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Up-regulation of fatty acid synthase induced by EGFR/ERK activation promotes tumor growth in pancreatic cancer

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ABSTRACT

Lipid metabolism is dysregulated in many human diseases including atherosclerosis, type 2 diabetes and cancers. Fatty acid synthase (FASN), a key lipogenic enzyme involved in *de novo* lipid biosynthesis, is significantly upregulated in multiple types of human cancers and associates with tumor progression. However, limited data is available to understand underlying biological functions and clinical significance of overexpressed FASN in pancreatic ductal adenocarcinoma (PDAC). Here, upregulated FASN was more frequently observed in PDAC tissues compared with normal pancreas in a tissue microarray. Kaplan–Meier survival analysis revealed that high expression level of FASN resulted in a significantly poor prognosis of PDAC patients. Knockdown or inhibition of endogenous FASN decreased cell proliferation and increased cell apoptosis in HPAC and AsPC-1 cells. Furthermore, we demonstrated that EGFR/ERK signaling accounts for elevated FASN expression in PDAC as ascertained by performing siRNA assays and using specific pharmacological inhibitors. Collectively, our results indicate that FASN exhibits important roles in tumor growth and EGFR/ERK pathway is responsible for upregulated expression of FASN in PDAC.

1. Introduction

Pancreatic cancer is the fourth most common cause of cancer-related mortality in USA [1]. Although significant achievements are made in treatment and diagnosis, the average 5-year survival rate of pancreatic cancer patients remains at less than 6% [2]. Therefore, discovery of novel therapeutic targets for treatment with pancreatic cancer is urgently needed.

Fatty acids are the major sources of energy and make up crucial components of membrane lipids, which also function as cellular signaling molecules [3]. In normal humans, fatty acids are synthesized to store excess energy derived from the metabolism of glucose. However, numerous studies have shown that fatty acid metabolism is dysregulated in many human diseases, such as type 2 diabetes, metabolic syndrome, atherosclerosis and cancers [4–6].

Fatty acid synthase (FASN) is a proficient multifunctional enzyme that capable of *de novo* lipogenesis through converting acetyl-CoA and malonyl-CoA to palmitate [7]. In normal human tissues, FASN is expressed at minimal levels. Under pathological conditions, however, over-expression of FASN is closely associated with the development of cardiovascular disease, insulin resistance of type 2 diabetes and many types of cancers [8]. Numerous clinical and basic studies have demonstrated that the cellular functions and clinical significance of FASN [9–14]. Neoplastic lipogenesis enhanced by FASN provides to a selective growth and survival advantage and up-regulated FASN correlates with poor prognosis and disease recurrence in several types of tumor [13,15–21]. Pharmacological inhibitors of FASN inhibit DNA replication and induce apoptosis in tumor cells; inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells and delays disease progression in a xenograft model of ovarian cancer [22,23]. However, limited knowledge is known about the functions of FASN in pancreatic cancer and the reason for its dysregulation.

In this study, we determined the roles of FASN in pancreatic cancer. We observed that FASN is over-expressed in pancreatic cancer compared with normal pancreas. For the first time, this study showed that knockdown or inhibition of FASN dramatically inhibits cell proliferation and promotes cell apoptosis. We further demonstrated that EGFR/ERK pathway contributes to elevated expression of FASN. Collectively, our data suggests that up-regulation of FASN induced by EGFR/ERK signaling is a critical factor in progression of pancreatic cancer.

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2. Materials and methods

2.1. Immunohistochemistry

The tissue microarray (TMA) containing 81 cases of PDAC specimens and 44 cases of normal pancreatic tissues used in this study was purchased from Shanghai Outdo Biotech Inc. All study protocols in collection of clinical specimens were approved by the Ethics Committee for Clinical Research in Shanghai. The section was deparaffinized in xylene and rehydrated with graded ethanol. After rehydration, endogenous peroxidase was neutralized with 0.3% hydrogen peroxide. Antigen was then retrieved by incubating the slide in target retrieval solution. After washed with phosphate buffered saline (PBS) for three times, the sections were incubated with specific anti-FASN antibody (BD Biosciences) diluted in a working solution (1:100) overnight at 4 °C, and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody for 30 min. Positive staining was visualized with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. Scoring was conducted by percent of positive cells according to 4 categories, where 0 was given for 0–10%, 1 for 11–35%, 2 for 36–70% and for 71–100%. And the staining score was defined as follows: 0–1, low expression, and 2–3, high expression.

2.2. Cell culture, reagents and transfection

Human Capan-2, CFPAC-1, BxPC-3, HPAC, PANC-1, AsPC-1 and SW1990 cells were cultured in specific media supplemented with 10% (v/v) fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 units/ml penicillin. All cell lines were cultured at 37 °C in a humidified incubator with 5% CO_2. EGF and Orlistat were purchased from Sigma–Aldrich. Erlotinib and PD98059 were purchased from Selleck. PANC-1 cells were transfected a mix of three siRNA targeting FASN as well as a negative control (GenePharma, Shanghai, China). Knockdown of FASN or EGFR was achieved with RNA interference. Different FASN or EGFR specific siRNAs were designed and synthesized according to the manufacturer's instructions (GenePharma, Shanghai, China). The transient transfection was performed in HPAC and AsPC-1 cells and FASN protein expression was measured by the Western blotting to validate interfere efficiency.

2.3. Quantitative real-time PCR

Total RNA from PDAC cell lines was isolated using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed were performed using the PrimeScript RT-PCR kit (Takara, Japan) according to the manufacturer's protocol. Real-time PCR was performed using an ABI Prism 7500 Sequence Detection System with SYBR Green Master Mix (Takara, Japan). The primers used in this study were as follows. FASN: forward 5′-CGACAGCACCAGCTTCGCCA-3′, reverse 5′-CACGCTGGCCTGCAGCTTCT-3′; β-actin: forward 5′-CATGTACGTTGCTATCCAGGC-3′, reverse 5′-CTCCTTAATGTCACGCACGAT-3′. The comparative Ct method was used to determine the relative mRNA levels.

2.4. Cell proliferation and apoptosis assay

Cell proliferation was performed using MTT colorimetric assay. Briefly, HPAC and AsPC-1 cells with corresponding treatments (siRNAs or the specific inhibitor Orlistat) were seeded in 96-well plate at 3000 cells per well and cultured overnight followed by addition of MTT (5 mg/ml) to a final concentration of 0.5 mg/ml and incubation of the plate at room temperature for 3 h. The absorbance at 570 nm was measured using a Multifunctional Microplate Reader. Cell apoptosis was evaluated by Annexin V/PI assay (BD Biosciences) and caspase-3/7 activity (Promega) according to manufacturer's instructions, respectively. Notably, all experiments were performed when cells were starved under serum-free media or treatment with different concentrations of Orlistat for 48 h.

2.5. Western blotting

Cell protein was extracted using a total protein extraction buffer (beyotime, China). Protein concentration was measured using a BCA Protein Assay Kit (Pierce Biotechnology). Cell lysates were separated by 6% SDS-PAGE gel electrophoresis. After transfer to a PVDF membrane and blocking with 1% BSA, samples were probed with one of the following antibodies: FASN (BD Biosciences), EGFR (Santa Cruz Biotechnology Inc.), p-Erk and Erk (Cell Signaling Technology Inc.), followed by species-specific secondary antibodies. Bound secondary antibodies were detected by Odyssey imaging system (LI-COR Biosciences, Lincoln, NE).

2.6. Statistical analysis

Statistical analyses and graphical representations were performed with SPSS 18.0 statistic software (SPSS Inc, Chicago, IL) and GraphPad Prism 5 (San Diego, CA) software. The influence of FASN expression on clinicopathological parameters of PDAC was analyzed by chi-square test. Overall survival rate was calculated according to the Kaplan–Meier method and the difference in survival curves was evaluated by the log-rank test. For all tests, $P < 0.05$ was considered as statistical significance.

3. Results

3.1. Increased expression of FASN indicates a poor prognosis in PDAC

To observe the protein expression of FASN in PDAC and normal pancreas, a tissue microarray (TMA) containing 81 cases of PDAC specimens and 44 cases of normal pancreatic tissues was analyzed by immunohistochemistry. We found FASN immunoreactivity was mainly distributed in cytoplasm (Fig. 1A); FASN protein was over-expressed in 48 (59.3%) PDAC cases, while high FASN expression was detected in only 4 (9.1%) normal pancreas cases; the difference of FSAN expression between PDAC and normal pancreas was statistically significant. (Fig. 1B). Notably, FASN expression was significantly associated with tumor size ($p = 0.048$) and TNM stage ($p = 0.033$), there were no correlations between FASN protein expression and age ($p = 0.502$), gender ($p = 0.102$), tumor location ($p = 0.456$) and neuronal invasion ($p = 0.441$) (Table 1). To determine the potential prognostic value of FASN in PDAC, the relationship between FASN expression and corresponding clinical follow-up information was analyzed. Kaplan–Meier analysis showed that high FASN expression was correlated with a decreased overall survival (Fig. 1C). Collectively, these findings suggest that up-regulated FSAN was associated tumor progression and predicted a poor prognosis in PDAC patients.

3.2. Effect of FASN knockdown or inhibition on cellular functions of PDAC cells

Previously and currently studies have demonstrated that elevated FASN is associated with tumor progression and poor prognosis in patients with PDAC [24,25]. Given FASN is a key

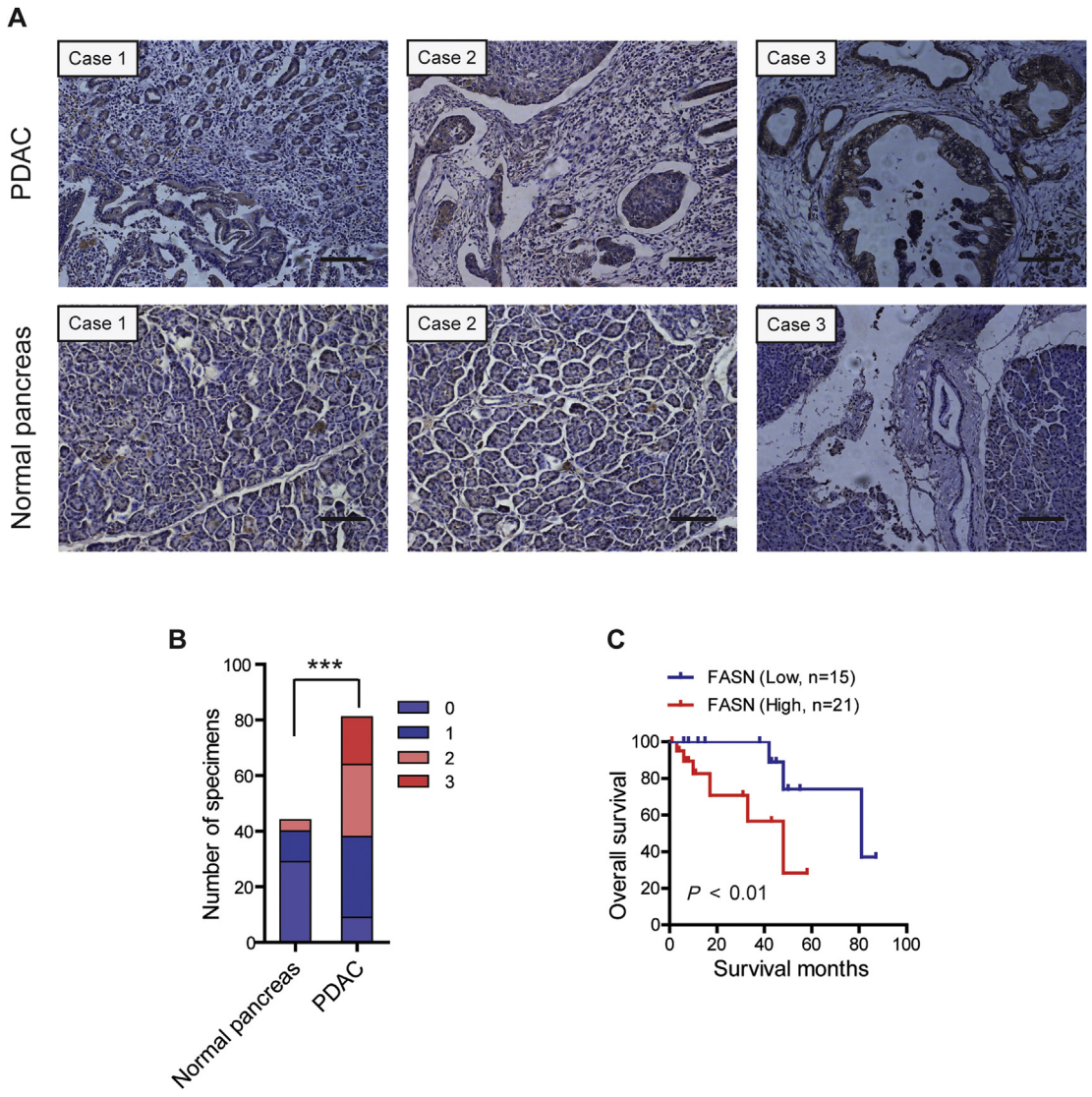

Fig. 1. Increased expression of FASN indicates a poor prognosis in PDAC. (A) Representative images of the FASN expression in PDAC and normal pancreas, scale bar: 100 μm. (B) Immunohistochemical analysis of FASN expression in a tissue microarray (***, $P < 0.001$). 0–3 indicates the scale of FASN expression levels. (C) Kaplan–Meier curves for PDAC patients grouped based on FASN expression in TMA2.

enzyme involved in synthesis of palmitate and lipogenesis, we hypothesized whether there is a correlation between FSAN and tumor growth. To confirm this hypothesis, we first detected the mRNA expression of FASN in seven PDAC cell lines (Fig. 2A). Then cellular functions of HPAC and AsPC-1 cells, two cell lines with higher FASN expression, were measured after transfected with three different siRNAs targeting FASN. The protein expression of FASN was markedly reduced after siRNAs treatment as evaluated by Western blotting (Fig. 2B). Next, we examined the effect of FASN knockdown on cell viability and cell apoptosis. As shown in Fig. 2, silencing of FASN significantly suppressed cell viability compared with the negative control cells (Fig. 2C, D). Cell apoptosis was measured by Annexin v/PI staining and Caspase-3/7 activity, respectively. The results showed that the ratio of apoptotic cells and caspase-3/7 activity were significantly increased after FASN knockdown (Fig. 2C, D). Meanwhile, we determined whether inhibiting FASN using its specific inhibitor orlistat also simulates these effects. As shown n Fig. 3, cell viability measured at 72 h was significantly deceased compared with normal control in a dose dependent manner. Similarly, the ratio of apoptotic cells and caspase-3/7 activity were also increased by orlistat treatment in a dose dependent manner. Taken together, inhibition of FASN expression by siRNAs or its function using pharmaceutical inhibitor suppresses tumor cell growth of pancreatic cancer.

Table 1
Correlations between FASN expression and clinicopathologic features in patients with PDAC.

Variable		Expression of FASN		*P* value
		Low (n = 33)	High (n = 48)	
Age	≤60 years	14	24	0.502
	>60 years	19	24	
Gender	Female	17	16	0.102
	Male	16	32	
Tumor location	Head	23	37	0.456
	Body + tail	10	11	
Tumor size	≤4 cm	25	26	0.048*
	>4 cm	8	22	
TNM stage	I	21	19	0.033*
	II–III	12	29	
Neuronal invasion	Yes	15	26	0.441
	No	18	22	

Asterisk represents the *P*-values with significant differences.

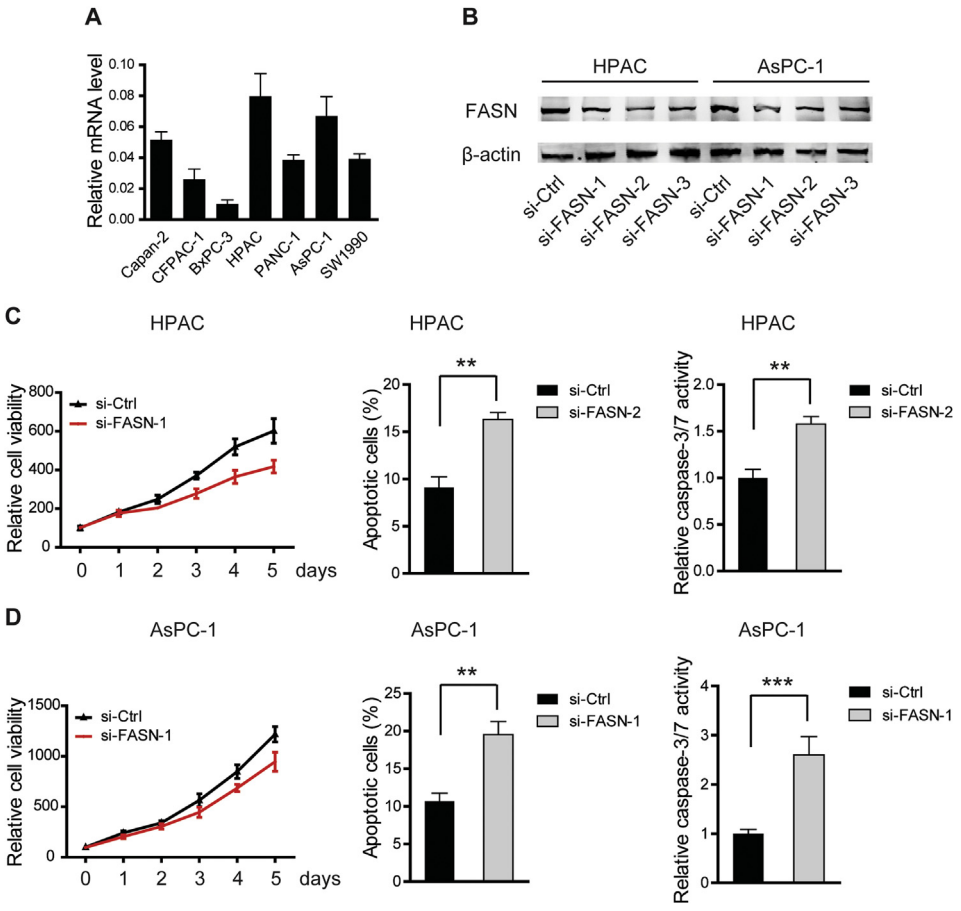

Fig. 2. Effect of FASN knockdown on cellular functions of PDAC cells. (A) Relative mRNA expression of FASN in PDAC cell lines. (B) The protein level of FASN was detected by Western blotting after treatment with targeted siRNAs. (C) Cell viability, cell apoptosis ratio and caspase-3/7 activity of HPAC cells were detected after FASN was silenced (si-Ctrl versus si-FASN-2; **, $P < 0.01$). (D) Cell viability, cell apoptosis ratio and caspase-3/7 activity of AsPC-1 cells were detected after FASN was silenced (si-Ctrl versus si-FASN-2; **, $P < 0.01$; ***, $P < 0.001$).

3.3. Activation of EGFR signaling contributes to elevated FASN expression in PDAC

In PDAC, the reason for elevated FASN remains unclear. Previously study has demonstrated that G protein-coupled estrogen receptor (GPER) mediates the up-regulation of FASN induced by 17β-Etradiol in cancer cells and cancer-associated fibroblasts and GPER mediates estrogen signals activating the epidermal growth factor receptor (EGFR)/ERK/AP1 transduction pathway [26]. EGFR is up-regulated in PDAC and its functions primarily have been closely associated with enhanced proliferation and invasiveness of PDAC cells [27,28]. Here we examined whether EGFR signaling contributes to increased FASN expression. Indeed, EGF significantly stimulates the protein expression of FASN and phosphorylation of Erk1/2; silencing EGFR in CFPAC-1 and BxPC-3 cells, two cell lines with lower FASN expression, EGF failed to induce FASN expression, suggesting that activation of EGFR signaling mediates this effect (Fig. 4A–D). Treatment with the EGFR inhibitor Erlotinib as well as the MEK inhibitor PD98059, EGF did not promote FASN protein expression (Fig. 4C, D). Taken together, these data indicate that the EGFR/ERK pathway mediates the expression of FASN induced by EGF in our current study.

4. Discussion

Alternations in cancer metabolism have long been recognized. The relationship between fatty acids and cancer progression was supported by numerous studies that cancer patients had an increase in metabolic rate and fatty acid oxidation [29,30]. FASN, a key lipogenic enzyme, is over-expressed in many human cancers including carcinomas of the colon [31–33], ovary [18], prostate [17], kidney [34], endometrium [35]. Specifically, up-regulated FASN exhibits a protective role against hypoxic conditions and harsh microenvironments, thus providing an advantageous to cancer cell growth and survival.

In the current study, we firstly observed that FASN expression was over-expressed in pancreatic cancer compared with normal pancreas. Meanwhile, Kaplan–Meier analysis indicated that patients with high FASN expression are associated with poor prognosis. Consistent with previous studies, these observations indicate that FASN may function as oncogene in pancreatic cancer during progression [24]. Previously, it has been demonstrated that FASN is an oncogenic stimulus for human cancer cell proliferation and suppressing its expression or inhibiting its function induces pronounced growth arrest and cell apoptosis [33,36]. These results indicate that FASN may be acted as a target for pancreatic cancer therapy.

Next, the cellular functions of FASN were analyzed in pancreatic cancer cell lines. Consistent with the result that FASN is closely associated with tumor size, we observed that knockdown or inhibition of FASN results in decreased cell viability and increased cell apoptosis in pancreatic cancer cell, thus promoting cell proliferation. Because over-expressed FASN favors fatty acid synthesis, which not only provides a source of energy but also constitutes a part of cell membrane and cellular signaling to cancer cells,

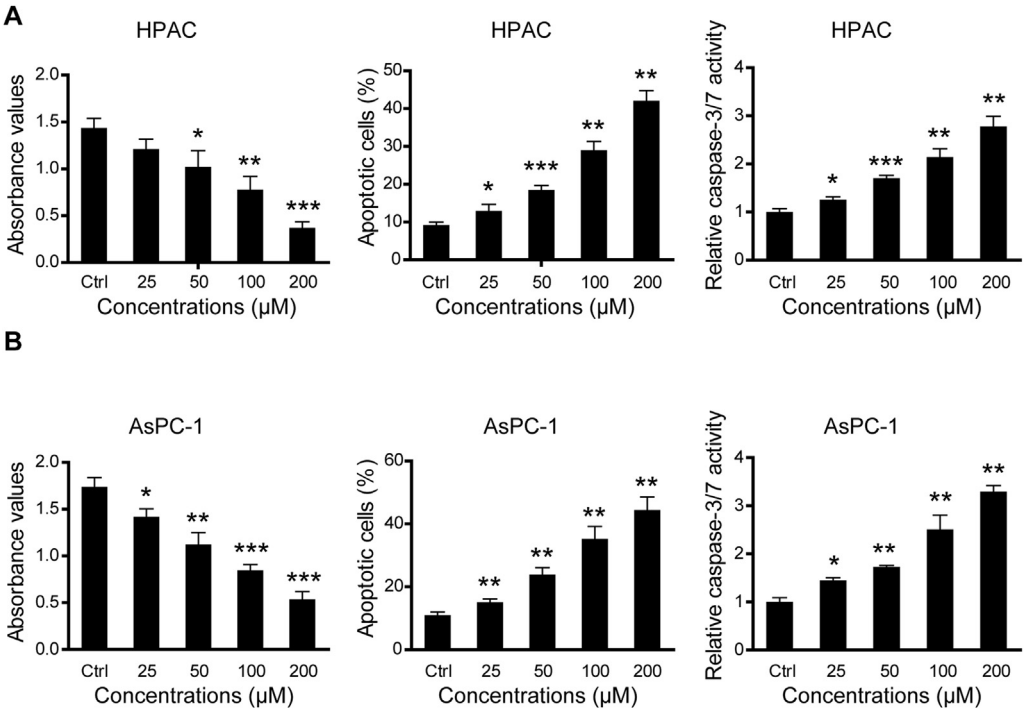

Fig. 3. Effect of FASN inhibition on cellular functions of PDAC cells. (A) Cell viability, cell apoptosis ratio and caspase-3/7 activity of HPAC cells were detected after FASN was inhibited (si-Ctrl versus orlistat; *, $P < 0.05$ **, $P < 0.01$; ***, $P < 0.001$). (B) Cell viability, cell apoptosis ratio and caspase-3/7 activity of AsPC-1 cells were detected after FASN was inhibited (si-Ctrl versus orlistat; *, $P < 0.05$ **, $P < 0.01$; ***, $P < 0.001$).

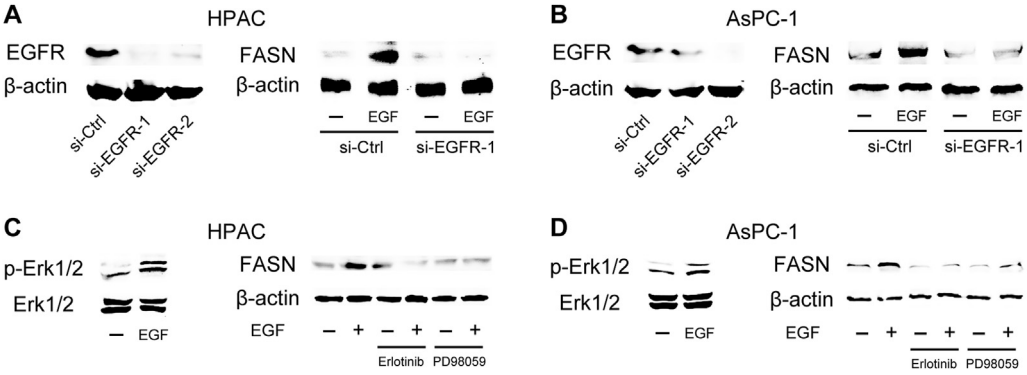

Fig. 4. Activation of EGFR signaling contributes to elevated FASN expression in PDAC. Increased FASN protein expression induced by EGF stimulation was reduced by EGFR knockdown in HPAC cells (A) and AsPC-1 cells (B). Increased FASN protein expression induced by EGF stimulation was reduced by Erlotinib and PD98059 treatment in HPAC cells (C) and AsPC-1 cells (D), respectively.

therefore providing cancer cells with advantages of growth and related mechanisms, and confirming that it would be beneficial to develop therapeutics targeting FASN.

When it comes to the regulation of over-expressed FASN, growth factors and the PI3K/AKT signaling were associated with modulation of FASN expression [37–39]. Given the crucial roles of EGFR in pancreatic cancer, whether the EGFR signaling accounts for FASN expression remains to be clarified. In this study, we demonstrated that EGF mediated activation of EGFR dramatically increased the expression of FASN and this effect was blocked by EGFR/ERK pathway induced by RNA interference or pharmaceutical inhibition. It implies that EGFR/ERK pathway can effectively regulate the expression of FASN in pancreatic cancer cells. Consistent with our results, Santolla et al. have demonstrated that up-regulation of FASN induced by 17β-Etradiol in cancer cells was mediated G protein-coupled estrogen receptor [26]. However, whether other signaling pathway involved in the regulation of FASN remains to be further explored.

In conclusion, our study demonstrated that FASN expression status can serve as an indicator for decreased survival. Meanwhile, we first identified that suppression of FASN results in growth arrest and EGFR/ERK pathway is responsible for elevated FASN expression. Therefore, FASN may be an important clinical marker and target of treatment for pancreatic cancer.

Conflict of interest

The authors declare that there is no conflict of interests.

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Transparency document

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