Biochemical and Biophysical Research Communications 464 (2015) 45–50

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Spondin 1 promotes metastatic progression through Fak and Src dependent pathway in human osteosarcoma

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ARTICLE INFO

Article history:
Received 18 May 2015
Accepted 28 May 2015
Available online 29 May 2015

Keywords:
Spondin 1
Fak
Src
Osteosarcoma

ABSTRACT

Spondin 1 (SPON1) is cell adhesion protein that involved in attachment of sensory neuron cells and outgrowth of neurites. Its cellular functions and related mechanisms in cancers, however, remain largely unexplored. In this study, we first identified that SPON1 acts a critical factor in the metastatic progression of osteosarcoma through analysis of a GEO dataset. Then we demonstrated that SPON1 was significantly up-regulated in 72 osteosarcoma specimens compared with benign osteochondroma samples and elevated SPON1 was positively correlated with MMP9 expression. Knockdown of SPON1 expression in two metastatic osteosarcoma cell lines, HKOS and KRIB, dramatically suppressed cell migration and invasion. Treatment with recombinant SPON1 protein in two non-metastatic osteosarcoma cell lines, HOS and U2OS, significantly promoted cell migration and invasion *in vitro*. Meanwhile, suppression of SPON1 in KHOS cells resulted in decreased pulmonary metastasis *in vivo*. Mechanistically, we determined that the effects of SPON1 on osteosarcoma cell motility were primarily mediated through Fak and Src dependent pathway. Taken together, our study provides evidence of the contributions of SPON1 and the Fak and Src signaling to the progression of osteosarcoma and suggests that this axis may represent a potential therapeutic target for osteosarcoma.

1. Introduction

Osteosarcoma is the most frequent primary malignant bone tumor in children and adolescents [1]. Due to high degree of malignancy, invasion and metastasis, however, only 30% of patients diagnosed with osteosarcoma will not survive for more than 5 years, and less than 50% will live beyond 10 years [2,3]. Pulmonary metastase is present in about 15–25% of patients and this contributes to the failure of chemotherapy and poor prognosis in osteosarcoma [4]. Therefore, it is of great importance to determine the molecular mechanisms that underlie this type of metastasis.

SPON1, also known as F-spondin and VSGP, is a secreted extracellular matrix glycoprotein that originally isolated from the embryonic floor plate of vertebrates [5–8]. Initially, treatment with recombinant SPON1 protein promotes neural cell adhesion and neuronal outgrowth, which indicates the functions of SPON1 in the modulation of axonal growth in the embryonic central nervous system [9]. Apart from neuronal tissues, SPON1 expression has recently been detected in several other tissues including ovary [10], embryonic growth plate cartilage [11], periodontal tissue [12] and osteoarthritic cartilage [13]. However, whether SPON1 is differentially expressed in tumors, especially in osteosarcoma, remains largely unexplored.

To address this problem, the aim of current study was to determine the expression pattern and cellular functions of SPON1 in osteosarcoma. We carried out immunohistochemical analysis of SPON1 expression in clinical osteosarcoma specimens and studied the relationship between SPON1 levels and MMP9 and ki67expression, respectively. The cellular migration and invasion affected by SPON1 were analyzed in high (HKOS and KRIB) and low (HOS and U2OS) metastatic potential osteosarcoma cell lines. And to demonstrate the mechanism on this effect induced by SPON1, Fak and Src pathway were inhibited using small interfere RNA (siRNA). Finally, our results imply that the SPON1/Fak/Src-related pathway might be an effectively therapeutic approach in the management of metastatic osteosarcoma.

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http://dx.doi.org/10.1016/j.bbrc.2015.05.092
0006-291X/

2. Materials and methods

2.1. Cell culture and reagent

Human osteosarcoma cell lines, KHOS, KRIB, HOS and U2OS, were grown in RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum, 100 U/mL, penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator under 5% CO_2 condition. Human recombinant SPON1 protein was purchased from R & D systems (Cat#3135-SP-025).

2.2. Transfection

All of the siRNAs (SPON1, Fak and Src) used in current study were synthesized by GenePharma (Shanghai, China). For transfection, KHOS or KRIB cells were transfected with 50 μM siRNAs using siRNA mate according to the manufacturer's instructions (GenePharma, Shanghai). The interference efficiency of targeted gene was demonstrated by western blotting or RT-PCR. Primer sequences used are as follows: FAK: forward: 5′-GCTTACCTTGACCCCAACTTG-3′, reverse: 5′-ACGTTCCATACCAGTACCCAG-3′; SRC: forward: 5′-GAGCGGCTCCAGATTGTCAA-3′, reverse: 5′-CTGGGGATGTAGCCTGTCTGT-3′; β-actin: forward: 5′-CATGTACGTTGCTATCCAGGC-3′, reverse: 5′-CTCCTTAATGTCACGCACGAT-3′. The relative mRNA expression of FAK or SRC was normalized to β-actin.

2.3. Immunohistochemistry

Two tissue microarray (OS208 and OS804) containing seventy-two cases of osteosarcoma tissues and twenty-four cases of Osteochondroma tissues were purchased from Xi-an Alenabio Inc (China). The tissue sections were deparaffinized with dimethylbenzene and rehydrated through grade ethanol. After three washes in phosphate-buffered saline (PBS), antigen retrieval was performed in a buffer containing 0.01 M sodium citrate-hydrochloric acid (pH = 6.0) for 15 min by microwave. After rinsing with PBS, the tissue sections were then rinsed in 0.3% peroxidase quenching solution (Invitrogen) to block endogenous peroxidase, followed by incubation with primary antibody against SPON1 (Abcam, USA, at 1:200 dilution), MMP9 (Abcam, USA, at 1:200 dilution) and ki67 (Proteintech, USA, at 1:100 dilution) overnight at 4 °C. After washing in PBS for three times, the sections were incubated with HRP-labeled anti-rabbit secondary antibody. The visualization signal was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB), and all of the slides were counterstained with hematoxylin. The total immunostaining score was calculated as the percentage of the positively stained tumor cells. The percent positivity was scored as follows: 0–10% scored 0; 10%–30% scored 1; 30%–60% scored 2; more than 60% scored 3. And scored at 0 and 1 was defined as low expression, while 2 and 3 was defined as high expression.

2.4. Western blotting

Treated and untreated osteosarcoma cells were harvested and washed twice with PBS and lysed in RIPA buffer (Beyotime, China) with freshly added 0.01% protease inhibitor cocktail (Sigma). Cell lysates were separated by SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane (Millipore). The blots were blocked with 5% skim milk, followed by incubation with antibodies against SPON1 (Abcam, Cat#ab170655), FAK (CST, Cat#13009), P-FAK (CST, Cat#3284), SRC (CST, Cat#2107), P-SRC (CST, Cat#12432) and β-actin (Proteintech, USA). Blots were then incubated with incubated with HRP-conjugated secondary antibodies (Abmart, China) and visualized using ECL Plus kit (Millipore).

2.5. Migration and invasion assays

The invasive potential of osteosarcoma cells was measured by transwell model (Corning, NY) according to the manufacturer's instructions. For migration assay, 2×10^4 cells in 100 μl medium were seeded into the upper compartment of the transwell inserts. The invasion assay was performed with matrigel-coated filters (BD Bioscience, USA). The lower chambers were filled with 700 μl of RPMI-1640 medium containing 2% FBS. After the cells were incubated for 16 h, the non-invading cells that remained on the upper surface were removed. The migrated and invaded cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of cells on the lower surface, was counted under a light microscope in six random fields. Each experiment was performed in triplicate.

2.6. In vivo tumorigenesis and metastasis

Cells stably interfered SPON1 or a control vector were collected and resuspended in 100 μl cold PBS. Then 5×10^5 KHOS cells were mixed with the same volume of matrigel and injected into the proximal tibia of each anesthetized nude mice (n = 5 animals/group). Six weeks after inoculation, orthotopic tumors and mouse lungs were harvested. The orthotopic tumors were weighed and the number of pulmonary metastatic tumor nodules was counted under an under a dissecting stereomicroscope. Finally, lung tissues were fixed with 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with H&E (hematoxylin and eosin). Animal experiments were performed in full accordance with the Third Hospital of Hebei Medical University.

2.7. Statistical analysis

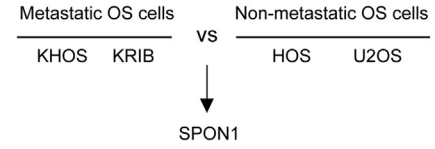
Data were presented as the means ± SD of three independent experiments. The SPSS software program (version 17.0; IBM Corporation) was used for statistical analysis. Graphical representations were performed with GraphPad Prism 5 (San Diego, CA) software. The chi-square test was used to analyze the expression of SPON1 between osteosarcoma and osteochondroma specimens. Correlations between SPON1and MMP9 or ki67 levels in osteosarcoma were analyzed by Spearman's rank correlation. All other data were analyzed using two-sided Student's t test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Up-regulated SPON1 expression is closely associated with osteosarcoma metastasis in clinical specimens

To explore the critical factors involved in metastatic osteosarcoma, we observed the differentially expressed genes (DEGs) between human metastatic osteosarcoma cells and non-metastatic osteosarcoma cells from a GEO dataset (GSE49003) (Fig. 1A). In these DEGs, we selected SPON1 for further study. We first detected SPON1 in four osteosarcoma cell lines. Expectedly, elevated SPON1 protein was found in metastatic osteosarcoma cells, KHOS and KRIB, compared with the non-metastatic osteosarcoma cells, HOS and U2OS (Fig. 1B). Immunohistochemical analysis showed that positive expression rate of SPON1 were significantly higher in osteosarcoma compared with osteochondroma samples (Fig. 1C). Moreover, there was significant positive correlation between SPON1 and MMP9 ($P < 0.001$, $R = 0.745$), but not ki67 ($P = 0.359$) in

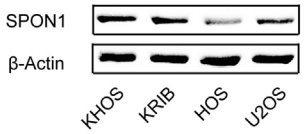
A



C

Sample	Low	High	P value
Osteosarcoma	24	48	0.001
Osteochondroma	18	6	

B



D

SPON1	MMP9			ki67		
	Low	High	P value	Low	High	P value
Low	18	6	0.000	5	19	0.359
High	2	46		15	33	

Spearman's rank correlation

Fig. 1. Up-regulated SPON1 expression is closely associated with osteosarcoma metastasis in clinical specimens. (A) Schematic description of the procedure that SPON1 was selected. (B) Expression of SPON1 in four osteosarcoma cell lines was measured. (C) Expression levels of SPON1 in osteosarcoma and osteochondroma specimens. (D) Correlation between SPON1expression and MMP9, ki67 levels in osteosarcoma.

osteosarcoma specimens (Fig. 1D). These results indicate that dysregulated SPON1 may favor metastatic progression of osteosarcoma.

3.2. SPON1 promotes migration and invasion of osteosarcoma cells

To investigate the implications of SPON1 in osteosarcoma, we performed cell migration and invasion assays when SPON1 was knockdown or treatment with human recombinant SPON1 protein. As shown in Fig. 2A and C, transfection of two SPON1 siRNAs (si-1, si-2) in HKOS and KRIB cells resulted in markedly decrease in SPON1 expression; knockdown of SPON1 resulted in significantly decreased migrated and invaded cells compared with negative control cells (Fig. 2B and D). We next determined the effects of SPON1 on tumor metastasis *in vivo*. As shown in Fig. 2E, stably suppression of SPON1 in KHOS cells did not affect tumor growth in xenograft. However, all the mice in the control group had gross more pulmonary metastatic lesions compared with the SPON1 knockdown group (Fig. 2F). Given SPON1 is a secreted protein, we further confirmed the effects of SPON1 on osteosarcoma cell migration and invasion by using recombinant human SPON1 protein (Fig. 3A). The results showed that recombinant SPON1 protein pronounced promoted HOS (Fig. 3B) and U2OS (Fig. 3C) cell migration and invasion in a dose-dependent manner. Collectively, these data above suggest that SPON1 promotes tumor metastasis both *in vitro* and *in vivo*.

3.3. SPON1 activates Fak and SRC signaling in osteosarcoma cells

Integrin-mediated cell-matrix adhesion exhibits an important role in the regulation of cell migration and invasion [14,15]. Previous reports have demonstrated that SPON1 inhibits angiogenesis through binding to integrin αvβ3 [16]. To determine whether integrins and their associated signaling are involved in the SPON1-mediated metastatic progression of osteosarcoma, two important integrin-associated signaling molecules, FAK and SRC, were examined. As shown in Fig. 4A and B, knockdown of SPON1resulted in decreased phosphorylation levels of Fak and Src in HKOS cells, while SPON1 protein treatment remarkably promoted the phosphorylation levels of Fak and Src in HOS cells. To further confirm the role of Fak and Src signaling in SPON1-induced metastatic progression, cell migration and invasion assay were performed after Fak and Src were silenced by siRNAs (Fig. 4C and D). Indeed, the enhanced effects of SPON1 on osteosarcoma cell migration and invasion were completely blocked by the silencing of Fak or Src in both HKOS and KRIB cells (Fig. 4E and F). Collectively, these results indicate that Fak and Src signaling is a critical mediator of SPON1-derived metastatic progression.

4. Discussion

Osteosarcoma is a solid malignant bone tumor characterized by a high rate of metastasis [17]. It has been estimated that about 20% of osteosarcoma patients are initially diagnosed with metastases and 25–50% of patients without metastases at initial diagnosis subsequently develop distant metastases [18]. Moreover, nearly 90% of osteosarcoma patients experience metastasis or relapse, even after completely surgical resection of the primary tumor [19]. Therefore, an extensive knowledge of the crucial factors involved in osteosarcoma metastasis, such as extracellular matrix protein, is required.

In current study, we identified SPON1 as a candidate gene contributing to metastatic progression of osteosarcoma from a GEO dataset. By Western blotting, we confirmed the differentially expressed pattern of SPON1 between metastatic osteosarcoma cells and non-metastatic osteosarcoma cells. By immunohistochemistry, we found up-regulated SPON1 levels are closely correlated with MMP9 expression, which involved in the dissemination of tumor cells from the primary tumor; and no correlation was observed between SPON1 and ki67, an indicator for tumor growth. Given HKOS and KRIB cell lines derived from metastatic osteosarcoma and with increased SPON1 expression, we performed knockdown assay to evaluate the implications of SPON1 on the ability of cell invasiveness. Consistent with the notion from immunohistochemical analysis, we found that metastatic behavior of in HKOS and KRIB cells was remarkably inhibited by silencing SPON1 both *in vitro* and *in vivo*. Similarly, treatment with SPON1 protein in the non-metastatic osteosarcoma cells, HOS and U2OS, cell invasiveness was significantly promoted. Based on these results, we speculated that SPON1 might function as a modulator in the metastatic potential of osteosarcoma cells.

A variety of recent studies have highlighted the role of extracellular matrix in key components of osteosarcoma progression, especially tumor metastasis. SPON1 is the prototype of the mindin-

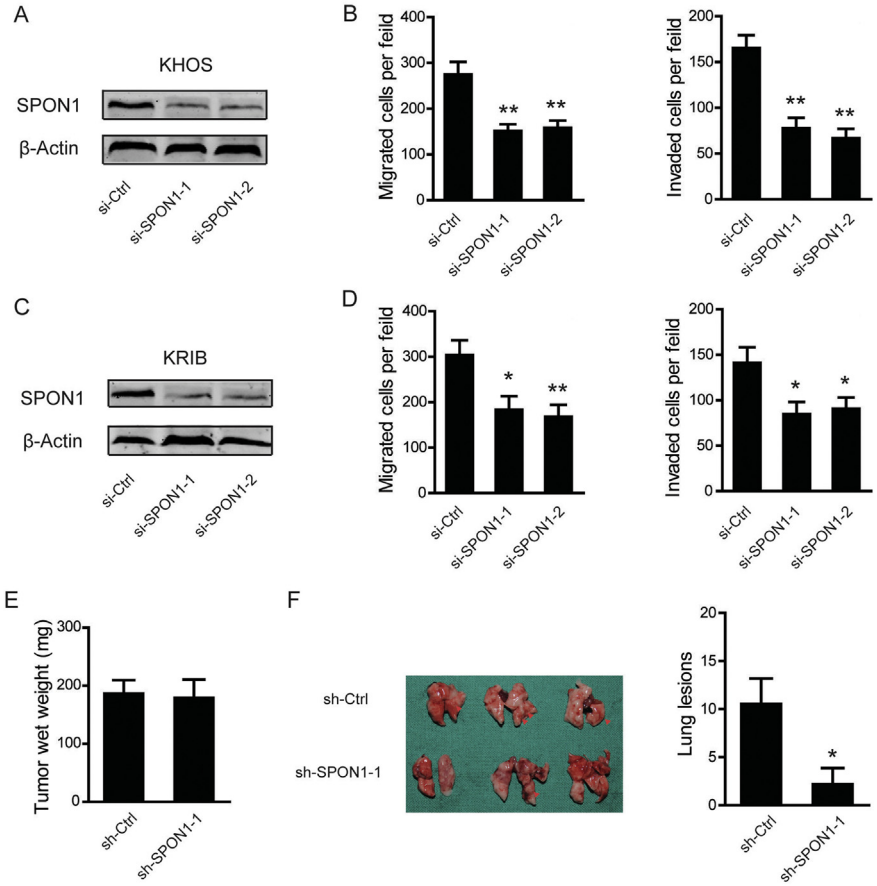


Fig. 2. Sliencing of SPON1 inhibits migration and invasion of osteosarcoma cells. (A) Interfere efficiency of SPON1 in KHOS cells as demonstrated by Western blotting. (B) Knockdown of SPON1 resulted in reduced cell migration and invasion ability in KHOS cells (si-Ctrl versus si-1 or si-2; **, $P < 0.01$). (C) Interfere efficiency of SPON1 in KRIB cells as demonstrated by Western blotting. (D) Knockdown of SPON1in KRIB cells resulted in decreased migrated and invaded cells (si-Ctrl versus si-1 or si-2; *, $P < 0.05$; **, $P < 0.01$). (E) Tumors were harvested at weeks 6 and tumor weights were measured. (F) Total lung lesions of mice from SPON1 or control group. The red arrows indicate macroscopic pulmonary metastatic lesions (sh-Ctrl versus sh-SPON1-1; *, $P < 0.05$). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

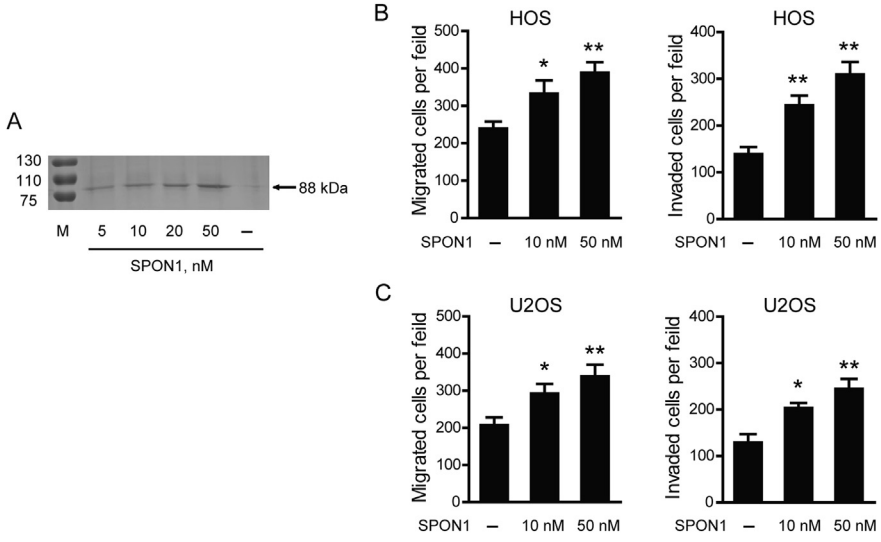


Fig. 3. Treatment of SPON1 protein promotes migration and invasion of osteosarcoma cells. (A) Different concentrations of recombinant human SPON1 protein were detected by Western blotting. Treatment of SPON1 protein resulted in increased cell migration and invasion ability in HOS (B) and U2OS cells (C). control versus 10 nM SPON1 or 50 nM SPON1; *, $P < 0.05$; **, $P < 0.01$.

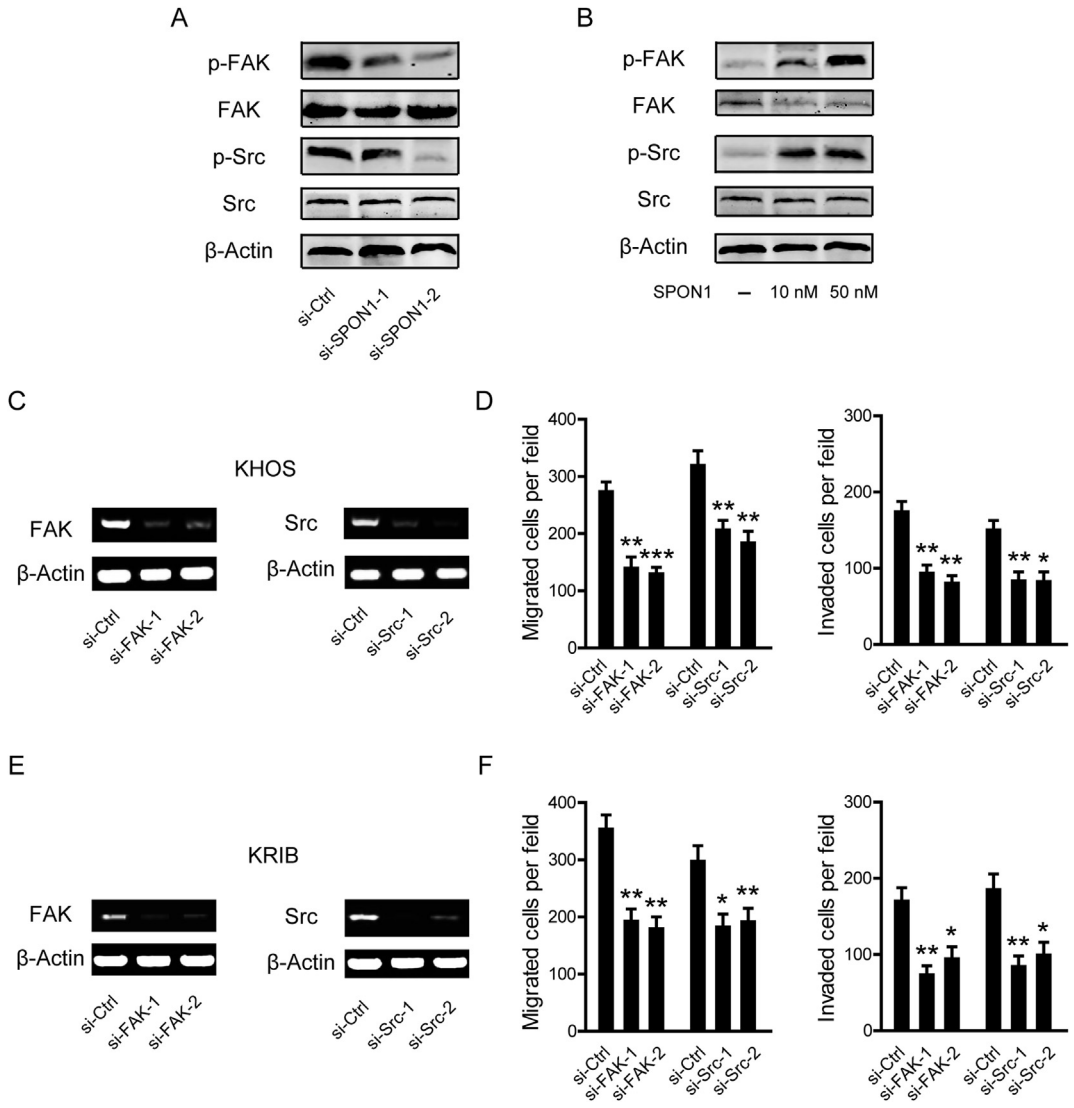


Fig. 4. SPON1 activates Fak and SRC signaling in osteosarcoma cells. Western blotting analysis of Fak and Src phosphorylation in SPON1-knockdown (A) and SPON1 protein treated cells (B). Interfere efficiency of Fak and Src in KHOS cells (C) and KRIB cells (D) as demonstrated by RT-PCR. Knockdown of Fak or Src in KHOS cells (E) and KRIB cells (F) resulted in decreased migrated and invaded cells (si-Ctrl versus si-1 or si-2; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

F-spondin family, which includes Mindin1, Mindin2 and M-spondin (SPON2) and belongs to extracellular matrix protein [5,7,20]. It encodes a secreted protein with six thrombospondin type 1 repeats (TSR) in the C-terminal half and many studies have confirmed its roles in neural cell adhesion and neurite outgrowth [20]. Several studies have demonstrated that SPON2 is associated with tumor progression in many types of cancers [21–24]. Note that the structurally resemblance between SPON1 and SPON2, it's convincible to meet the role of SPON1 in osteosarcoma. Different from previous reports in SPON2 [25], we found that the phosphorylation levels of Fak and Src was remarkably enhanced by SPON1, thus promoting cell invasiveness. However, whether this effect induced by SPON1 was duo to activation of αvβ3 intergrin pathway remains to be further demonstrated.

To best of our knowledge, this is the first study to systemically determine the expression pattern and cellular functions of SPON1 in tumors. Our data provides evidence of the effects of the SPON1/Fak/Src pathway in metastatic progression of osteosarcoma and indicates the critical roles of Spondin family in tumor development and progression.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgment

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.092.

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