Fyn/heterogeneous nuclear ribonucleoprotein E1 signaling regulates pancreatic cancer metastasis by affecting the alternative splicing of integrin β1

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Abstract. Pancreatic cancer is characterized by a dense desmoplastic reaction in which extracellular matrix proteins accumulate and surround tumor cells. Integrins and their related signaling molecules are associated with progression of pancreatic cancer. In the present study, the association between the metastasis of pancreatic cancer and the expression of hnRNP E1 and integrin β1 was evaluated. *In vitro* and *in vivo* experiments were designed to study the mechanism underlying the regulation of integrin β1 splicing by the Fyn/hnRNP E1 spliceosome. Expression of hnRNP E1 and integrin β1A were associated with metastasis of pancreatic cancer. Inhibition of Fyn activity upregulated the expression of P21-activated kinase 1 and promoted the phosphorylation and nuclear localization of hnRNP E1, leading to the construction of a spliceosome complex that affected the alterative splicing of integrin β1. In the hnRNP E1 spliceosome complex, hnRNP A1 and serine/arginine-rich splicing factor 1 were responsible for binding to the pre-mRNA of integrin β1. Suppression of Fyn activity and/or overexpression of hnRNP E1 decreased the metastasis of pancreatic cancer cells. In pancreatic cancer, the present study demonstrated a novel mechanism by which Fyn/hnRNP E1 signaling regulates pancreatic cancer metastasis by affecting the alternative splicing of integrin β1. hnRNP E1 and integrin β1A are associated with the metastasis of pancreatic cancer and may be novel molecular targets for pancreatic cancer treatment.

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Introduction

Pancreatic cancer is characterized by a dense desmoplastic reaction in which extracellular matrix (ECM) proteins accumulate and surround tumor cells. These ECM proteins not only provide physical support but also affect the biological properties of pancreatic cancer cells (1-3). Recent studies have demonstrated that the integrin family of heterodimeric transmembrane glycoproteins, each of which are composed of an α- and a β-subunit, and their related signaling molecules are associated with the progression of pancreatic cancer (4-7). Among these signaling molecules, the Src family kinases (SFKs), a group of non-receptor tyrosine kinases, are overexpressed in 70% of human pancreatic cancer cases and their activities are critical for tumor progression (8-11).

Fyn, a key member of the SFKs, mediates 'outside-in' signaling from the ECM-integrin interaction to affect cellular processes, including T-cell receptor differentiation, cell adhesion, migration and apoptosis regulation (12-15). Our previous study showed that the activation of Fyn promotes cell proliferation and metastasis in pancreatic cancer (16). However, the role of Fyn in 'inside-out' signaling to regulate pancreatic cancer metastasis is not completely understood.

Integrins are heterodimers of two type-I membrane glycoproteins, α and β, which bind to each other non-covalently. Currently, seventeen α subunits and eight β subunits have been identified in vertebrates, and different components of integrin signaling have been shown to be vital for cell survival and cancer metastasis (17-19). It is well established that alternatively spliced variants of the α- and β-subunits contribute to the variety of biological functions of the integrin receptors (20). In endothelial cells, alternative splicing of integrin αv have been analyzed by Gauck *et al* (21) who found Cdc2-like kinases as well as DNA topoisomerase I to modulate the differential isoform expression of Cyr61 and its receptor integrin αv, the protein expression and secretion in resting as well as in TNF-α-stimulated human microvascular endothelial cells. Moreover, these processes affected the endothelial cell proliferation and pro-angiogenic tube formation by HMEC-1. In pancreatic cancer, the integrin β1-ECM interaction has been associated with metastasis (4). Four splice variants (A, B, C and D), which differ only in the amino acid sequence of the

cytoplasmic domain, have been identified in the integrin β1 family, and the β1A isoform is widely expressed among all mammalian species assessed so far. The expression of splice variants B, C and D, which can inhibit β1A-mediated focal adhesion formation, cell spreading and motility, has been found to be downregulated or even lost in various tumor tissues, and may be involved in tumor progression (22-24). However, the formation of splice variants of integrin β1 is not yet fully understood.

Splicing of individual precursor messenger ribonucleic acid (pre-mRNA) is determined by spliceosome proteins including serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) (25). The SR proteins and hnRNPs function as *trans*-acting factors by binding to exonic splicing enhancers (ESEs) or exonic splicing silencers (ESSs) to regulate alternative splicing (26-29). Recent reports have shown that hnRNP E1 binds to the CD44 pre-mRNA, which affects its alternative splicing (30). However, the participation of spliceosome proteins in integrin β1 splicing regulation and the resultant effect on cancer metastasis are unknown.

As a group of RNA-binding proteins, hnRNPs regulate the splicing and transportation of mRNA and participate in growth regulation and carcinogenesis (31-33). Expression of hnRNP E1 has been detected in several cancer types (34,35). However, the mechanism by which hnRNP E1 regulates tumor metastasis is not clear. Our preliminary study indicated that inhibition of the activity of Fyn significantly increased hnRNP E1 expression in human pancreatic cancer cells (36). To determine whether Fyn modulates pancreatic cancer metastasis via hnRNP E1, the present study evaluated the association between the metastasis of pancreatic cancer and the expression of hnRNP E1 and integrin β1 in human pancreatic cancer tissues. Subsequently, the mechanism of integrin β1 splicing regulation by Fyn/hnRNP E1 signaling in pancreatic cancer cells was investigated using multiple experimental approaches.

Materials and methods

Antibodies. Rabbit monoclonal antibodies against SRC family-phospho Y418 (reactive to Fyn-pY419, ab40660; 1:1,000 for western blot analysis), integrin β1 (reactive to the extracellular domain, ab179471, 1:1,000 for western blot analysis) and p21-activated kinase 1 (ab40852, 1:1,000 for western blot analysis), rabbit polyclonal antibodies against serine/arginine-rich splicing factor 1 (ab38017, 1:1,000 for western blot analysis) and SRp20 (ab73891, 1:1,000 for western blot analysis) and a mouse monoclonal antibody against hnRNP A1 (ab5832, 1:1000 for western blot analysis) were purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibodies against hnRNP E1 (sc-28725, 1:500 for western blot analysis) and β-actin (sc-130657, 1:5,000 for western blot analysis) were purchased from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX, USA). Mouse monoclonal antibodies against Fyn (P2992, 1:1000 for western blot analysis) and green fluorescent protein (GFP) (SAB5300167, 1:5000 for western blot analysis) were purchased from Sigma-Aldrich (St. Louis, MQ, USA). A rabbit polyclonal antibody against phosphothreonine (71-8200, 1:500 for western blot analysis) was purchased from Invitrogen/Thermo Fisher Scientific (Waltham, MA, USA). Goat anti-rabbit IgG rhodamine-conjugated (31670) and goat anti-rabbit (31460) or anti-mouse (31430) IgG (1:5,000 for western blot analysis) peroxidase-conjugated antibodies were purchased from Pierce Biotechnology (Rockford, IL, USA).

Adenoviral expression of kinase-dead Fyn (KdFyn) and hnRNP E1-GFP (E1-GFP). The KdFyn recombinant adenovirus was previously prepared in our laboratory (16). The full-length hnRNP E1 coding sequence was obtained from HEK293 RNA using a PrimeScript™ RT-PCR kit (RR014A; Takara Bio, Dalian, China) according to the manufacturer's protocol. The polymerase chain reaction (PCR) products were identified by sequencing and cloned into the multiple cloning site of the pEGFP-N2 plasmid (6081-1 BD; Biosciences, Bedford, MA, USA) to construct an hnRNP E1-GFP fusion protein. The hnRNP E1-GFP recombinant adenovirus was generated using the AdEasy Vector System (240009; Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The GFP recombinant adenovirus was used as a control vector in the cell study. Sequences of the hnRNP E1-GFP fusion protein PCR primers are listed in Table I.

Cell lines and tissue samples. The human pancreatic cancer cell lines AsPC1, BxPC3, CFPAC1 and Panc1, and the HEK293 cell line, were cultured in RPMI-1640 medium (11875093; Thermo Fisher Scientific, Waltham, MA, USA), Iscove's modified Dulbecco's medium (12440053; Thermo Fisher Scientific) or Dulbecco's modified Eagle's medium (41965062; Thermo Fisher Scientific), each supplemented with 10% fetal bovine serum (FBS, 12657; Thermo Fisher Scientific) and antibiotic-antimycotic (15240-062; Thermo Fisher Scientific). All cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) characterized according to the cell line authentication testing and used within 6 months after resuscitation.

A total of 152 pancreatic cancer specimens from our institution were used in the present study, including 76 specimens that had been cryopreserved in liquid nitrogen, which were used for RNA analysis, and 76 paraffin-embedded specimens (8330; Thermo Fisher Scientific), which were used for histological analysis. Each pancreatic cancer specimen was reviewed by two pathologists.

All human studies were reviewed and approved by the Ethics Committee of Southwest Hospital and were therefore performed in accordance with the ethical standards described in the 1975 Declaration of Helsinki, as revised in 1983. Informed consent was obtained from all individual participants that were included in the study.

Cell staining for confocal microscopy. Cells were cultured to 50-70% confluence on glass-bottom tissue culture dishes (150680; Thermo Fisher Scientific) and then washed with phosphate-buffered solution (PBS, 10010023; Thermo Fisher Scientific). The cells were fixed with 2% paraformaldehyde (FB002; Thermo Fisher Scientific), followed by the addition of glycine buffer (0.1 mM glycine) (G7126; Sigma-Aldrich) for paraformaldehyde quenching and blocked in 1% bovine serum albumin (BSA, TS-38839; Thermo Fisher Scientific) in PBS for 1 h. The hnRNP E1 rabbit polyclonal antibody (sc-28725; Santa Cruz Biotechnology) was diluted in PBS (1:200) with 1% BSA and incubated with the cells at 4˚C overnight. The

Table I. Sequences of the integrin β1, β1A, β1C, hnRNP E1-GFP and β-actin PCR primers.

	Forward primer	Reverse primer	Size (bp)	EFF%
Integrin β1A	AGAATCCAGAGTGTCCCACTGG	TTTCCCTCATACTTCGGATTG	238	92.4
Integrin β1C	TCTGTCGCCCAGCCTGGAGTG	TTTCCCTCATACTTCGGATTG	172	96.3
integrin β1	CCTCATAACAGTCCTGTGCCTAGAAT	CCAGCCAATGTGGTGAAACCC	270	91.2
hnRNP E1-GFP	GC(AGATCT)CTCGCCATGGATGCCGGTGT	CA(GAATTC)GCCCTTCTCAGAGGAAAGCCTGG	1078	93.5
β-actin	CGGGAAATCGTGCGTGAC	TGGAAGGTGGACAGCGAGG	443	90.7

EFF%, amplification efficiency.

cells were then washed with cold PBS three times prior to incubation with rhodamine-conjugated goat anti-rabbit IgG (1:500; Pierce Biotechnology) for 45 min at room temperature. The labeled cells were washed with cold PBS and imaged using a confocal microscope (Carl Zeiss LSM780; Carl Zeiss Microscopy GmbH, Jena, Germany). The images were analyzed using ZEN 2012 software (Carl Zeiss Microscopy GmbH).

Cell invasion assay. Cell invasion assays were performed using 24-well Transwell inserts with 8 μm pore size (PI8P01250; Merck Millipore, Billerica, MA, USA) coated with a thin layer of matrigel (356234; BD Biosciences, Bedford, MA, USA) (1.5 μg/mm^2). Cells (1x10^5 cells in 300 μl serum-free medium) were seeded into the upper chamber, and the lower compartment was filled with 500 μl of complete medium. After 24 h at 37˚C, non-invading cells were removed by wiping the upper side of the membrane. Invading cells were fixed, stained and counted.

Flow cytometry. BxPC3 pancreatic cancer cells were cultured in RPMI-1640 medium with 10% FBS and antibiotic-antimycotic, prior to harvesteding by trypsinization (25200056; Thermo Fisher Scientific). Subsequently, the cells were washed with PBS containing 1% normal goat serum (01-6201; Thermo Fisher Scientific) and incubated with integrin β1 rabbit monoclonal antibody (ab179471, 1:500; Abcam) at 4˚C for 1 h, followed by rhodamine-conjugated goat anti-rabbit IgG (31670, 1:500; Pierce Biotechnology) for 30 min. The stained cells were resuspended in 100 μl PBS and analyzed with a Becton Dickinson FACSort flow cytometer.

Immunoprecipitation and RNA-protein immunoprecipitation. Immunoprecipitation was performed by incubating 1 μg hnRNP E1 rabbit polyclonal antibody (sc-28725; Santa Cruz Biotechnology) or Fyn mouse monoclonal antibody (P2992; Sigma-Aldrich) with 500 μg extracted proteins overnight at 4˚C with constant rotation. The immunocomplexes were captured by adding protein A agarose (15918014; Invitrogen/Thermo Fisher Scientific) for 1 h at 4˚C with constant rotation. The immunoprecipitates were analyzed by western blotting.

For RNA-protein immunoprecipitation, 1 mg extracted proteins were pre-cleared with 20 μl protein A/G plus agarose (20423; Invitrogen/Thermo Fisher Scientific) and then incubated with 5 μg of hnRNP E1 rabbit polyclonal antibody (sc-28725; Santa Cruz Biotechnology), SF2/ASF rabbit polyclonal antibody (ab38017; Abcam) or hnRNP A1 mouse monoclonal antibody (ab5832; Abcam) overnight at 4˚C with constant rotation. The co-precipitated RNA was then extracted by phenol (AM9712; Thermo Fisher Scientific)/chloroform (1.02444; Sigma-Aldrich) and used for further analysis.

Immunohistochemical staining. Specimens were fixed in formalin (9990916; Thermo Fisher Scientific), embedded in paraffin and cut into 3-mm sections. Sections were deparaffinized in xylene (9990501; Thermo Fisher Scientific), rehydrated in a graded series of ethanol (E7023; Sigma-Aldrich) solutions and incubated in 3.0% hydrogen peroxide (TA-060-HP; Thermo Fisher Scientific) in methanol (960055; Thermo Fisher Scientific) for 30 min to block endogenous

peroxidase activity. Slides were heated at 120˚C in an autoclave in 10 mM sodium citrate (pH 6.0) (71497; Sigma-Aldrich) for 130 sec and then cooled to room temperature. After blocking with 10% goat serum for 30 min, the sections were incubated overnight at 4˚C with the hnRNP E1 rabbit polyclonal antibody (sc-28725, 1:200; Santa Cruz Biotechnology). Negative controls were obtained by omitting the primary antibody. The sections were incubated with peroxidase-conjugated anti-mouse/rabbit immunoglobulins (K5007; Dako EnVision™ System; Dako, Copenhagen, Denmark) according to the manufacturer's protocol for 60 min at 37˚C. The peroxidase reaction was developed with 3,3'-diaminobenzidine as the chromogen, followed by counterstaining with hematoxylin.

Mass spectrometry. Briefly, 5 mg extracted proteins were pre-cleared with 50 μl protein A/G Plus agarose and then incubated with 10 μg hnRNP E1 rabbit polyclonal antibody (sc-28725; Santa Cruz Biotechnology) overnight at 4˚C with constant rotation. The immunocomplexes were captured by adding protein A/G Plus agarose for 1 h at 4˚C. The immunoprecipitates were sent to the BGI (Shenzhen, China) for mass spectrometry analysis.

Nude mouse xenograft model. Male athymic nu/nu mice, 4-6 weeks of age, were obtained from the Beijing Animal Facility and provided with food and water *ad libitum*. Tumor cells ($2x10^5$ tumor cells in 100 μl of sterile PBS) were transplanted into each mouse via subcutaneous or intrasplenic injection. Animals were euthanized after 5-8 weeks, and the subcutaneous tumors and liver were resected. Primary tumor mass was determined by measuring the wet weight of the resected tumors and metastasis was determined as the incidence of tumor nodes present in the liver.

Procedures involving animals and their care were conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University.

RNA extraction and quantitative real-time-polymerase chain reaction (qRT-PCR). Total RNA was extracted from tissues or cultured cells using RNAiso Plus reagent (9108; Takara) according to the manufacturer's protocol. The RNA was stored at -80˚C, and reverse transcription of the extracted RNA was performed using the PrimeScript™ RT reagent kit with genomic DNA Eraser (RR047A; Takara) according to the manufacturer's protocol. The cDNA was stored at -20˚C. qRT-PCR assays were performed to detect integrin β1 expression using the One Step SYBR® PrimeScript™ RT-PCR kit (RR086A; Takara) according to the manufacturer's protocol. The results were normalized to the expression of β-actin. For quantitative measurement of integrin β1 expression, qRT-PCR was performed using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycling conditions: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The qRT-PCR results were analyzed and expressed relative to a pancreatic cancer sample and converted to fold-change value. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide and imaged with a video camera (Vilber Lourmat, Marne-la-Vallée, France). The primers used and the sizes of all the PCR products for integrin β1 and β-actin are presented in Table I.

Small interfering RNA (siRNA) transfection. Target-specific siRNAs for hnRNP E1 (sc-43843), hnRNP A1 (sc-270345), SF2/ASF (sc-38319) and SRp20 (sc-38338) were purchased from Santa Cruz Biotechnology and transfected into pancreatic cancer cells according to the manufacturer's protocol. In a 6-well tissue culture plate (PIDL06P05; Merck Millipore), $2x10^5$ cells were seeded per well in 2 ml antibiotic-free normal growth medium supplemented with FBS. The cells were incubated at 37˚C in a CO_2 incubator until the cells were 60-80% confluent. For each transfection, 2-8 μl of siRNA duplexes were diluted into 100 μl siRNA transfection medium (31985070; Thermo Fisher Scientific), and 2-8 μl siRNA transfection reagent (11668019; Thermo Fisher Scientific) was diluted into 100 μl siRNA transfection medium. The siRNA duplex was added directly to the diluted transfection reagent using a pipette and mixed gently by pipetting the solution up and down, followed by incubation for 45 min at room temperature. The cells were washed once with 2 ml of siRNA transfection medium, and 0.8 ml of siRNA transfection medium was added to each tube containing the siRNA transfection reagent mixture. The solutions were mixed gently and overlaid onto the washed cells. The cells were incubated for 5-7 h at 37˚C in a CO_2 incubator. Subsequently, 1 ml of normal growth medium containing two times the normal serum and antibiotic concentration (2X normal growth medium) was added without removing the transfection mixture. The cells were incubated for an additional 18-24 h. The medium was aspirated and replaced with fresh 1X normal growth medium. The cells were assayed using the appropriate protocol at 72 h after the addition of normal growth medium (2X normal growth medium) in the step above.

Scratch wound assay. Cells were seeded in 6-well plates (PIDL06P05; Merck Millipore) at a density of >80% confluence. Cells were transfected with KdFyn, E1-GFP or hnRNP E1 siRNA or the control vector 24 h after seeding. Wounds were made in confluent monolayers 48 h after transfection by using a pipette tip under vacuum suction. A straight line was drawn across the bottom of each well, and then pictures were captured with the line at the bottom of the viewing field. Wounds were measured from the exact vertical middle of each image such that the initial measurement and the measurement 24 h later were taken from the same vertical spot in each well. Representative images are shown for each group, and results are reported as the percentage of the wound closed.

Western blot analysis. Whole cell protein was extracted with lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol), and 30 μg protein, determined using a BCA protein assay kit (23227; Pierce Biotechnology), was resolved on a 12% acrylamide gel and then transferred onto nylon membranes (LC2003; Thermo Fisher Scientific). The blots were incubated overnight at 4˚C with 1% blocking solution in TBS buffer (50 mM Tris-HCl and 150 mM NaCl) and 0.1% Tween-20. The membranes were then incubated with the primary antibodies. After 2 h at room

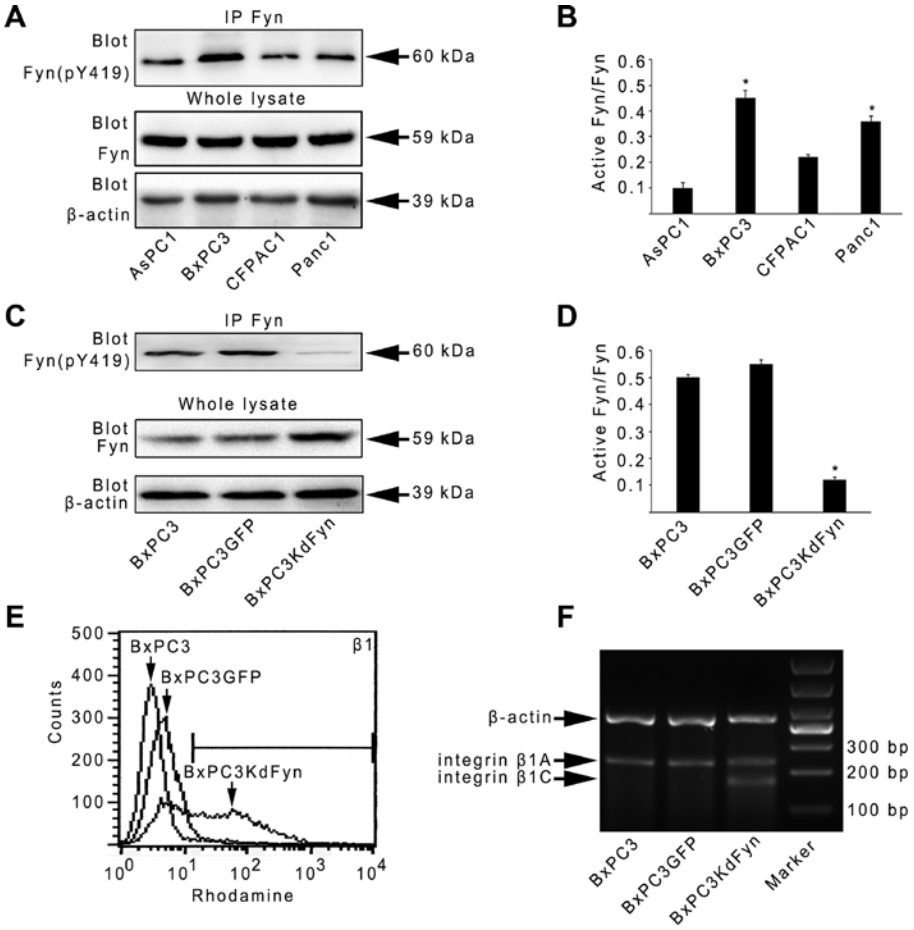

Figure 1. Activity of Fyn affects alterative splicing of integrin β1. (A-D) Active Fyn was analyzed by detecting the expression of tyrosine 419-phosphorylated Fyn in different pancreatic cancer cell lines. (E) Expression of integrin β1 was analyzed by fluorescence-activated cell sorting using an antibody specific for the extracellular amino acid sequence of integrin β1. (F) Splicing of integrin β1 was analyzed in BxPC3, BxPC3GFP and BxPC3KdFyn cells. *P<0.05. Data are presented as the mean ± standard deviation from ≥3 independent experiments. Sequences of the PCR primers for integrin β1A and C (Fig. 4A) and β-actin are listed in Table I.

temperature, the membranes were washed twice with TBS plus 0.1% Tween-20 and 0.5% blocking buffer and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody at room temperature. Following incubation with the appropriate secondary antibodies, the membranes were washed and the signals were visualized with Amersham ECL plus Western blotting detection reagents (28-9829-42 AB; GE Healthcare Bio-Sciences AB, Stockholm, Sweden). The specific bands on the autoradiograms were quantitated by densitometry.

Statistical analysis. All analyses were performed using SPSS 19.0 software. The correlation between categorical variables was evaluated using the χ^2 test. Normally distributed data were analyzed using a Student's t-test; otherwise, the non-parametric Mann-Whitney test was applied. P<0.05 was considered to indicate a statistically significant difference.

Results

Activity of Fyn affects alterative splicing of integrin β1. To determine whether the activity of Fyn affects the expression of integrin β1 in pancreatic cancer cells, the expression of active Fyn was analyzed in four pancreatic cancer cell lines, and BxPC3 pancreatic cancer cells were selected for the investigation of integrin β1 expression when Fyn activity was suppressed. As shown in Fig. 1A-E, BxPC3 cells expressed higher levels of active Fyn than the other three pancreatic cancer cells. In the KdFyn-transfected BxPC3 cells (BxPC3KdFyn), the expression of integrin β1 did not decrease; however, the level of a certain subtype of integrin β1 was increased, suggesting that suppressing Fyn activity may result in a change in integrin β1 splicing. Therefore, the splicing of integrin β1 in BxPC3 cells with different activity levels of Fyn was further analyzed. Integrin β1B and D were not expressed in these pancreatic cancer cells, and decreasing the activity of Fyn significantly promoted the expression of integrin β1C (Fig. 1F). These results suggested that the activity of Fyn affected the alterative splicing of integrin β1.

Fyn regulates phosphorylation and nuclear localization of hnRNP E1 via P21-activated kinase 1 (PAK1), thus affecting the alterative splicing of integrin β1. To determine whether Fyn regulates the biological function of hnRNP E1, the protein levels of hnRNP E1 in BxPC3 cancer cells with different Fyn activities were evaluated. The expression of hnRNP E1 was revealed to be increased ~2-fold in BxPC3KdFyn cells compared with BxPC3GFP or BxPC3 cells (Fig. 2A and B). It has been shown that PAK1, one of the evolutionarily

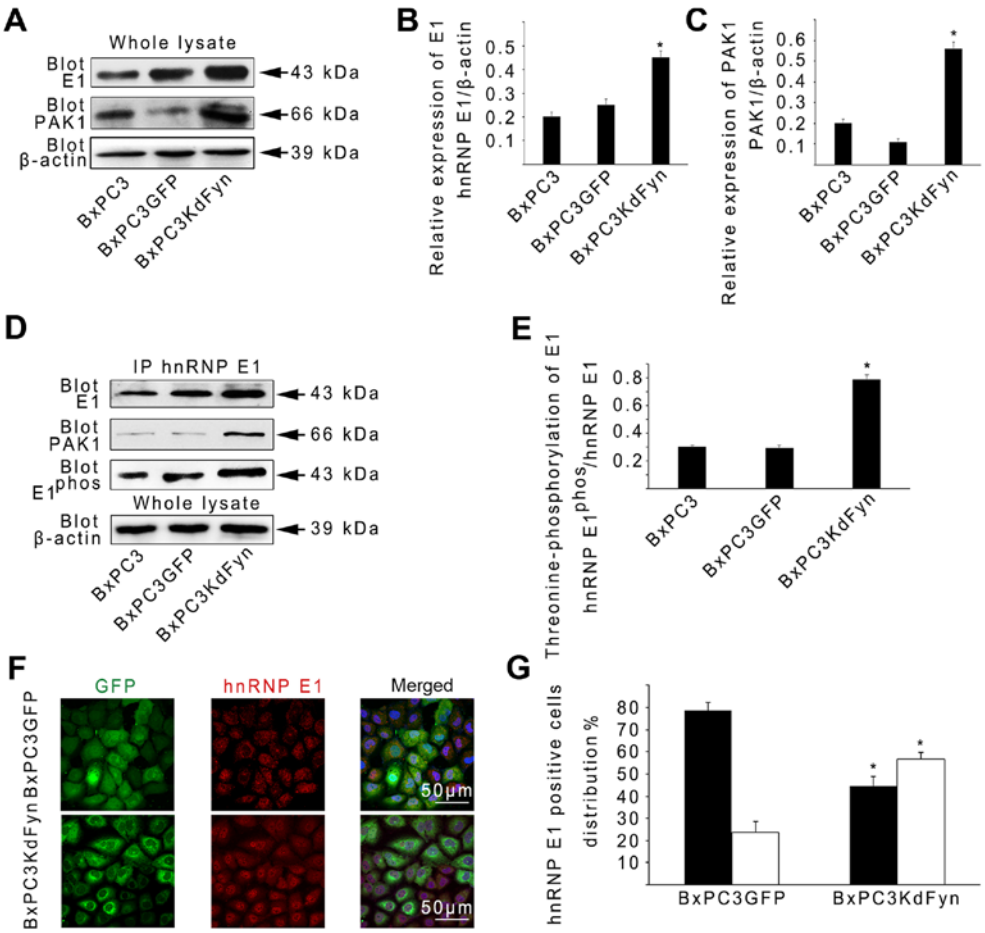

Figure 2. Activity of Fyn regulates the phosphorylation and nuclear localization of hnRNP E1 via PAK1. (A-C) Western blot analysis revealed the relative expression levels of hnRNP E1 and PAK1 protein among BxPC3, BxPC3GFP and BxPC3KdFyn cells. β-actin blotting was used as a loading control. (D and E) Co-immunoprecipitation analysis showed the threonine-phosphorylation of hnRNP E1 and the interaction of hnRNP E1 with PAK1 among BxPC3, BxPC3GFP and BxPC3KdFyn cells. (F and G) Confocal microscopy analysis showed that the nuclear distribution of hnRNP E1 was increased in BxPC3KdFyn cells compared with BxPC3GFP cells. *P<0.05. Data are presented as the mean ± standard deviation from ≥3 independent experiments.

conserved families of serine/threonine protein kinases, contributes to the threonine-phosphorylation of hnRNP E1 thus activating hnRNP E1 for RNA processing (37). To further explore whether the inhibition of Fyn activity affects PAK1 expression, thus promoting hnRNP E1 phosphorylation, PAK1 expression was analyzed in BxPC3 cells with different Fyn activity levels. PAK1 expression was found to be increased significantly in BxPC3KdFyn cells compared with the control cells (Fig. 2A and C). Subsequently, co-immunoprecipitation was used to analyze the threonine-phosphorylation of hnRNP E1 and the interaction between hnRNP E1 and PAK1 in BxPC3 cells with different Fyn activity levels. As shown in Fig. 2D and E, threonine-phosphorylation of hnRNP E1 was increased ~2.5-fold in BxPC3KdFyn cells compared with BxPC3 or BxPC3GFP cells. In addition, inhibition of Fyn activity promoted the interaction of hnRNP E1 with PAK1 in BxPC3KdFyn cells. Phosphorylated hnRNP E1 is involved in nuclear localization and mediates a variety of functions associated with mRNA splicing and stability (37,38). Therefore, the effect of Fyn activity on hnRNP E1 nuclear localization was next investigated using confocal immunofluorescence, revealing that the nuclear distribution of hnRNP E1 was increased ~2-fold in BxPC3KdFyn cells compared with BxPC3GFP cells (Fig. 2F and G). These results suggested that Fyn regulated the phosphorylation and nuclear localization of hnRNP E1 via PAK1 in pancreatic cancer cells.

However, to date, the cellular function of hnRNP E1 in integrin β1 splicing has not been reported. Therefore, whether changes in hnRNP E1 expression could modulate the splicing of integrin β1 was next investigated. A GFP-hnRNP E1 fusion protein was expressed from an adenoviral system in BxPC3 cells (BxPC3E1-GFP) (Fig. 3A). The expression of integrin β1C was markedly increased in BxPC3E1-GFP cells (Fig. 3B). Furthermore, knockdown of the expression of hnRNP E1 by transfection specific hnRNP E1 siRNA in the BxPC3KdFyn cells (BxPC3KdFyn+siE1) decreased the expression of integrin β1C (Fig. 3C-G). It was also noted that inhibition of the expression of hnRNP E1 did not affect PAK1 expression, but significantly decreased the phosphorylation of hnRNP E1 (Fig. 3E). In addition, inhibition of the activity of Fyn or overexpression of hnRNP E1 in the Panc1 pancreatic cancer cells which expressed active Fyn (Fig. 1A) also promoted the splicing of integrin β1C (Fig. 3H and I). These results suggested that Fyn affected the splicing of integrin β1 by regulating the phosphorylation and nuclear localization of hnRNP E1.

hnRNP E1 spliceosome regulates integrin β1 splicing by using hnRNP A1 and SF2/ASF to recognize the pre-mRNA of

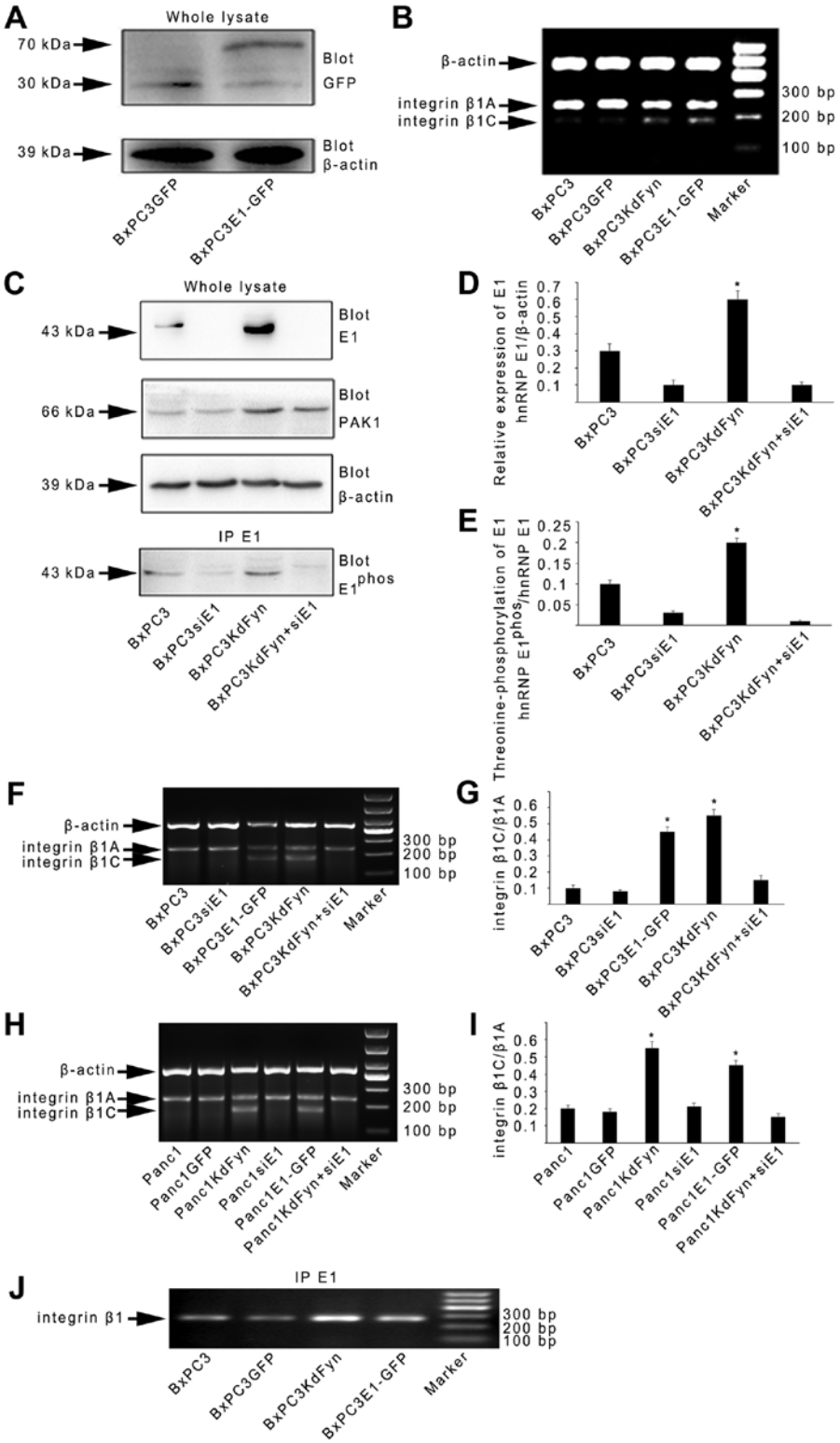

Figure 3. Fyn regulates the alternative splicing of integrin β1 via hnRNP E1. (A) Co-immunoprecipitation analysis showed the expression of GFP-hnRNP E1 fusion protein in BxPC3 cells. (B, F and G) Splicing of integrin β1 was analyzed under different Fyn activity levels or hnRNP E1 expression levels in BxPC3 cells. (C-E) Knockdown of hnRNP E1 expression decreased the phosphorylation of hnRNP E1 in BxPC3 cells. (H and I) Splicing of integrin β1 was analyzed under different Fyn activity levels or hnRNP E1 expression levels in Panc1 cells. (J) RNA immunoprecipitation showed the interaction between hnRNP E1 and the pre-mRNA of integrin β1. *P<0.05. Data are presented as the mean ± standard deviation from ≥3 independent experiments. Sequences of the PCR primers for integrin β1 pre-mRNA, which cover the exon and intron sequences of integrin β1C (Fig. 4A) are listed in Table I.

integrin β1. It is established that splicing is performed by the spliceosome, which is composed of five small ribonucleoproteins (snRNPs) and hundreds of additional proteins, including the SR proteins and the hnRNPs. The SR proteins and hnRNPs function as *trans*-acting factors by binding to the ESEs or ESSs of target pre-mRNAs to regulate alternative splicing (39-43). As the results of the present study identified that hnRNP E1 participated in the regulation of integrin β1 splicing, whether

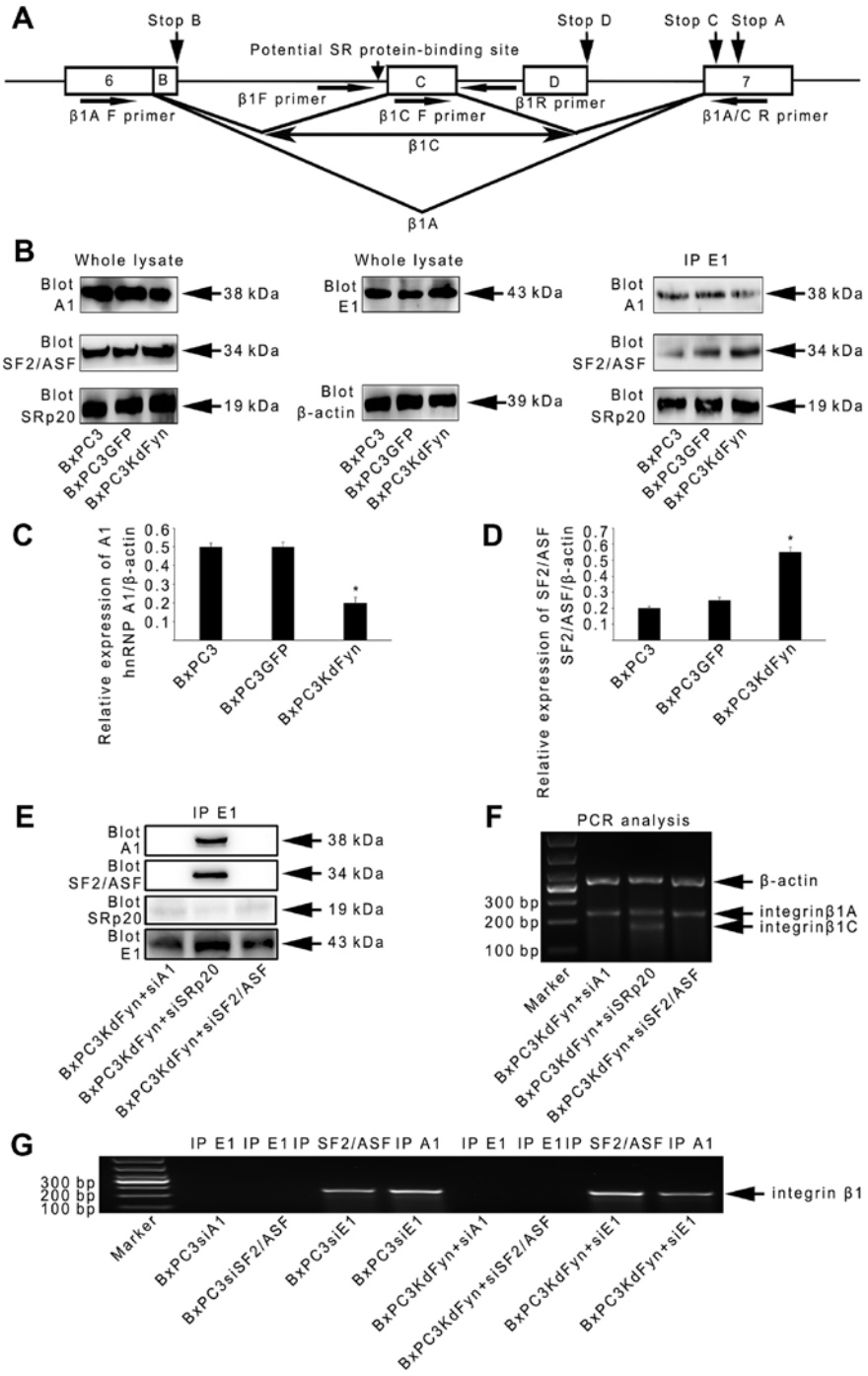

Figure 4. hnRNP E1 spliceosome regulates integrin β1 splicing by hnRNP A1 and SF2/ASF. (A) Schematic representation of the genomic sequence of integrin β1. Exon sequences are shown as boxes and introns as solid lines. The splicing pattern used to generate β1A and C transcripts are depicted, and the potential SR protein-binding site is indicated with a vertical arrow. The positions of the stop codons for the various splice variants are also indicated by arrows. The horizontal arrows indicate the primers used in PCR experiments (Table I). (B-D) Western blot analysis and co-immunoprecipitation were used to analyze the expression levels and abundance of hnRNP E1-specific spliceosome components among BxPC3, BxPC3GFP and BxPC3KdFyn cells. β-actin blotting was used as a loading control. (E and F) Co-immunoprecipitation and splicing analysis were used to explore the functions of hnRNP A1 and SF2/ASF on the construction of the hnRNP E1 spliceosome complex and the splicing of integrin β1. (G) RNA immunoprecipitation showed that hnRNP A1 and SF2/ASF, but not hnRNP E1, were directly bound to the pre-mRNA of integrin β1. *P<0.05. Data are presented as the mean ± standard deviation from ≥3 independent experiments.

hnRNP E1 binds to the pre-mRNA of integrin β1 was further analyzed by RNA immunoprecipitation. As shown in Fig. 3J, the binding of hnRNP E1 to integrin β1 pre-mRNA in BxPC3 pancreatic cancer cells with different Fyn activity or hnRNP E1 protein levels suggested that hnRNP E1 may be a component of the spliceosome complex that regulates integrin β1 splicing. Therefore, the components of the hnRNP E1 spliceosome complex extracted from BxPC3KdFyn cells were next analyzed by mass spectrometry. This revealed two SR proteins (SRSF1 and SRSF3, also known as SF2/ASF and SRp20) and six other hnRNPs (hnRNP A1, A3, C1, K, M and U) in the hnRNP E1 spliceosome complex (Table II). Previous studies

Table II. Proteins identified by mass spectrometry in the hnRNP E1 spliceosome of BxPC3KdFyn cells.

Group ID	Protein ID	Protein score	Protein mass	Coverage	No. of unique peptide	No. of unique spectrum	Isoelectric point	Description
1	IPI00003865	972.09	71082.31	0.243034056	11	24	5.16	HSPA8 isoform 1 of heat shock cognate 71 kDa protein
2	IPI00216049	704.64	51229.51	0.265658747	9	17	5.18	*HNRNPK*, isoform 1 of heterogeneous nuclear ribonucleoprotein K
3	IPI00017617	526.07	69617.93	0.140065147	7	9	9.21	DDX5 probable ATP-dependent RNA helicase DDX5
4	IPI00911039	440.23	64169.98	0.015358362	1	1	5.19	HSPA1B, HSPA1A cDNA FLJ54408, highly similar to heat shock 70 kDa protein 1
5	IPI00304925	440.12	70294.14	0.014040562	1	2	5.31	HSPA1B, HSPA1A heat shock 70 kDa protein 1A/1B
6	IPI00479217	335.99	89665.43	0.079404467	5	8	5.51	*HNRNPU*, isoform short of heterogeneous nuclear ribonucleoprotein U
7	IPI00171903	323.1	77749.42	0.115068493	6	9	9.1	*HNRNPM*, isoform 1 of heteronuclear ribonucleoprotein M
8	IPI00215965	316.85	38837.09	0.112903226	3	6	9.46	*HNRNPA1*, isoform A1-B of heterogeneous nuclear ribonucleoprotein A1
9	IPI00420014	295.88	246006.24	0.033707865	5	5	5.95	SNRNP200 isoform 1 of U5 small nuclear ribonucleoprotein 200 kDa helicase
10	IPI00216592	292.33	32374.89	0.160409556	4	10	4.69	*HNRNPC*, isoform C1 of heterogeneous nuclear ribonucleoproteins C1/C2
11	IPI00419373	278.6	39798.67	0.137566138	4	5	9.31	*HNRNPA3*, isoform 1 of heterogeneous nuclear ribonucleoprotein A3
12	IPI00300371	236.25	136575.15	0.045193098	4	4	4.91	SF3B3 isoform 1 of splicing factor factor 3B subunit 3
13	IPI00552938	208.36	22271.46	0.178571429	3	6	4.95	RBMX heterogeneous nuclear ribonucleoprotein G isoform 2
14	IPI00215884	208.03	27841.86	0.14516129	3	5	10.77	*SRSF1*, isoform ASF-1 of serine/arginine-rich splicing factor 1
15	IPI00641829	206.99	51103.09	0.0248307	1	1	5.67	SNORD84, DDX39B isoform 2 of spliceosome RNA helicase DDX39B
16	IPI00328840	178.33	27540.9	0.189393939	3	5	11.6	THOC4 THO complex subunit 4
17	IPI00007423	173.48	28941.37	0.079681275	2	2	3.67	ANP32B isoform 1 of acidic leucine-rich nuclear phosphoprotein 32 family member B
18	IPI00017964	167.06	14021.35	0.317460317	3	4	10.98	SNRPD3 small nuclear ribonucleoprotein Sm D3
19	IPI00010204	141.72	19545.99	0.140243902	2	4	12.15	*SRSF3*, Serine/arginine-rich splicing factor 3
20	IPI00031556	104.61	53809.32	0.044210526	2	2	9.49	U2AF2 isoform 1 of splicing factor U2AF 65 kDa subunit
21	IPI00396435	91.92	91673.47	0.031446541	2	3	7.48	DHX15 putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15
22	IPI00009328	86.29	47126.29	0.03406326	1	2	6.69	EIF4A3 eukaryotic initiation factor 4A-III

Table II. Continued.

Group ID	Protein ID	Protein score	Protein mass	Coverage	No. of unique peptide	No. of unique spectrum	Isoelectric point	Description
23	IPI00001757	80.34	19933.75	0.097701149	1	2	5.43	RBM8A isoform 1 of RNA-binding protein 8A
24	IPI00007928	79.34	274738.05	0.003426124	1	1	9.14	PRPF8 Pre-mRNA-processing-splicing factor 8
25	IPI00026089	70.91	146479.37	0.013803681	1	1	7.1	SF3B1 splicing factor 3B subunit 1
26	IPI00302850	64.13	13273.36	0.092436975	1	1	12.09	SNRPD1 small nuclear ribonucleo-protein Sm D1
27	IPI00016610	60.99	37987.14	0.04494382	1	1	7.11	*PCBP1*, Poly(rC)-binding protein 1 (hnRNP E1)
28	IPI00003519	60.81	110335.65	0.012345679	1	1	4.6	EFTUD2 116 kDa U5 small nuclear ribonucleoprotein component
29	IPI00027285	54.91	24764.75	0.033333333	1	1	11.75	SNRPB isoform SM-B' of small nuclear ribonucleoprotein associated proteins B and B'
30	IPI00016572	54.82	8547.45	0.157894737	1	1	9.41	SNRPG small nuclear ribonucleo-protein G
31	IPI00305068	43.69	107656.09	0.015940489	1	1	8.4	PRPF6 Pre-mRNA-processing factor 6
32	IPI00221106	41.48	100279.02	0.013407821	1	1	5.37	SF3B2 splicing factor 3B subunit 2
33	IPI00329791	39.34	117802.7	0.010669253	1	1	9.87	DDX46 probable ATP-dependent RNA helicase DDX46
34	IPI00019380	38.82	92863.93	0.013924051	1	1	6.4	NCBP1 nuclear cap-binding protein subunit 1
35	IPI00012442	38.16	52189.1	0.036480687	1	1	5.21	G3BP1 Ras GTPase-activating protein-binding protein 1
36	IPI00008943	37.53	54348.96	0.022964509	1	1	6.21	DDX19B isoform 1 of ATP-dependent RNA helicase DDX19B
37	IPI00013180	36.66	17558.8	0.0625	1	1	9	BUD31 protein BUD31 homolog
38	IPI00010158	34.07	14758.48	0.061068702	1	2	4.73	CHRAC1 chromatin accessibility complex protein 1
39	IPI00029266	32.15	10853.66	0.119565217	1	1	9.86	SNRPE small nuclear ribonucleo-protein E

showed a potential SR protein-binding site in the pre-mRNA of integrin β1 for the splicing of β1C (Fig. 4A (44). Therefore, whether these two SR proteins and three other hnRNPs (hnRNP A1, C1 and M, which have been demonstrated to participate in splicing regulation) affect integrin β1 splicing was examined in this study.

Generally, the alternative splicing of genes is determined by SR proteins and hnRNPs in a concentration-dependent manner (45). Therefore, we analyzed the expression of these SR and hnRNP proteins and their abundance in the hnRNP E1 complex in pancreatic cancer cells. As shown in Fig. 4B-D, the expression of SF2/ASF was significantly increased in BxPC3KdFyn cells compared with control cells. However, the expression of SRp20 did not significantly differ among these groups of cells. With regard to the three hnRNPs, only hnRNP A1 was obviously decreased in BxPC3KdFyn cells compared with control cells. In addition, the level of hnRNP A1 was decreased in the hnRNP E1 complex in BxPC3KdFyn cells when the SF2/ASF level was increased (Fig. 4B). These results suggested that hnRNP A1 and SF2/ASF may serve important roles in the hnRNP E1 spliceosome complex.

To explore the functions of hnRNP A1 and SF2/ASF in the spliceosome, target-specific siRNAs were used to knock down their expression in BxPC3KdFyn cells. As shown in Fig. 4E and F, knockdown of hnRNP A1 or SF2/ASF in BxPC3KdFyn cells completely eliminated the construction of the hnRNP E1 complex and ultimately prevented the expression of integrin β1C. By contrast, knockdown of SRp20

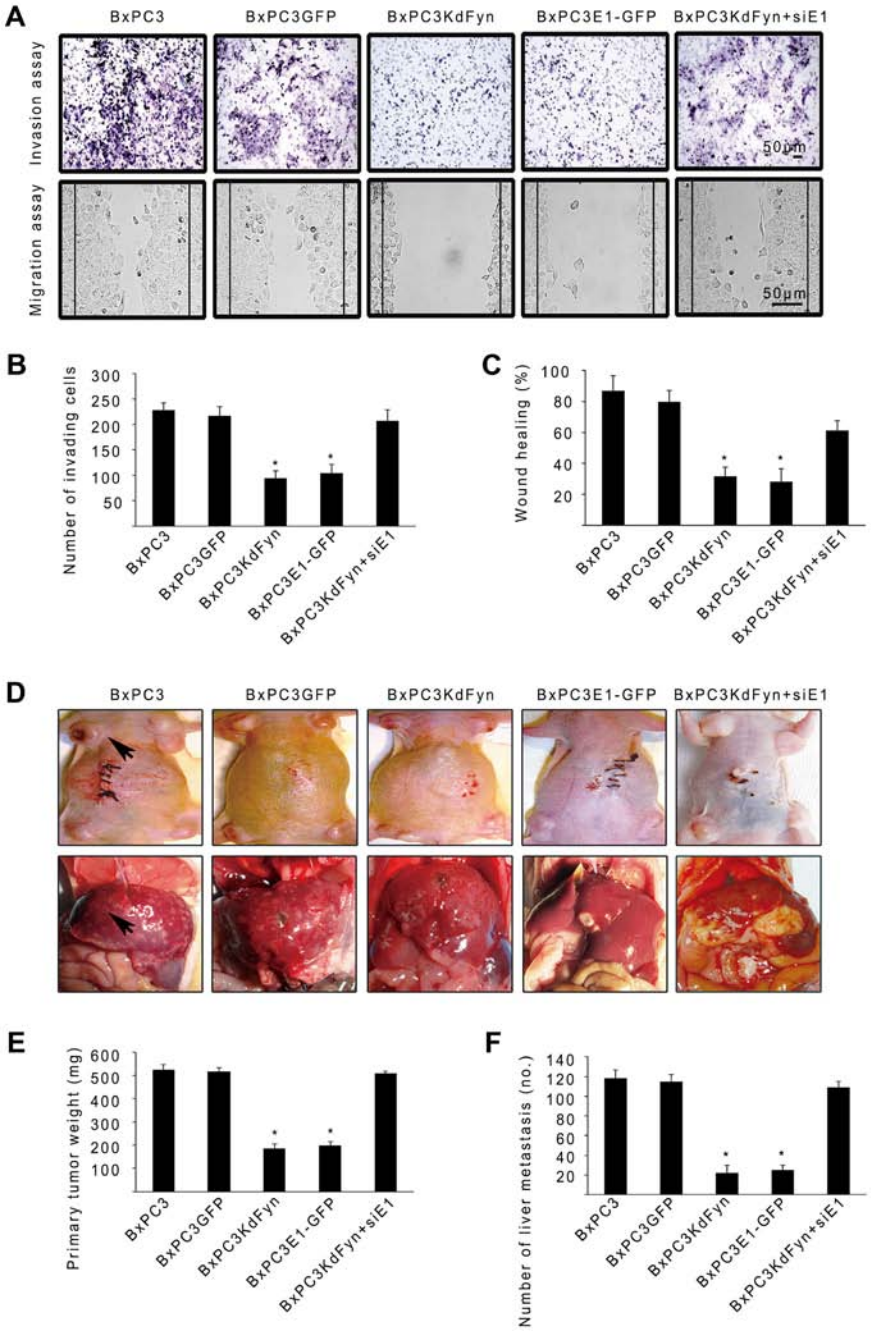

Figure 5. Effect of Fyn and hnRNP E1 on the invasion and metastasis of pancreatic cancer cells. (A-C) Transwell and wound-healing assays revealed that the activity of Fyn and/or the expression of hnRNP E1 significantly affected the invasion and migration abilities of BxPC3 pancreatic cancer cells *in vitro*. (D-F) An assay of the subcutaneous tumor mass and liver metastatic nodules revealed that the activity of Fyn and/or the expression of hnRNP E1 significantly affected the tumorigenesis and metastasis of BxPC3 pancreatic cancer cells *in vivo*. Each group of xenograft experiments included 5 mice. $^{*}P<0.05$. Data are presented as the mean ± standard deviation from ≥3 independent experiments.

did not affect the interaction of hnRNP E1 with hnRNP A1 and SF2/ASF, and did not alter the splicing of integrin β1 in BxPC3KdFyn cells. Therefore, in the spliceosome complex of BxPC3KdFyn cells, hnRNP A1, hnRNP E1 and SF2/ASF were key proteins responsible for integrin β1 splicing.

To further determine whether hnRNP A1, hnRNP E1 and SF2/ASF have direct roles in the post-transcriptional modification of integrin β1 pre-mRNA, endogenous complexes of RNA with hnRNP A1, E1 and SF2/ASF were extracted from BxPC3KdFyn cells to analyze the presence of integrin β1 pre-mRNA. hnRNP A1 and SF2/ASF, but not hnRNP E1, were directly bound to the pre-mRNA of integrin β1 (Fig. 4G). These results suggested that hnRNP E1 spliceosome regulated integrin β1 splicing by using hnRNP A1 and SF2/ASF to recognize the pre-mRNA of integrin β1 in pancreatic cancer cells.

Effect of Fyn and hnRNP E1 on the invasion and metastasis of pancreatic cancer cells in vitro and in vivo. Transwell and wound-healing assays were employed to determine the effect of Fyn and hnRNP E1 on the invasion and migration of pancreatic cancer cells *in vitro*. As shown in Fig. 5A-C, inhibition of the

Table III. Associations between the expression of integrin β1A, hnRNP E1 and the categorical clinicopathological parameters of pancreatic carcinoma.

	Integrin β1A mRNA expression		Cytoplasmic hnRNP E1 protein expression		
Parameters	Median expression (range)	P-value	Low	High	P-value
Age (years)		0.183			0.805
<65	44.343 (24.355-82.093)		27	29	
≥65	28.273 (17.489-58.197)		9	11	
Sex		0.847			0.092
Male	37.853 (18.741-74.376)		5	12	
Female	28.671 (23.728-79.674)		31	28	
Tumor size (cm)		0.517			0.961
≤2	55.170 (23.557-80.806)		16	18	
>2	29.847 (18.257-74.357)		20	22	
Tumor location		0.601			0.108
Head	35.482 (21.230-74.376)		34	33	
Body and tail	64.364 (27.496-79.674)		2	7	
Lymph node metastasis		**0.001**			**0.004**
Negative	28.051 (10.770-57.022)		27	17	
Positive	70.630 (34.876-96.331)		9	23	
Vascular invasion		0.150			0.258
Negative	36.222 (25.467-77.838)		30	29	
Positive	29.816 (5.563-64.032)		6	11	
Neural invasion		0.147			0.426
Negative	29.361 (9.563-56.324)		11	9	
Positive	40.934 (25.618-80.162)		25	31	
Duodenal invasion		0.328			0.417
Negative	27.934 (9.758-63.527)		8	6	
Positive	37.038 (24.087-78.756)		28	34	
Hepatic metastases		**0.005**			**0.011**
Negative	29.847 (16.785-69.192)		35	31	
Positive	72.487 (64.115-106.964)		1	9	
Differentiation		0.56			0.404
Well	49.798 (25.015-69.084)		1	4	
Moderate	35.482 (18.741-80.892)		26	25	
Poor	28.189 (25.769-30.836)		9	11	
T-stage (UICC)		0.419			0.077
T1, 2	35.482 (25.467-77.188)		26	21	
T3, 4	39.998 (12.114-76.003)		10	19	
Tumor stage (UICC)		**0.002**			**0.001**
I	28.632 (13.919-44.015)		21	8	
II	46.717 (18.839-82.530)		14	22	
III, IV	76.003 (54.517-117.941)		1	10	

The bold values indicate P-values <0.05, UICC Union for International Cancer Control.

activity of Fyn or overexpression of hnRNP E1 significantly decreased the number of invading cells and the migration of BxPC3 pancreatic cancer cells. By contrast, knockdown of the expression of hnRNP E1 in the BxPC3KdFyn cells promoted the invasion and migration of tumor cells. Subsequently, the effects of Fyn and hnRNP E1 on pancreatic cancer cell tumorigenesis and metastasis were investigated *in vivo* by subcutaneous or intrasplenic injection of $2x10^5$ tumor cells

Table IV. Correlation between the expressions of β1A (mRNA) and hnRNP E1 (protein).

		Spearman rank correlation analysis	
	n	Correlation coefficient	P-value
hnRNP E1 protein expression β1A mRNA expression	76	0.556	<0.01

into nude mice. After 5-8 weeks, the mice were sacrificed to detect subcutaneous tumors and liver metastases, the findings revealed that inhibition of Fyn activity or overexpression of exogenous hnRNP E1 within BxPC3 pancreatic cancer cells significantly decreased the primary tumor mass and liver metastases. Furthermore, knockdown of hnRNP E1 in the BxPC3KdFyn cells promoted the growth of the primary tumor mass and liver metastases (Fig. 5D-F). These results suggested that Fyn/hnRNP E1 signaling regulated the invasion and metastases of pancreatic cancer cells *in vitro* and *in vivo*.

Expression of hnRNP E1 and integrin β1 are associated with metastasis of pancreatic cancer. Our data showed that Fyn, hnRNP E1 and integrin β1 were involved in the metastases of pancreatic cancer cells. Therefore, we next analyzed the expression of these proteins in pancreatic cancer using the Human Protein Atlas database and pancreatic cancer tissues.

In the Human Protein Atlas database, the expression of hnRNP E1 was positive in pancreatic cancer and the malignant cells exhibited moderate to strong cytoplasmic positivity (http://www.proteinatlas.org/ENSG00000169564-PCBP1/cancer/tissue/pancreatic+cancer). In pancreatic cancer tissues obtained from our institution, the mRNA expression of integrin β1A was correlated with lymph node metastasis (P=0.001), hepatic metastasis (P=0.005) and the tumor stage (P=0.002) of pancreatic cancer. By contrast, only cytoplasmic and not nuclear, expression of hnRNP E1 was correlated with lymph node metastasis (P=0.004), hepatic metastasis (P=0.011) and the tumor stage of pancreatic cancer (P=0.001) (Table III). In addition, the cytoplasmic expression of hnRNP E1 was positively correlated with the mRNA expression of integrin β1A (Table IV). Therefore, the expression of hnRNP E1 and integrin β1A were associated with metastasis of pancreatic cancer.

Discussion

The present study revealed a novel mechanism by which Fyn/hnRNP E1 signaling regulates pancreatic cancer metastasis by affecting the alternative splicing of integrin β1. The results demonstrated that inhibition of Fyn activity upregulated the expression of PAK1 and promoted the phosphorylation and nuclear localization of hnRNP E1, which affected the alterative splicing of integrin β1. Subsequently, an hnRNP E1 spliceosome complex including SR proteins and hnRNPs was shown to regulate the alternative splicing of integrin β1. In this spliceosome complex, hnRNP A1, hnRNP E1 and SF2/ASF were key proteins responsible for integrin β1 splicing. Importantly, hnRNP A1 and SF2/ASF directly bound to the pre-mRNA of integrin β1 to achieve post-transcriptional regulation. *In vivo* and *in vitro* studies demonstrated that suppression of Fyn activity and/or overexpression of hnRNP E1 significantly decreased the invasion and metastasis of pancreatic cancer cells. Finally, the expression of hnRNP E1 and integrin β1A were associated with the metastasis of pancreatic cancer.

Alternative splicing of the mRNA encoding certain integrin subunits increases diversity within the integrin family, and alters the function of the specific integrin (22). Previous studies have shown that splicing of integrin αv affected the proliferation and pro-angiogenic properties of human endothelial cells (21). Four splice variants (A, B, C and D) have been identified in the integrin β1 family, and the β1A isoform is known to be widely expressed among all mammalian species tested to date. Splice variants B, C and D which inhibit β1A-mediated focal adhesion formation, focal adhesion kinase phosphorylation, fibronectin matrix assembly, cell spreading and motility, have been shown to be downregulated or even absent in many types of tumor tissues and may be involved in tumor progression (22,46-49). However, the formation of splice variants of integrin β1 has not been fully described. In the present study, inhibition of Fyn activity and/or overexpression of hnRNP E1 was found to significantly promote the splicing of the integrin β1C variant in pancreatic cancer cells and decrease the invasion and migration of tumor cells *in vitro*, thus eventually suppressing tumorigenesis and metastasis of pancreatic cancer cells *in vivo*. In human pancreatic cancer tissues obtained from our institution, the expression of hnRNP E1 and integrin β1A were associated with the metastasis of pancreatic cancer. These results suggest that Fyn and hnRNP E1 affect pancreatic cancer metastasis by participating in the regulation of integrin β1 splicing.

As a member of the SFKs, Fyn has been shown to affect many cellular processes, including mRNA splicing (50,51). However, the mechanism by which Fyn regulates integrin β1 alternative splicing has not been reported. In the present study, the activity of Fyn was demonstrated to regulate the alternative splicing of integrin β1 via hnRNP E1. Inhibition of Fyn activity significantly promoted the phosphorylation and nuclear localization of hnRNP E1, which affected the alterative splicing of integrin β1 in pancreatic cancer cells. In addition, overexpression of hnRNP E1 significantly promoted the splicing of integrin β1C. By contrast, knockdown of hnRNP E1 expression eliminated integrin β1C splicing induced by KdFyn. These results suggest that Fyn acts though hnRNP E1 to regulate the alternative splicing of integrin β1. Additionally, inhibition of Fyn activity upregulated PAK1, which phosphorylated hnRNP E1 through direct interaction. Therefore, Fyn functions through PAK1 to induce phosphorylation and nuclear localization of hnRNP E1, which regulates integrin β1 alternative splicing.

The different components of the spliceosome determine the splicing of individual pre-mRNAs and assist in the accurate recognition of splice sites (52,53). The hnRNPs are involved in processing heterogeneous nuclear RNAs into mature mRNAs, as well as functioning as *trans*-acting factors in the regulation of gene expression under the phosphorylation of serine and threonine residues or methylation of arginine

residues (54). The SR proteins, which are phosphorylated by the SR-specfic protein kinase family, including cdc2-like kinase, are key determinants of exon identity, and function as molecular adaptors to link the pre-mRNA to the splicing machinery (55-58). In the present study, hnRNP E1 was found to form a complex with SR proteins and hnRNPs, which coordinately regulated the alternative splicing of integrin β1 in pancreatic cancer cells. In this spliceosome complex, hnRNP E1, hnRNP A1 and SF2/ASF served key roles in the regulation of integrin β1 splicing. Firstly, the amounts of hnRNP A1 and SF2/ASF in the hnRNP E1 spliceosome complex were demonstrated to be inversely correlated with one another among BxPC3 cells with different Fyn activity levels, suggesting that hnRNP A1 and SF2/ASF may have opposing roles in integrin β1 splicing. This phenomenon is consistent with a previous report, which showed that these two proteins have competing roles in regulation of alternative splicing of the Ron gene (59). Additionally, in the present study, knockdown of either hnRNP A1 or SF2/ASF completely eliminated the formation of the hnRNP E1 spliceosome complex and ultimately prohibited the alternative splicing of integrin β1 in pancreatic cancer cells, suggesting that hnRNP A1 and SF2/ASF were the key proteins responsible for integrin β1 splicing. Furthermore, RNA immunoprecipitation revealed that hnRNP A1 and SF2/ASF, but not hnRNP E1, directly bound to the pre-mRNA of integrin β1, suggesting that the hnRNP E1 spliceosome complex acts via binding of hnRNP A1 and SF2/ASF to the pre-mRNA of integrin β1. Therefore, the hnRNP E1 spliceosome regulates integrin β1 splicing via hnRNP A1- and SF2/ASF-mediated recognition of the pre-mRNA of integrin β1.

In conclusion, the results of the present study provide a potential explanation for how the Fyn/hnRNP E1 spliceosome signaling regulates pancreatic cancer metastasis. Inhibition of Fyn activity promoted the phosphorylation and nuclear localization of hnRNP E1, leading to construction of the hnRNP E1 spliceosome complex, thus resulting in alterative splicing of integrin β1. In the hnRNP E1 spliceosome complex, hnRNP A1 and SF2/ASF are responsible for binding to the pre-mRNA of integrin β1 to achieve posttranscriptional modification. Suppression of Fyn and/or overexpression of hnRNP E1 significantly decreased the invasion and metastasis of pancreatic cancer cells. Thus, by altering integrin β1 splicing, the Fyn/hnRNP E1 spliceosome signal modulates the metastasis of pancreatic cancer.

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