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Fyn stimulates the progression of pancreatic cancer *via* Fyn-GluN2b-AKT axis

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Abstract. – OBJECTIVE: To assess the expression levels of Fyn in human tissue samples and pancreatic cancer cells and explore the potential mechanisms of Fyn in pancreatic cancer progression.

MATERIALS AND METHODS: Quantitative PCR and immunohistochemical (IHC) assays were performed to detect the expression of Fyn in 30 cancer tissue samples from pancreatic cancer patients and corresponding adjacent normal tissues. In addition, the potential correlations between Fyn expression levels and clinical pathological features were assessed. We further detected the effects of Fyn on the proliferation, apoptosis, migration, and invasion of the pancreatic cancer cells through colony formation assay, flow cytometry (FCM) assay, wound healing assay, and transwell assay, respectively. The potential effects of Fyn on tumor growth were assessed using an animal model.

RESULTS: We demonstrated the possible involvement of Fyn in the progression of pancreatic cancer. We found that Fyn was upregulated in human pancreatic cancer tissues and cells, and we analyzed the correlations between Fyn expression and the clinicopathological features, including metastasis staging (p=0.010*) and tumor size (p=0.025*) of patients with pancreatic cancer. Our data further confirmed that Fyn affects cell proliferation, apoptosis, migration, and invasion of pancreatic cancer cells *via* the phosphorylation of GluN2b and regulation of AKT signaling pathway. We also demonstrated that Fyn promoted tumor growth of pancreatic cancer cells *in vivo*.

CONCLUSIONS: We investigated the potential involvement of Fyn in the progression of pancreatic cancer, and therefore indicated Fyn as a possible therapeutic target for pancreatic cancer.

Key Words:
Pancreatic cancer, Fyn, Proliferation, Migration, Therapeutic target.

Introduction

Pancreatic cancer is known as the most lethal malignant tumor[1]. In decades, unlike the improvement in the survival rate for most types of cancers, the 5-year total survival rate for patients with pancreatic cancer is still less than 8%, and only 2% for advanced pancreatic cancer patients[2,3]. Approximately 90% of pancreatic cancers are ductal adenocarcinoma originating in the epithelium, and metastasis is the major cause of death in pancreatic cancer patients[4,5]. The main treatments for pancreatic cancer include surgery, chemotherapy, and cyberknife therapy. However, these treatments have little effect on pancreatic cancer[6]. In recent years, targeted therapy and immunotherapy have played an important role in the treatment of pancreatic cancer[7,8]. However, to effectively treat this malignant tumor, new therapeutic targets are still urgently needed.

SRC family kinases (SFKs) are a series of non-receptor protein tyrosine kinases, such as SRC, FRK, and FYN[9,10]. SFKs can integrate multiple signaling pathways to mediate cellular processes, such as cell proliferation, migration, and differentiation[11]. Of note, previous studies[12,13] provided evidence that SFKs are involved in the progression of a variety cancer types, such as bladder cancer and lung cancer. As a member of SFKs, Fyn is also overexpressed in various types of cancers[14]. Fyn is a 59 kDa protein which contains 4 domains, including SH1-4[15]. It was reported that Fyn affected the progression of Alzheimer's disease *via* the regulation of Aβ production, and also mediated the development and signal transduction[16]. Notably, Fyn may affect the progression and development of multiple cancers, such as breast cancer and prostate cancer[17,18]. However, the possible role of Fyn in the pathogenesis of pancreatic cancer remains unclear.

AKT is a serine/threonine protein kinase involved in the regulation of apoptosis, proliferation, differentiation, and other cellular activities[19]. AKT itself can be phosphorylated by a variety of regulatory proteins, thereby activating itself and leading to tumor development[20]. N-methyl-d-aspartic acid receptor (NMDAR) can be coupled

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with ion channels to form a receptor channel complex, which can affect tumor progression through downstream signal transduction. GluN2b, as a member of NMDARs, has been shown to regulate the phosphorylation of AKT, and the potential role of this phosphorylation process in tumors, especially pancreatic cancer, is still unknown[21].

In this study, we demonstrated the possible involvement of Fyn in the progression of pancreatic cancer. We found that the expression of Fyn was upregulated in human pancreatic cancer tissues and cells, and also investigated the correlations between Fyn expression and the clinicopathological features of patients with pancreatic cancer. Our data further confirmed that Fyn affects cell proliferation, apoptosis, migration, and invasion of pancreatic cancer cells *in vitro via* the phosphorylation of GluN2b and the regulation of AKT signaling pathway. We also demonstrated that Fyn promoted tumor growth of pancreatic cancer cells *in vivo*. These results therefore provide a promising therapeutic target for the treatment of pancreatic cancer.

Materials and Methods

Antibodies, Plasmids, and Primers

Rabbit anti-Fyn antibody (For IHC assays, 1:200 dilution; for Immunoblot assays, 1:2000 dilution, ab184276, Abcam, Cambridge, MA, USA), rabbit anti-MCM2 antibody (1:2000 dilution, ab4461, Abcam, Cambridge, MA, USA), rabbit PCNA antibody (1:1500 dilution, ab92552, Abcam, Cambridge, MA, USA), rabbit Bcl-2 antibody (1:1000 dilution, ab32124, Abcam, Cambridge, MA, USA), rabbit cleaved caspase-3 antibody (1:1000 dilution, ab2302, Abcam, Cambridge, MA, USA), rabbit anti-AKT antibody (1:2000 dilution, ab18785, Abcam, Cambridge, MA, USA), rabbit anti-AKT (phospho T308) antibody (1:1000 dilution, ab38449, Abcam, Cambridge, MA, USA).

The quantitative PCR primer sequences of Fyn are as follows: forward, 5'-GTCATGGCAACCCGCTAAAC-3' and reverse, 5'-CACTGGAGAACTCCTGGACAT-3'. The quantitative PCR primer sequences of GAPDH are as follows: 5'-CGACCACTTTGTCAAGCTCA-3' and reverse, 5'-GGTTGAGCACAGGGTACTTTATT-3'.

pcDNA3.1-vector and pcDNA3.1-Fyn plasmids, Fyn shRNA plasmids (Ready-to-package AAV), and GluN2b shRNA plasmids were all bought from Addgene, MA, USA.

Immunohistochemistry

Tumor tissues were obtained from The First Hospital of Jilin University. To explore the expression levels of Fyn and GluN2b phosphorylation in human pancreatic cancer tissues, we performed immunohistochemistry (IHC) assays. Briefly, the sections were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature and subsequently blocked with 2% bovine serum albumin (BSA) for 20 min. The slides were subsequently incubated with Fyn or phosphorylated GulN2b antibodies at room temperature for 2 h. Then, the sections were incubated with biotinylated secondary antibody for 1.5 h, and diaminobenzidine was used as a chromogen substrate.

Cell Culture and Transfection

The indicated cells lines: HPDE6-C7, QGP1, PANC-1, BxPC-3, SW1990, were all purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and maintained in Dulbecco's Modified Eagle's Medium (DMEM) culture medium, supplemented with 10% of fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator.

The shRNA plasmids were transfected into pancreatic cancer cells by the use of Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA), following the manufacturers' protocols. Stable knockdown cell clones were screened by adenovirus infection and used for the *in vivo* assays.

Quantitative PCR Assays

TRIzol (15596026, Invitrogen, Carlsbad, CA, USA) reagent was used to extract total mRNA from human pancreatic cancer cells or tissues. Subsequently, the RNA was reverse transcribed into cDNA by reverse transcriptase (M1701, Promega, Madison, WI, USA). Quantitative PCR was performed using SYBR Ex Taq kit (638319, TaKaRa, Otsu, Shiga, Japan), and the expression levels of Fyn were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunoblot Assays

Cells and tissues were first lysed by lysis buffer. Then, the total protein samples were separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred onto the polyvinylidene difluoride (PVDF) membranes, the membranes were blocked with 5% fat-free milk in Tris-Buffered Saline and Tween-20 (TBST)

and incubated with the primary antibodies for the detection of Fyn, GluN2b, AKT, MCM2, PCNA, Bcl-2, cleaved caspase-3, and the phosphorylated GluN2b and AKT at room temperature for 2 h. Then, PVDF membranes were washed with TBST for 4 times and after washing, the PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugate secondary antibodies for 45 min. After washing, signals were detected using an enhanced chemiluminescence (ECL) kit.

Colony Formation Assays

A total number of 1000 cells were seeded into a 6-well culture plate and transfected with shRNA plasmids. Then, the cells were maintained in a 3°C, 5 % CO_2 incubator for 24 h. After cultured for 2 weeks, cells were fixed with paraformaldehyde (PFA) for 20 min at room temperature, stained with 0.2% crystal violet buffer for 30 min, and washed with phosphate-buffered saline (PBS) twice. Next, the colonies were photographed, and the numbers were manually counted.

MTT Assays

Pancreatic cancer cells were seeded into 96-well plates, transfected with control or Fyn shRNA plasmids, and maintained for 48 h. Pancreatic cancer cells were then incubated with MTT agent for 4 h and washed with PBS twice. Then, the OD value was measured respectively with a microplate reader at 562 nm wavelength.

Wound Healing Assays

Both QGP1 and PANC-1 cells were transfected with the plasmids, maintained for 48 h, and mechanically wound was made using a 20-μL pipette tip. Subsequently, cancer cells were washed with PBS to remove debris, and the complete culture medium was added to stimulate wound healing. Photographs were respectively taken at 0 h and 24 h, and the relative extent of wound healing was calculated.

Transwell Assays

Pancreatic cancer cells were transfected with plasmids for 48 h and re-suspended in serum-free DMEM culture medium. The upper chambers of filters (8.0 μm membrane pores) were containing 20% matrigel (in DMEM medium) and incubated at 37°C for 30 min. Approximately 105 cells in 200 μL of medium were then seeded into the upper chambers of the inserts and induced to migrate toward the bottom chambers containing complete medium. After 24 h, cells in the top chamber were removed using cotton swabs, and the remaining cells were fixed in 4% PFA at room temperature for 20 min, stained with 0.2% crystal violet buffer for 30 min. Then, the relative cell number was manually counted.

Tumor Growth Assays

All animal assay processes in this study were approved by the Ethics Committee of The First Hospital of Jilin University (Approval no. 2017036). QGP1 cells were infected with control or Fyn shRNA adenovirus to stably deplete proteins. After infection, approximately 5×10^5 control or Fyn depleted QGP1 cells were subcutaneously implanted into athymic nude mice to induce tumor formation. After 1 week, mouse weight was measured, tumor volume was also measured every week. After 4 weeks, all tumors were collected from mice.

Statistical Analysis

GraphPad 6.0 was used for statistical analysis in this study. All data were represented as mean ± SEM. Additionally, the correlation analysis between clinical pathological features and Fyn expression was performed through χ^2-analysis. Kaplan-Meyer analysis was performed to evaluate the prognosis. Student's *t*-test was used for statistical comparisons. *indicates $p<0.05$, and **indicates $p<0.01$.

Results

Fyn is Abnormal High Expression in Human Pancreatic Cancer Tissues

To explore the role of Fyn in the progression of pancreatic cancer, we first assessed its mRNA and protein expression levels in human pancreatic cancer tissues. A total of 30 pancreatic cancer patients were enrolled in the study, and the mRNA levels or protein expression levels of Fyn in tumor tissues or corresponding normal tissues were detected through quantitative PCR assays, immunoblot assays, and IHC assays, respectively. The results showed that the expression of Fyn mRNA was higher in tumor tissues, compared to adjacent normal tissues (Figure 1A). To further detect the expression difference of Fyn between tumor tissues and normal tissues, we performed IHC assays. As expected, the IHC assay results demonstrated that the expression of Fyn was much higher in human pancreatic cancer tissues, while the expression levels of Fyn in normal tissues were lower than tumor tissues (Figure 1B, C).

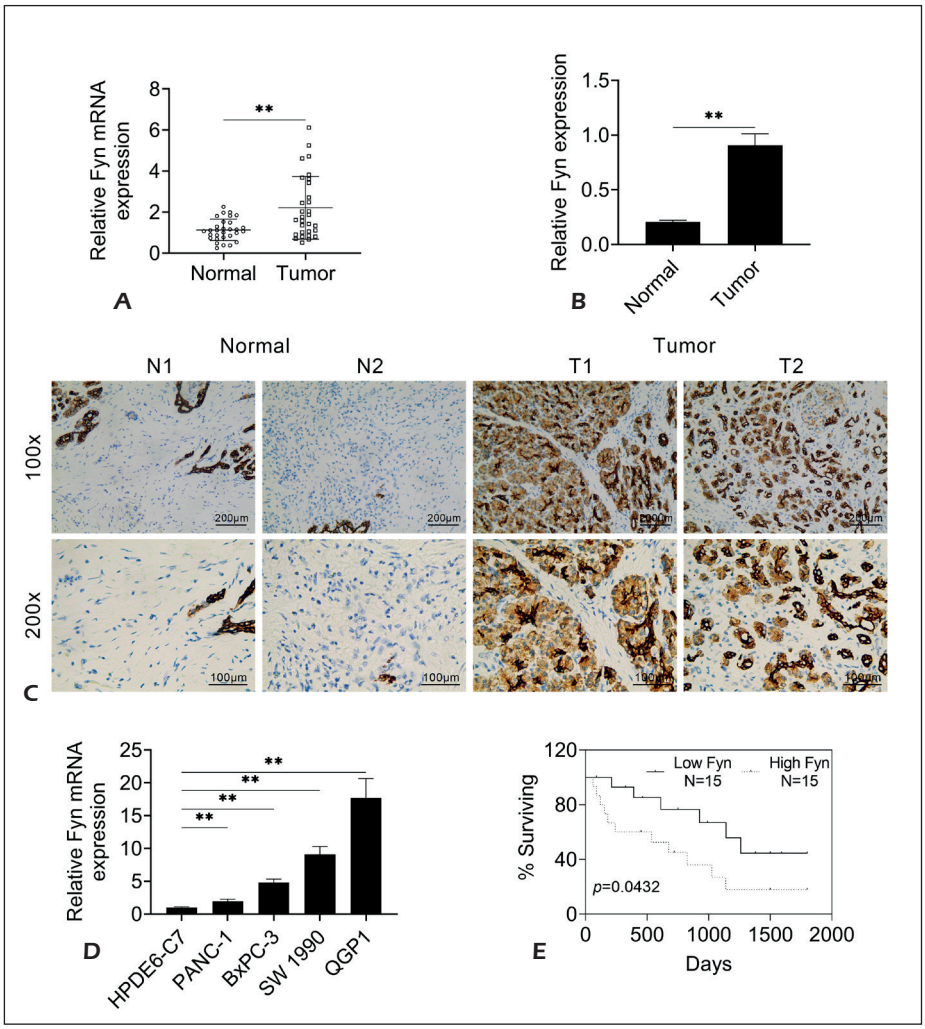

Figure 1. Fyn was highly expressed in human pancreatic cancer tissues and cells and correlated with the prognosis of patients. **A**, Quantitative PCR assays revealed the obviously increased mRNA levels of Fyn in 30 human pancreatic cancer tissues. **B**, Immunoblot assays exhibited the Fyn expression difference between pancreatic cancer tissues and normal tissues. **C**, Immunohistochemical assays were performed, and the representative photographs of Fyn expression in pancreatic cancer tissues and the corresponding normal tissues were showed (magnification 100× and 200×, respectively). **D**, Immunoblot assays indicated the expression of Fyn in HPDE6-C7, PANC-1, BxPC3, SW1990, and QGP-1 cells. E, KM-Plot analysis of survival rates between Fyn low and high expression patients was exhibited.

Subsequently, we revealed the mRNA levels of Fyn in normal pancreatic cell lines, HPDE6-C7, and pancreatic cancer cell lines, including PANC1, BxPC3, SW1990, and QGP1. Consistent with the results in pancreatic tumor tissue, we found that Fyn was significantly upregulated in pancreatic cancer cells, compared with normal pancreatic cells (Figure 1D). Therefore, these results demonstrated that the expression of Fyn was upregulated in both pancreatic tumor tissue and pancreatic cancer cell lines.

Fyn Expression is Associated With the Clinicopathological Characteristics and the Prognosis of Patients with Pancreatic Cancer

According to the expression levels of Fyn mRNA in tumor tissues of 30 patients with pancreatic cancer, the patients were divided into Fyn low expression or high expression groups. 15 of patients (50%) exhibited Fyn high expression, and the remaining showed low expression (Table I). We then performed clinical pathological characteristics analysis to explore the role of Fyn in cancer progression.

We found there was no significance between Fyn expression and clinical parameters such as patient sex (p=0.713), TNM staging (p=0.129), and differentiation (p=0.082, Table I). By comparison, we noticed that Fyn expression was markedly correlated with clinicopathological features, including metastasis staging (p=0.010*) and tumor size (p=0.025*, Table I).

Through K-M survival analysis, we found that Fyn expression was also associated with survival rates of 30 patients with pancreatic cancer (p=0.0432, Figure 1E), suggesting the correlation of Fyn expression and the prognosis of patients with pancreatic cancer.

Table I. Relationship between Fyn expression and the clinical pathological characteristics of patients with pancreatic cancer.

Parameters	No. of patients	Fyn mRNA expression		*p*-value*
		Low (< median)	High (≥ median)	
Patients	30	15	15	
Sex				0.713
Male	17	8	9	
Female	13	7	6	
TNM staging				0.129
T1	4	3	1	
T2	10	6	4	
T3	6	4	2	
T4	10	2	8	
Staging				0.010*
Non-metastasis	17	12	5	
Metastasis	13	3	10	
Differentiation				0.082
High	3	2	1	
Middle	15	10	5	
Low	12	3	9	
Tumor size				0.025*
≤3 cm	12	9	3	
>3 cm	18	6	12	

Fyn Affects the Proliferation, Apoptosis, Migration, and Invasion of Pancreatic Cancer Cells In Vitro

To further explore the possible role of Fyn in the progression of pancreatic cancer, we downregulated Fyn by its shRNAs and overexpressed it by using its plasmids in pancreatic cancer cells and then detect the related effects *in vitro*. Quantitative PCR assays confirmed the evident decrease of Fyn expression caused by the transfection of three different shRNA plasmids targeted Fyn in QGP1 cells (Figure 2A, left). Meanwhile, transfection of pcDNA3.1-Fyn significantly increased the mRNA levels of Fyn in PANC-1 cells, compared with pcDNA3.1-vector-transfected cells (Figure 2A, right).

Then, we conducted MTT assays and colony formation assays to investigate the impact of Fyn on cell proliferation. MTT assay results showed that Fyn depletion by its shRNA plasmids markedly decreased the proliferation of QGP1 cells, whereas overexpression of Fyn promoted cell proliferation in PANC-1 cells (Figure 2B). Colony formation assays results showed that Fyn ablation decreased colony numbers, while its over-expression markedly increased the relative colony numbers in QGP1 and PANC-1 cells, respectively (Figure 2C). Immunoblot also confirmed that Fyn expression was positively correlated with the expression of MCM2 and PCNA, two proliferation related cell markers, in QGP1 or PANC-1 cells (Figure 2F). These results demonstrated that Fyn played a promotion role in the proliferation of pancreatic cancer cells *in vitro*.

Furthermore, we performed wound healing assays and transwell assays to detect its possible effect of Fyn on the migration and invasion of pancreatic cancer cells. Of note, we found that downregulation of Fyn remarkably inhibited the migration and invasion of QGP1 cells (Figure 2D and 2E, left), indicated by wound healing and transwell assays. Overexpression of Fyn stimulated the migration and invasion of PANC-1 cells, with the increased migration rates and invasion rates, respectively (Figure 2D and 2E, right).

We next detected the effect of Fyn on the apoptosis of pancreatic cells, the results demonstrated that the apoptosis of QGP1 pancreatic cancer cells was enhanced by the transfection of Fyn shRNA plasmids, while the suppression of apoptosis was observed in Fyn-overexpressed PANC-1 cells, suggesting the inhibition role of Fyn on pancreatic cancer apoptosis (Figure 2G). Also, through immunoblot assays, we found an evident decrease of Bcl-2 and increase of cleaved caspase-3 in Fyn-depleted QGP1 cells, and the opposite change in Fyn-overexpression PANC-1 cells (Figure 2H).

Therefore, our *in vitro* data have demonstrated the critical role of Fyn on pancreatic cancer cell proliferation, apoptosis, migration, and invasion.

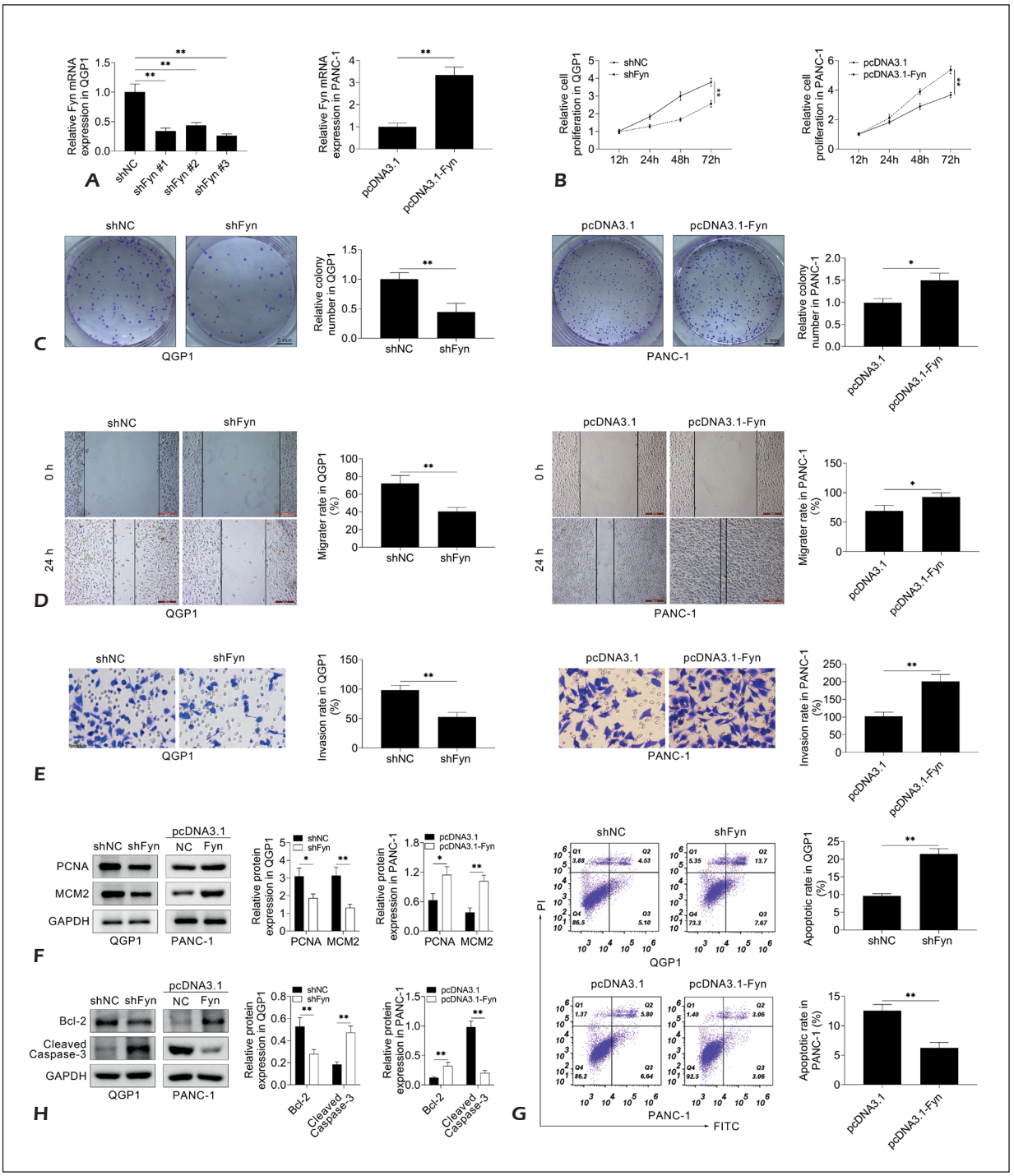

Figure 2. Fyn promotes the proliferation, migration, and invasion, and suppresses the apoptosis of pancreatic cancer cells in vitro. **A**, Quantitative PCR assays revealed the significantly dropped expression levels of Fyn caused by the transfection of three different shRNA plasmids targeted Fyn in QGP1 cells (left), and the increased Fyn mRNA levels caused by the transfection of pcDNA3.1-Fyn plasmids in PANC-1 cells (right). **B**, Results of MTT assays showed the inhibition of cell proliferation caused by Fyn ablation and promotion of cell proliferation caused by the overexpression of Fyn, in QGP1 and PANC-1 cells, respectively. **C**, Representative photographs showed the results of colony formation assays of QGP1 or PANC-1 cells transfected with the indicated plasmids. The relative proliferation levels were quantified. Scale bar, 5 mm. **D**, Wound healing assays were performed using QGP1 or PANC-1 cells transfected with the indicated plasmids, and the relative wound width was detected (200× magnification). **E**, Transwell assays using QGP1 or PANC-1 cells transfected with the indicated plasmids were performed and the extent of relative transwell migration was quantified (400× magnification). **F**, Immunoblot assays indicated the expression level of GAPDH, PCNA, and MCM2 in QGP1 or PANC-1 cells transfected with the indicated plasmids. **G**, Cell apoptosis assays showed the difference of apoptosis levels of QGP1 or PANC-1 cells transfected with the indicated plasmids. **H**, Immunoblot assays indicated the expression level of GAPDH, Bcl-2, and cleaved caspase-3 in QGP1 or PANC-1 cells transfected with the indicated plasmids. Results are presented as mean ± SEM, *p<0.05, **p<0.01.

Fyn Mediates AKT Signaling Pathway Through the Regulation of GluN2b Phosphorylation

To study the molecular mechanism of Fyn affecting the progression of pancreatic cancer *in vitro*, we focused on GluN2b, a protein kinase. GluN2b is known to affect the occurrence and development of a variety of tumors[6,22], and it has been reported that Fyn can regulate the phosphorylation of GluN2b, thereby affecting the development of tumors. Notably, through immunoblot assays, we did not find difference of GluN2b expression between control and Fyn depleted QGP1 cells, whereas the expression of phosphorylated GluN2b was decreased by the depletion of Fyn in QGP1 cells (Figure 3A). Meanwhile, overexpression of Fyn induced the up-regulation of GluN2b phosphorylation in PANC-1 cells (Figure 3B). We therefore demonstrated that Fyn could regulate the phosphorylation of GluN2b in pancreatic cancer cells.

It has been known that GluN2b is an upstream regulatory protein of the AKT signaling pathway[21], so we next examined whether the regulation of Fyn on the phosphorylated GluN2b affect the AKT signaling pathway. We tested this hypothesis through immunoblot assays. According to the results, we found that Fyn depletion led to the significant decrease of AKT phosphorylation in QGP1 cells, whereas the expression of total AKT was not affected (Figure 4A). Similarly, Fyn overexpression in PANC-1 cells also resulted in the increase of AKT phosphorylation and GluN2b phosphorylation, with the modest change of total AKT expression (Figure 4B). Thus, we demonstrated that Fyn mediated AKT signaling pathway through the regulation of GluN2b phosphorylation.

Fyn Contributes to the Progression of Pancreatic Cancer Via Fyn-GluN2b-AKT Axis

As was known that AKT signaling pathway widely participates in the progression and development of various types of tumors, and here we found the regulation of Fyn on AKT pathway through GluN2b[19]. Therefore, we further detected whether Fyn affects the proliferation, apoptosis, migration, and invasion of pancreatic cancer cells through GluN2b-AKT signaling pathways.

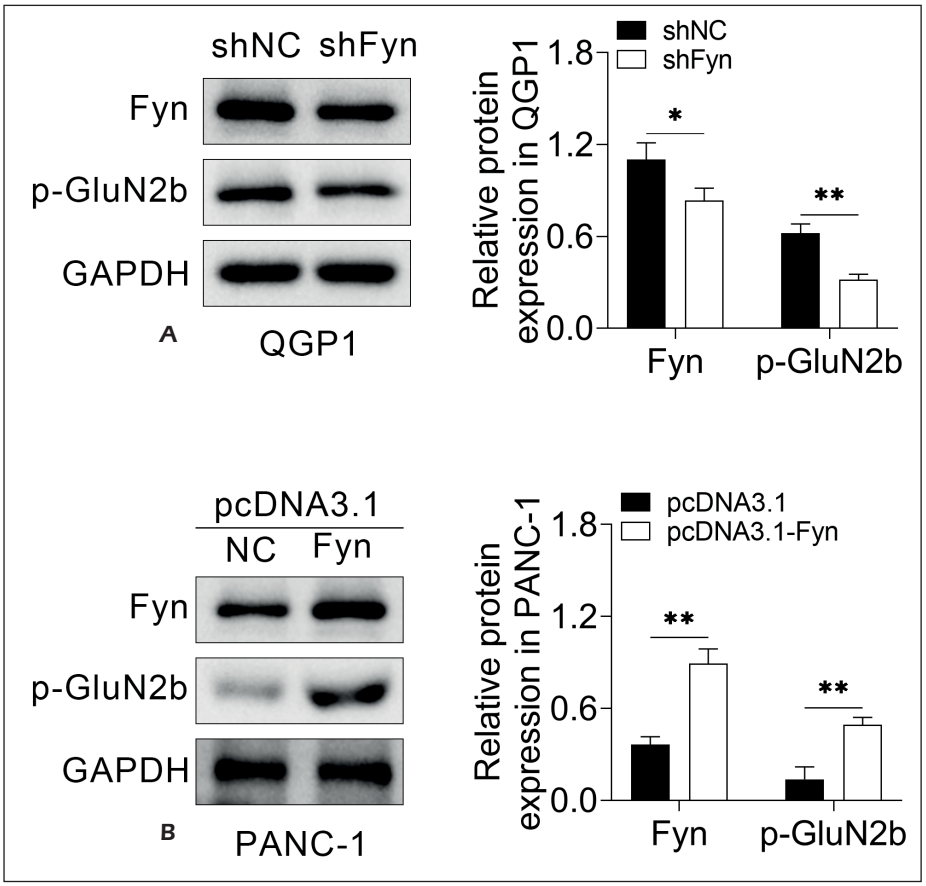

Figure 3. Fyn mediates the phosphorylation of GluN2b in pancreatic cancer cells. **A**, Immunoblot assays indicated the levels of Fyn, GluN2b, GAPDH, and the phosphorylation of GluN2b in QGP1 cells transfected with control or Fyn shRNA plasmids. **B**, Immunoblot assays indicated the levels of Fyn, GluN2b, GAPDH, and the phosphorylation of GluN2b in PANC-1 cells transfected with pcDNA3.1-vector or pcDNA3.1-Fyn plasmids. Results are presented as mean ± SEM, * $p<0.05$, ** $p<0.01$.

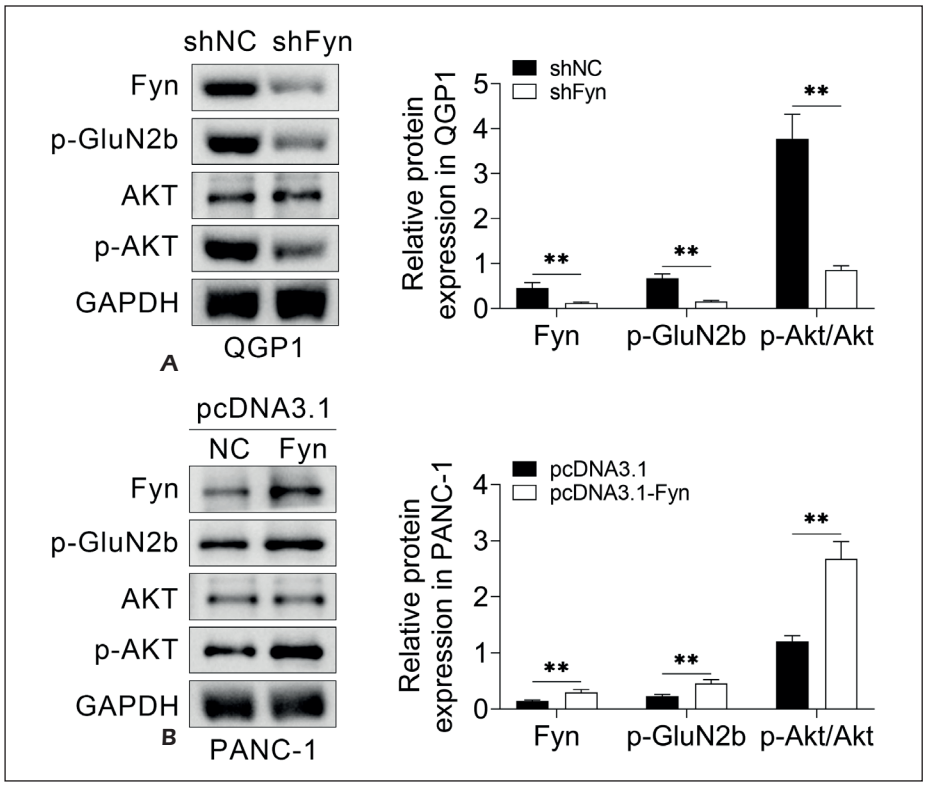

Figure 4. Fyn mediates the AKT signaling pathway via the phosphorylation of GluN2b in pancreatic cancer cells. **A**, Immunoblot assays indicated the levels of Fyn, AKT, GAPDH, and the phosphorylation of AKT and GluN2b in QGP1 cells transfected with control or Fyn shRNA plasmids. **B**, Immunoblot assays indicated the levels of Fyn, AKT, GAPDH, and the phosphorylation of AKT and GluN2b in PANC-1 cells transfected with pcDNA3.1-vector or pcDNA3.1-Fyn plasmids. Results are presented as mean ± SEM, * $p<0.05$, ** $p<0.01$.

We then used the shRNA plasmids targeted GluN2b to deplete its expression in pancreatic cancer cells. To verify our expectations, QGP1 cells were divided into four groups, including control, Fyn, GluN2b shRNA transfection, and the co-transfection of Fyn and GluN2b shRNA groups. Through immunoblot assays, we found the phosphorylation of GluN2b was significantly decreased in Fyn and GluN2b shRNA-transfected cells, as well as in the co-transfection cells (Figure 5A). However, no difference was found between Fyn and GluN2b depletion groups, as well as co-transfection group (Figure 5A), suggesting the regulation of Fyn on the phosphorylation of GluN2b. Similarly, we found the decrease of AKT phosphorylation in Fyn and GLuN2b depletion groups, and in co-transfection group (Figure 5A). However, co-transfection of Fyn and GluN2b shRNA plasmids did not further decrease the phosphorylation levels of AKT, suggesting that the phosphorylation of AKT is mediated by the phosphorylation of GluN2b (Figure 5A).

In a similar manner, we examined the effects of co-transfection of Fyn and GluN2b shRNA on cell proliferation. Both MTT assay and colony formation assay demonstrated that transfection of Fyn or GluN2b shRNA plasmids alone significantly inhibited cell proliferation, while the co-transfection of Fyn and GluN2b shRNA plasmids did not further suppress cell proliferation (Figure 5B, C), suggesting that Fyn did regulate pancreatic cancer cell proliferation through the AKT signaling pathway.

Subsequently, cell apoptosis was also analyzed among control, Fyn, GluN2b shRNA transfection, and co-transfection groups. Our data indicated that depletion of Fyn and GluN2b stimulated the apoptosis of QGP-1 cells *in vitro*. However, the co-transfection of Fyn and GluN2b did not further promote QGP-1 cell apoptosis (Figure 5D), compared to the Fyn and GluN2b transfected alone groups.

Similarly, through wound healing and transwell assays, we also noticed the impairment of cell migration and invasion capacity caused by the depletion of Fyn or GluN2b alone, whereas the co-transfection of Fyn or GluN2b did not further suppress cell migration or invasion (Figure 5E-F).

The results of immunoblot assays also verified our previous results. We found Fyn or GluN2b depletion alone decreased the expression of Bcl-2, MCM2, and PCNA, and increased the expression of cleaved caspase-3. However, Fyn and GluN2b co-transfected cells exhibited a modest change in the expression of these proteins, consistent with the previous data (Figure 5G).

In conclusion, we demonstrated that Fyn promoted the progression of pancreatic cancer via the Fyn-GluN2b-AKT axis.

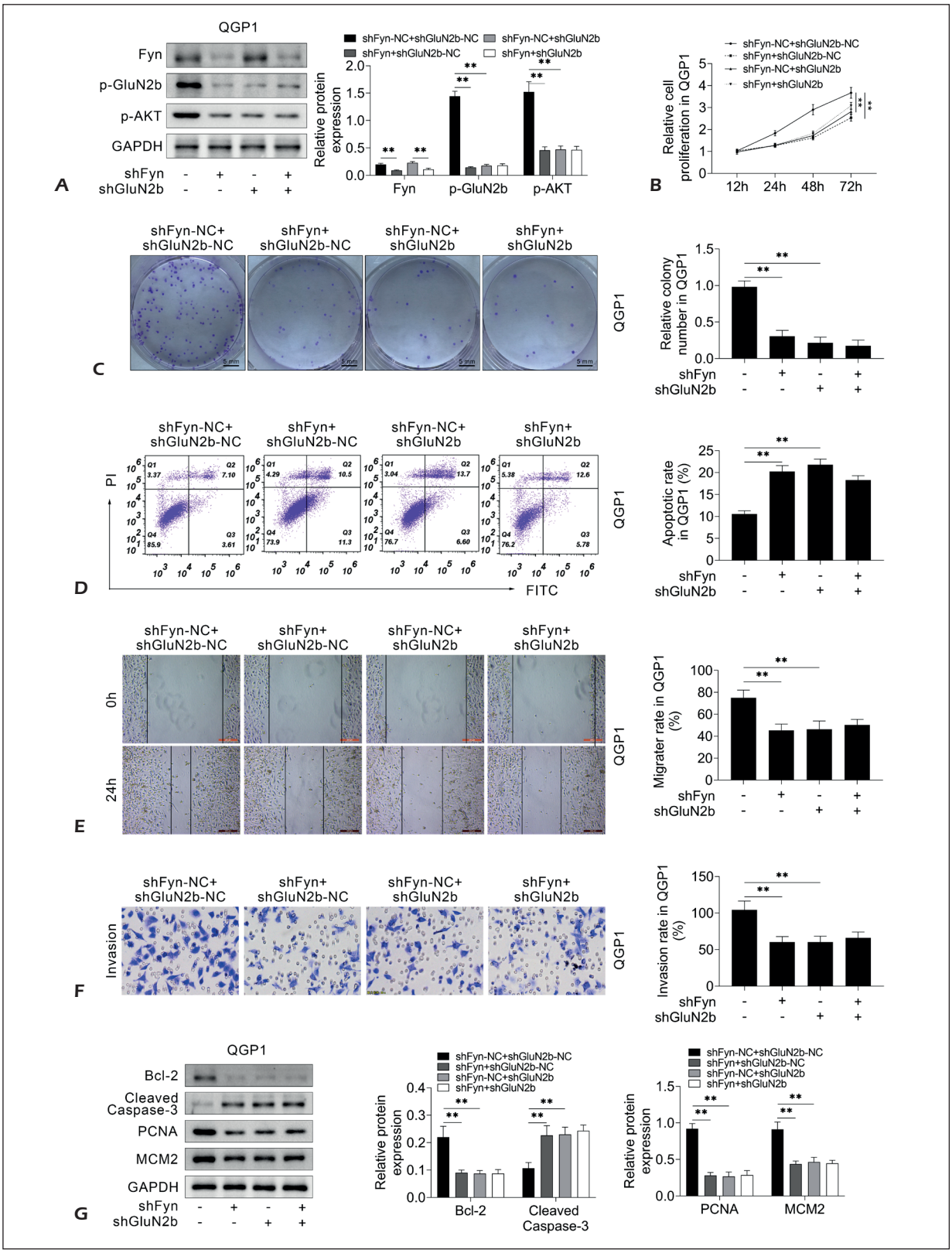

Figure 5. Fyn contributes to the progression of pancreatic cancer via Fyn-GluN2b-AKT axis. **A**, Immunoblot assays indicated the levels of Fyn, GAPDH, and the phosphorylation of GluN2b and AKT in QGP1 cells transfected with the indicated plasmids. **B**, Results of MTT assays showed the difference of cell proliferation in QGP1 cells transfected with the indicated shRNA plasmids, respectively. **C**, Representative photographs showed the results of colony formation assays of QGP1 cells transfected with the indicated shRNA plasmids. The relative proliferation levels were quantified. Scale bar, 5 mm. **D**, Cell apoptosis assays showed the difference of apoptosis levels of QGP1 cells transfected with the indicated shRNA plasmids. **E**, Wound healing assays were performed using QGP1cells transfected with the indicated shRNA plasmids, and the relative wound width was detected (200× magnification). **F**, Transwell assays using QGP1cells transfected with the indicated shRNA plasmids were performed and the extent of relative transwell migration was quantified (400× magnification). **G**, Immunoblot assays indicated the expression level of PCNA, MCM2, Bcl-2, cleaved caspase-3, and GAPDH in QGP1cells transfected with the indicated shRNA plasmids. Results are presented as mean ± SEM, *p<0.05, **p<0.01.

Fyn Promotes Tumor Growth of Pancreatic Cancer Cells In Vivo

According to our *in vitro* data, Fyn depletion resulted in the impairment of both the proliferation and motility of pancreatic cancer cells, and the induction of cell apoptosis. We then detected the potential role of Fyn in tumor growth of pancreatic cancer cells *in vivo*.

QGP-1 cells were infected with control or Fyn shRNA adenovirus to stably deplete its expression. Subsequently control or Fyn depletion cells were injected into nude mice, respectively. After 1 week, tumors were observed, and the volume was detected every week. Representative photographs of tumors were acquired, and the growth curves were shown in Figure 6A. According to the results, the volume of tumors isolated from Fyn ablation mice was significantly smaller than control (Figure 6A). Furthermore, we performed immunoblot assays and found that the levels of phosphorylated GluN2b and AKT were all decreased (Figure 6B). The expressions of Bcl-2, MCM2, and PCNA in tumors of knockdown groups were remarkably decreased, whereas cleaved caspase-3 was increased, compared with tumors in control groups (Figure 6B).

Immunohistochemistry assays further confirmed the evident decrease of Fyn expression and GluN2b phosphorylation in Fyn depletion tumors (Figure 6C). Therefore, all these data revealed that Fyn was involved in the regulation of tumor growth of pancreatic cancer cells *in vivo*.

Discussion

Pancreatic cancer is a complex disease with an average of 63 mutations per tumor cell and high heterogeneity[23]. Due to this, targeted therapy for pancreatic cancer is very difficult[24]. Some devel-

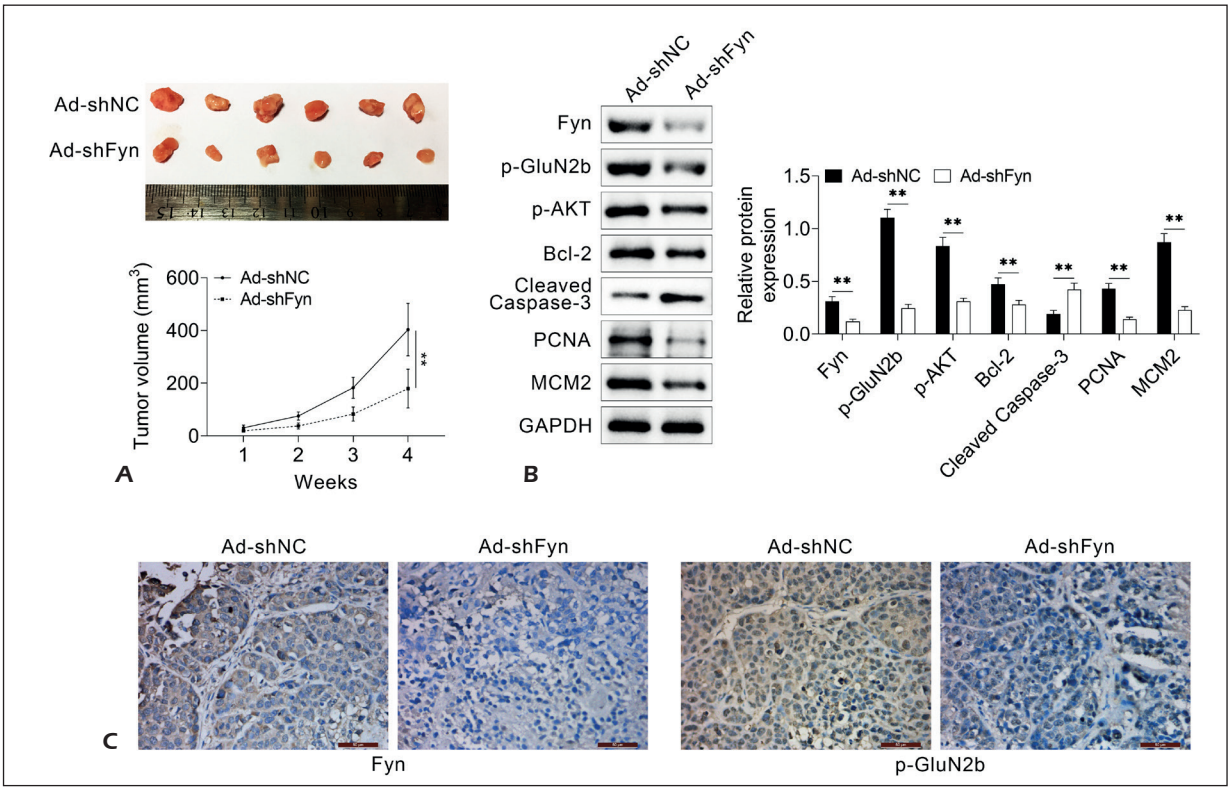

Figure 6. Knockdown of Fyn impaired tumor growth of pancreatic cancer cells *in vitro*. **A**, QGP1 cells were infected with Fyn or control shRNA adenovirus, and subsequently implanted into nude mice. After 1 week, tumors were isolated, and volume was calculated every week. After 4 weeks, all tumors were isolated (n=6 in each group). Representative images of tumors were shown *(left)*, and tumor growth curves were calculated based on the average volume of 6 tumors for each group *(right)*. **B**, Immunoblot assays confirmed the expression of Fyn, Bcl-2, cleaved caspase-3, PCNA, MCM2, and the phosphorylation levels of GluN2b and AKT in tumor tissues from control or Fyn-depleted mice. C, Immunohistochemical assays indicated the expression level of Fyn and GluN2b phosphorylation in control or Fyn depletion tumor tissues isolated from mice (magnifications 200×). Results are presented as mean ± SEM, ** $p<0.01$.

oped therapeutic targets, such as KRAS, HER2, and IGF1R, have been well studied and have the potential to develop as targeted therapy drugs for pancreatic cancer[25]. As was known, due to the high heterogeneity of pancreatic cancer, more therapeutic targets are urgently needed[26]. In this study, we revealed that a member of SFKs, Fyn, serves as a novel molecular target for the treatment of pancreatic cancer. Indeed, previous studies[27] have found the potential anti-tumor effect of Fyn inhibitor, which has the potential as a targeted therapy for pancreatic cancer.

In this study, we systematically investigated the possible regulatory effects and molecular mechanism of Fyn in pancreatic cancer. Through IHC assays and quantitative PCR assays, we analyzed the expression levels of Fyn in tumor tissues and normal tissues of 30 patients with pancreatic cancer. Of note, we found the significant high expression of Fyn in human pancreatic cancer tissues compared to normal tissues, suggesting the critical role in the pathogenesis of pancreatic cancer. Then, quantitative PCR assays results showed the high expression of Fyn in pancreatic cancer cells, compared to normal pancreatic cells, which is consistent with the clinical data. Furthermore, we investigated the link between clinical pathological features, the prognosis, and Fyn expression in patients with pancreatic cancer. Our results indicated that the clinical characteristics, including metastasis staging and tumor size, which reflect the degree of tumor development, were affected by Fyn expression levels. Similar to our findings, other studies[28] demonstrated the effects of Fyn on the prognosis and clinical features of tumor patients. Fyn expression was correlated with the prognosis of breast cancer, and also affected the mesenchymal phenotypes of basal type breast cancer cells[17,29]. In addition, Fyn was up-regulated in thyroid carcinoma and played a critical role in tumorigenesis[28]. These researches all confirmed the potential regulatory role of Fyn in tumor development.

By MTT assays, colony formation assays, FCM assays, wound healing assays, and transwell assays, we further proved that Fyn promoted the proliferation, apoptosis, migration, and invasion of pancreatic cancer cells. Mechanical studies demonstrated that Fyn phosphorylates GluN2b, which in turn activates the AKT signaling pathway, affecting a variety of tumor cell processes, and these results were verified in animal models. Fyn has different regulatory mechanisms in the development of other tumors. Fyn ablation blocks the migration and invasion of cholangiocarcinoma cells via the activation of AMPK/mTOR signaling pathway[30]. In addition, Fyn could stimulate the progression of breast cancer via epithelial-mesenchymal transition (EMT)[29]. Du et al[31] reported that a transcription factor, KLF5, promoted cell migration by up-regulating Fyn to affect bladder cancer. These authors have demonstrated that Fyn, as a member SFKs, plays a promotion role in different types of tumor progression.

AKT pathway is widely involved in the regulation of cancer progression and metastasis[32]. Previous studies[33] have witnessed further insight into the role of AKT signaling in cancer. AKT-mediated phosphorylation enhanced protein stability and transcription activity of ZNF322A, thereby promoting the progression of lung cancer[19]. TRIM11 was involved the regulation of proliferation and glycolysis of breast cancer cells via targeting AKT pathway[34]. These reports, together with our findings in this study, confirmed the critical regulatory effect of AKT pathway on cancer progression. Therefore, inhibitors targeting the AKT pathway have the promising potential of developed as an anti-tumor drug.

Conclusions

We found that Fyn was highly expressed in human pancreatic cancer tissues and cells, and its expression was associated with the prognosis and clinicopathological features of patients with pancreatic cancer. We further demonstrated that Fyn promoted the progression of pancreatic cancer *in vitro* and *in vivo* via Fyn-GluN2b-AKT axis. Therefore, Fyn has the potential to serve as a novel and promising therapeutic target for the treatment of pancreatic cancer.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Authors' Contributions

GML conceived and designed the experiments, WD and SJS analyzed and interpreted the results of the experiments, JJQ performed the experiments.

Ethics Approval and Consent to Participate

All animal protocols for animal experiments in this study were approved by the Ethics Committee of The First Hospital of Jilin University (Approval no. 2017036).

Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article

Conflict of Interests

The authors declare that they have no conflict of interests.

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