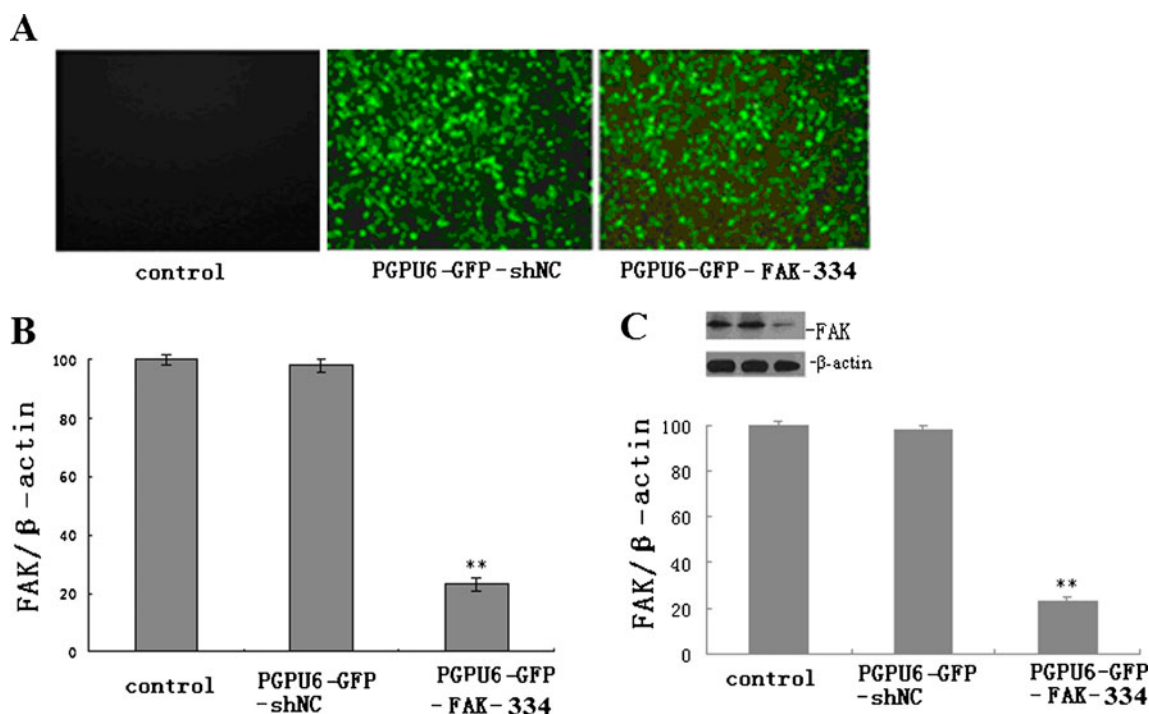
### Cell culture, plasmid construction and transient transfection

The human osteosarcoma cells (SAOS-2) were maintained in 1640 medium containing 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in a fully humidified incubator (US AutoFlow, NuAire, MN) at 37 °C with 5 % CO<sub>2</sub>. The cells were kept in an exponential growth phase during experiments. The plasmids expressed the nucleotide fragment that RNAi to FAK, in the sense (5'- GCGA GTATTAAGGTCTTTCA -3') (shRNA-334) and mismatch sense nucleotide fragment (5'- GTTCTCCGAACGTGTCA CGT -3') (shNC), were selected from the human FAK gene (GenBank Accession No. L13616) and were constructed by GenePharma Inc, Shanghai, China. Transient transfection with PGPU6/GFP/shNC (shNC) or PGPU6/GFP/FAK-334 (shRNA-334) was performed in DMEM/serum free medium

with Lipofectamine 2000 (Invitrogen, USA) at a final concentration of 0.2 µmol/l at 37 °C for 6 h. Medium was then replaced with Lipofectamine-free DMEM containing 20 % FBS for another 24 h. Cells were then incubated at 37 °C for an additional 48 h in DMEM or DMEM + cisplatin with 10 % FBS. The transfection rate was measured by fluorescence microscopy (Becton Dickinson).

### Fluorescent quantitative RT-PCR

Total RNA was prepared from cells harvested using Trizol (Invitrogen, Carlsbad, California) and purified over RNAeasy columns (QIAGEN, Valencia, California) according to the manufacturer's protocol. The RNA quality and quantity were evaluated by spectrophotometry and gel electrophoresis. Total RNA was reverse transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California) according to the manufacturer's directions. Transcript levels were evaluated by quantitative PCR using the comparative threshold method. The PCR reactions were carried out on cDNA templates corresponding to 16.5 ng of total RNA using intron-spanning primers and an intercalating dye in iQ SYBR Green Supermix reaction mixture (Bio-Rad Laboratories, Hercules, California). Thermal cycling conditions were 10 min at 95 °C followed by



**Fig. 1** Expression of FAK in the SAOS-2 cells. SAOS-2 cells transiently transfected with PGPU6/GFP/shNC or PGPU6/GFP/FAK-334 was performed in DMEM/serum-free medium with Lipofectamine 2000, respectively, and the expression of FAK in SAOS-2 cells was shown by fluorescence microscopy and down-regulated tested by fluorescent quantitative RT-PCR and Western blotting in PGPU6/GFP/FAK-334 group. **a**

The expression of green fluorescent protein in control, PGPU6/GFP/shNC, or PGPU6/GFP/FAK-334 group. **b** Fluorescent quantitative RT-PCR was performed to detect mRNA expression of FAK in control, PGPU6/GFP/shNC, or PGPU6/GFP/FAK-334 contrast to the β-actin, respectively. **c** Western blotting showed expression of FAK in control, PGPU6/GFP/shNC, or PGPU6/GFP/FAK-334 contrast to the β-actin, respectively

40 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 75 °C, and a final extension step for 6 min at 72 °C performed in an iCycler (Bio-Rad) with an optical module. Each PCR product was confirmed to be a single band by melt curve analysis and visualization on agarose gel. Replicates were performed for each gene and condition assayed. Cycle threshold values were determined using iCycler Optical System Software 3.0 (Bio-Rad) with the default settings for a PCR baseline subtracted curve fit model. The  $\beta$ -actin values showed little variation across all samples.

#### Proliferation assay

Cell proliferation inhibition was analyzed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 20  $\mu$ l MTT (5 mg/ml) was added into 96-well after transfected with shRNA-334 or shNC or shRNA-334 + cisplatin for 72 h. Five hours later, 100- $\mu$ l DMSO was added to each well to dissolve formazan crystals. Absorbance was checked at 490 nm using an enzyme-linked immunosorbent assay reader (SpectraMax, Molecular Devices, Sunnyvale, CA). Three separate experiments were operated and the data were determined by comparison to DMSO-treated control cells.

#### Detection of apoptosis with flow cytometer

SAOS-2 cells transfected with shNC or shRNA-334 or shRNA-334 + cisplatin were washed twice with PBS, adjusted to 100  $\mu$ l of the solution and transferred to a 1-ml centrifuge tube ( $1 \times 10^5$  cells). Then, 10  $\mu$ l of Annexin V-APC and 10  $\mu$ l of 7-AAD were added and cells were gently vortexed. Cells were then incubated for 15 min at room temperature (25 °C) in the dark and 400  $\mu$ l of  $1 \times$  binding buffer was added to each tube. Finally, cells were analyzed by flow cytometry (FCM) (Becton Dickinson).

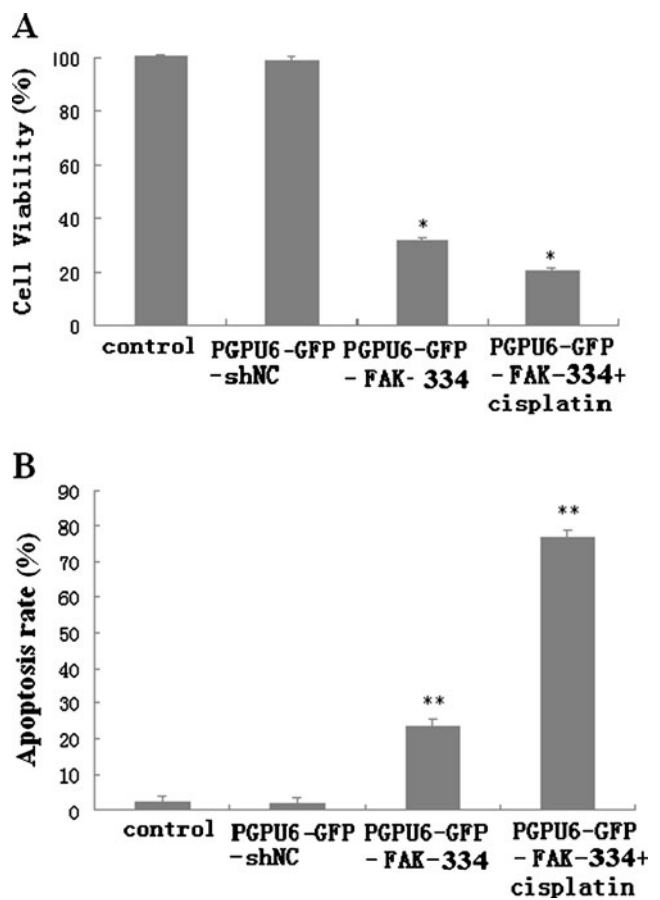
#### Western blot analysis

Cells treated as described above were harvested by scraping on ice into 1 ml RIPA buffer (50-mM Tris, 150-mM NaCl, 1 % Triton X-100, and 0.5 % deoxycholate) containing 50 mg/ml leupeptin, 10 mg/ml aprotinin, 2-mM ethylenediamine tetraacetic acid, and 1-mM vanadate. Lysates were cleared by centrifugation (14,000 g) at 4 °C for 1 min. The supernatant protein concentration was determined using a Micro BCA Protein Assay Reagent Kit. Normalized lysates were boiled in electrophoresis SDS sample buffer, run on a 10 % SDS-PAGE gel, and transferred to nitrocellulose. Membranes were blocked for 30 min in Tris-buffered saline (TBS) containing 0.1 % bovine serum albumin, 0.05 % Tween-20 (TBST), and 2 % cold fish gelatin. The membrane was probed with primary antibodies at 37 °C for 2 h. The antibodies used were anti-FAK (1:400 dilution), anti-P53, anti-cleaved poly-ADP ribose

polymerase (PARP), anti-Bcl-2 and Bax, anti-cleaved -caspase-3, 9, anti- $\beta$ -actin (1:1000 dilution). After washing twice with TBST (pH 7.4), they were incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at 37 °C. The membranes were washed again with TBST, and the protein bands were visualized by a chemiluminescence detection kit (Beyotime Institute of Biotechnology, Shanghai, China). All Western blots were performed at least three times for each experiment.

#### Statistical analysis

Data are presented as mean values  $\pm$  standard deviation.  $P < 0.05$  was considered statistically significant.



**Fig. 2** The apoptosis induced by down-regulation of FAK in SAOS-2 cells. MTT and flow cytometric analysis was performed for cells, respectively. **a** MTT assay was used to determine the cell proliferation. ShRNA-334-treated cells displayed a significant reduction in cell proliferation by  $71.4 \pm 1.7$  % comparison with the control group ( $P < 0.05$ ). Additionally, significant reduction increase at  $80.5 \pm 1.5$  % in shRNA-334 + cisplatin group compared with control group ( $P < 0.05$ ). **b** To verify the apoptosis induced by down-regulation of FAK, flow cytometric analysis was performed for cells, respectively. The flow cytometry assays showed shRNA-334 group increase in apoptosis at  $19.8 \pm 1.8$  % compared with the control group ( $P < 0.01$ ). Additionally, apoptosis significant increase at  $75.3 \pm 1.6$  % in shRNA-334 + cisplatin group compared with control group ( $P < 0.01$ )

## Results

### Down regulation of the expression of FAK

To assess the effect of shRNA-334 or shNC on FAK expression, SAOS-2 cells transfected with PGPU6/GFP/shNC or PGPU6/GFP/FAK-334 was performed in DMEM/serum-free medium with Lipofectamine 2000 (Invitrogen, USA), respectively (Fig. 1a). Fluorescent quantitative RT-PCR and western blot analysis was performed to detect expression of FAK, and FAK mRNA and protein levels were significantly reduced in the shRNA-334 group contrast to the control ( $P < 0.01$ ) (Fig. 1b, c).

### Inhibitory rate of SAOS-2 cell growth

To assess the effect of down regulation on FAK expression, SAOS-2 cells transiently transfected with PGPU6/GFP/shNC or PGPU6/GFP/FAK-334 was performed in DMEM/serum-free medium with Lipofectamine 2000 (Invitrogen, USA), respectively. MTT assay was used to determine the cell proliferation. ShRNA-334-treated cells displayed a significant reduction in cell proliferation by  $71.4 \pm 1.7\%$  comparison with the control group ( $P < 0.05$ ). Additionally, significant reduction increase at  $80.5 \pm 1.5\%$  in shRNA-334 + cisplatin group compared with control group ( $P < 0.05$ ) (Fig. 2a).

### Detection of apoptotic cells by flow cytometry

To verify the apoptosis induced by down-regulation of FAK, flow cytometric analysis was performed for cells, respectively.

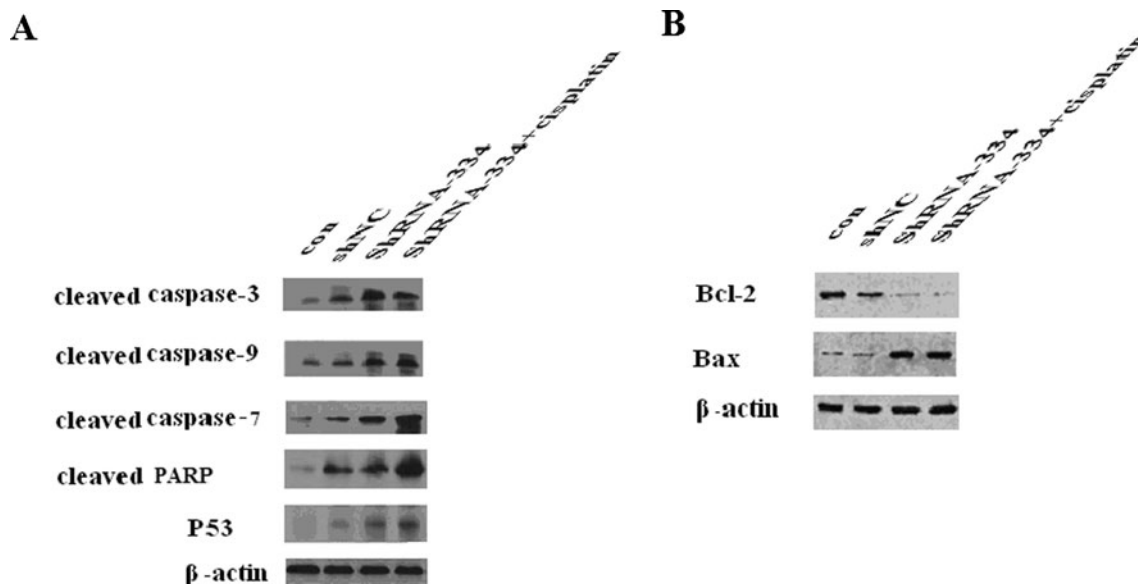
The flow cytometry assays showed shRNA-334 group increase in apoptosis at  $19.8 \pm 1.8\%$  compared with control group ( $P < 0.01$ ). Additionally, apoptosis significant increase at  $75.3 \pm 1.6\%$  in shRNA-334 + cisplatin group compared with control group ( $P < 0.01$ ) (Fig. 2b).

### Western blot analysis

To determine the mechanism responsible for down-regulation of FAK-mediated apoptosis, the apoptotic protein expressions were evaluated by western blot analysis. PARP-specific proteolytic cleavage by caspases is considered to be characteristic of apoptosis, the cleavage of PARP and caspase was evaluated. The results of Western blot analysis for cleaved PARP, cleaved caspase-9, cleaved caspase-7, cleaved caspase-3, P53, Bcl-2 and Bax proteins were shown in Fig. 3. It can be seen that shRNA-334 group resulted in the up-regulation of cleaved PARP, cleaved caspase-9, cleaved caspase-3, P53 and Bax proteins, which are all involved in apoptosis. In contrast, there was a decrease in the expression of Bcl-2 protein in shRNA-334 group and shRNA-334 + cisplatin group.

## Discussion

Osteosarcoma, the most common type of malignant bone tumor, occurs predominantly in adolescents and young adults. Until recently, a 5-year survival rate of 20 % for treatment through surgical intervention alone was considered acceptable (because approximately 80 % of patients had pulmonary



**Fig. 3** Effects of down-regulation of FAK on the expression of apoptosis-related proteins. Western blotting of protein release in SAOS-2 cells after transfection with plasmids expressing PGPU6/GFP/shNC or PGPU6/GFP/FAK-334 for 48 h. Representative western blotting for the

expression of cleaved caspase-3, cleaved caspase-9, cleaved caspase-7, cleaved PARP, P53, bcl-2, and bax in SAOS-2 cells.  $\beta$ -actin ( $\sim 44$  kDa) was used as a loading control. Results are representative of two independent experiments

metastasis at time of presentation) [17, 18]. Currently, the standard treatment for OS is neoadjuvant chemotherapy. However, the effectiveness of cytotoxic drugs often declines, due to acquired chemoresistance. Finding new therapeutic agents to target the malignant behavior of osteosarcoma cells is, therefore, important for improving the prognosis.

Emerging novel strategies of cancer treatment are based on the selective down-regulation of specific targets involved in the neoplastic progression. FAK seems to be a relevant target for such therapeutic intervention. FAK is a member of the tyrosine kinase family that functions as a potent inhibitor of apoptosis. Previous studies have demonstrated that enhanced expression of FAK in various cancer cells, such as human leukemia cells [19], medullary thyroid carcinoma cells [20], and ovarian carcinoma cells [21], is associated with enhanced resistance of these cells toward chemotherapy or other apoptosis stimuli. Accordingly, low expression of FAK can evoke an apoptotic response. In the present study, we have shown that FAK expression levels were significantly down-regulated in SAOS-2 cells, after shRNA-334 treatment, whereas no significant changes in shNC-treated cells were observed. These results indicate that the shRNA-334 used in the present study effectively down-regulated of FAK expression in SAOS-2 cells. Cell proliferation and FCM revealed that apoptotic cell death was abundant in shRNA-334 or shRNA-334 + cisplatin treated cells but almost completely absent in shNC-treated or control cells.

How does the down-regulation of FAK expression induce cell death in SAOS-2 cells? Generally speaking, apoptosis is initiated by either an extrinsic (activated caspase-8, 10) or an intrinsic pathway (activated caspase-9) [22]. The extrinsic pathway can directly activate caspase-8 through death receptors on the cell surface. The intrinsic pathway regulates apoptotic cascades by a signaling convergence in the mitochondrion, which results in the alteration of the MMP, the release of cytochrome C into the cytosol. In the presence of Cytochrome C and dATP, Caspase-9 and Apaf-1 can be combined, and prompted Caspase-9 activation. Cytochrome C release and Caspase-9 activation is essential for activation of other caspase such as Caspase-3 and lead to subsequent DNA fragmentation [23, 24]. To understand the association between down-regulated of FAK and apoptosis, we examined levels of various apoptotic markers. Through the western blot analysis of caspase-9 and -3, we showed that down-regulation of FAK-induced cell death in SAOS-2 cells was caspase dependent. It is well documented that disruption of nucleolar morphology results in activation of the p53 stress–response pathway [25]. We showed that after down-regulation of FAK expression, the p53 stress–response pathway is also activated followed by up-regulation of Bax/Bcl-2. Similar to the role of caspase in the control of cell death, we observed a decrease in expression of bcl-2, as well as an increase in expression of bax in SAOS-2 cells after treatment with shRNA-334. This change in bcl-2 and bax expression may facilitate the initiated apoptosis.

In a clinical setting, the RNAi alone may not be effective enough to kill all the osteosarcoma cells in a tumor. Therefore, chemotherapeutic agent was investigated for whether it could enhance the cytotoxicity. We chose the anticancer drug, cisplatin, of which antitumor effects have been well documented. In the presence of cisplatin, down-regulation of FAK had additive cytotoxic effects in vitro. Consistent with our results; the use of combined approaches in cancer therapy seems to be more efficient than a single type of treatment [26]. Based on these finding, our study supports the idea that combined cancer treatment approaches such as inhibition of FAK could substantially augment the sensitivity of SAOS-2 cell to chemotherapy.

In summary, we have shown that shRNA-334 is an effective inhibitor of FAK. Moreover, inhibition of FAK in combination with cisplatin effectively inhibited SAOS-2 cell growth by formation of apoptosomes mechanisms. Based on these findings, FAK represents an attractive therapeutic target in osteosarcoma and supports further consideration of anti-FAK therapies for clinical development.

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**Conflicts of interest** None

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