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Original Research Article

Knockdown of pancreatic adenocarcinoma upregulated factor (PAUF) suppresses proliferation, migration, invasion, and cancer stem cell properties in lung cancer cells

Liangchao Dong[1], Weiwei Li[2], Xiaoli Zhang[3]*

[1]Department of Thoracic Surgery, [2]Department of Oncology, The First People's Hospital of Tianmen City, Tianmen City, Hubei Province 431700, [3]Department of Pathology, The First Affiliated Hospital of University of South China, Hengyang City, Hunan Province 421001, China

For correspondence: **Email: zhangxiaoli6111@126.com; **Tel:** +86-0734-8578939*

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Abstract

Purpose: *To investigate the role of pancreatic adenocarcinoma up-regulated factor (PAUF) in lung cancer.*
Method: *Proliferation of lung cancer cell lines (A549 and H1299) was determined using MTS and Edu staining assays. Wound healing and transwell assays were performed to evaluate cell migration and invasion abilities. Lung cancer stem cell (CSC) marker expressions, including CD133, CD44, ALDH1, SOX2, and Oct4, were determined by western blot assay.*
Results: *Knockdown of PAUF significantly inhibited A459 and H1299 cell proliferation ($p < 0.01$). The wound healing and transwell assay results indicated that depletion of PAUF markedly suppressed H1299 and A549 cell migration and invasion, compared with the control cells ($p < 0.01$). Knockdown of PAUF reduced distinct CSC marker expression, suggesting inhibition of CSC phenotypes, and reduced phosphorylated focal adhesion kinase (FAK), phosphorylated Src, and phosphorylated extracellular signal-regulated kinase (ERK), but not total FAK, Src, and ERK. These results suggested that knockdown of PAUF deactivated the FAK/Src/ERK signal pathway.*
Conclusion: *Knockdown of PAUF inhibits lung cancer cell proliferation, migration, invasion, and CSC properties via deactivation of FAK/Src/ERK signal pathway. These results may provide a novel strategy for the development of lung cancer therapeutics.*

Keywords: *PAUF, Proliferation, Migration, Invasion, Cancer stem cell, Lung cancer*

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INTRODUCTION

Lung cancer is a malignant tumor and causes high incidence and mortality rates worldwide [1]. Approximately 15 % of lung cancer cases are small cell lung cancer (SCLC) and 85 % are non-small cell lung cancer (NSCLC) [2]. The NSCLCs can be subdivided into adenocarcinoma, large-cell carcinoma, squamous cell carcinoma, and bronchoalveolar carcinoma. Compared with

SCLC cells, NSCLC cells have slower proliferation rates and later diffusion metastasis [3]. The five-year survival rate after lung cancer diagnosis in the United States is 17.4 % and is even lower in developing countries [4]. Approximately 40 % of NSCLC patients have metastatic or locally advanced disease, and 90 % of lung cancer-related deaths are caused by metastasis [5]. Cancer stem cells (CSCs) are tumor cell subsets that regulate tumor maintenance and spread, which can result in unlimited proliferation, self-renewal, and differentiation [6]. Given the characteristics of CSCs, they are potential therapeutic targets for cancer. Pancreatic adenocarcinoma up-regulated factor (PAUF) is a novel protein that participates in cancer tumorigenesis and metastasis [7-9]. Expression of PAUF is closely associated with a poor prognosis for cervical cancer patients [9]. It alters mitotic functions and Wnt/beta-catenin signaling, resulting in colorectal cancer progression [10]. In pancreatic cancer, PAUF enhances vascular permeability and angiogenesis [11]. It has also been found to be an endogenous ligand for toll-like receptor 4, leading to activation of JNK, AKT and ERK signaling in human leukemia cells [8,12]. Given these findings, it has been hypothesized that PAUF might participate in lung cancer. However, the potential role of PAUF in lung cancer remains unknown.

In this study, two representative lung cancer cell lines, A549 and H1299, were used investigate the role of PAUF in lung cancer *in vitro*. Cells were transfected with two short hairpin (sh) PAUFs (shPAUF#1 and shPAUF#2). The functions of PAUF on H1299 and A549cell proliferation, migration, invasion, and CSC phenotypes were examined, laying a foundation for the role of PAUF in lung cancer.

EXPERIMENTAL

Cell culture and transfection

The H1299 and A549 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium (12491-015, Gibco, New York, USA) supplemented with 10 % fetal bovine serum (FBS; 10099141, Gibco) at 37 °C with 5 % CO_2. The human PAUF shRNA sequences shPAUF#1 (5'-ACACCAGCAAGGACCGCTATT-3'), shPAUF#2 (5'-CCTTGGCATCAAGAGCATT-3'), or shNC (5'-GGAATCTCATTCGATGCATAC-3') were inserted into the GV248 lentiviral vector (Genechem, Shanghai, China). Subsequently, virus was resuspended, added to a 6-well plate, and cultured with H1299 and A549 cells for 72 hours. The cells were then selected and used for the experiments.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

After transfection, the H1299 and A549 cells were cultured in a 96-well plate. After 24, 48, and 72 hours, an MTS assay kit (ab197010, Abcam, Cambridge, UK) was used to evaluate cell viability. After adding 20 µL of reagent, absorbance (490 nm) of each well of cells was measured.

5-ethynyl-2'-deoxyuridine (Edu) staining assay

The cell proliferation assay was performed with EdU Proliferation Assay Kit (ab222421, Abcam). After transfection, approximately 5×10^3 cells/well H1299 and A549 cells were seeded into 96-well plates. After a 24-hour cell culture, each well was added with EdU solution for 2-h incubated at 37 °C. After DAPI staining, images were captured with fluorescence microscopy (Leica, Germany).

Migration wound healing assay

After transfection, the H1299 and A549 cells were scratched using a sterile 200-µL pipette tip and subsequently rinsed twice using phosphate buffered saline (PBS). After incubation for 24 hours, the cells were visualized using light microscopy at 0 and 24 hours after scratching.

Transwell invasion assay

The invasion assay was evaluated with transwell assay with Matrigel. A total of 5×10^4 A549 or H1299 cells were placed in the upper chamber; 600 µL medium was added to the lower chamber. After 24 hours, the newly-established cells on the surface of the lower membrane were fixed, stained, photographed, and counted.

Tumorsphere culture

After transfection, approximately 2×10^3 cells/well H1299 and A549 cells were seeded in 6-well plates (Corning, New York, USA). One-half of the medium was replaced every 3 days. After 15 days, the representative images of spheroid formation were taken.

Quantitative real time PCR assay

The total RNA was extracted by RNA easy extraction kit (74104, Qiagen, Germany). Real-

time PCR was performed with QIAGEN OneStep RT-PCR Kit (210210, Qiagen). The result was normalized to the corresponding GAPDH mRNAs to obtain a relative quantitation value. The primer sequences used are presented in Table 1.

Table 1: Primers sequence used in this study

Gene	Primer	Sequence
PAUF	Forward	5'-CACCTGGGCAGGGAAGATGTA-3'
	Reverse	5'-GCTCAGTGGTCGGCTCCTCT-3'
GAPDH	Forward	5'-ATGGGGAAGGTGAAGGTCGG-3'
	Reverse	5'-GACGGTGCCATGGAATTTGC-3'

Western blot

Total proteins from cells were extracted using radioimmunoprecipitation assay lysis buffer and separated using SDS-PAGE gels. Subsequently, the blots were transferred onto PVDC membranes (Millipore, Massachusetts, USA). After that, the membranes were then incubated with primary antibody against PAUF (1:900; ab104162, Abcam), CD133 (1:900; ab216323, Abcam), CD44 (1:900; ab189524, Abcam), OCT4 (1:900; ab181557, Abcam), SOX2 (1:900; ab92494, Abcam), ALDH1 (1:900; ab52492, Abcam), pERK1/2 (1:900; ab214362, Abcam), ERK1/2 (1:900; ab54230, Abcam), pFAK (1:900; ab81298, Abcam), FAK (1:900; ab40794, Abcam), pSrc (1:900; ab40660, Abcam), Src (1:900; ab109381, Abcam), and GAPDH (1:2000; ab9485, Abcam) at 4°C overnight.

Thereafter, the membranes were blocked with Goat Anti-Rabbit IgG H&L (HRP) (1:6000; ab6721, Abcam) or Rabbit Anti-Mouse IgG H&L (HRP) (1:6000; ab6728, Abcam) for 2 hours at room temperature. Protein expression was visualized using BeyoECL Plus (P0018M, Beyotime, China).

Statistical analysis

All data are analyzed with SPSS 20.0 (USA) and presented as mean ± standard deviation (SD) values. Statistically significant differences were analyzed using the student's t-test or one-way analysis of variance (ANOVA), respectively. The *p*-value less than 0.05 was considered statistically significant.

RESULTS

Knockdown of PAUF inhibited cell proliferation in H1299 and A549 cells

To examine the role of PAUF in lung cancer, two shPAUFs, shPAUF#1 and shPAUF#2, were synthesized. ShPAUF#1 or shPAUF#2 significantly decreased the PAUF mRNA levels in H1299 and A549 cells (Figure 1 A). Consistent with the results in Figure 1 A, western blot assay revealed that shPAUF#1 or shPAUF#2 markedly reduced PAUF protein levels in H1299 and A549 cells (Figure 1 B). The MTS assay results indicated that knockdown of PAUF suppressed cell viability of the H1299 and A549 cells (Figure 1 C). The Edu staining assay results indicated that knockdown of PAUF reduced the Edu-positive cells in the H1299 and A549 cells (Figure 1 D). Taken together, these findings indicated that knockdown of PAUF inhibited H1299 and A549 cell proliferation.

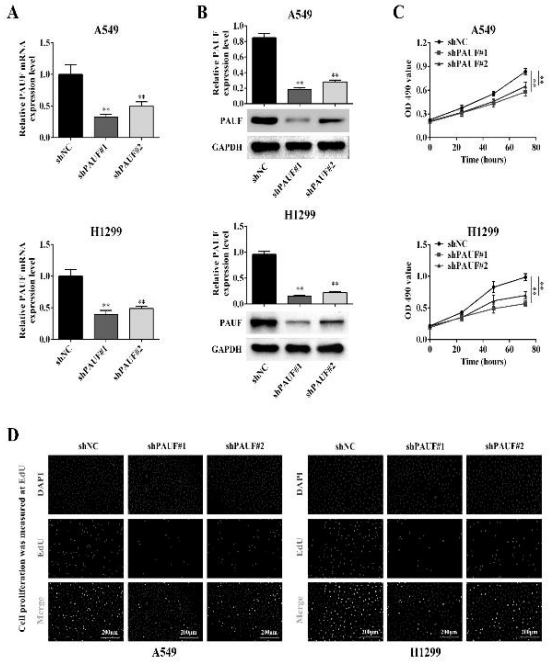

Figure 1: Knockdown of PAUF inhibited cell proliferation in H1299 and A549 cells. H1299 and A549 cells were transfected with shNC, shPAUF#1, or shPAUF#2. Then, the mRNA and protein levels of PAUF in H1299 and A549 cells were determined by qRT-PCR(**A**) and western blot (**B**). (**C**) The MTS assay results indicated that knockdown of PAUF decreased cell viability of H1299 and A549 cells. (**D**) The Edu staining assay results confirmed that cell proliferation was inhibited by knockdown of PAUF. Each experiment was repeated three times; $^{**}p < 0.01$; NC, negative control

Depletion of PAUF suppressed H1299 and A549 cell migration and invasion

To verify the function of PAUF on lung cancer migration and invasion, wound healing and transwell assays were performed. In the H1299 and A549 cells, knockdown of PAUF cells resulted in lower migration potential, compared with control cells (Figure 2 A). The transwell assay results indicated that knockdown of PAUF cells resulted in migration of fewer cells through the membrane in both the H1299 and A549 cells (Figure 2 B). These results indicated that depletion of PAUF suppressed H1299 and A549 cell migration and invasion.

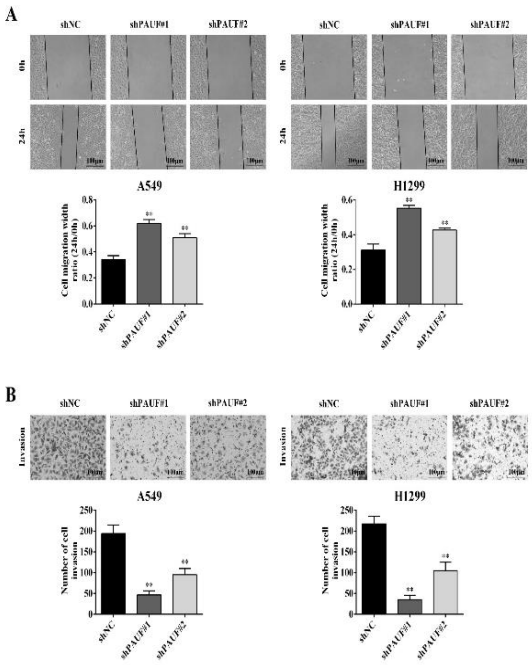

Figure 2: Depletion of PAUF suppressed H1299 and A549 cell migration and invasion. (**A**) The wound healing assay results indicated that knockdown of PAUF decreased cell migratory ability in H1299 and A549 cells. (**B**) The transwell assay results suggested that knockdown of PAUF reduced H1299 and A549 cell invasive ability. Each experiment was repeated three times; $^{**}p < 0.01$; NC, negative control

Knockdown of PAUF inhibited CSC phenotypes

Cancer stem cells are characterized by tumorsphere formation *in vitro*. Therefore, the tumorsphere formation assay was used to evaluate CSC phenotypes *in vitro*. The tumorsphere formation assay revealed that knockdown of PAUF in H1299 and A549 cells resulted in lower sphere-forming ability (Figure 3A). To further determine CSC characteristics of sphere-forming cells, expression of distinct lung CSC markers (a series of proteins that can identify CSCs), including CD133, CD44, ALDH1, SOX2, and Oct4, were evaluated using western blot assays. Knockdown of PAUF reduced expression of ALDH1, CD133, CD44, SOX2, and Oct4 in H1299 and A549 cells (Figure 3B). These findings indicated that knockdown of PAUF inhibited CSC phenotypes *in vitro*.

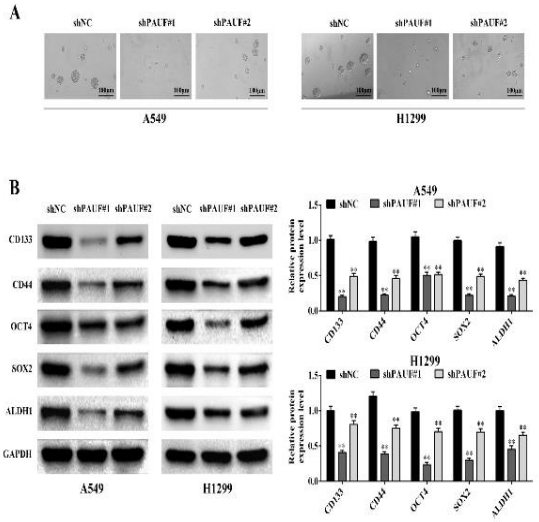

Figure 3: Knockdown of PAUF inhibited CSC phenotypes**.** (**A**) After transfection, sphere formation assays revealed that shPAUF H1299 and A549 cells had lower sphere-forming ability than shNC cells. (**B**) The CD133, CD44, OCT4, SOX2, and ALDH1 expressions were measured by western blot to identify CSCs in H1299 and A549 cells. Each experiment was repeated three times; $^{**}p < 0.01$; NC, negative control

Depletion of PAUF deactivated the FAK/Src/ERK signal pathway

The FAK/Src/ERK pathway has a role in tumor proliferation, invasion, and migration in many cancers. To elucidate the involvement of the FAK/Src/ERK pathway in PAUF-mediated cell migration and invasion, the expressions of key proteins were evaluated by western blot assays. The results revealed that depletion of PAUF reduced the levels of phosphorylated FAK, phosphorylated Src, and phosphorylated ERK, but not total FAK, Src, and ERK in H1299 and A549 cells (Figure 4). Based on the calculated ratios of pERK1/2:ERK1/2, pFAK:FAK, and pSrc:Src, PAUF knockdown significantly lessened the values of pERK1/2:ERK1/2, pFAK:FAK, and pSrc:Src, suggesting that the FAK/Src/ERK signal pathway was inactivated. Taken together, these results indicated that depletion of PAUF inactivated the FAK/Src/ERK signal pathway.

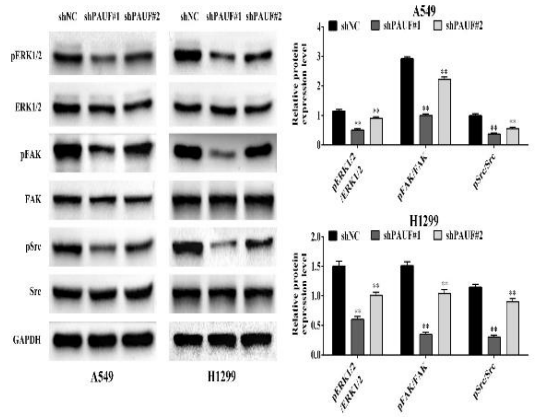

Figure 4: Depletion of PAUF inactivated the ERK/FAK signal pathway. Levels of pERK1/2, ERK1/2, pFAK, FAK, Src, and pSrc were determined by western blot in H1299 and A549 cells. The pERK1/2:ERK1/2, pFAK:FAK, and pSrc:Src ratios were calculated. Each experiment was repeated three times; $^{**}p < 0.01$; NC, negative control

DISCUSSION

In this study, we found that knockdown of PAUF inhibited A459 and H1299 cell proliferation, suppressed H1299 and A549 cell migration and invasion, and reduced the expression of distinct lung CSC markers, including ALDH1, CD133, CD44, SOX2, and Oct4, suggesting that PAUF knockdown inhibited CSC phenotypes. Finally, knockdown of PAUF reduced the levels of phosphorylated FAK, phosphorylated Src, and phosphorylated ERK but not the overall expression of FAK, Src, or ERK in H1299 and A549 cells. These findings indicated that knockdown of PAUF inactivated the FAK/Src/ERK signal pathway. Therefore, knockdown of PAUF may potentially serve as a new therapeutic strategy for lung cancer.

The novel gene PAUF is up-regulated in human pancreatic cancer cells [7]. In pancreatic cells, PAUF promotes expression of cyclin-D1, c-Jun, and β-catenin, leading to rapid cell proliferation. Conversely, knockdown of PAUF expression results in reduced proliferation [13]. Overexpression of PAUF induces cell activation and proliferation in ovarian cancer cells, whereas PAUF knockdown inhibits cell activation and proliferation [14]. In colorectal cancer cells, PAUF-small interfering (si)RNA inhibits proliferation, promotes apoptosis, and arrests cell division at the G0/G1 stage [15]. Therefore, PAUF has important roles in cancer cell proliferation. Investigations about the roles of PAUF in lung cancer are limited; however, this study found that knockdown of PAUF suppressed H1299 and A549 cell proliferation. This finding contributes new information that increases the understanding of PAUF in lung cancer.

Given that metastasis is a common malignant phenotype of lung cancer, previous research has focused on the mechanisms and related genes and proteins that might be important in lung cancer metastasis, but the process is not completely understood [16]. We found that knockdown of PAUF significantly reduced H1299 and A549 cell migration and invasion. Similarly, PAUF was found to enhance the migratory and invasive potential of pancreatic cancer cells by upregulating CXCR4 expression [8,15]. Our results were consistent with this finding. These results suggested that PAUF not only can affect the tumorigenesis of pancreatic cancer but also of lung cancer, and provides a novel direction for new therapeutic strategies for lung cancer.

During treatment with chemotherapy, while conventional cytotoxic therapies eliminate the bulk of tumor cells, CSCs that present with a resistant tumor phenotype continue to proliferate and survive [17]. These CSCs have special characteristics that allow self-renewal, differentiation, and tumor growth promotion, which can weaken the efficiency of chemotherapy treatment [6]. Elimination of CSCs in combination with conventional therapies may improve the efficacy of lung cancer treatment, which demonstrates the essential function of CSCs in lung cancer. Study findings also reveal a strong relationship between drug resistance and cellular expression of stem-like markers in lung cancer [18], and CD133, CD44, ALDH1, SOX2, and Oct4T have been indicated as markers for CSCs in lung cancer [19-22]. We found that PAUF knockdown reduced distinct lung CSC marker expression. This result suggested that PAUF expression was associated with CSC phenotypes. While the mechanism of PAUF in CSC phenotypes remains unknown, these findings are the first to reveal the association between PAUF and CSC phenotypes.

Previous study results have reported that PAUF elicits activation of the ERK, JNK, and AKT signaling cascades [8]. It is also positively correlated with activation and expression of FAK in pancreatic cancer cells [23]. Similarly, in this study, knockdown of PAUF inactivated the FAK/Src/ERK signal pathway. Intensive studies of the ERK pathway revealed it has vital roles in the molecular signaling network that modulates proliferation, growth, survival, and differentiation in lung cancer [24]. In addition, FAK and Src can form the FAK-Src complex, which is activated in

many tumor cells and produces signals resulting in tumor growth and metastasis [25]. Although we found that knockdown of PAUF inhibited lung cancer cell proliferation, migration, invasion, and CSC phenotypes via inactivation of the FAK/Src/ERK signal pathway, the potential mechanisms relevant to the FAK/Src/ERK signal pathways in lung cancer are complex and require more investigation in the future. This study, to the best of our knowledge, is the first to find that modulation of PAUF occurs in lung cancer, as well as pancreatic cancer cells. It can be hypothesized that PAUF also participates in or regulates tumorigenesis of multiple cancers. Therefore, investigations about the regulatory mechanisms of PAUF in other cancers are needed. Altogether, through investigating the molecular mechanisms of PAUF in lung cancer, this study provided novel insights that can be applied to development of therapies to treat lung cancer.

CONCLUSION

Knockdown of PAUF inhibits lung cancer cell proliferation, migration, invasion, and CSC phenotypes via inactivation of the FAK/Src/ERK signal pathway. The findings reveal the molecular regulatory mechanisms of PAUF in lung cancer tumorigenesis and thus, provides novel insights into the development of new therapeutics for lung cancer.

DECLARATIONS

Acknowledgement

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Competing interests

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article. The liabilities pertaining to claims relating to the content of this article will be borne by the authors. Liangchao Dong and Weiwei Li designed the study and supervised the data collection; Xiaoli Zhang analyzed the data; and Liangchao Dong, Weiwei Li, and Xiaoli Zhang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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