Int J Clin Exp Med 2016;9(7):12687-12695
www.ijcem.com /ISSN:1940-5901/IJCEM0021351

Original Article

PEBP4 is upregulated in gastric cancer and promotes the growth and migration of cancer cells

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Received December 7, 2015; Accepted March 19, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: Phosphatidylethanolamine-binding protein 4 (PEBP4) is a member of the PEBP family and has been reported to be upregulated in various cancers. However, its expression pattern and biological functions in gastric cancer are unknown. In this study, we investigated the expression and biological roles of PEBP4 in gastric cancer cells. Our results showed that the expression of PEBP4 was significantly up-regulated in gastric cancer tissues and cell lines. In addition, knockdown of PEBP4 inhibits cell proliferation, migration and invasiveness, as well as the epithelial-mesenchymal transition (EMT) phenotype. Furthermore, knockdown of PEBP4 greatly inhibited the levels of p-PI3K and p-Akt in gastric cancer cells. These results suggest that knockdown of PEBP4 inhibits gastric cancer development via inactivation of the PI3K/Akt signaling pathway, and PEBP4 may serve as a potential molecular target for the treatment of gastric cancer.

Keywords: Phosphatidylethanolamine-binding protein 4 (PEBP4), gastric cancer, proliferation, invasion, PI3K/Akt pathway

Introduction

Gastric cancer is the fourth most commonly diagnosed malignancy and the second most common cause of cancer-related mortality worldwide [1]. Despite recent advances in early detection and targeted therapy, the median survival time for advanced gastric cancer patients still appears to remain at 7 months [2, 3]. Thus, understanding of the molecular mechanisms of gastric cancer invasion and metastasisis crucial for developing novel and more effective therapeutic approaches.

Phosphatidylethanolamine-binding protein 4 (PEBP4) is a member of the PEBP family. PEBP family members have a similar domain structure that consists of a large β-sheet flanked by smaller β-sheets and 2 α-helices at the C terminus in different species [4]. The human PEBP4 gene is located on 8p21.3, and the encoded product is about 23 KD. It was reported that PEBP4 plays important roles in multiple biological processes, including the synthesis of cell membrane, myoblast differentiation, and the neural development [5-7]. Recently, PEBP4 has been reported to be upregulated in various cancer types, and upregulation of PEBP4 promoted the proliferation, invasion and drug resistance, as well as inhibited the apoptosis of cancer cells [8-10]. However, its expression pattern and biological functions in gastric cancer are unknown. Therefore, in this study, we investigated the expression and biological roles of PEBP4 in gastric cancer. To elucidate the biological function of PEBP4 in gastric cancer, we silenced the expression of PEBP4 by specific siRNA in gastric cancer cell lines and tested the effects of PEBP4 on cell proliferation, migration andinvasion. Our results show that PEBP4 inhibitsgastric cancer tumorigenesis and metastasis through dysregulation of PI3K/Akt signaling pathway.

Materials and methods

Sample collection

Gastric cancer tissues were obtained from 11 patients undergoing surgical treatment at the

Department of Oncology, the Second Hospital of Jilin University (China), during the period from 2012 to 2014. All of the patients received primary treatment by surgery followed by adjuvant radiotherapy, chemotherapy, or hormone therapy. Healthy tissue samples were from non-pathologic areas distant from tumors in surgical specimens. The fresh tissue specimens were immediately snap-frozen and stored in liquid nitrogen until use. A protocol for the use of patient samples was approved by the Biomedical Ethics Committee of the Second Hospital of Jilin University and written informed consent was obtained from each patient.

Cell culture

Three human gastric cancer cell lines (TSGH, N87 and AGS) and a normal gastric mucosal epithelial cell line (GES-1) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and sodium pyruvate (1 mM) in a humidified incubator with 5% CO_2 at 37°C.

siRNA knockdown of PEBP4

Small interfering RNA (siRNA) targeting PEBP4 or its corresponding negative control was designed and synthesized by Guangzhou RiboBio (Guangzhou, China). For transfection, gastric cancer 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. Following transfection, cells were incubated at 37°C in a CO_2 incubator for 48 h before being harvested for the assays described above.

Cell proliferation assay

Cell proliferation was confirmed by quantifying cell numbers using a cell counter (Kaihong, Beijing, China), and BrdU incorporation into DNA was quantified using the Cell Proliferation ELISA BrdU kit (Takara Biotechnology, Dalian, China). Cells were transfected with siRNA against PEBP4 and scramble siRNA. After 24 h of serum starvation, the cells were incubated in the presence of BrdU at 37°C in a humidified incubator containing 5% CO_2 for 24 or 48 h. The amount of BrdU incorporation into the cells was measured at 450 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell migration and invasion assays

The invasive and migration behaviors of indicated cells were analyzed by Transwell chamber (Corning Costar Corp., Cambridge, MA, USA) assay with or without coated Matrigel (BD Biosciences, Bedford, MA, USA). TSGH and AGS cells transfected with siRNA-PEBP4 or siRNA-scramble (1×10^5 cells/ml) suspended in RPMI medium were added to the upper chamber. The lower chamber of the Transwell was filled with 500 μl DMEM containing 10% FBS as a chemoattractant. After 24 h incubation, cells on the surface of upper chamber were removed by scraping with a cotton swab. The invaded/migrated cells on the lower surface of the filter were washed, fixed, stained with Giemsa, and counted under a microscope. Experiments were repeated at least three times.

RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from gastric cancer tissues and cells using Trizol reagent (Abcam, Cambridge, UK) according to the manufacturer's instructions. cDNA was synthesized from the extracted RNA (5 μg) using the EasyScript First-Strand cDNA Synthesis SuperMix kit (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed using the following primers: PEBP4, 5'-ACTGGGTCT CATGATGGTGG-3' (sense), and 5'-CTCCATCCAGGAGGTGAT CT-3' (antisense); and β-actin, 5'-TTAGTTGCGTTACACCCTTTC-3' (sense) and 5'-ACCTTCACCGTTCCAGTTT-3' (antisense). The PCR conditions included an initial denaturation step of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and a final elongation step of 72°C for 10 min. Data were analyzed using the formula: $R = 2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$.

Western blot

Total protein was extracted from gastric cancer tissues and cells, then washed with ice-cold PBS and lysed with RIPA Cell Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein concentrations were determined by the

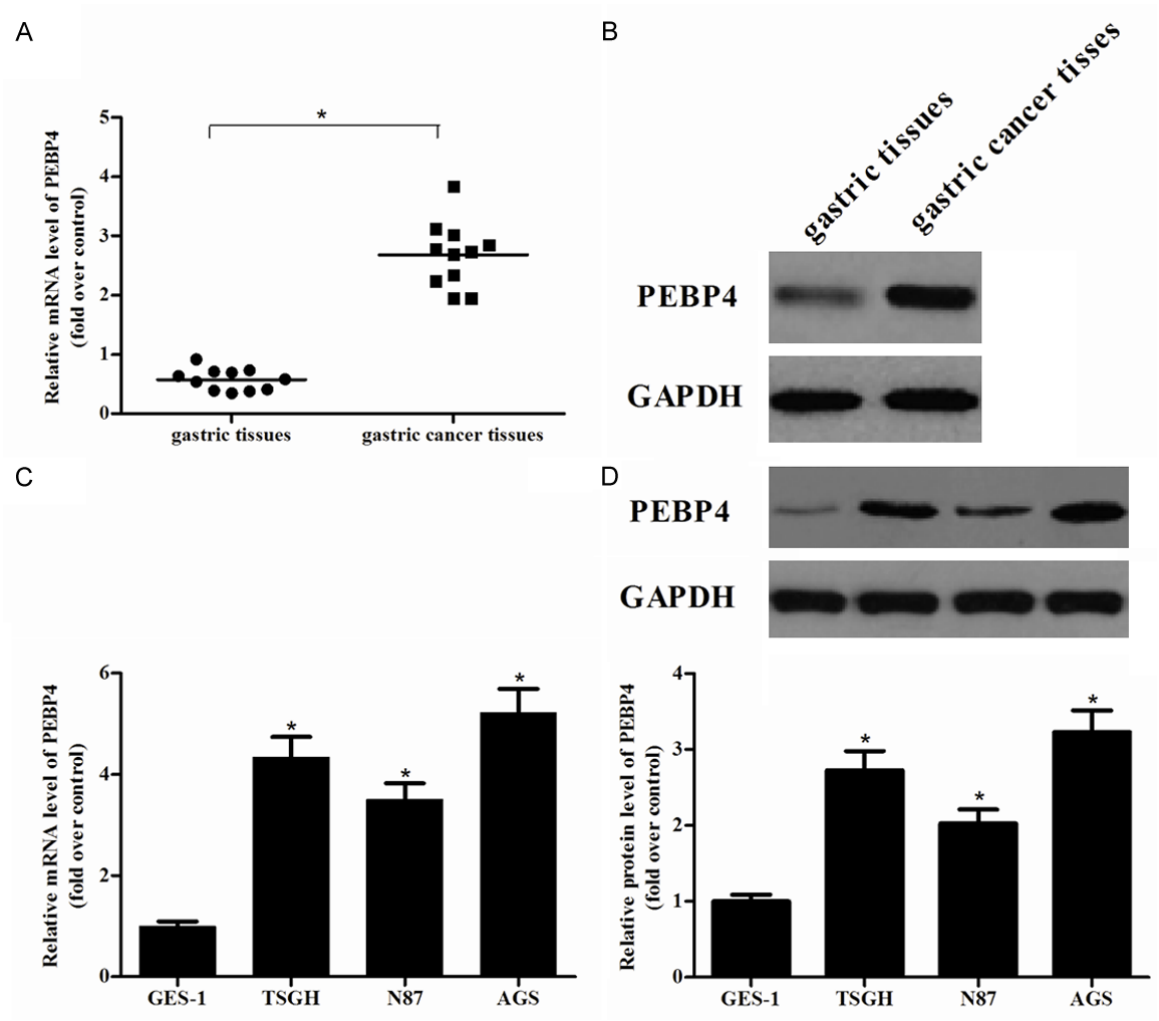

Figure 1. Expression of PEBP4 was up-regulated in gastric cancer tissues and cell lines. A. QRT-PCR analysis of PEBP4 mRNA in 11 human gastric cancer tissues and paired adjacent normal gastric tissues. PEBP4 mRNA levels in human gastric cancertissues were obviously higher than that in normal gastric tissues; B. Western blot analysis of PEBP4 protein in gastric cancertissues and normal gastric tissues; *P<0.05 compared with the normal gastric tissues. C. The representative mRNA expression of PEBP4 in human gastric cancer cell lines; D. The representative Western image of PEBP4 protein in human gastric cancer cell lines. Data is expressed as mean ± SD. Experiments were performed in triplicate. *P<0.05 compared with the GES-1 group.

BCA method. Fifty micrograms of protein per lane was separated on 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA). The membrane was treated by shaking and blocking at room temperature with 2% nonfat dry milk in Tris-buffered saline (TBS) for 1 h followed by incubation in primary antibodies (anti-PEBP4, anti-E-cadherin, anti-N-cadherin, anti-PI3K, anti-p-PI3K, anti-p-Akt, anti-Akt and anti-GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Following washing, the sites of antibody binding were visualized via chemiluminescence (Boehringer Mannheim GmbH, Mannheim, Germany) andBandScan 5.0 software was used for the quantification of all the proteins after western blot analysis.

Statistical analysis

All results are reported as means ± SD. Statistical analysis involved Student's t-test for the comparison of two groups or one-way ANOVA for multiple comparisons. P<0.05 was considered to be significant.

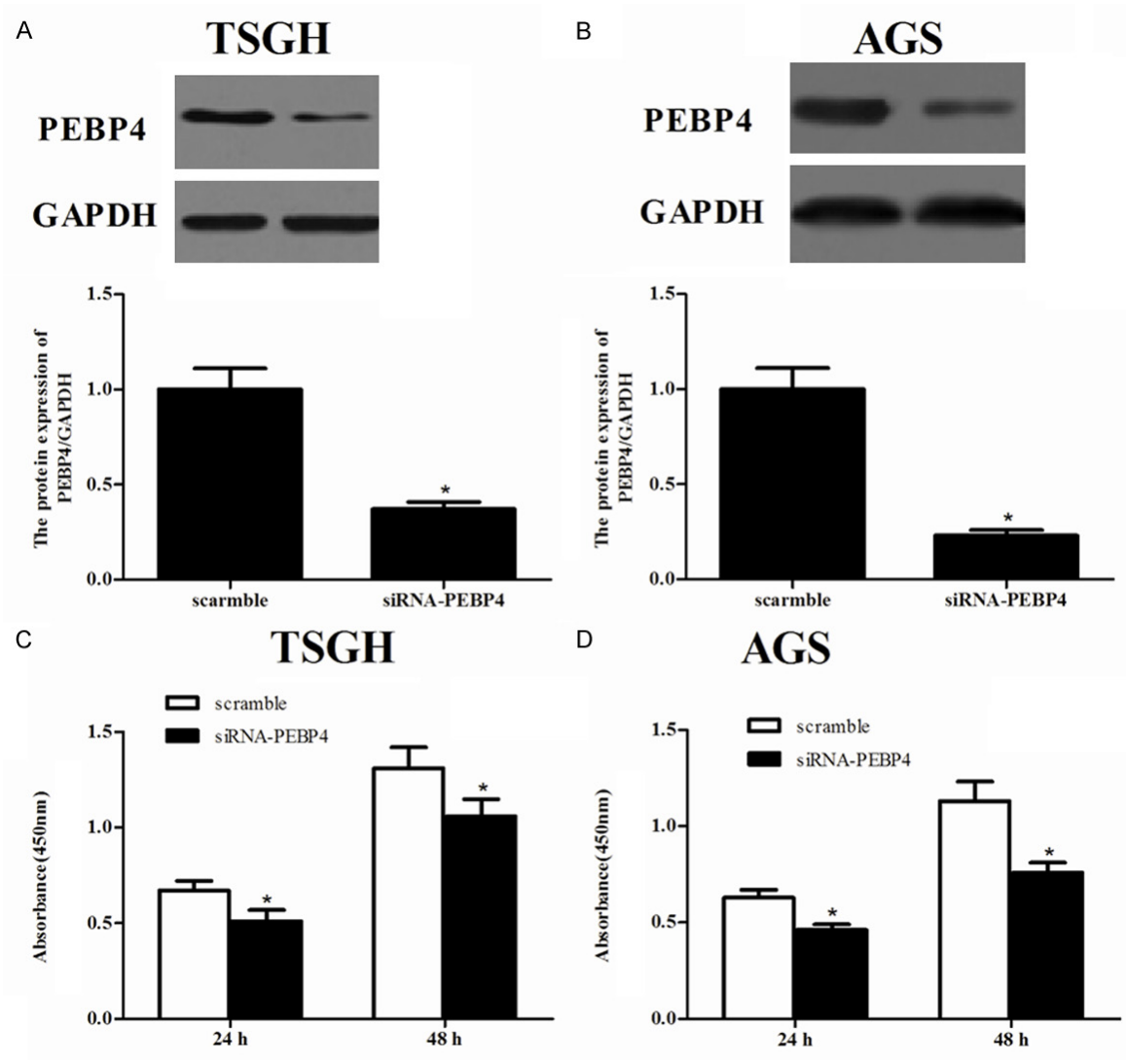

Figure 2. Knockdown of PEBP4 inhibited the proliferation of gastric cancer cells. TSGH and AGS cells were transfected with siRNA against PEBP4 and scramble siRNA for 24 h, respectively. A, B. Western blot of analysis of PEBP4 protein in TSGH and AGS cells, respectively. C, D. Proliferation of TSGH and AGS cells was detected by the BrdU assay. Data is expressed as mean ± SD. Experiments were performed in triplicate. *P<0.05 compared with the scramble group.

Results

PEBP4 is highly expressed in gastric cancer tissues and cell lines

To explore the potential role of PEBP4 in the tumorigenesis of gastric cancer, we detected the mRNA levels of PEBP4 in 11 paired human gastric cancer tissues and the corresponding adjacent normal tissues using RT-qPCR. As shown in **Figure 1A**, the mRNA levels of PEBP4 in human gastric cancer tissues were significantly higher than those in the adjacent normal gastric tissues. Western blot analysis showed that the expression of PEBP4 protein was obviously increased in human gastric cancer tissues, as compared with the normal gastric tissues (**Figure 1B**). Similarly, the expression of PEBP4 mRNA and protein was also increased in gastric cancer cell lines (**Figure 1C** and **1D**).

Knockdown of PEBP4 inhibited the proliferation of gastric cancer cells

To investigate the effect of PEBP4 on cell proliferation, TSGH and AGS cells were transfected with siRNA-PEBP4 or siRNA-scramble. Western blot results confirmed a remarkable downregu-

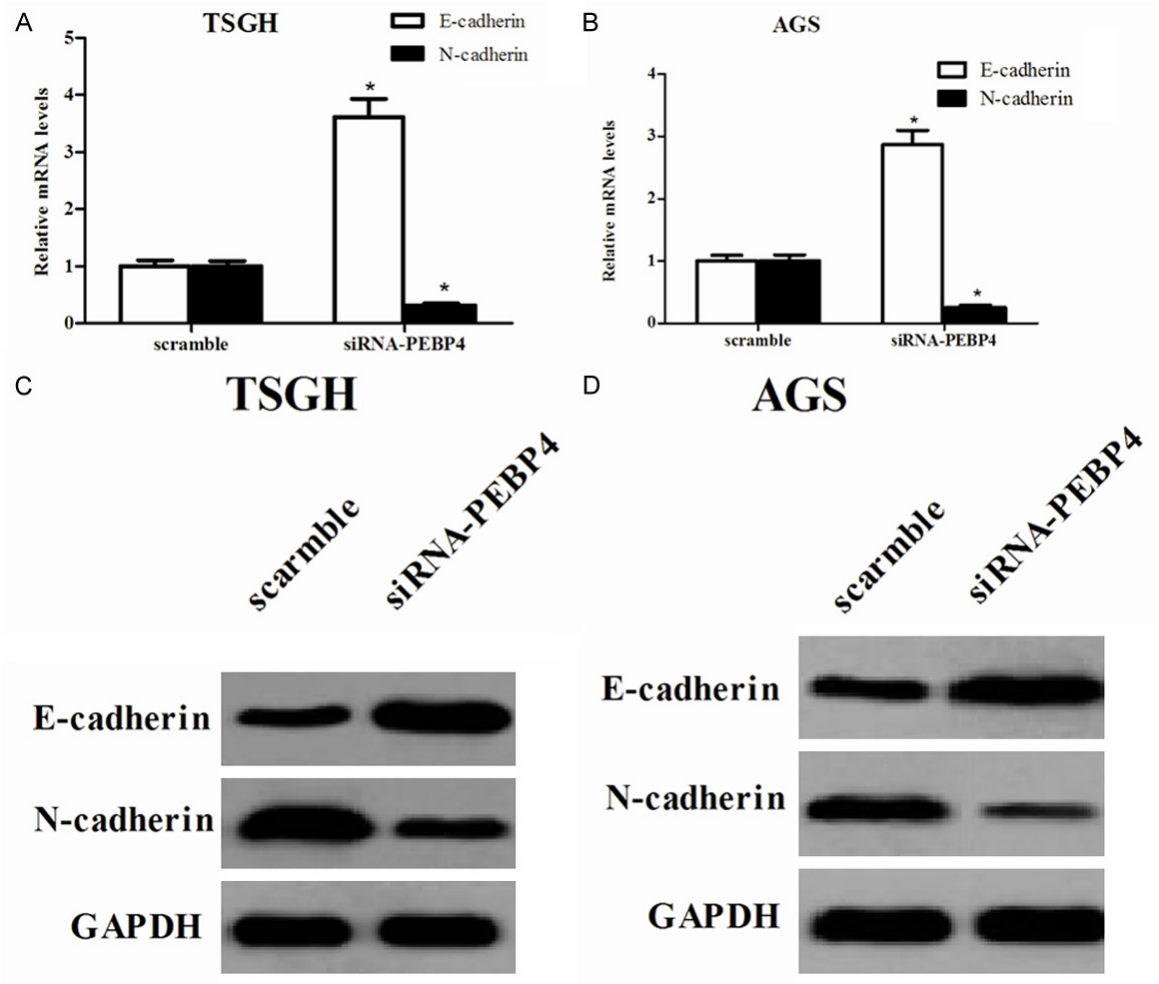

Figure 3. Knockdown of PEBP4 inhibited epithelial-mesenchymal transition (EMT) phenotype in gastric cancer cells. TSGH and AGS cells were transfected with siRNA against PEBP4 and scramble siRNA for 24 h, respectively. A, B. Total RNA was isolated from the indicated cells, the expression of E-cadherin and N-cadherin was analyzed using RT-qPCR. C, D. Total protein was isolated form the indicated cells, and EMT markers were measured using western blot analysis. Data is expressed as mean ± SD. Experiments were performed in triplicate. **P*<0.05 compared with the scramble group.

lation of PEBP4 expression in TSGH cells. The siRNA-PEBP4 obviously reduced PEBP4 protein levels to 36.7% of the control. Similarly, siRNA-PEBP4 also significantly decreased the expression of PEBP4 in AGS cells. Then, cell proliferation was evaluated using a BrdU incorporation assay. Knockdown of PEBP4 greatly suppressed the proliferation of both types of cells in a time-dependent manner (**Figure 2C** and **2D**).

Knockdown of PEBP4 inhibited epithelial-mesenchymal transition (EMT) phenotype in gastric cancer cells

We investigated the effect of PEBP4 on EMT phenotype in gastric cancer cells using RT-qPCR and Western blot. As shown in **Figure 3A**, RT-qPCR results indicated that knockdown of PEBP4 greatly increased the expression of E-cadherin mRNA, and decreased the expression of N-cadherin mRNA in TSGH cells. Similar results were observed in AGS cells (**Figure 3B**). Furthermore, western blot analysis showed that knockdown of PEBP4 obviously increased the expression of E-cadherin protein, and decreased the expression of N-cadherin protein in TSGH and AGS cells, respectively (**Figure 3C** and **3D**).

Knockdown of PEBP4 inhibited the migration and invasion of gastric cancer cells

We next determined the effect of PEBP4 on gastric cancer cell migration and invasion. As

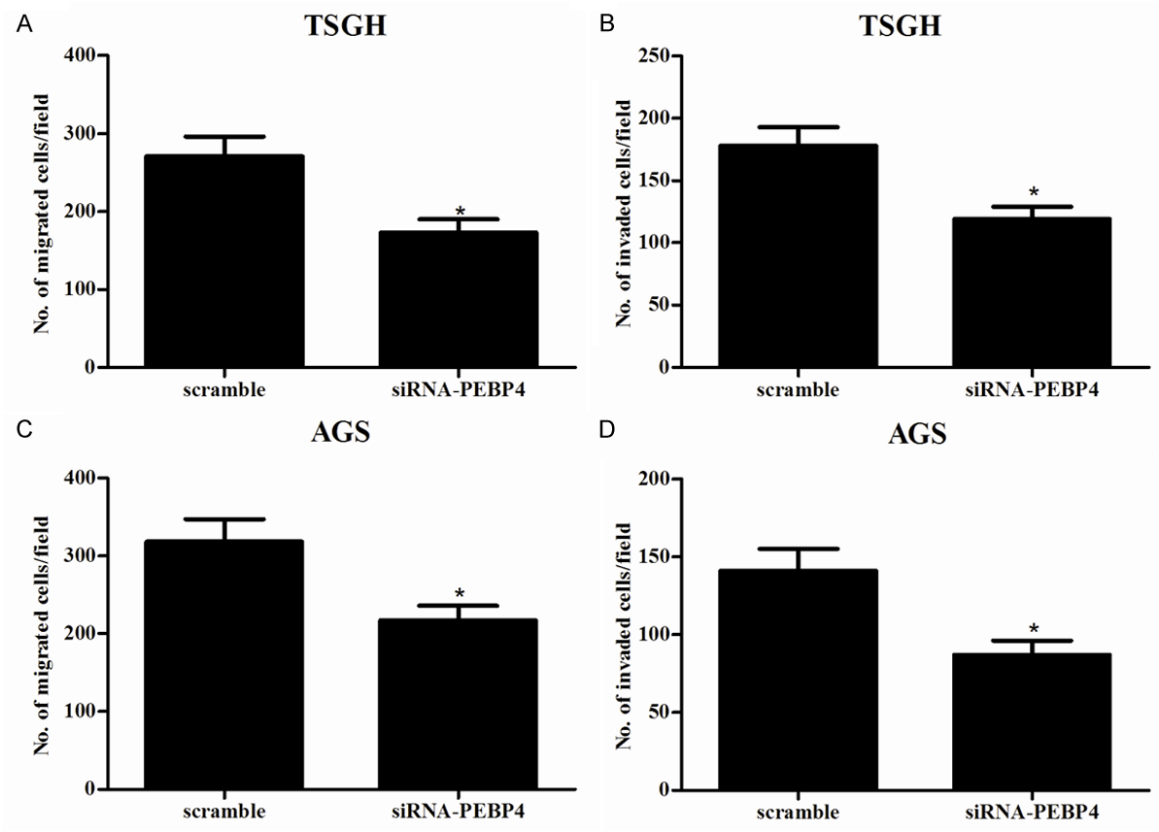

Figure 4. Knockdown of PEBP4 inhibited the migration and invasion of gastric cancer cells.TSGH and AGS cells were transfected with siRNA against PEBP4 and scramble siRNA for 24 h, respectively. A, C. The migration of the indicated cells was evaluated by a Transwell assay, cells that have passed through Matrigel for 24 h were counted in five representative microscopic fields. B, D. The invasiveness of the indicated cells was evaluated by a Matrigel-coated Transwell assay cells that have passed through Matrigel for 24 h were counted in five representative microscopic fields. Data is expressed as mean ± SD. Experiments were performed in triplicate. *P<0.05 compared with the scramble group.

shown in **Figure 4A**, for TSGH cells transfected with siRNA-PEBP4, the number of migrating cells significantly decreased by approximately 37%. In addition, siRNA-PEBP4 greatly suppressed the invasion of TSGH cells compared with the scramble group (**Figure 4B**). Similarly, knockdown of PEBP4 also inhibited the migration and invasion in AGS cells (**Figure 4C** and **4D**).

Knockdown of PEBP4 inhibited the activation of PI3K/Akt signaling pathway

Furthermore, we explored the cell signaling that may be involved in PEBP4-induced gastric cancer cell proliferation and invasion. In AGS cells transfected with siRNA-PEBP4, we observed that knockdown of PEBP4 greatly inhibited the expression of p-PI3K. Consistent with the changes in PI3K levels that we observed, the levels of p-Akt was obviously lower in siRNA-PEBP4-transfected cells than that in scramble cells, whereas the level of Akt remained unchanged (**Figure 5**).

Discussion

In this study, we provide the first evidence that upregulation of PEBP4 in gastric cancer tissues and cell lines. We show that knockdown of PEBP4 inhibits cell proliferation, migration and invasiveness, as well as the EMT phenotype. In addition, knockdown of PEBP4 greatly inhibited the expression levels of p-PI3K and p-Akt in gastric cancer cells.

Extensive evidences on PEBP4' tumorigenic functions have been documented [8-11]. Zhang *et al*. confirmed that the mRNA and protein level of PEBP4 was elevated in pancreatic duc-

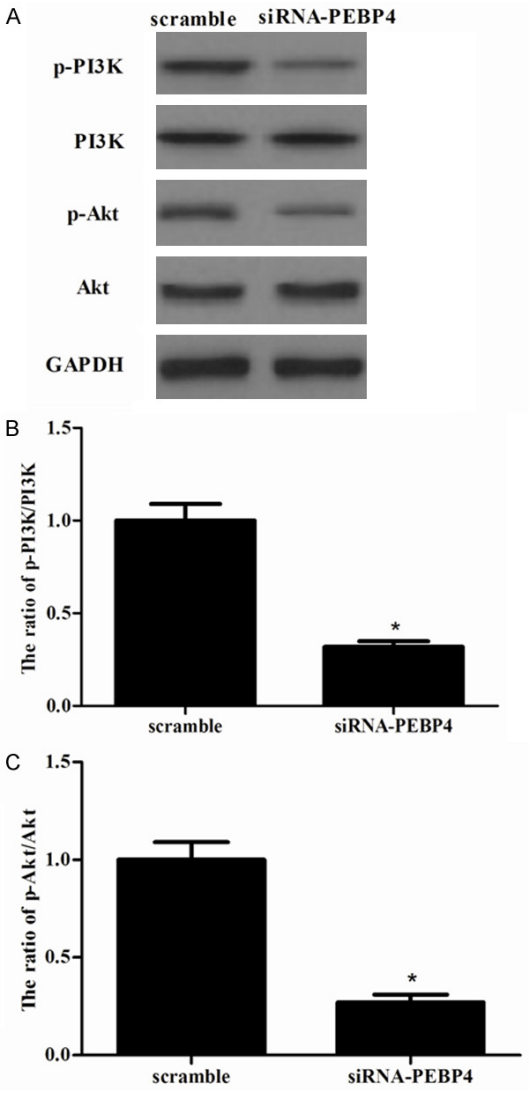

Figure 5. Knockdown of PEBP4 inhibited the activation of PI3K/Akt signaling pathway. AGS cells were transfected with siRNA against PEBP4 and scramble siRNA for 30 min, respectively. A. The levels of phosphorylated PI3K, total PI3K, phosphorylated Akt, and total Akt were analyzed using western blot analysis. B, C. The relative protein expression levels of p-PI3K, and p-Akt were quantified using BandScan 5.0 softwareand normalized to GAPDH. Data is expressed as mean ± SD. Experiments were performed in triplicate. *P<0.05 compared with the scramble group.

tal adenocarcinoma (PDAC) samples. Forced expression of PEBP4 in PDAC cell lines promoted cell growth and migration, while knockdown of PEBP4 using siRNA resulted in drastic attenuation of PDAC cancer cell growth, migration, and metastasis of the cancer cells [10]. Yu *et al.* determined that PEBP4 was highly expressed in lung cancer cells, and PEBP4 enhanced lung cancer cell proliferation and invasion ability and inhibited apoptosis [12]. Liu *et al*. found that PEBP4 expression in colorectal cancer tissues was obviously higher than that in the normal peri-carcinoma tissues, the expression in the cancer tissues from the patients with positive lymph node and distant metastasis was greatly higher than that from the patients who were negative for metastasis, and downregulation of PEBP4 significantly reduced the number of colorectal cancer cells that passed through the Transwell chamber [13]. In line with these results, in this study, we found that the expression of PEBP4 was significantly upregulated in gastric cancer tissues and cell lines. Downregulation of PEBP4 significantly inhibited the proliferation of gastric cancer cells. These findings suggest that stable knockdown of PEBP4 in gastric cancer cells can suppress tumor growth.

EMT is an important process during development by which epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility [14]. Reduction of E-cadherin expression has a crucial role in tumor progression to invasive cancer and is also one of the well-established hallmarks of EMT [15]. In this study, we found that knockdown of PEBP4 strikingly increased the expression level of E-cadherin, but decreased the expression level of N-cadherin in TSGH and AGS cells. These results suggest that knockdown of PEBP4 prevents EMT progression in gastric cancer cells, thus inhibiting the migration and invasion of gastric cancer cells.

The PI3K/Akt pathway plays a critical role in tumor initiation, progression and metastasis [16-18]. It is frequently activated in gastric cancers, and contributes to gastric cancer cell proliferation, metastasis, apoptosis and EMT [19-21]. It was reported that activated Akt enhances the epithelial-mesenchymal transition, down-regulates E-cadherin transcription, and increases cell motility and invasion [22], and pretreatment of gastric cancer cells with PI3K/Akt kinase inhibitor significantly inhibited bone morphogenetic protein (BMP)-2-induced EMT and invasiveness [23]. Moreover, PEBP4 was found to interact with Akt and promoted the phosphorylation of Akt at serine 473 [10]. One study reported that hPEBP4 overexpression in

tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-sensitive human prostate cancer cells promoted Akt activation, in contrast, silencing of hPEBP4 in TRAIL-resistant human prostate cancer cells inhibited Akt activation [24]. In this study, we found that knockdown of PEBP4 obviously inhibited the phosphorylation of PI3K and Akt in gastric cancer cells. These results support the notion that knockdown of PEBP4 inhibits cell invasion and EMT through suppressing PI3K/Akt signaling pathway.

In our study, we found that knockdown of PEBP4 greatly inhibited the expression levels of p-PI3K and p-Akt in AGS cells. These results suggest that knockdown of PEBP4 inhibits gastric cancer development via inactivation of the PI3K/Akt signaling pathway, and PEBP4 may serve as a potential molecular target for the treatment of gastric cancer.

Disclosure of conflict of interest

None.

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