Original Article

PEBP4 silencing inhibits TGF-β1-induced epithelial-mesenchymal transition of osteosarcoma cells by suppressing the PI3K/Akt pathway

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Abstract: Phosphatidylethanolamine-binding protein 4 (PEBP4) is a member of the PEBP family and has been reported to be upregulated in various cancer types. However, the roles of PEBP4 in osteosarcoma (OS) are unknown. The aim of this study was to investigate the effects of PEBP4 during the transforming growth factor β1 (TGF-β1)-induced epithelial-to-mesenchymal transition (EMT) in OS cells. In this study, we found that PEBP4 was highly expressed in human OS tissues and cell lines, and TGF-β1 treatment significantly increased the expression of PEBP4 in OS cells. In addition, knockdown of PEBP4 significantly inhibited the EMT process and cell migration/invasion induced by TGF-β1 in OS cells. Mechanistically, knockdown of PEBP4 down-regulated the levels of PI3K and Akt phosphorylation induced by TGF-β1. Taken together, the present study showed that knockdown of PEBP4 significantly inhibits TGF-β1-induced EMT by blocking the function of the PI3K/Akt pathway under treatment with TGF-β1 of OS cells, and that PEBP4 may be a potential therapeutic target for the treatment of OS.

Keywords: Phosphatidylethanolamine-binding protein 4 (PEBP4), osteosarcoma (OS), epithelial-mesenchymal transition (EMT)

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor and occurs frequently in children and adolescents [1]. During the last decade, the development of multiple therapeutic strategies for OS including wide tumor excision, multi-agent chemotherapy and radiotherapy has significantly improved the prognosis of patients with this malignancy [2, 3], however, patients with metastatic disease and local relapse still have poor outcomes with survival rates of approximately 20% [4].

The epithelial-mesenchymal transition (EMT) is a crucial step in the initiation of the metastatic spread of many tumor cells into distal organs [5]. During EMT procedure, the actin cytoskeleton is reorganized and cells acquire increased cell-matrix contacts, leading to dissociation from surrounding cells and enhanced migration and invasion [6]. Recent studies have indicated that transforming growth factor β1 (TGF-β1) acts to induce EMT in various tumors, including OS [7-9]. Thus, inhibiting TGF-β1-induced EMT may be a therapeutic approach for the treatment of OS.

Phosphatidylethanolamine-binding protein 4 (PEBP4) is a member of the PEBP family and has been reported to be expressed in the mammalian testis, heart, skeletal muscle, thyroid, liver, spinal cord, brain, adrenal gland and bone marrow [10]. Previous studies showed that PEBP4 plays important roles in spermatogenesis, myoblast differentiation, membrane biosynthesis, neurodevelopment and apoptosis [1, 11]. Recently, emerging studies have indicated that PEBP4 is highly expressed in various malignancies such as breast cancer, lung cancer, endometrial carcinoma, colorectal cancer, and pancreatic ductal adenocarcinoma (PDAC) [12-16]. For example, Zhang et al. reported that the expression level of PEBP4 was elevated in PDAC samples, and forced expression of PEBP4 in PDAC cell lines promoted cell growth and migra-
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tion, while downregulation of PEBP4 in PDAC cells inhibited the growth, migration, and metastasis of the cancer cells [16]. However, its roles of PEBP4 in OS are unknown. The aim of this study was to investigate the effects of PEBP4 during the TGF-β1-induced EMT in OS cells, and the underlying mechanism was also explored.

Materials and methods

Tissue specimens

A total of 12 pairs of OS tissues and their matched adjacent normal bone were obtained from patients who underwent surgery at First Affiliated Hospital of Shantou University Medical College (China). All the tissues were immediately stored in liquid nitrogen until use. This study was approved by the Ethics Committee of First Affiliated Hospital of Shantou University Medical College, and all patients provided informed consent.

Cell culture and treatment

The human OS cell lines (U2OS and SaOS2) were obtained from American Type Culture Collection and maintained in DMEM medium, which was supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C with 5% CO2. Then, A549 cells were treated with TGF-β1 (5 ng/mL) for different times.

Small interfering RNA and transfection

Small interfering RNA (siRNA) targeting PEBP4 or its corresponding negative control was designed and synthesized by Guangzhou RiboBio (Guangzhou, China). For transfection, SaOS2 cells were seeded in each cell of a 24-well micro-plate, grown for 24 h to reach 30%-50% confluence, and then transfected with 2 μl (20 μM) siRNA using a Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The transfection efficiency was examined by RT-PCR and Western blot.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from OS tissues and cells using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol, and first-strand cDNA was synthesized from 5 μg total RNA using random primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). qPCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) and SLAN Real-Time PCR System (Hongshi Medical Technology, Shanghai, China). The specific primers for PEBP4 were sense, 5’-ACTGGGTCTCATGATGG-TGG-3’; and antisense, 5’-CTCCATCCAGGAG-GTATCT-3’; and for β-actin were sense, 5’-GATCATTGCTCCTCCTGAGC-3’ and antisense, 5’-ACTCTCTGTCTGGATCAAC-3’. These primers were all synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR procedure was as followed: polymerase activation for 30 s at 95°C, 35 cycles of amplification each consisting of 95°C for 5 s, 60°C for 20 s, and 1 cycle of dissociation consisting of 94°C for 15 s, 59°C for 30 s, and 95°C for 15 s. Relative quantification of gene expression was performed using the 2ΔΔCt method and with β-actin mRNA as an internal control.

Western blot

Total protein was extracted from OS tissues and cells and lysed in cell lysis buffer (Beyotime, Haimen, China). Protein concentrations were measured by BCA Protein Assay kit. Equal amount of the proteins from each extract were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred electrophoretically using a PVDF membrane. The membrane was then incubated with 5% non-fat dry milk in Tris-buffered saline (TBS) to block non-specific binding at room temperature for 1 h. Subsequently, the membrane was incubated with the following primary antibodies: PEBP4, E-cadherin, N-cadherin, vimentin, PI3K, p-PI3K, Akt, p-Akt and GAPDH (Cell Signaling Technology, Inc., Boston, MA, USA) overnight at 4°C. The membranes were rinsed 3 times with PBST and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Boster Corporation, Wuhan, China), the target protein was visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA). The protein gray intensity was detected by using BandScan 0.5 software (ProZyme, San Leandro, CA, USA).

In vitro migration and invasion assays

Migration and invasion assays were performed using transwell chambers. For migration assay,
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$1 \times 10^4$ infected with OS cells in 200 μl of serum-free DMEM were added to the upper chamber of transwells (BD Bioscience, USA). The lower chamber of the transwell plates were filled with 500 μl of DMEM medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). After a 24 h incubation at 37°C, cells on the upper surface of the filters were removed using cotton swabs. Then, the cells on the lower surface of the filter were fixed, stained and examined under a microscope. The average number of migrated cells from five random optical fields and triplicate filters was determined. The invasion assay was done by the same procedure, except that the membrane was coated with Matrigel to form a matrix barrier. The experiments were performed in triplicate.

**Statistical analysis**

Data were processed as mean ± SD. The differences were analyzed by the Student’s $t$ test or one-way analysis of variance and Student’s $t$ test. A $P$ value of $<0.05$ was considered to be statistically significant.

**Results**

PEBP4 is highly expressed in OS tissues and cell lines

To verify the expression of PEBP4 in OS, we determined the mRNA and protein expression levels of PEBP4 in OS tissues and cell lines. As shown in **Figure 1A**, the expression of PEBP4
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mRNA in OS tissues was obviously up-regulated compared to that of controls. Western blot analysis showed that the expression of PEBP4 protein was also significantly increased in OS tissues (Figure 1B). Similarly, we observed that the mRNA and protein expressions of PEBP4 were higher in OS cell lines than in the normal bone cells (Figure 1C and 1D).

**TGF-β1 induces upregulation of PEBP4 expression in SaOS2 cells**

It has been reported that TGF-β1 promotes tumor progression. PEBP4 is highly expressed in OS tissues and cell lines. Therefore, we examined the response of PEBP4 to TGF-β1 in SaOS2 cells. As shown in Figure 2A, after 6 h, TGF-β1 (5 ng/ml) greatly increased the mRNA level of PEBP4, reaching a peak around 48 h. Moreover, consistent with the mRNA level, the level of PEBP4 protein was also increased by TGF-β1, in a time-dependent manner (Figure 2B).

**Knockdown of PEBP4 reverses TGF-β1-mediated EMT in SaOS2 cells**

Because EMT is one of the critical steps of tumor cell invasion, we then investigated whether PEBP4 participates in the EMT process in TGF-β1-stimulated SaOS2 cells. To downregulate PEBP4 expression in SaOS2 cells, a siRNA-PEBP4 was transfected into SaOS2 cells. As shown in Figure 3A, the expression of PEBP4 was obviously decreased in siRNA-PEBP4-transfected SaOS2 cells. Next, we analyzed the mRNA and protein expression levels of an epithelial marker (E-cadherin) and two mesenchymal markers (N-cadherin and vimentin). Both qRT-PCR (Figure 3B) and western blot analysis (Figure 3C) demonstrated that TGF-β1 stimulation greatly suppressed the level of E-cadherin and increased the levels of N-cadherin and vimentin. Whereas, knockdown of PEBP4 significantly increased expression of E-cadherin, but decreased the expression of N-cadherin and vimentin in TGF-β1-stimulated SaOS2 cells.

**Knockdown of PEBP4 reverses TGF-β1-induced migration and invasion in SaOS2 cells**

Invasion and metastasis are the most important hallmarks of malignant cancer cells. To explore the effect of PEBP4 on the migration ability of SaOS2 cells under TGF-β1 conditions, a transwell assay was performed. As shown in Figure 4A, a significantly increased migration was observed in SaOS2 cells treated with TGF-β1, as compared with untreated control cells. Whereas, knockdown of PEBP4 greatly suppressed TGF-β1-induced migration in SaOS2 cells. Additionally, a Matrigel invasion assay was conducted to investigate the effect of PEBP4 on the invasion ability of SaOS2 cells. Consistently, knockdown of PEBP4 significantly decreased TGF-β1-induced invasion in SaOS2 cells (Figure 4B).

**Knockdown of PEBP4 inhibits phosphorylation of PI3K and Akt in TGF-β1-stimulated SaOS2 cells**

The PI3K/AKT pathway is known to play an important role in human cancer initiation and progression, and is also associated with the induction of EMT [17]. Thus, we further examined whether and how PEBP4 regulates the PI3K/Akt pathway to suppress TGF-β1-mediated EMT in SaOS2 cells. As shown in Figure 5, TGF-β1 significantly promoted the phosphorylation of PI3K and Akt, however, knockdown of PEBP4 inhibited TGF-β1-induced the phosphorylation of PI3K and Akt in SaOS2 cells.
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Figure 3. Knockdown of PEBP4 reverses TGF-β1-mediated EMT in SaOS2 cells. A. siRNA targeting PEBP4 and its corresponding negative control were transfected into SaOS2 cells. B. Cells transfected with siRNA-PEBP4 or scramble were treated with TGF-β1 (5 ng/ml) for 48 h and then mRNA expression levels of EMT markers were detected by RT-qPCR. C. Western blotting analysis of same samples used in B was shown. GAPDH served as a loading control. All quantitative data are the mean ± SD of three independent experiments. *P<0.05, compared with control group; #P<0.05, compared with TGF-β1 group.

Figure 4. Knockdown of PEBP4 reverses TGF-β1-induced migration and invasion in SaOS2 cells. Cells transfected with siRNA-PEBP4 or scramble were treated with TGF-β1 (5 ng/ml) for 48 h. A. The migration of SaOS2 cells was evaluated by a Transwell assay, and cell numbers for each count were plotted in the graph. B. The invasiveness of SaOS2 cells was evaluated by a Matrigel-coated Transwell assay, and cell numbers for each count were plotted in the graph. All quantitative data are the mean ± SD of three independent experiments. *P<0.05, compared with control group; #P<0.05, compared with TGF-β1 group.
Discussion

In this study, the expression of PEBP4 was found to be upregulated in OS samples and cell lines, and TGF-β1 significantly increased the expression of PEBP4 in SaOS2 cells. In addition, knockdown of PEBP4 inhibited TGF-β1-induced EMT process, as well as migration and invasion in SaOS2 cells. Moreover, knockdown of PEBP4 inhibits phosphorylation of PI3K and Akt in TGF-β1-stimulated SaOS2 cells.

Previous studies have documented that PEBP4 plays an important role in tumorigenesis. Liu et al. [15] reported that the expression of PEBP4 mRNA and protein in colorectal cancer tissue was significantly higher than that in the normal pericarcinoma tissue. Yu et al. [18] found that PEBP4 mRNA and protein expression in lung squamous cell carcinoma tissues of patients with lymph node metastasis were significantly higher than those in patients without lymph node metastasis. However, the role of PEBP4 in human OS is still unclear. Here, our results are consistent with previous reports that found PEBP4 expression to be highly expressed in OS samples and cell lines, and TGF-β1 significantly increased the expression of PEBP4 in SaOS2 cells. These findings suggested the oncogenic roles of PEBP4 in the progression of OS.

EMT is known to be a central mechanism responsible for invasiveness and metastasis of various cancers [19]. TGF-β1 has emerged as a potent secreted factor that drives cancer progression, not only through its immunosuppressive and proangiogenic roles, but also as a potent inducer of epithelial plasticity leading to EMT [20, 21]. Previous studies demonstrated that TGF-β1 significantly decreases E-cadherin expression and concomitantly increases N-cadherin and vimentin expression [22]. Our present study demonstrated that knockdown of PEBP4 inhibited TGF-β1-induced EMT process, as well as migration and invasion in SaOS2 cells. Previous studies indicated that PEBP4
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plays an important role in tumor cell migration and invasion [17, 18], and this is also confirmed in our study. Our findings suggest that knockdown of PEBP4 inhibits TGF-β1-induced EMT and suppresses OS cell invasion and metastasis.

A growing body of evidence demonstrates that the PI3K/Akt signaling pathway plays important roles in the progression of various human cancer types through modulation of many biological processes, including cell growth, EMT, invasion and metastasis [23-25]. PI3K is activated by oncogenes, and activated PI3K promotes cancer cell growth and invasion. Akt is a vital node of signaling elicited by PI3K and growth factor receptors. Phosphorylation of serine 473 and threonine 308 residues leads to the full activation of Akt kinase activity. After activation, Akt enhances the EMT, down-regulates E-cadherin transcription, and increases cell motility and invasion [26]. One study reported that TGF-β1 treatment induced the activation of PI3K-Akt-iKBα-NF-κB-Snail pathway which leads to the decline of E-cadherin in lung cancer cells [27]. Most interestingly, in a recent study, PEBP4 was found to interact with Akt and promoted the phosphorylation of Akt in PDAC cells [16]. Consistent with the previous studies, in this study, we showed that TGF-β1 significantly activated the PI3K/Akt axis, whereas, knockdown of PEBP4 greatly inhibited phosphorylation of PI3K and Akt in TGF-β1-stimulated SaOS2 cells. These results suggest that PEBP4 silencing inhibits TGF-β1-induced EMT of OS cells by suppressing the PI3K/Akt signaling pathway.

In summary, our study is the first to show that PEBP4 silencing inhibits TGF-β1-induced EMT of OS cells by suppressing the PI3K/Akt signaling pathway. Thus, PEBP4 might be a potential therapeutic target for the treatment of OS.

Disclosure of conflict of interest

None.

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