

Basic Study

Silencing profilin-1 inhibits gastric cancer progression *via* integrin β 1/focal adhesion kinase pathway modulation

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titative real-time polymerase chain reaction (qRT-PCR) and Western blot were performed to detect PFN1 expression in clinical gastric carcinoma and adjacent tissues, and the association of PFN1 expression with patient clinicopathological characteristics was analyzed. PFN1 was knocked down to investigate the role of this protein in cell proliferation and metastasis in the SGC-7901 cell line. To explore the underlying mechanisms, the expression of integrin β 1 and the activity of focal adhesion kinase (FAK) and the downstream proteins extracellular-regulated kinase (ERK)1/2, P38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), AKT and mammalian target of rapamycin (mTOR) were measured through Western blot or qRT-PCR analysis. Fibronectin (FN), a ligand of integrin β 1, was used to verify the correlation between alterations in the integrin β 1/FAK pathway and changes in tumor cell aggressiveness upon PFN1 perturbation.

RESULTS: Immunohistochemical, Western blot and qRT-PCR analyses revealed that PFN1 expression was higher at both the protein and mRNA levels in gastric carcinoma tissues compared with the adjacent tissues. In addition, high PFN1 expression (53/75, 70.4%) was correlated with tumor infiltration, lymph node metastasis and TNM stage in gastric cancer, but not with gender, age, location, tumor size, or histological differentiation. *In vitro* experiments showed that PFN1 knockdown inhibited the proliferation of SGC-7901 cells through the induction G₀/G₁ arrest. Silencing PFN1 inhibited cell migration and invasion and down-regulated the expression of matrix metalloproteinase (MMP)-2 and MMP9. Moreover, silencing PFN1 reduced the expression of integrin β 1 at the protein level and inhibited the activity of FAK, and the downstream effectors ERK1/2, P38MAPK, PI3K, AKT and mTOR. FN-promoted cell proliferation and metastasis *via* the integrin β 1/FAK pathway was ameliorated by PFN1 silencing.

Abstract

AIM: To investigate the role of profilin-1 (PFN1) in gastric cancer and the underlying mechanisms.

METHODS: Immunohistochemical analysis, quan-

CONCLUSION: These findings suggest that PFN1 plays a critical role in gastric carcinoma progression, and these effects are likely mediated through the integrin β 1/FAK pathway.

Key words: Gastric cancer; Profilin-1; Integrin β 1; Focal adhesion kinase; Fibronectin

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Core tip: The expression of profilin-1 (PFN1) has been detected in many types of human cancers and has also been associated with tumor malignancy. However, the role of PFN1 in gastric carcinoma is unclear. The results of the present study suggest an important role for PFN1 in gastric cancer. PFN1 is overexpressed in gastric cancer, associated with tumor infiltration, lymph node metastasis and TNM stage. Furthermore, we demonstrated that PFN1 silencing inhibits gastric cancer cell proliferation, migration and invasion through the integrin β 1/focal adhesion kinase pathway.

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INTRODUCTION

Gastric cancer is the second most common cause of cancer deaths worldwide^[1]. Traditional therapies for gastric cancer include surgical resection and chemotherapy, but these therapies are non-curative for patients diagnosed with advanced gastric cancer. Therefore, more effective treatments are urgently needed for this aggressive malignancy. The molecular mechanisms of gastric cancer progression should be clarified to identify potential therapeutic markers. The dysregulation of the actin cytoskeleton is a hallmark of tumor transformation, which is controlled through actin-binding proteins that regulate the nucleation, branching, elongation, bundling, severing, and capping of the actin filament^[2,3]. Thus, identifying actin-binding proteins might facilitate the discovery of novel targets for gastric cancer therapies.

Profilin-1 (PFN1), an important actin-binding protein, was first identified more than 30 years ago in the calf thymus^[4]. A biological role for PFN1 in cancer has recently emerged, and this protein has been implicated in almost every cellular function, including proliferation, survival, motility, endocytosis and membrane trafficking, mRNA splicing and gene transcription^[5]. It has been recently shown that PFN1 is underexpressed in some human solid cancers,

such as breast, pancreas, and liver carcinomas, while the overexpression of this protein can inhibit proliferation and migration of these cancer cells, suggesting that PFN1 might be a tumor suppressor protein^[6-8]. In contrast, some reports have shown PFN1 overexpression in other cancers, such as renal cell carcinoma and laryngeal carcinoma^[9-16]. These differences suggest that PFN1 might be involved in different tumorigenic mechanisms in different tissue types. However, few studies have demonstrated a role for PFN1 in gastric cancer.

Integrins are heterodimeric cell-surface molecules that associate the actin cytoskeleton to the cell membrane on one side and mediate cell-matrix interactions on the other side^[11]. Actin-binding proteins mediate the adhesion of cells to extracellular matrices and cell survival through the association of integrins with the cortical actin cytoskeleton^[12]. Recently, studies have reported that PFN1 facilitates staurosporine-triggered apoptosis through the regulation of the concentration of integrin β 1 associated with the cytoskeleton on the cell surface^[13]. However, the contribution of the integrin β 1 downstream signaling pathway to the biological role of PFN1 has not been clarified.

In the present study, we examined PFN1 expression in gastric cancer tissues and gastric cancer cell lines and analyzed the influence of PFN1 expression on patient clinicopathological characteristics. Furthermore, PFN1 was knocked down to determine the role of this protein in the proliferation and metastasis of SGC-7901 cells. To explore the underlying mechanisms, the expression of integrin β 1 and the activity of focal adhesion kinase (FAK) and downstream proteins extracellular-regulated kinase (ERK)1/2, P38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), AKT and mammalian target of rapamycin (mTOR) were measured through Western blot or quantitative real-time polymerase chain reaction (qRT-PCR). Moreover, fibronectin (FN), a ligand of integrin β 1, was used to verify the association between alterations in the integrin β 1/FAK pathway and changes in tumor cell aggressiveness upon PFN1 perturbation.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Ethics Committee of the Scientific and Ethical Committee of Second Military Medical University, and informed consent form was obtained from all participants.

Patient specimens

All tissue specimens, including 75 gastric cancer tissues and the corresponding non-plastic gastric mucosal tissues for immunohistochemistry and another 30 frozen gastric cancer and paired adjacent

normal tissues (not included in immunohistochemistry tissues) for qRT-PCR and Western blot, were obtained from patients during surgery at Shanghai Changzheng Hospital. All noncancerous human gastric tissue samples were obtained from gastrectomies of adjacent gastric cancer margins greater than 5 cm. All patients underwent surgical resection and were not treated with neoadjuvant chemotherapy or radiotherapy.

Immunostaining and evaluation

Immunohistochemical staining was performed using the EnVision system (Dako Carpinteria, CA, United States). TMA sections were submerged in citrate buffer (pH 6.0) and microwaved at 99 °C for 10 min for antigenic retrieval. PFN1 expression was detected using a primary antibody against PFN1 (1:200, ab133529, Epitomics, United States), and the immunostaining was evaluated as previously described^[14]. Two pathologists blinded to the findings of the other researcher independently analyzed the immunohistochemical staining. A semiquantitative estimation of the staining was conducted using a composite score obtained from the product of the staining intensity and relative abundance of positive cells. The intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). The abundance of positive cells was graded from 0 to 4 (0, < 5% positive cells; 1, 5%-25%; 2, 26%-50%; 3, 51%-75%; and 4, > 75%). A composite score in cancer tissues greater than that in normal tissues was considered high expression, and a composite score in cancer tissues less than or equal to that in normal tissues was considered low expression.

Cell lines and cell culture

The following human gastric carcinoma cell lines were used: AGS purchased from the American Type Culture Collection; SH-10-TC obtained from the Riken Bio Resource Center; MKN28, SGC-7901, BGC-803, BGC-823, N87 and gastric epithelial cells (GES) provided by the Institute of Biochemistry and Cell Biology of the Chinese Academy of Science. All cells were maintained in RPMI 1640 (Biowest) medium supplemented with 10% fetal bovine serum (FBS; Biowest), 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 1 mmol/L sodium pyruvate, with or without 10 µg/mL FN (Sigma) at 37 °C in 5% CO₂. The cells were passaged using trypsin-EDTA (0.05% trypsin and 0.53 mmol/L tetrasodium EDTA).

Small interfering RNA (siRNA) preparation and cell transfection

The siRNAs against human PFN1 were chemically synthesized at Shanghai GenePharma Co., Ltd. The siRNA sequences for the indicated genes were designed as previously described^[16]. According to the manufacturer's specifications, the cells were transfected with siRNA-PFN1 in 6-well plates using

Lipofectamine 2000 (Invitrogen, Gaithersburg, MD). Briefly, before transfection, 2.0×10^5 cells were grown to 30%-50% confluence in 2 mL of growth medium without antibiotics. The Lipofectamine 2000, diluted in Opti-MEM I Reduced Serum medium (Gibco), was used to supplement the dsRNA mixture, and the mixture was incubated for 25 min. Subsequently, 6 µL of 20 µmol/L dsRNA formulated with 8 µL of Lipofectamine 2000 was added to a final volume of 2 mL Opti-MEM I Reduced Serum medium. The cells were incubated at 37 °C in a CO₂ incubator for 24-48 h. The medium was changed after 6 h.

Western blot analysis

The harvested cells and human tissue specimens were lysed in a buffer containing 50 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, phosphatase inhibitors (100 mmol/L Na₃VO₄, 10 mmol/L NaF) and protease inhibitor (1 mmol/L phenyl methylsulphonyl fluoride) to obtain whole cell lysates. The insoluble tissue debris was removed through centrifugation at $13000 \times g$ at 4 °C for 15 min. The supernatant was collected, and the protein concentration was quantified using a protein assay reagent (BCA, Beyotime, Shanghai). The proteins were separated through SDS-PAGE, transferred to a nitrocellulose membrane and incubated with antibodies against total and phosphorylated FAK (pY397; Cell Signaling Technology, United States), total and phosphorylated AKT (Ser473; Cell Signaling Technology), total and phosphorylated mTOR (Ser2448; Cell Signaling Technology), total and phosphorylated PI3K (Tyr485 and Tyr199; Cell Signaling Technology), total and phosphorylated ERK1/2 (Thr202, Thr204; Cell Signaling Technology), PFN1 (Abcam, Cambridge, United Kingdom), matrix metalloproteinase (MMP)-2 (Abcam), MMP9 (Abcam), and integrin β1 (Abcam) at 4 °C overnight. The immunocomplexes were visualized using a horseradish peroxidase conjugated antibody followed by incubation with a chemiluminescence reagent (Millipore, Billerica, MA, United States) and exposure to a photographic film. The Western blot was quantified and analyzed using Quantity One software.

qRT-PCR analysis

Total RNA was isolated from frozen tissues, and the cells were treated with TRIzol (Takara, Dalian, China). The RNA quality (A₂₆₀/A₂₈₀ ratio) and quantity were determined using a standard spectrophotometer. One microgram of total RNA was used for cDNA synthesis using the RevertAid™ First Strand cDNA Synthesis Kit 1622 (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Appropriate forward and reverse primers were used in the reverse transcription-polymerase chain reactions (RT-PCRs) for cDNA amplification to detect the transcripts of interest. The primer sequences for GAPDH, PFN1 and integrin β1 have been previously described^[13,16]. The following PCR

conditions were used for the amplification: 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 55–58 °C for 30 s, and 72 °C for 45 s, and a final cycle at 72 °C for 10 min. qRT-PCR was conducted using a 7300 Real-time PCR System (Applied Biosystems). Standard curves were plotted for each optimized assay, to generate a linear plot of the threshold cycle (Ct) against log (dilution). The concentration of each target was quantified based on the concentration obtained from the standard curve and presented in arbitrary units. The quantity of each target was normalized against the quantity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Cell proliferation assay

The cells (1×10^4 /mL) were plated onto 96-well plates. At 24, 48 and 72 h post-transfection with PFN1 siRNA, cell viability was determined using the cell counting kit-8 (CCK-8) assay (RS Biotechnology, Shanghai) according to the manufacturer's protocol. The experiments were performed in triplicate.

Colony formation assay

Colony formation assays were performed as previously described^[15]. Briefly, the cells (500 per well) were cultured in 6 well plates for 2 wk. The colonies were counted and photographed using Quantity One software. All experiments were performed in triplicate.

Cell cycle analysis

SGC-7901 cells were transfected with PFN1 siRNA, NC and controls and harvested at 48 h post-transfection. The collected cells were fixed in 70% ethanol at 4 °C overnight. The cells were subsequently labeled with propidium iodide (PI) in the presence of RNase A. The fractions of cells in the G₀/G₁, S and G₂/M phases were analyzed using flow cytometry. The experiments were performed in triplicate.

Transwell assay

Transwell assays were performed using polycarbonate transwell filters (Corning, 8 µm) placed over the bottom chambers, which were filled with culture medium containing 20% FBS. Briefly, 1×10^5 cells in 0.5 mL of serum-free RPMI 1640 medium were placed in the upper chamber, and the lower chamber was loaded with 0.8 mL of medium containing 20% FBS. After 24 h, the cells on the upper surface of the well were removed, and the cells on the lower surface were fixed in cold methanol and stained with 0.4% crystal violet. For each experiment, the number of transmigrated cells in five random fields on the underside of the filter were counted and photographed, and three filters were independently analyzed.

Invasion assay

The invasion assays were performed using BD Matrigel invasion chambers (BD, 8 µm). Briefly, 1×10^5 cells in 0.5 mL of serum-free RPMI 1640 medium were

placed in the upper chamber, and the lower chamber was loaded with 0.8 mL of medium containing 20% FBS. After 24 h, the cells on the upper surface of the well were removed, and the cells on the lower surface were fixed in cold methanol and stained with 0.4% crystal violet. For each experiment, the number of transmigrated cells in five random fields on the underside of the filter were counted and photographed, and three filters were independently analyzed.

Statistical analysis

Data are expressed as mean \pm SD. Statistical analyses were performed using Student's *t*-test and analysis of variance. Pearson's χ^2 test was applied to examine the relationships between different variables. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

PFN1 expression in gastric cancer

All tissue microarray block sections used in this study contained both normal and malignant epithelium. Figure 1A shows representative images of PFN1 immunostaining in gastric cancer tissue samples. A semiquantitative estimation of the staining was represented as a composite score obtained from the product of the staining intensity and relative abundance of positive cells. Among the 75 paired gastric cancer samples, 53 (70.4%) cancer tissue samples showed higher PFN1 expression than the matched adjacent normal tissues. Moreover, the staining score ($P < 0.01$) showed that PFN1 expression was higher in gastric cancer tissues than in the adjacent normal tissues (Figure 1B). These results were consistent with those obtained from the qRT-PCR and Western blot analyses using another 30 tissue sample pairs (Figure 1C, D). Furthermore, the associations between PFN1 expression and the clinicopathological factors of gastric cancer are shown in Table 1. High PFN1 expression was associated with tumor infiltration ($P < 0.05$), lymph node metastasis ($P < 0.05$) and TNM stage ($P < 0.05$), but not with gender, age, location, tumor size, or histological differentiation ($P > 0.05$).

PFN1 expression in gastric cancer epithelial cell lines

The expression of PFN1 in the gastric cancer epithelial cell lines AGS, MKN28, SGC-7901, BGC-823, N87, SH-10-TC, BGC-803 and GES was determined by Western blot and qRT-PCR. Among these cell lines, the gastric cancer epithelial cells exhibited relatively higher levels of PFN1 expression than the gastric epithelial cell lines (Figure 2). These results indicated that the expression of PFN1 in gastric cancer cells was relatively high, consistent with the levels detected in tissues.

Knockdown of PFN1 inhibits gastric cancer cell proliferation

To address the potential role of PFN1 in the tumorigenesis of gastric cancer, SGC-7901 cells with

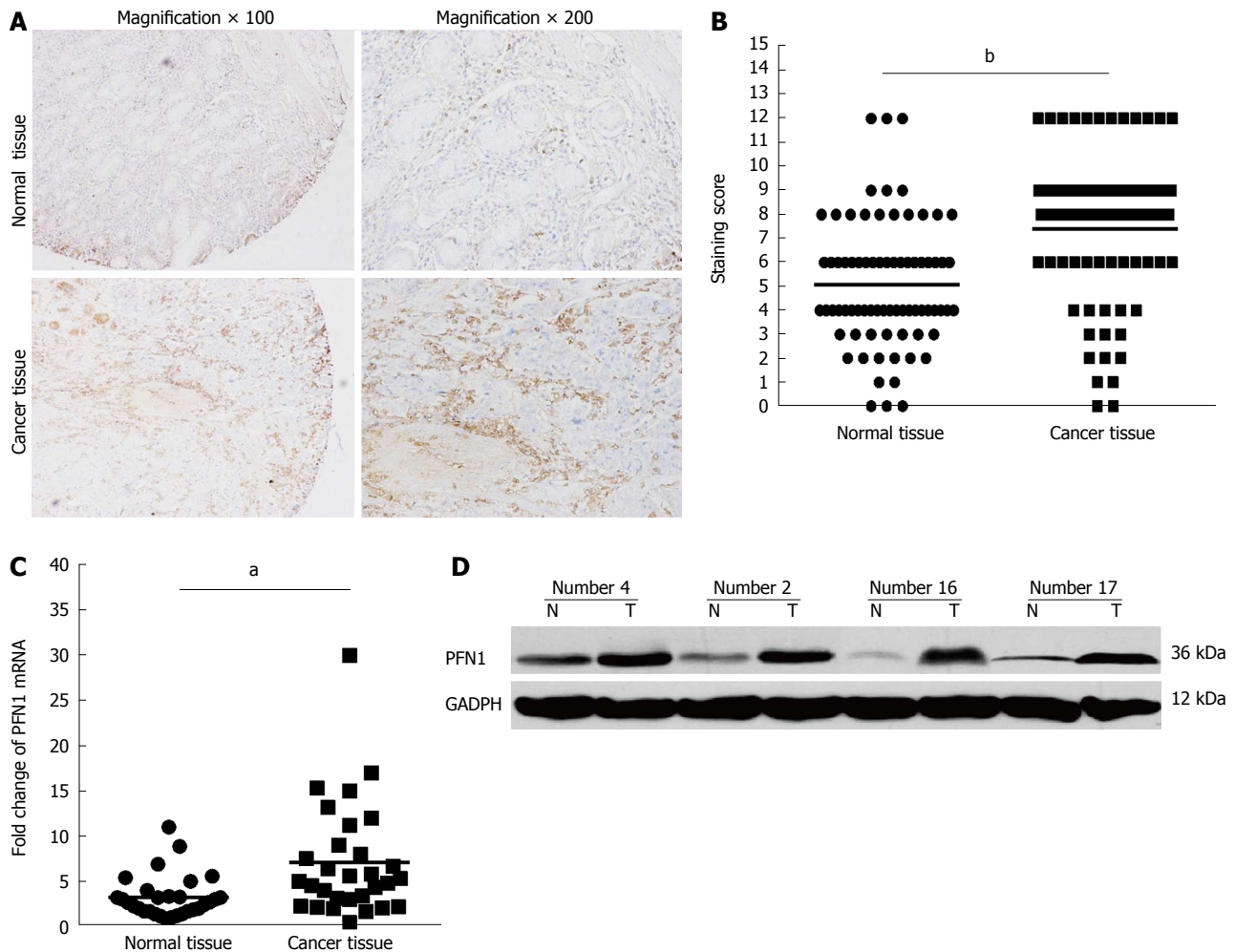


Figure 1 Analysis of profilin-1 expression in human gastric cancer and adjacent normal tissues. A: Immunohistochemical staining for profilin-1 (PFN1) in human gastric cancer and adjacent normal tissues; B: The PFN1 staining score in human gastric cancer and adjacent normal tissues; C and D: Quantitative real-time polymerase chain reaction (C) and Western blot analyses (D) of PFN1 expression in another set of gastric cancer tumor tissues compared with paired adjacent normal tissues ($n = 30$) assessed. T: Tumor tissues; N: Adjacent normal tissues. $^aP < 0.05$, $^bP < 0.01$ vs paired adjacent normal tissues. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

relatively higher PFN1 expression were transfected with PFN1-siRNA. The down-regulated expression of PFN1 was evidenced through Western blot (Figure 3A). The effects of PFN1 knockdown on the proliferation of SGC-7901 cells were evaluated using CCK-8 kit. As shown in Figure 3B, silencing PFN1 significantly inhibited cell growth in PFN1-siRNA transfected cells relative to control and vector transfectants at 48 and 72 h ($P < 0.05$). However, significant differences were not observed at 24 h ($P > 0.05$). In addition, the colony formation assay revealed that the PFN1-siRNA transfected cells exhibited a significantly lower colony-forming efficiency ($P < 0.05$) (Figure 3C). Moreover, cell apoptosis and the cell cycle were assessed using flow cytometry to determine the mechanisms underlying the observed tumor suppression in response to PFN1 silencing. PFN1 silencing resulted in higher G_0/G_1 phase populations compared with the untreated control and NC-transfectants (Figure 3D), but no

influence on cell apoptosis was observed (data not shown). Taken together, these results indicate that silencing PFN1 inhibits proliferation through the induction of G_0/G_1 arrest in SGC-7901 cells.

Knockdown of PFN1 inhibits gastric cancer cell migration

The migration of SGC-7901 cells was assessed using transwell migration assays. The micrographs obtained from a typical transwell experiment (Figure 4A) indicated that fewer cells transmigrated in the PFN1 siRNA-treated group compared with those in the untreated control and NC-transfected groups. Because MMPs are important mediators of tumor cell invasiveness and metastasis, we investigated the impact of gene silencing on MMP expression. The knockdown of *PFN1* reduced MMP2 and MMP9 expression (Figure 4B). These results suggested that PFN1 mediates gastric cancer cell migration and invasion.

Table 1 Correlation between profilin-1 expression and clinicopathological factors *n* (%)

Parameter	Cases	PFN1 expression		<i>P</i> value
		High expression	Low expression	
Gender				0.073
Male	50	32 (60.4)	18 (81.8)	
Female	25	21 (39.6)	4 (18.2)	
Age (yr)				0.572
> 60	51	35 (66)	16 (72.7)	
≤ 60	24	18 (34)	6 (27.3)	
Location				0.457
Cardia	15	9 (60)	6 (40.0)	
Corpus	26	18 (69.2)	8 (30.8)	
Antrum	34	26 (76.5)	8 (23.5)	
Size (diameter) (cm)				0.601
< 6	41	30 (56.6)	11 (50.0)	
≥ 6	34	23 (43.4)	11 (50.0)	
Differentiation				0.236
I and II	33	21 (39.6)	12 (54.5)	
III and IV	42	32 (60.4)	10 (45.5)	
Invasion depth				0.046
T0-T2	19	10 (18.9)	9 (41.0)	
T3-T4	56	43 (81.1)	13 (59.0)	
Nodal metastasis				0.038
Negative	27	15 (28.3)	12 (54.5)	
Positive	48	38 (71.7)	10 (45.5)	
TNM stage				0.036
I and II	29	16 (30.1)	13 (59.0)	
III and IV	46	37 (69.9)	9 (41.0)	
Total	75	53 (70.4)	22 (39.6)	

PFN1: Profilin-1; TNM: Tumor-node-metastasis.

PFN1 silencing decreases the expression of integrin β 1 and inhibits the FAK signaling pathway

To elucidate the molecular mechanisms underlying tumor inhibition in response to PFN1 silencing, we assessed integrin β 1 expression using Western blot and qRT-PCR. The results showed that PFN1 silencing did not affect the integrin β 1 mRNA level (Figure 5B), but led to the down-regulation of integrin β 1 protein expression (Figure 5A). The FAK signaling pathway plays an important role in the response to integrin-mediated cell proliferation, cell motility and migration. The expression of phospho-FAK and the downstream effectors phospho-ERK1/2, phospho-P38, phospho-PI3K and phospho-AKT were down-regulated after PFN1 silencing (Figure 5C). Because mTOR is a direct target of AKT, mTOR expression was also examined using Western blot, and the results showed that activities of these proteins were inhibited through PFN1 silencing (Figure 5C).

Effect of FN is ameliorated through PFN1 silencing

FN, an integrin β 1 ligand, was used to verify the association between alterations in the integrin β 1/FAK pathway and changes in tumor cell aggressiveness through PFN1 silencing. The activation of the FAK signaling pathway through FN *via* integrin β 1 binding promoted cell proliferation, migration, and invasion in different cell types. The induction of FAK activity through FN was ameliorated after PFN1 silencing

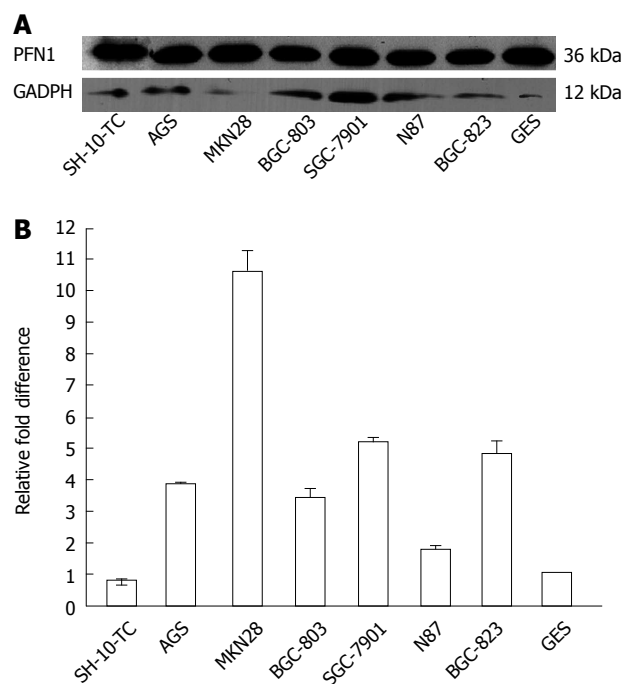


Figure 2 Analysis of profilin-1 expression in human gastric cancer cell lines. Western blot (A) and quantitative real-time polymerase chain reaction analyses (B) revealed the expression of profilin-1 (PFN1) in gastric cancer epithelial cell lines. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

(Figure 6A). Moreover, the effect of FN on the cell proliferation, migration and invasion was ameliorated after PFN1 silencing (Figure 6B and C).

DISCUSSION

PFN1 is a 12-15 kDa protein that plays important roles in cellular functions. This protein regulates signaling-dependent actin polymerization *via* actin monomer binding at a ratio of 1:1 to form the profilin-actin complex, which participates in a variety of cellular functions, including growth and division, cell adhesion and motility, signal transduction and formation, and the maintenance of actin-binding protein-dependent cell morphology^[17,18]. The results of the present study suggest that increased PFN1 expression promotes gastric cancer progression through stabilizing integrin β 1 *via* changes in the FAK pathway.

The immunohistochemistry data revealed that PFN1 expression significantly increased from normal tissues to primary tumors. This result was also verified by qRT-PCR and Western blot in another set of paired gastric cancer tissues. We demonstrated that PFN1 is dysregulated in gastric cancer and gastric cancer cell lines, which is consistent with the findings of Tanaka *et al.*^[19]. In addition, the high expression of PFN1 was associated with tumor infiltration, lymph node metastasis and TNM stage. These results indicate that PFN1 plays an important role in gastric cancer progression.

To determine the biological function of this protein in gastric cancer, SGC-7901 cells with high PFN1

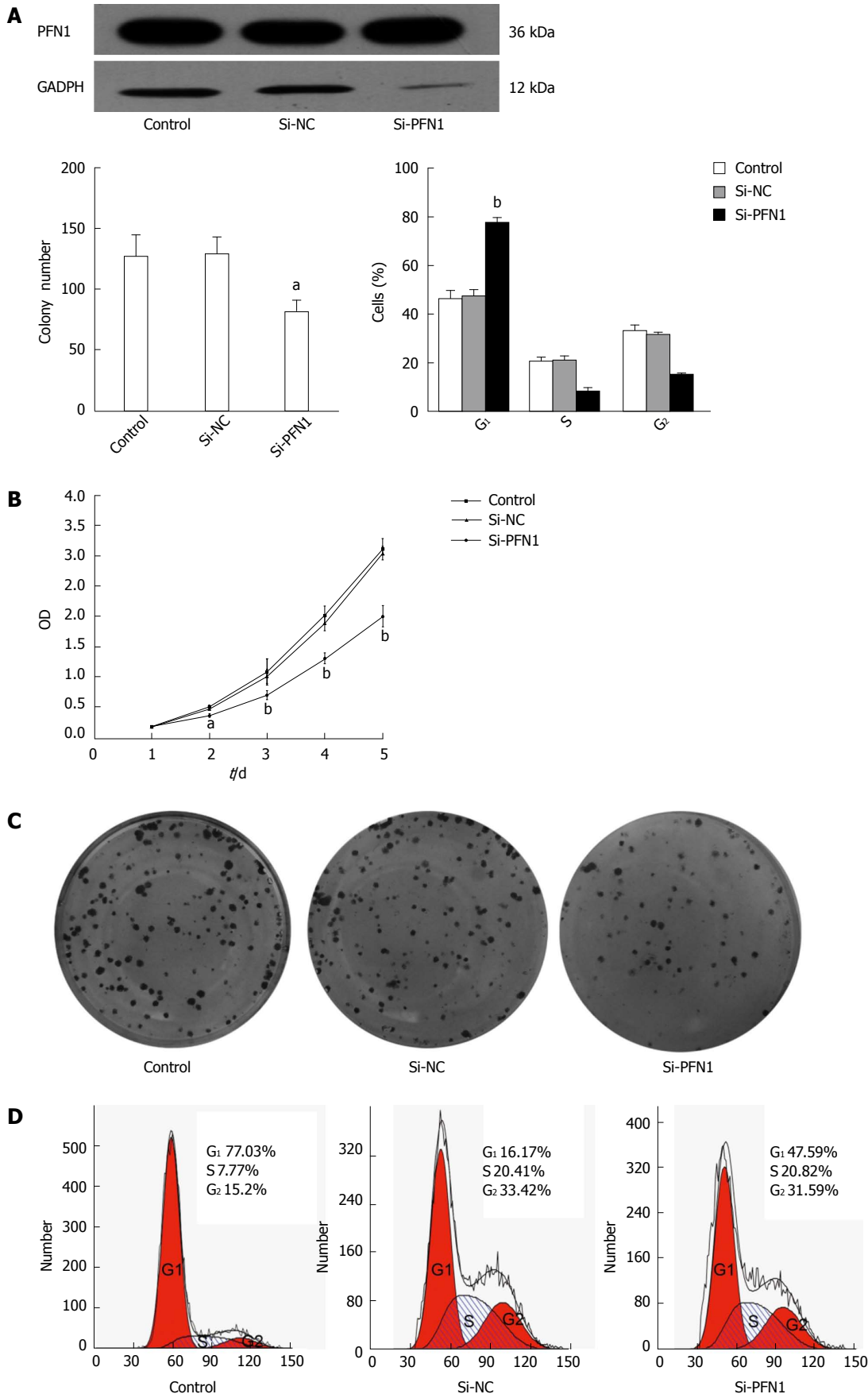


Figure 3 Profilin-1 knockdown inhibits tumor growth. A: Expression of profilin-1 (PFN1) was assessed in the PFN1-transfected cells, the untreated control and the negative control (NC)-transfectants by Western blot; B: Cell proliferation levels were measured using the cell counting kit-8 on 1, 2, 3, 4 and 5 d. The optical density (A) represents the proliferative characteristics of the treated cells; C: PFN1 silencing resulted in significantly lower colony-forming efficiency; D: Cell-cycle distribution was analyzed by flow cytometry on SGC-7901 cells. The results obtained from three independent experiments are shown. ^a*P* < 0.05, ^b*P* < 0.01 vs other groups. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Si: Silencing.

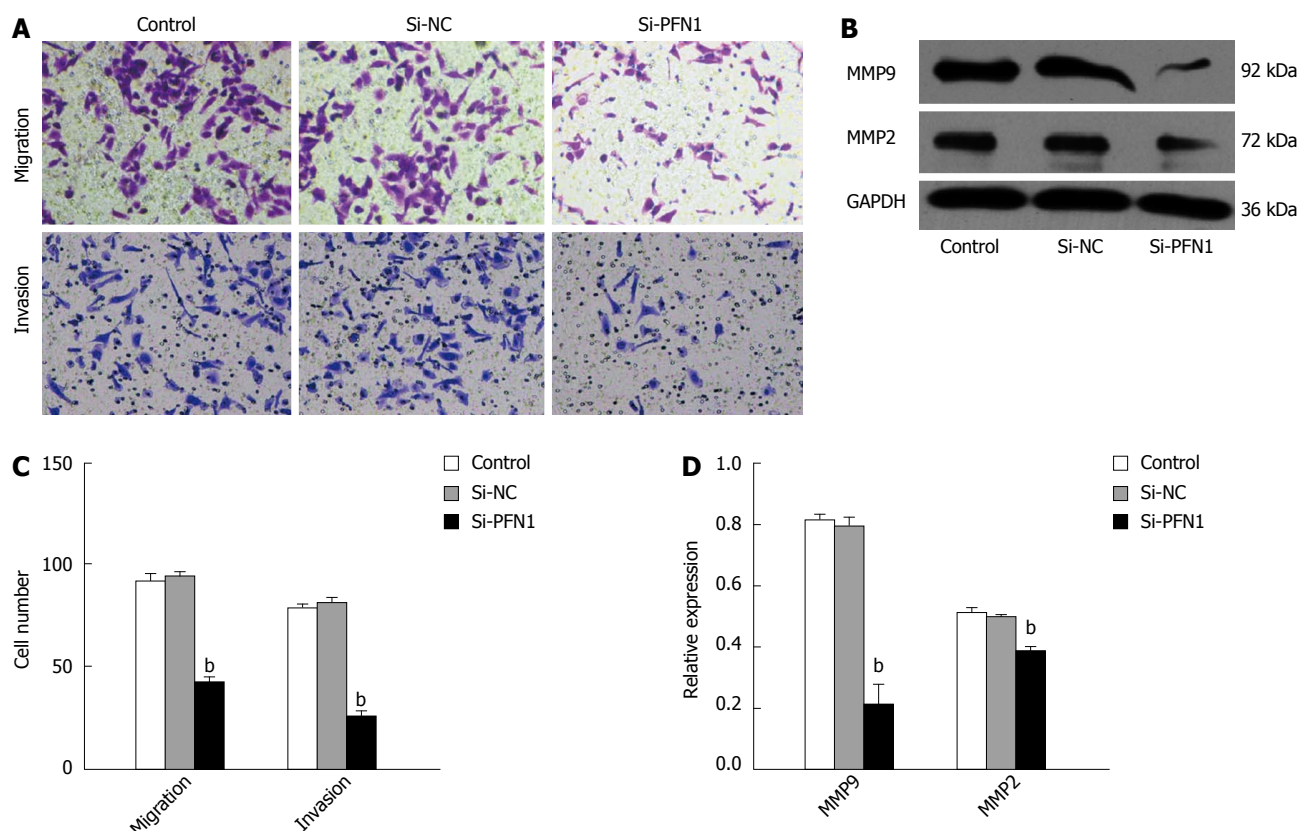


Figure 4 Effects of profilin-1 on gastric cancer cell migration *in vitro*. A: The cell migration and invasion were assessed using transwell and invasion assays, respectively; B: The protein expression of matrix metalloproteinase (MMP)-2 and MMP9 was detected by Western blot. All results were obtained from three independent experiments. ^b $P < 0.01$ vs other groups. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PFN1: Profilin-1; NC: Negative control; Si: Silencing.

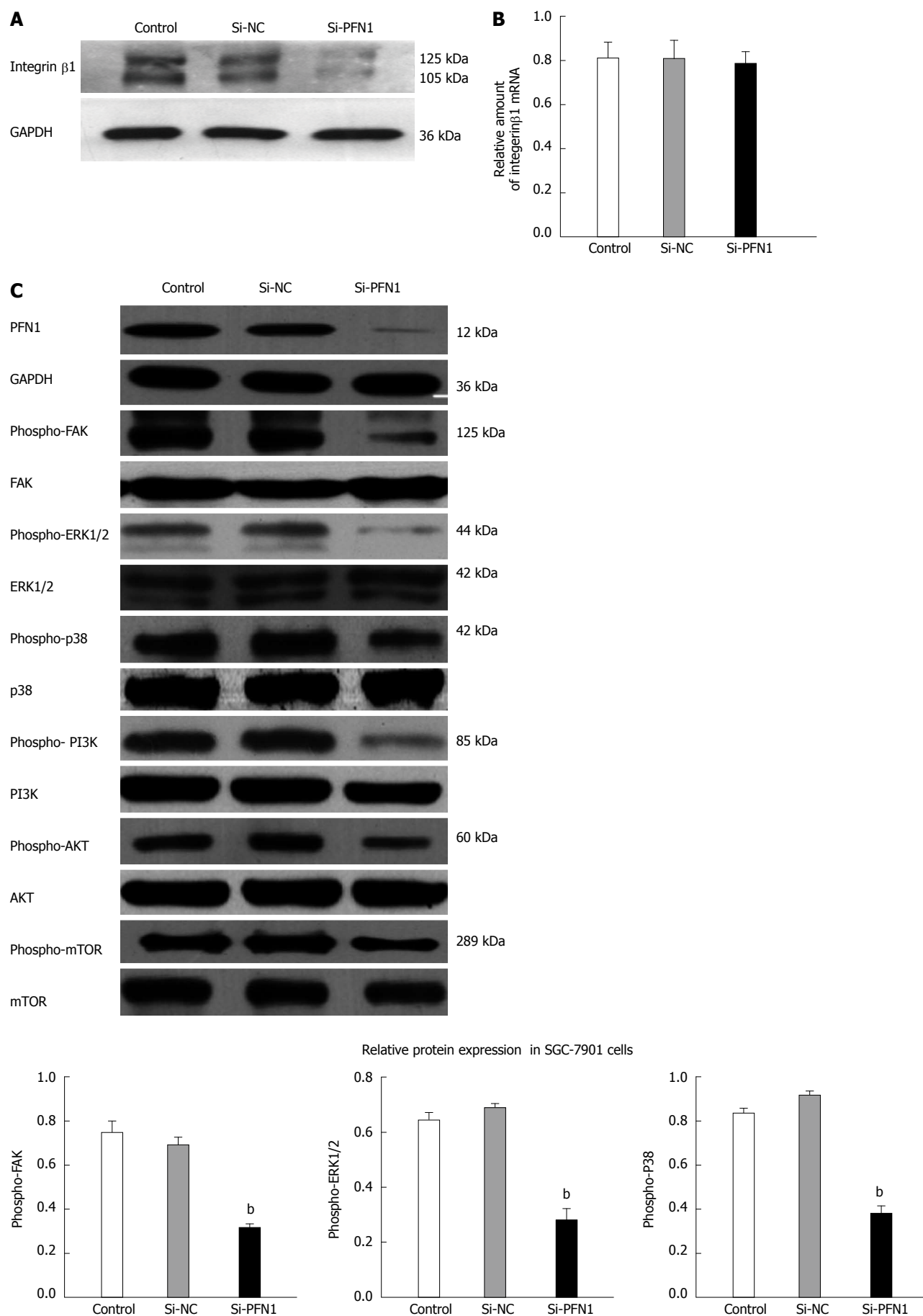
expression were subjected to *PFN1* knockdown. The results of the cell proliferation and colony formation assays demonstrated that *PFN1* silencing significantly inhibited cell growth. This result is consistent with association of *PFN1* overexpression with TNM stage. In addition, *PFN1* silencing also reduced cellular proliferation in other cancer cell lines, such as MCF7, HeLa, and SKOV3^[20]. To further examine the function of this protein in gastric cancer, the effects of *PFN1* on cell apoptosis and cell cycle distribution were analyzed using flow cytometry. The results showed that *PFN1* silencing reduced the number of S- and G₂-phase cells and increased the number of G₀/G₁-phase cells, indicating a G₀/G₁ arrest. However, cell apoptosis was not affected after *PFN1* silencing (data not shown). These results suggest that *PFN1* silencing inhibits the proliferation of gastric cancer cells through the induction of G₀/G₁ arrest.

In addition, *PFN1* silencing significantly decreased gastric cancer cell migration and invasion. Consistently, blocking *PFN1* decreased bladder cancer cell motility through reduced actin (F-actin) polymerization^[21]. Furthermore, we observed that *PFN1* silencing reduced the cellular expression of MMP2 and MMP9, two key enzymes involved in the degradation of the extracellular matrix (ECM). Because the ECM exerts biochemical and mechanical barriers to cell movement, the degradation of this scaffold is an important process

in cancer cell metastasis^[22]. The data presented here support these observations, suggesting that high *PFN1* expression plays an essential role in the gastric cancer progression.

Indeed, the results of the present study showed that *PFN1* silencing decreased integrin $\beta 1$ at the protein level. A recent study suggested that *PFN1* contributes to the quantity of integrin $\beta 1$ linked to the cytoskeleton on the cell surface by promoting actin polymerization to increase the amount of F-actin^[13]. Therefore, the alterations in integrin $\beta 1$ expression at the protein level may reflect a decrease in the F-actin concentration after *PFN1* silencing. Integrins including integrin $\beta 1$ are non-kinase receptors, and their binding to the ECM and subsequent activation require kinases to initiate signal transduction. Integrin $\beta 1$ signaling occurs primarily through the recruitment and activation of the tyrosine protein kinase FAK, and FAK promotes cancer cell proliferation and metastasis upon integrin $\beta 1$ binding, which consequently results in the activation and auto-phosphorylation of FAK^[23,24]. In the present study, we revealed that *PFN1* silencing might inhibit the activation of FAK by down-regulating integrin $\beta 1$ expression.

Activating FAK ensures successive signaling events, as this protein binds to the SH2 domain of PI3K, thereby transporting the catalytic subunit of PI3K to the membrane, where it catalyzes the phosphorylation



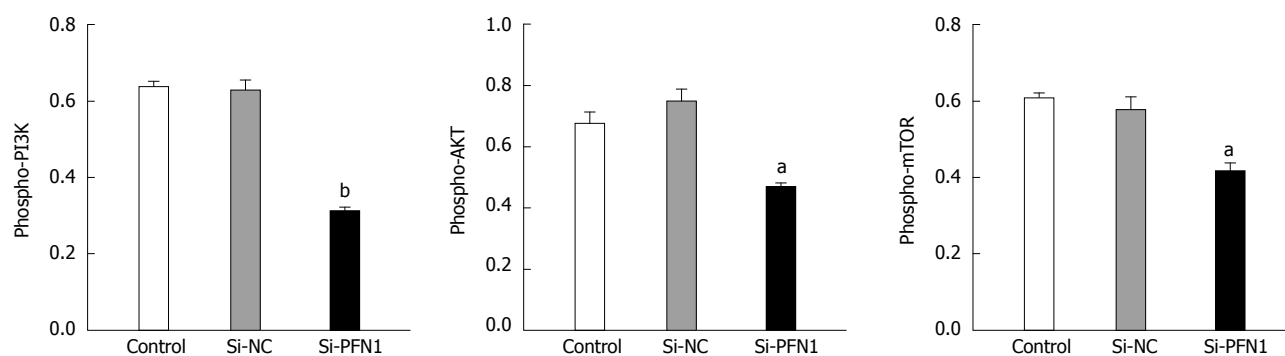


Figure 5 Profilin-1 silencing regulates the expression of integrin β 1 and its downstream molecular targets. A and B: Expression of integrin β 1 protein and mRNA was detected by Western blot and quantitative real-time polymerase chain reaction analyses, respectively; C: The effect of profilin-1 (PFN1) knockdown on its downstream targets in the focal adhesion kinase (FAK) signaling pathway. The quantity of PFN1 was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the phosphorylation of other proteins was normalized against the respective total protein. All results were obtained from three independent experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs other groups. ERK: Extracellular-regulated kinase; mTOR: Mammalian target of rapamycin; PI3K: Phosphatidylinositol 3-kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NC: Negative control; Si: Silencing.

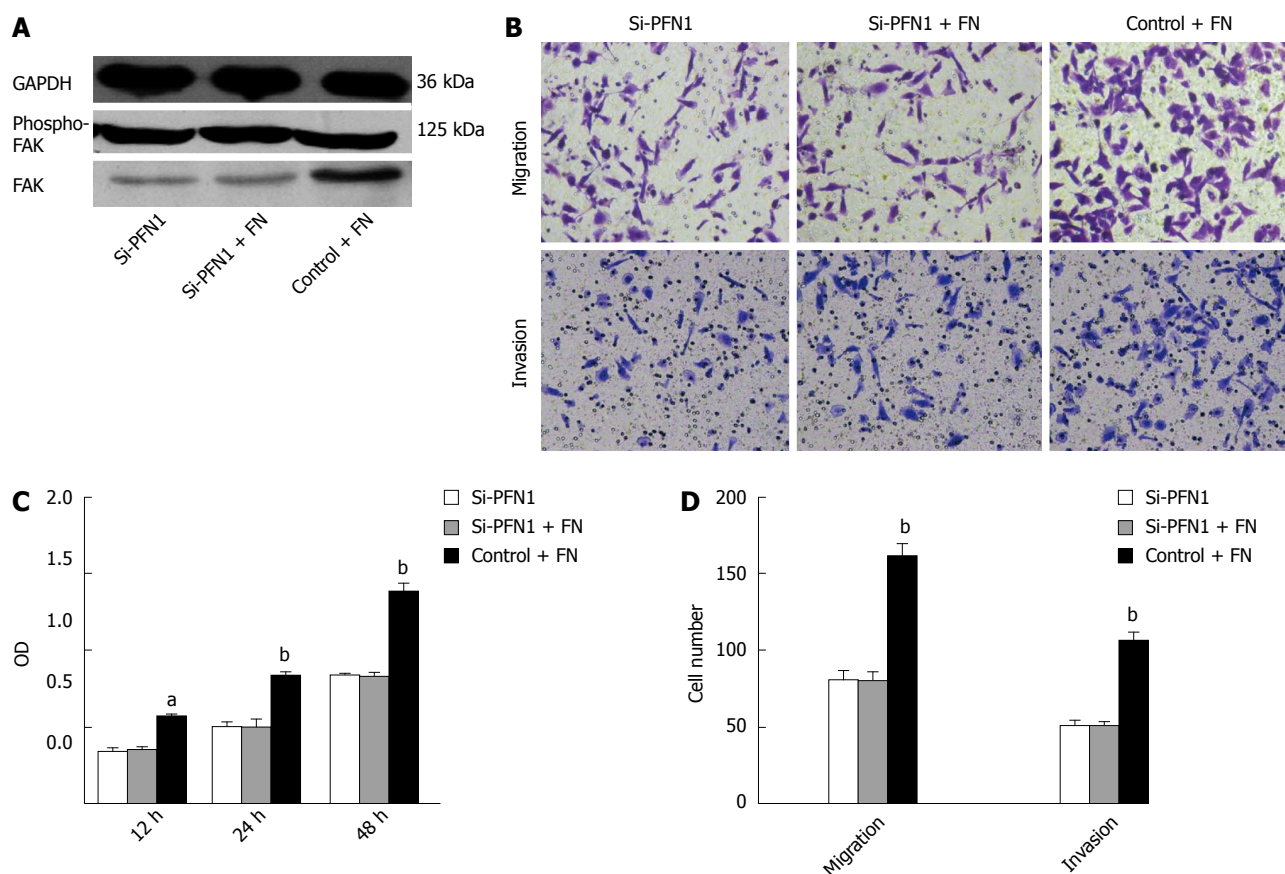


Figure 6 The effect of fibronectin is ameliorated after profilin-1 silencing. A: SGC-7901 cells with and without profilin-1 (PFN1) silencing were seeded onto media with or without fibronectin (FN) (10 μ g/mL) for 24 h. The expression of phosphorylated focal adhesion kinase (FAK) was evaluated by Western blot; B: SGC-7901 cells with and without PFN1 silencing were seeded on media with or without FN (10 μ g/mL) at the indicated time points. Cell proliferation levels were measured using the cell counting kit-8 at 12, 24 and 48 h. The optical density (A) represents the proliferative characteristics of the treated cells; C: SGC-7901 cells with and without PFN1 silencing were suspended in media with or without FN (10 μ g/mL) and seeded onto transwell plates. The cell migration was assessed 24 h later. For the invasion assays, the cells were seeded onto BD Matrigel invasion chambers. ^a $P < 0.05$, ^b $P < 0.01$ vs other groups. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Si: Silencing.

of AKT^[25]. mTOR is a direct target of AKT oncogenic signaling, involved in cell growth, tumorigenesis, and cell invasion in various types of cancers, including gastric cancer^[26]. In addition, it can also activate the ERK1/2/P38MAPK signaling pathway by binding to ERK1/2 with a specific sequence^[27]. Moreover, FAK

inactivity leads to the inhibition of ERK1/2, P38MAPK, PI3K and AKT activity. Therefore, we examined changes in the expression of these molecules, and the results showed that the activity of the enzymes was inhibited after PFN1 silencing^[28]. Previous studies have indicated that FAK/PI3K/AKT and FAK/MAPK

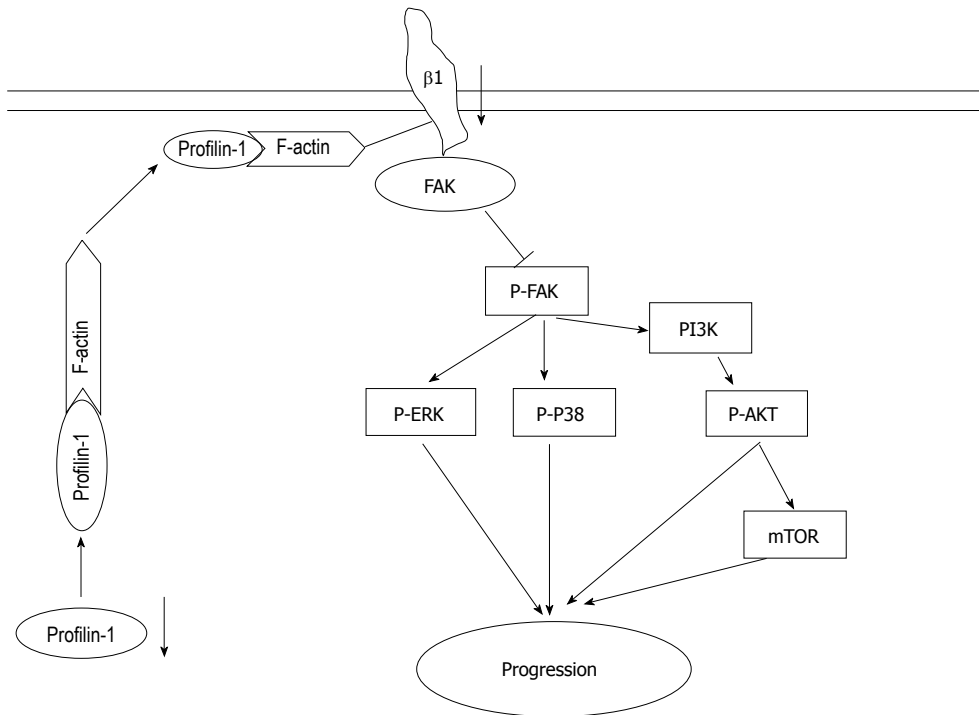


Figure 7 Schematic diagram of the mechanism underlying the inhibition of gastric cancer progression through profilin-1 silencing. Profilin-1 (PFN1) silencing decreases actin filament assembly and reduces the binding among PFN1, actin and integrin $\beta 1$, which inhibits the focal adhesion kinase (FAK) activation through the down regulation of integrin $\beta 1$ expression. FAK inhibition through profilin-1 silencing inhibits gastric cancer progression via mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways. mTOR: Mammalian target of rapamycin.

are involved in the regulation of MMP2 and MMP9 activities in different cell types^[29]. Consistently, MMP2 and MMP9 expression was down-regulated after PFN1 depletion. Taken together, these results suggest that PFN1 silencing might inhibit gastric cancer progression through the integrin $\beta 1$ /FAK signaling pathway.

Moreover, FN, an integrin $\beta 1$ ligand, was used to verify the association between alterations in the integrin $\beta 1$ /FAK pathway and changes in tumor cell aggressiveness upon PFN1 perturbation. The integrin $\beta 1$ /FAK signaling pathway, activated through FN binding to integrin $\beta 1$, promotes cell proliferation, migration, and invasion^[30,31], and this effect can be ameliorated through the inhibition of the FAK pathway using an integrin $\beta 1$ neutralizing antibody^[31,32]. In the present study, this effect was also ameliorated through the down-regulation of integrin $\beta 1$ protein expression via PFN1 silencing. Integrin $\beta 1$ is a non-kinase receptor, and the subsequent activation of this receptor requires FAK to initiate signal transduction. In addition, the FN-mediated activation of FAK is ameliorated after PFN1 silencing through down-regulation of integrin $\beta 1$ expression. Thus, the effect of FN on cell proliferation, migration and invasion could be ameliorated through PFN1 silencing via the integrin $\beta 1$ /FAK pathway. These results suggest that PFN1 silencing inhibits gastric cancer progression through the integrin $\beta 1$ /FAK pathway (Figure 7).

Traditionally, PFN1 has been considered indispensable for the pro-proliferation and pro-migration

of cells through actin cytoskeleton remodeling via actin polymerization^[32,33]. The data in present study are consistent with this role for PFN1; however, these results are different from those in other studies concerning cancers, such as breast^[34], pancreas^[35], and liver cancers^[8]. These differences suggest that PFN1 might be involved in different tumorigenic mechanisms in different tissue types. In addition, the recent study reported that PFN1 had contrasting effects on early vs late steps of breast cancer metastasis, and the loss of PFN1 significantly inhibited the metastatic outgrowth of disseminated breast cancer cells, which had a completely different tumor microenvironment than the primary tumor^[2]. These results suggested that this contradiction was critically influenced through signaling from the tumor microenvironment. Mediated through PFN1 and the ECM of tumor microenvironment, integrin $\beta 1$ acts as a bridge connecting the actin cytoskeleton, and this interaction might play an important role in the tumor cell response to the loss of PFN1 expression. Consistently, blocking integrin signaling significantly inhibits metastatic outgrowth of breast cancer cells^[2]. Therefore, profilin-1 silencing inhibits gastric cancer progression through integrin $\beta 1$ /FAK pathway modulation.

In conclusion, the present study underscores an important role for PFN1 in gastric cancer. PFN1 overexpression in gastric cancer is associated with tumor infiltration, lymph node metastasis and TNM stage. Furthermore, we demonstrated that PFN1

silencing inhibits gastric cancer progression through the integrin β 1/FAK pathway.

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COMMENTS

Background

Gastric cancer is the second most common cause of cancer deaths worldwide. Traditional therapies for gastric cancer include surgical resection and chemotherapy, but these therapies are non-curative for those diagnosed with advanced gastric cancer. Therefore, more effective treatments are urgently needed for this aggressive malignancy.

Research frontiers

Recent studies have suggested that profilin-1 (PFN1) might be involved in different tumorigenic mechanisms in different tissue types. However, there are few studies concerning the role of PFN1 in gastric cancer.

Innovations and breakthroughs

This study underscores an important role for PFN1 in gastric cancer. PFN1 overexpression in gastric cancer is associated with tumor infiltration, lymph node metastasis and tumor-node-metastasis (TNM) stage. Furthermore, authors demonstrated that PFN1 silencing inhibits gastric cancer cell proliferation, migration and invasion through the integrin β 1/focal adhesion kinase (FAK) pathway.

Applications

The findings in the present support the idea that PFN1 might be a novel target for gastric cancer therapy.

Terminology

PFN1, an important actin-binding protein, was first identified more than 30 years ago in the calf thymus and this protein was considered to play an indispensable role in cell pro-proliferation and pro-migration through actin cytoskeleton remodeling via actin polymerization.

Peer-review

In this study, the authors demonstrated that profilin-1 plays important roles in gastric cancer, associated with tumor infiltration, lymph node metastasis and TNM stage. The authors concluded that PFN1 promotes the progression of gastric cancer via modulation of the integrin β 1/FAK pathway. This article is concise and well organized.

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