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Original Articles

YY1 inhibits the migration and invasion of pancreatic ductal adenocarcinoma by downregulating the FER/STAT3/MMP2 signaling pathway

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis and a high mortality rate. The transcription factor YY1 acts as an inhibitor of many types of tumors. We found that YY1 knockdown promoted the invasion and migration of PANC-1 and BxPC-3 cells; FER knockdown partially restored the promotion of pancreatic cancer caused by YY1 knockdown. In vivo experiments yielded the same results. According to luciferase reporter gene, electrophoretic mobility shift (EMSA) and chromatin immunoprecipitation (ChIP) assays, YY1 directly binds to the FER promoter region. Moreover, higher level FER expression results in a worse TNM stage and prognosis for patients with PDAC. Furthermore, by downregulating FER, YY1 inhibits the formation of the STAT3-MMP2 complex, thereby suppressing expression of MMP2 and ultimately inhibiting the migration and invasion of pancreatic cancer. Our study demonstrates that the YY1/FER/STAT3/MMP2 axis is associated with the progression of pancreatic cancer and may provide a new therapeutic target for the treatment of pancreatic cancer.

1. Introduction

Pancreatic cancer is the fourth most common cause of cancer death and has a low early detection rate and high malignancy [1]. Some pancreatic cancer patients with nonspecific symptoms, such as abdominal pain and bloating, are diagnosed with advanced-stage cancer [2]. After intervention with surgical treatment, chemotherapy and radiation therapy, 5-year survival rate of pancreatic cancer is still less than 8% [3]. Furthermore, 60% of patients exhibit distant metastasis within 24 months after surgery. Overall, the prognosis of pancreatic cancer is very poor [4,5], and there is an urgent need to study the development of pancreatic cancer and to seek novel therapeutic targets.

The transcription factor Yin-Yang1 (YY1) is a 65-kDa member of the GLI-Kruppel zinc finger protein family that participates in various biological functions, including embryogenesis, cell proliferation, DNA repair, differentiation, and apoptosis. YY1 promotes or inhibits the expression of downstream genes depending on the cellular environment, cofactors and target genes [6–10]. In most tumor analyses, YY1 expression is significantly higher than in corresponding normal tissues, and the transcription rate is higher in metastatic tumors, such as prostate cancer, ovarian cancer, colorectal cancer, breast cancer, liver cancer, and lymphoma [11–17]. In addition, YY1 is expressed at low levels in some melanomas and pediatric osteosarcoma [18]. Our previous study suggested that YY1 performs a negative function in pancreatic cancer, and higher level expression of YY1 predicted a better outcome in PDAC patients [9]. Therefore, the mechanism by which YY1 affects pancreatic cancer needs to be clarified.

Feline sarcoma-related (FER) is a member of the nonreceptor tyrosine kinase family that is expressed in a variety of cells and tissues [19,20]. FER is present in the cytoplasm and contains F-BAR (Fer/CIP4 homology-Bin/amphiphysin/RVS), FX (extended F-BAR), SH2 (Src homology 2), and protein tyrosine kinase domains [21,22]. The SH2 domain in FER is activated by certain ligands, such as EGFR (epidermal growth factor receptor) [23]. FER is activated by the combination of FX

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domain binding to PA (phosphatidic acid) and other cytokines, such as erythropoietin and steel factor. These activities are related to the multifunctional roles of FER in cellular adhesion and migration [24–27]. FER induces STAT3 (signal transducer and activator of transcription 3) activation via IFN-γ (gamma interferon), forming a FER/STAT3 complex that influences cell cycle progression and promotes the stagnation of cells in the G1 phase [28]. Previous studies have shown that FER is highly expressed in lung cancer, liver cancer, breast cancer, and ovarian cancer [29–32]. However, to the best of our knowledge, FER has not been reported in pancreatic cancer; thus, it is necessary to further elucidate the molecular mechanism of FER in pancreatic cancer.

In this study, we found that YY1 plays an important role as a tumor suppressor in the migration and invasion of pancreatic cancer. Moreover, our research indicates that YY1 inhibits the migration and invasion of pancreatic cancer by downregulating the FER/STAT3/MMP2 signaling pathway.

2. Materials and methods

2.1. Cell lines and culture

Four human pancreatic ductal adenocarcinoma (PDAC) cell lines (BxPC-3, COLO-357, CFPAC-1, and PANC-1) and a normal human pancreatic ductal cell line (HPNE) were purchased from the Shanghai Cell Bank (Shanghai, China). Stable YY1 overexpressing and YY1 knockdown cells were previously produced by transfection into the BxPC-3 and PANC-1 cell lines (BxPC-3-YY1-OE, BxPC-3-YY1 shRNA, PANC-1-YY1-OE and PANC-1-YY1 shRNA). Their corresponding control cell lines (BxPC-3-vector, BxPC-3-scramble shRNA, PANC-1-vector and PANC-1-scramble shRNA) were also prepared [33,34].

The HPNE cells were grown in keratinocyte serum-free medium supplemented with epidermal growth factor and bovine pituitary extract according to the recommendation of the ATCC. The PDAC cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal calf serum (FBS) (Wisent Inc, Montreal, Qc, Canada), 10 mM HEPES (Sigma, Louis, MO, USA), 2 mM L-glutamine (Sigma), 1 mM pyruvate sodium (Sigma), 100 units/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

2.2. Preparation of FER-overexpressing/FER-knockdown cells

Obio Technology Corp., Ltd. (Shanghai, China) constructed the FER-overexpression lentiviruses. Briefly, the full-length coding region of human FER was sub-cloned into the pLenti-EF1a-EGFP-P2A-Puro-CMV-FER-3 × Flag vector (System Biosciences, Mountain View, CA, USA) and verified by sequencing (Supporting Information Table S1). The supernatant of the cultured 293 T cells was collected to infect the BxPC-3 cells, BxPC-3-YY1-OE cells, PANC-1 cells and PANC-1-YY1-OE cells. Stable cell lines were selected by culturing in medium containing 5 μg/ml puromycin (Sigma). FER expression was confirmed by qRT-PCR and Western blotting.

Obio Technology Corp., Ltd. (Shanghai, China) constructed the FER-knockdown lentiviruses. Briefly, the full-length coding region of human FER was sub-cloned into the pLKD-CMV-EGFP-2A-Puro-U6-shRNA (FER) vector (System Biosciences, Mountain View, CA, USA) and verified by sequencing (Supporting Information Table S2). The supernatant of the cultured 293 T cells was collected to infect the BxPC-3 cells, BxPC-3-YY1 shRNA cells, PANC-1 cells and PANC-1-YY1 shRNA cells. Stable cell lines were selected by culturing in medium containing 5 μg/ml puromycin (Sigma). FER expression was confirmed by qRT-PCR and Western blotting.

2.3. Quantitative RT-PCR (qRT-PCR)

The total RNA samples were isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After spectrophotometric quantification, 1 μg of total RNA in a final volume of 20 μl was used for reverse transcription (RT) with an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The cDNA was then reverse-transcribed using a TaqMan Gene Expression Assay (ThermoFisher Scientific, Rockford, IL, USA) on a StepOne Plus RT PCR System (ThermoFisher Scientific) according to the manufacturer's protocol. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression of YY1/FER (target) and β-actin (control). Each quantitative PCR was performed in triplicate and independently repeated three times.

The primer sequences used are listed in Supporting Information Table S3.

2.4. Western blotting

Protein aliquots were electrophoresed by 8%–10% SDS-PAGE and then transferred onto a PVDF membrane (Bio-Rad) [35]. The membranes were incubated with 5% nonfat dry milk in 0.1% Tween (TBST) buffer at room temperature for 2 h to block nonspecific protein interactions, and then, the membrane was incubated in fresh blocking buffer with primary antibodies for 4 °C overnight. Following washing with TBST, the membrane was incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The antibodies against YY1, FER, STAT3, pho-STAT3, and MMP2 were obtained from Abcam (Cambridge, MA, USA), while the β-actin antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Each blot was independently repeated three times.

2.5. Patients and pancreatic tissues

Forty-eight pancreatic tumor tissue samples were obtained from the First Affiliated Hospital of Nanjing Medical University, China (2009–2014). All patients provided written informed consent, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. The collected specimens were fixed in 4% formalin and embedded in paraffin. Forty-eight patients were followed up with regularly until November 1, 2018. The overall survival was defined as the time between surgery and death or the last follow-up. None of the patients died within one month of surgery.

2.6. Tissue microarrays and immunohistochemistry

To verify the expression of YY1 and FER in the PDAC tissues, a tissue microarray (TMA) containing 48 pancreatic cancer tumor tissue samples (2 mm tissue core per replicate) was constructed by Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). Immunohistochemistry (IHC) was performed according to the manufacturer's protocol. After dewaxing with xylene, the samples were rehydrated with ethanol. The tissue samples were incubated with 3% H_2O_2 for 5 min and incubated with sodium citrate buffer (pH 6.0) for 20 min. Finally, the sections were incubated with polyclonal antibodies against YY1 and FER overnight at 40 °C, followed by incubation with the secondary antibodies. Two independent pathologists who were blinded assessed the positivity and intensity of the tissue sections. The expression levels were assessed based on the staining intensity (0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining) and the positive cell ratio (0 for < 10%, 1 for 10 to < 50%, and 2 for ≥50% cell). The histochemistry score (H-SCORE) was calculated as follows: H-SCORE = (percentage of cells with weak staining × 1) + (percentage of cells with medium staining × 2) + (percentage of cells with strong staining × 3).

2.7. Cell wound healing assay

A wound healing assay was used to evaluate cell migration. Forty-eight hours after transfection, a monolayer was scraped with a 200 μl pipette tip to produce lesions of a constant length. After washing with PBS to remove the loose cells, phase images were taken by inversion fluorescence microscopy (Olympus, Japan). ImageJ software was used to measure the relative wound areas.

2.8. Cellular transwell assay

Cells were uniformly used to inoculate the upper layer of the Transwell membrane, and culture medium (750 μl) containing 10% serum was used as a chemoattractant to induce the migration of cells to the other side of the membrane. The cells that passed through the membrane were collected after incubation at 37 °C for 24 h to assess migration. According to the manufacturer's protocol, Matrigel (BD Bioscience Pharmingen) was spread on the upper layer, and the remaining procedure was the same as described above. After 48 h, the cells were collected to assess invasion. Approximately 3×10^4 PANC-1 cells were prepared, while 5×10^4 cells BxPC-1 cells were prepared to assess their migration and invasion. After incubation, the cells above the membrane were gently wiped off with cotton-tipped swabs, and the cells that passed through the membrane were stained with 0.1% crystal violet for 40 min. Finally, the cells in five random areas under 100 × magnification were counted, and representative images were captured under a microscope. All experiments were repeated three times.

2.9. Cell count kit-8 (CCK-8) assay

A CCK-8 assay kit (Dojindo, Japan) was used to detect cell proliferation. The cells were seeded into 96-well plates at 2.5×10^3 cells/well. At the same time of each day, 10 μl of CCK-8 reagent and 100 μl of complete medium were mixed and added to each well. After incubation in the dark for 2 h at 37 °C, the absorbance of each well was measured at 450 nm by a microplate reader. Each sample consisted of five duplicate wells and was independently repeated three times.

2.10. Construction of reporter gene plasmids

A luciferase reporter construct containing the human FER promoter was prepared using the pGL3-Basic vector (Promega, Madison, WI, USA). A DNA fragment of the FER promoter region (including restriction enzyme sites) synthesized by GenScript Biotechnology Co., Ltd. (Nanjing, China) was subcloned into the KpnI and XhoI sites of the pGL3-Basic vector to construct the pGL3-FER-promoter (pFER) recombinant plasmid. A mutant construct, i.e., pFER-YY1-M, containing the FER promoter in which the presumed YY1 binding site was mutated at CAACATGGTAAA, was also constructed.

2.11. Cell transient transfection and luciferase assay

Transfections were performed using Lipofectamine 3000 according to the manufacturer's protocol. Cells were seeded into 6-well cell culture plates (3×10^5 cells/well) 1 day before transfection as described above. The cells were constructed with 1 μg of the luciferase reporter plus 1 ng of the Renilla luciferase reporter vector pRL-SV40 and used as an internal control for each experiment. After 48 h, the cells were washed with PBS and lysed using 1 × passive lysis buffer. The firefly and Renilla luciferase activities were measured with a Promega Dual-Luciferases Reporter Assay Kit (Promega). Each experiment was performed in triplicate and independently repeated three times.

2.12. Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were isolated from the YY1-overexpressing BxPC-3 cells with NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific) according to the manufacturer's instructions. The protein concentrations were determined with a DC Protein Assay kit (Bio-Rad). The nuclear extracts were stored at −80 °C until use. An EMSA was performed using a DIG Gel Shift Kit (Roche) according to the manufacturer's protocol. The probe sense sequences used for EMSA were as follows: wild-type probe, 5′-TTCAGATAAAAATATGGCATATT TGGG-3′, which corresponded to the FER promoter; mutant probe, 5′-AAACGGCCATCACTGAAGAG-3′. Double-stranded (ds) probes were synthesized, and the 3′-end of the wild-type probe was labeled with digoxigenin-11-ddUTP. The nuclear extracts (5 μg of protein) were incubated with 1 μg poly [d(I–C)], the binding buffer included in the kit and DIG-labeled wild-type probe in the presence or absence of an unlabeled probe for 15 min at room temperature. The bound DNA complexes were separated by 5% nondenaturing polyacrylamide gel electrophoresis and transferred to a nylon membrane (Roche). The nylon membranes were cross-linked, and chemiluminescent detection was performed using CSPD. The signals were recorded using the FluorChemE system.

For the supershift analyses, the YY1 antibody (4 μg, Abcam) was added to the nuclear extracts in gel shift buffer (above) for 1 h at 4 °C, followed by the addition of the probe. The subsequent protocol used was the same as that described above.

2.13. Chromatin immunoprecipitation assay (ChIP)

Chromatin immunoprecipitation (ChIP) was carried out with a Magna Chromatin Immunoprecipitation kit (Millipore, Darmstadt, Germany). Immunoprecipitation was performed with anti-YY1 antibody. The final purified DNA fragment was subjected to PCR analysis using Hot-Start Taq DNA polymerase (TaKaRa, Dalian, China; 32 cycles). The primers used were as follows: sense TTTCAGCCTCAGAATT GGT, antisense TAGACTCCCGAAGAGGTAAGA (255 bp product length). PCR products were analyzed using gel electrophoresis. ChIP data were shown as the percentage of the input normalized to control purifications.

2.14. Animal study

Four-week-old male athymic mice (BALB/cA-nu) were ordered from the Animal Center of Nanjing Medical University (Nanjing, China). All animal experiments were conducted in compliance with animal protocols approved by Nanjing Medical University and were carried out at the Animal Center of Nanjing Medical University. In total, 5 groups with 10 mice per group were used for the construction of a tail vein metastasis model. Stable cells (PANC-1-YY1 shRNA, PANC-1-YY1 shRNA + FER shRNA, PANC-1-scramble shRNA, PANC-1-FER-OE, or PANC-1-vector) were injected into the tail vein of each mouse. After four weeks, the mice were euthanized, and the lungs and livers were removed and fixed with 4% paraformaldehyde. Three 6 μm sections (first collected in every 10 slices) were stained with HE to assess the presence of lung and liver metastasis.

2.15. Statistical analysis

The statistical analysis was performed by SPSS software (Version 22.0) and GraphPad Prism (version 5.0). The quantitative data are presented as the mean ± SD. The differences between the means of two samples were analyzed by Student's t-test. The correlations between FER expression and various clinicopathological or serological variables were analyzed by the Mann-Whitney *U* test. A receiver operating characteristic (ROC) curve analysis was performed to determine

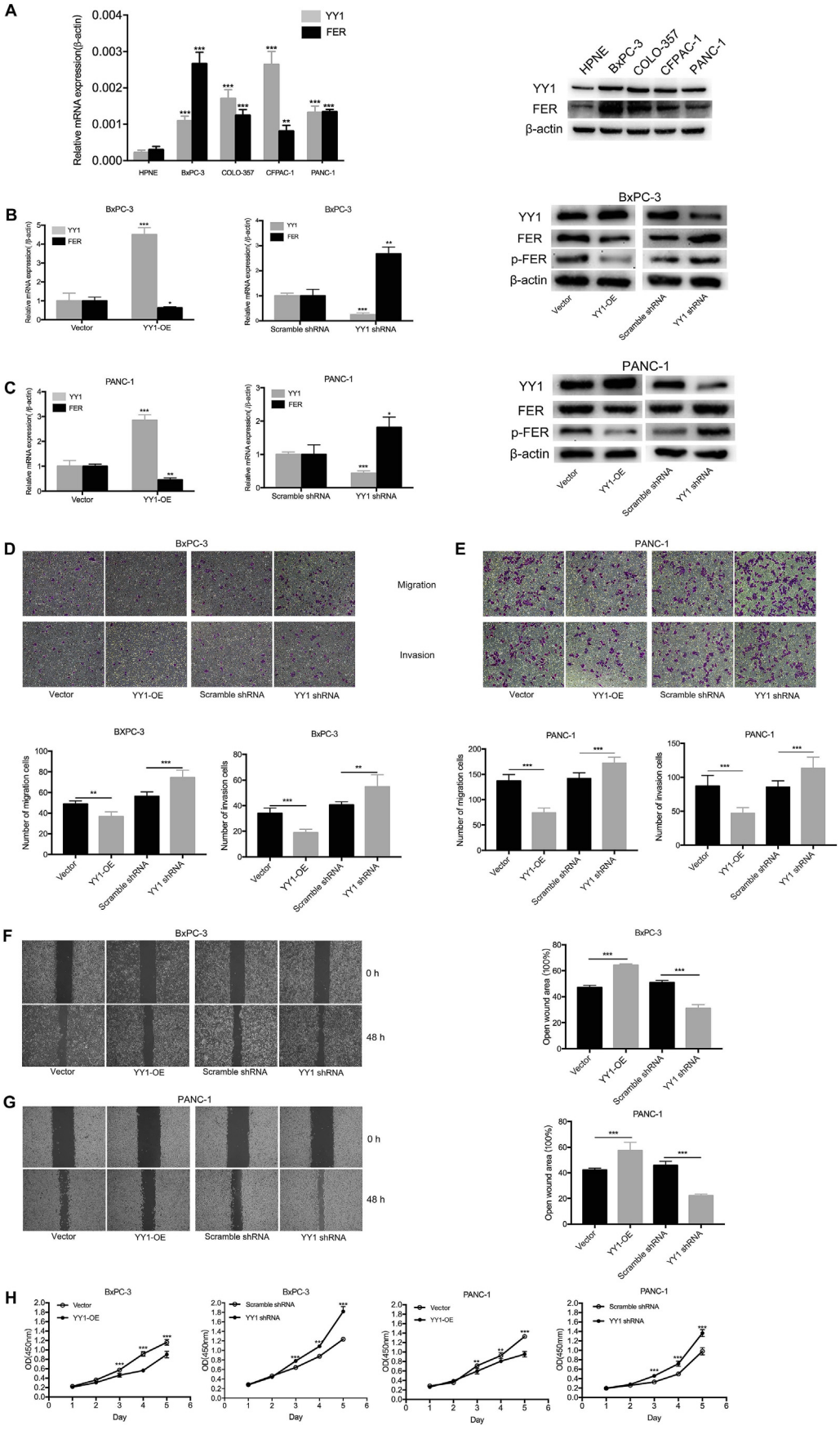

Fig. 1. YY1 inhibits the migration, invasion and proliferation of pancreatic cancer cells. (A) The relativeexpression of YY1 and FER in different PDAC celllines was detected by quantitative RT-PCR and Western blotting. (B and C) YY1 and FER expression level in YY1-overexpressing or YY1 knockdown cells were measured by quantitative RT-PCR and Western blotting. (D and E) Cell migration and invasion assays were performed. The upper chambers were seeded with various cell lines. The membranes of the chambers were stained with 0.1% crystal violet. Scale bar, 100 μm. (F and G) Wound healing assays were performed. Different cell lines were transfected with lentiviruesfor 0 and 48 h. Magnification, 200 × ; scale bar, 100 μm. (H) CCK-8 assays were performed.The numbers of cells per well were measured by the absorbance at 450 nm. (*represents $p < 0.05$, **represents $p < 0.01$, and *** represents $p < 0.001$ compared with the control group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

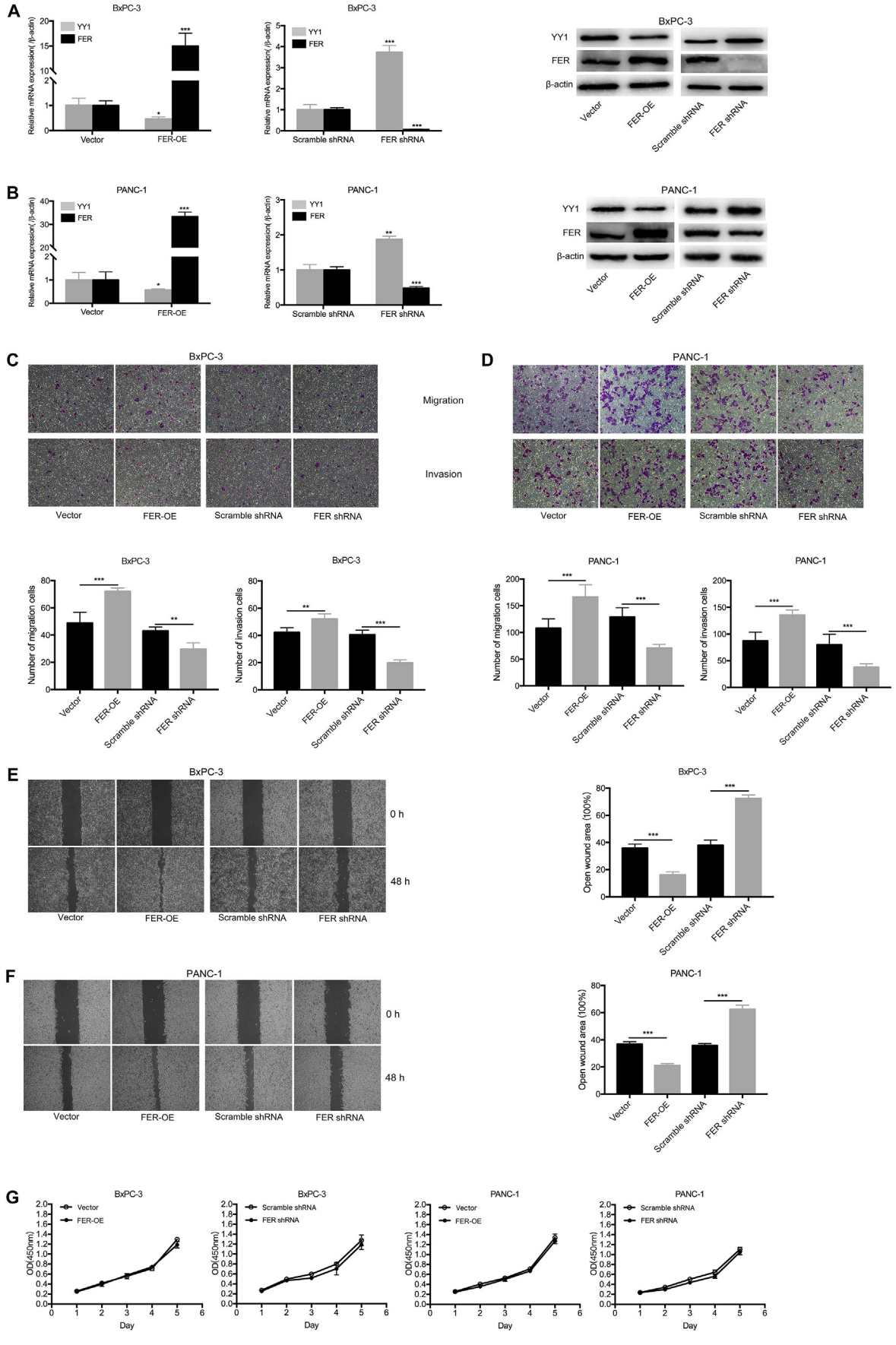

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Fig. 2. FER promotes the migration and invasion of pancreatic cancer cells. (A and B) YY1 and FER expression levels in FER-overexpressing or FER knockdown cells were measured by quantitative RT-PCR and Western blotting. (C and D) Cell migration and invasion assays were performed. The upper chambers were seeded with various cell lines. The membranes of the chambers were stained with 0.1% crystal violet. Scale bar, 100 μm. (E and F) Wound healing assays were performed. Different cell lines were transfected with lentiviruses for 0 and 48 h. Magnification, 200 × ; scale bar, 100 μm. (G) CCK-8 assays were performed.The numbers of cells per well were measured by the absorbance at 450 nm.(*represents $p < 0.05$, **represents $p < 0.01$, and *** represents $p < 0.001$ compared with the control group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the FER expression level cut-off value for the survival analysis. Survival distributions and overall survival rates were determined using the Kaplan-Meier method, and the significance of the differences between the survival rates was calculated by the Log-rank test. All data were representative of at least three independent experiments, and a difference was considered statistically significant at*$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$.

3. Results

3.1. Overexpression of YY1 inhibits the migration, invasion and proliferation of pancreatic cancer cells in vitro

The YY1 and FER expression levels in four PDAC cell lines (BxPC-3, COLO-357, CFPAC-1, and PANC-1) and a normal human pancreatic ductal cell line (HPNE) were measured by qRT-PCR and WB. The YY1 and FER expression levels were elevated in all cancer cell lines compared to the HPNE cells (Fig. 1A). Finally, BxPC-3 and PANC-1 cell lines were chosen for the subsequent studies.

As shown in Fig. 1B–C, the expression of FER and p-FER was reduced or increased in stable overexpressing or knocking down YY1 cell lines. Cellular Transwell assays and wound healing assays were used to assess the effect of YY1 on cell motility. The results of the Transwell assays showed that YY1 overexpression significantly attenuated the migration of BxPC-3 and PANC-1 cells compared with the control group, while YY1 knockdown enhanced cell migration (Fig. 1D–E). In the wound healing assays, the wound healing rate of YY1-overexpressing cells was significantly slower than that of the control group, while the effect in the YY1 knockdown cells was the reverse. These results indicated that YY1 negatively regulates the migration of BxPC-3 and PANC-1 cells (Fig. 1F–G). Compared with the control group, YY1 knockdown promoted the invasion of BxPC-3 and PANC-1 cells in the Transwell assays. In contrast, YY1 overexpression inhibited the invasion of pancreatic cancer cells (Fig. 1D–E).

Cell Counting Kit-8 (CCK-8) assays were used to elucidate the effects of YY1 on the proliferation of pancreatic cancer cells. Compared with the cells transfected with the control vector, BxPC-3-YY1-OE and PANC-1-YY1-OE cells exhibited significantly lower cell proliferation. In contrast, YY1 knockdown increased cell proliferation (Fig. 1H). These results indicated that YY1 overexpression may inhibit the proliferation of pancreatic cancer cells.

3.2. Knockdown of FER inhibits the migration and invasion of pancreatic cancer cells in vitro

To further investigate whether the activation of FER by YY1 is a key factor in the migration and invasion of pancreatic cancer, we used lentiviruses to overexpress or knockdown FER expression. The expression of YY1 and FER in BxPC-3, PANC-1 and stable YY1 knockdown or YY1 overexpressing cells was shown in Fig. 2A, B, 5A and 5B. We confirmed their expression levels using qRT-PCR and WB.

Firstly, we verified the expression of YY1 and FER in BxPC-3 and PANC-1 cells transfected with virus. In the case of FER overexpression, YY1 was knocked down, whereas in the case of FER knockdown, YY1 was overexpressed (Fig. 2A–B). As shown in Fig. 2C–D, the cellular transwell assays showed that FER overexpression promoted the migration of BxPC-3 and PANC-1 cells, while FER knockdown inhibited cell migration. The wound healing assays showed that FER overexpression accelerated wound healing rate, while FER knockdown had the opposite effect (Fig. 2E–F). In addition, FER overexpression promoted the invasion of the BxPC-3 and PANC-1 cells, vice versa (Fig. 2C–D). These results indicated that FER overexpression has a positive effect on the migration of pancreatic cancer cells as opposed to YY1 overexpression.

Furthermore, the results of the CCK-8 assays showed that the proliferation of BxPC-3 and PANC-1 cells did not differ significantly from that in the control group regardless of whether FER was overexpressed or knocked down (Fig. 2G). These results indicated that the effect of FER on pancreatic cancer cells is mainly on migration and invasion rather than on proliferation in pancreatic cancer cells.

3.3. FER is a functional target of YY1

Further studies revealed that the migration and invasion capacity inhibited by YY1 overexpression might be recovered by upregulating FER (Fig. 3A–C), while the FER knockdown had the opposite effect on that of the YY1 knockdown cells (Fig. 3B–D). However, in the CCK-8 assays, the FER overexpression or knockdown had no significant effect on the proliferation of pancreatic cancer cells inhibited by YY1 (Fig. 3E). These results indicate that FER is a functional target of YY1 and that YY1 inhibits the migration and invasion of pancreatic cancer cells by downregulating FER in vitro.

3.4. Correlation between FER expression and patient survival

Forty-eight pancreatic cancer patients were enrolled for the survival analysis. Fifteen patients died, and the remaining thirty-three patients were alive at the last follow-up (November 1, 2018). The immunohistochemistry (IHC) results showed that FER, which exists in the cytoplasm, was expressed in both the cancer tissues and adjacent tissues. However, FER was more highly expressed in the cancer tissues (Fig. 4A, $p < 0.001$). To determine the cut-off value of FER expression level for the survival analysis, the patients were divided into the following two groups based on the length of overall survival: short-term survivors (≤24 months) and long-term survivors (> 24 months). A threshold of 154.226 was chosen as the cut-off for high and low FER expression as it was on the receiver operating characteristic (ROC) curve. The curve was closest at (0.0, 1.0). This cut-off value maximized both the sensitivity and specificity of the survival outcome (Fig. 4B). The area under the ROC curve (AUC) was 0.7513 (95% CI: 0.6157–0.8869, $p = 0.0031$).

The Kaplan-Meier survival curves showed that the patients with high level of FER expression (≥154.226) had lower postoperative survival than those with low level of FER expression (< 154.226) (Fig. 4C, $p = 0.0431$, log rank test). These results indicated that high level expression of FER predicts a poor outcome in patients with pancreatic cancer.

We also analyzed the correlations between the clinical pathological or serological characteristics and FER expression. As shown in Table 1, FER expression was statistically correlated with the TNM staging, especially N staging ($p = 0.008$ and $p = 0.008$, respectively).

3.5. YY1 binds to and inactivates the FER promoter

In our previous study, BxPC-3 cells were used for ChIP-Sequencing to further investigate the potential binding sites of YY1 that affects

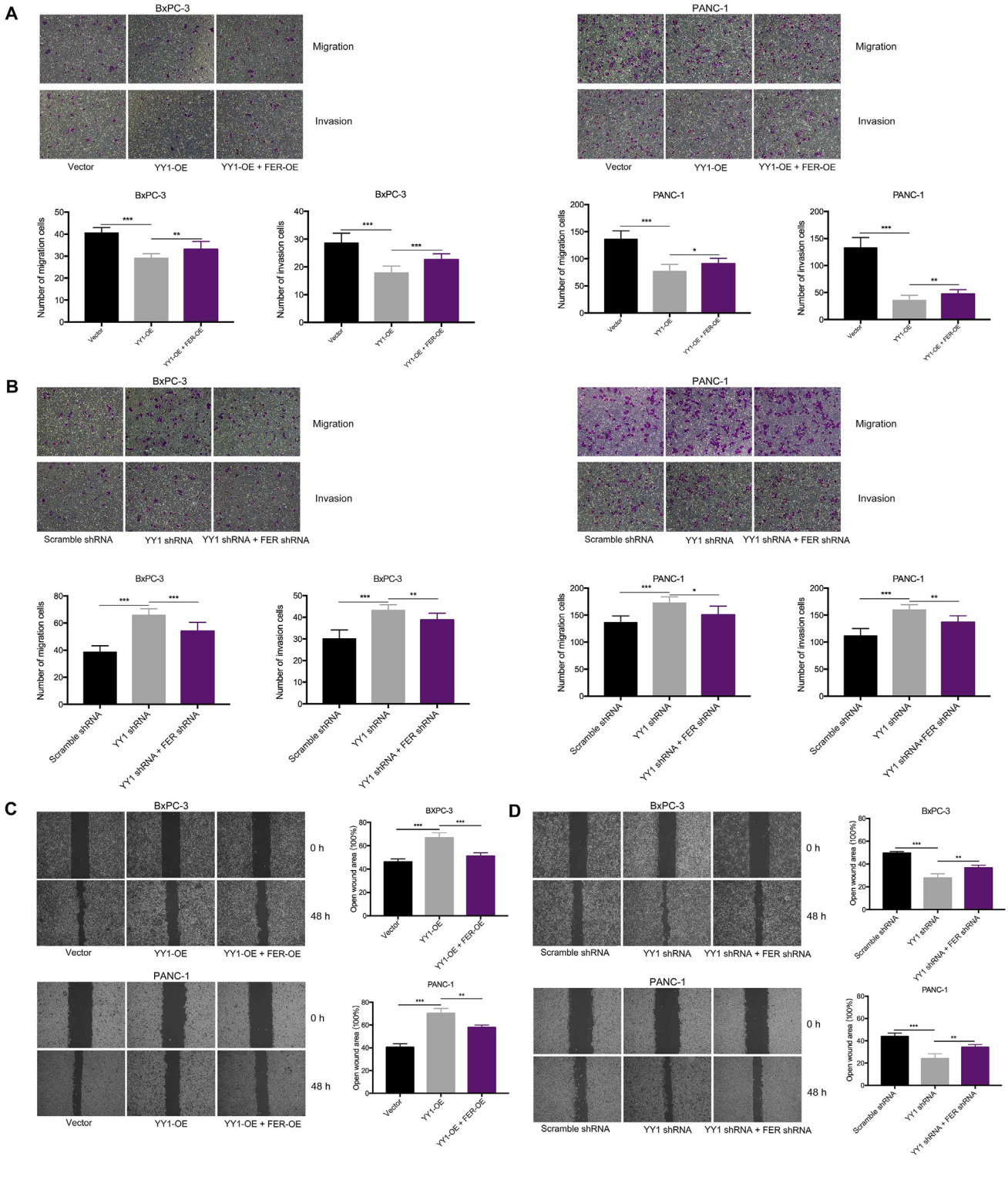

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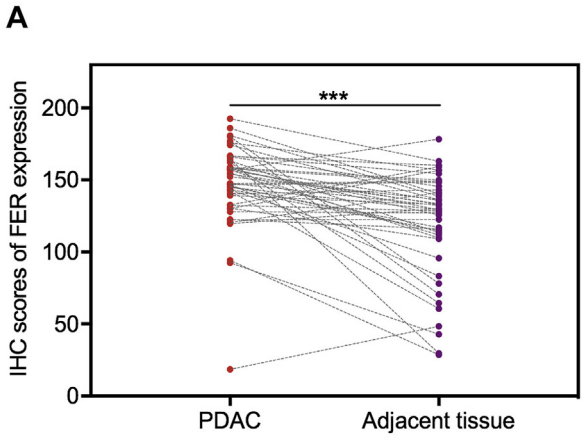
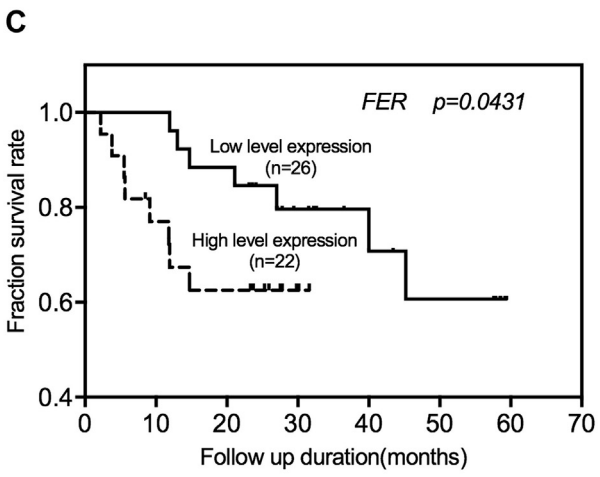
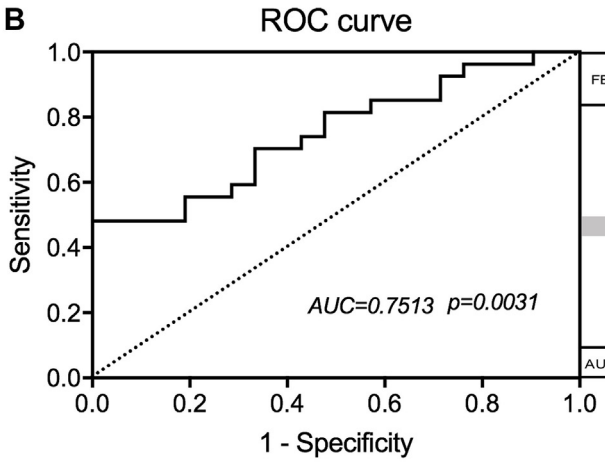
Fig. 3. FER is a functional target of YY1. (A and B) Cell migration and invasion assays were performed. BxPC-3-YY1-OE or PANC-1-YY1-OE cells were transfected with FER-overexpression lentiviruses (A) and BxPC-3-YY1-shRNA or PANC-1-YY1-shRNA cells were transfected with FER-knockdown lentiviruses (B). The membranes of the chambers were stained with 0.1% crystal violet. Scale bar, 100 μm. (C and D) Wound healing assays were performed. BxPC-3-YY1-OE or PANC-1-YY1-OE cells were transfected with FER-overexpression lentiviruses (C) and BxPC-3-YY1 shRNA or PANC-1-YY1 shRNA cells were transfected with FER-knockdown lentiviruses (D) for 0 and 48 h. Magnification, 200 × ; scale bar, 100 μm. (E and F) CCK-8 assays were performed to analyze proliferation in BxPC-3-YY1-OE or PANC-1-YY1-OE cells transfected with FER-overexpression lentiviruses (E) and BxPC-3-YY1 shRNA or PANC-1-YY1 shRNA cells transfected with FER-knockdown lentiviruses (F). (*represents $p < 0.05$, **represents $p < 0.01$, and *** represents $p < 0.001$ compared with the control group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)







FER expression level (95% CI)	sensitivity	1-specificity	*distance to the point [0.0, 1.0] on ROC curve
145.77	0.5556	0.2381	0.504165618
146.719	0.5556	0.2857	0.528314158
146.886	0.5926	0.2857	0.497593459
147.403	0.5926	0.3333	0.52636836
147.745	0.6296	0.3333	0.498282099
152.204	0.6667	0.3333	0.47135738
154.226	0.7037	0.3333	0.445962532
154.786	0.7037	0.381	0.482653799
156.63	0.7037	0.4286	0.521048606
157.007	0.7407	0.4286	0.500933578
157.231	0.7407	0.4762	0.54222037
157.589	0.7778	0.4762	0.525489562
157.851	0.8148	0.4762	0.510945672

AUC: area under the ROC curve; *$[(1\text{-sensitivity})^2+(1\text{-specificity})^2]1/2$

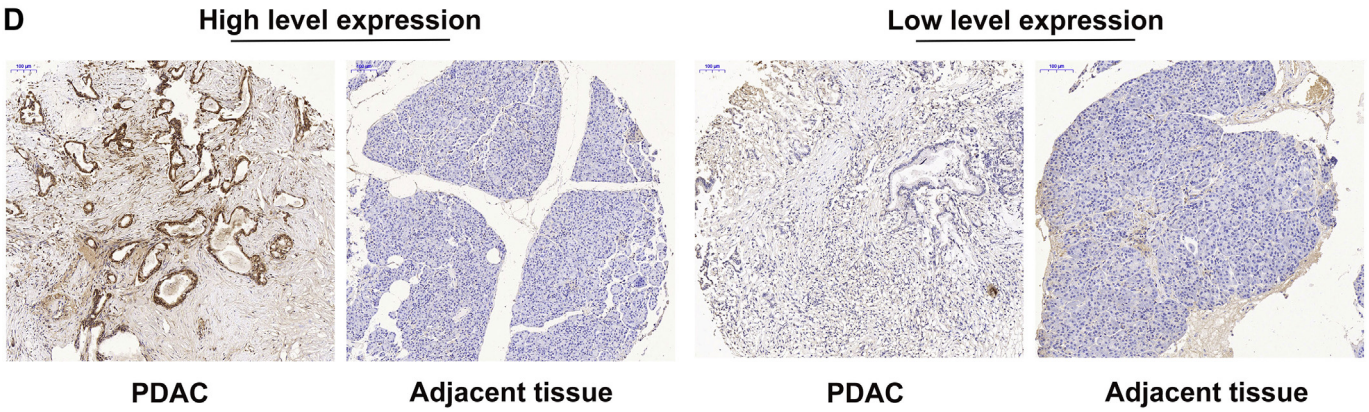


Fig. 4. Correlation between FER expression and patient survival. (A) A boxplot showing the results of the immuno histo chemistry (IHC) analysis of FERprotein expression in 48 cancer tissues and their adjacent tissues. (B) ROC curve of FER expression and cut-off value selection for high and low levels of FER expression. (C) Kaplan-Meier survival curves of 48 patients with pancreatic cancer according to their FER expression as determined by IHC. (D) Higher expression levels of FER were observed in pancreatic cancer than in adjacent tissues by IHC. Scale bar, 100 μm. (*** represents $p < 0.001$ compared with the control group).

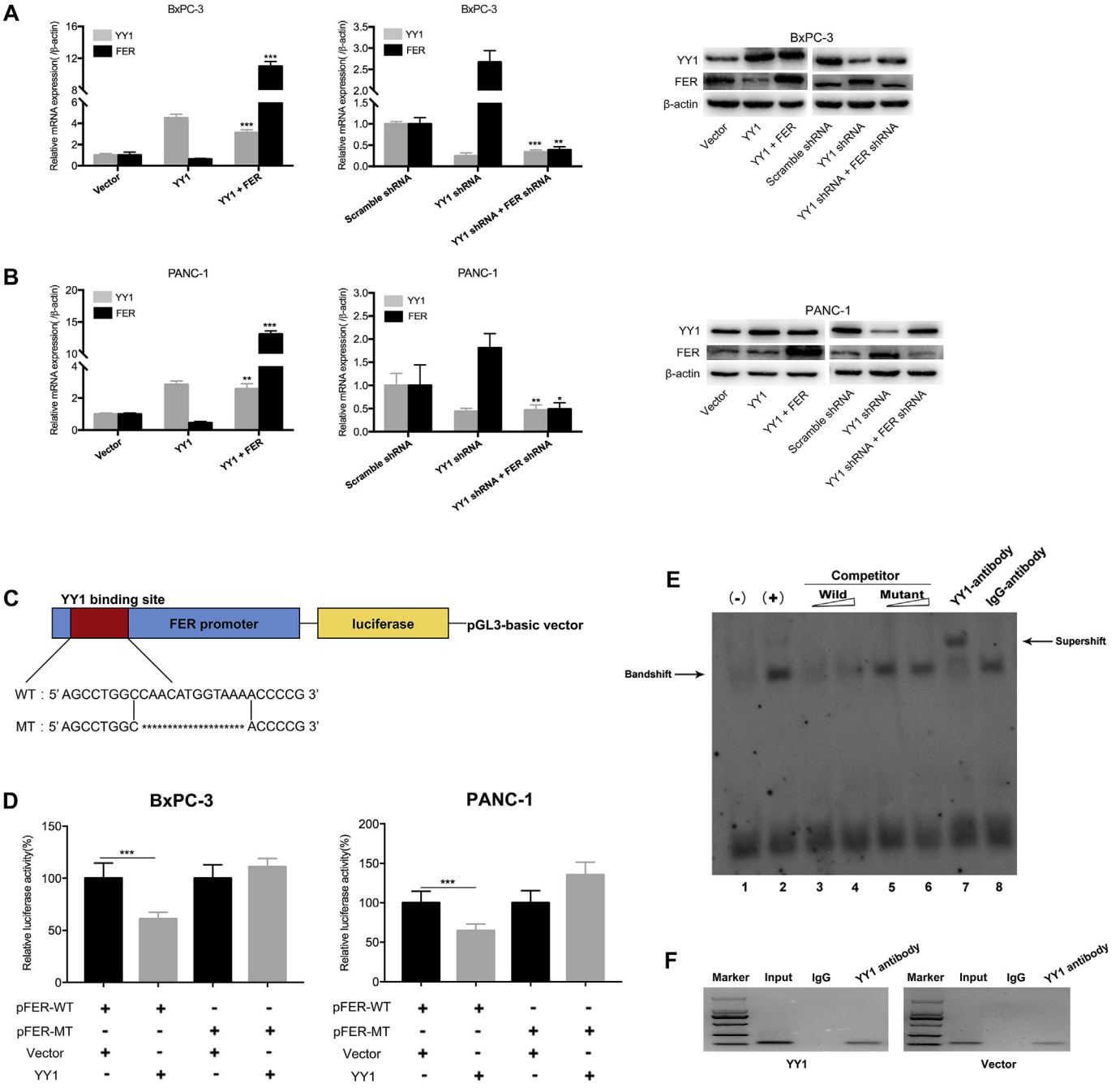


Fig. 5. YY1 directly binds to the FER promoter to regulate the expression of FER. (A and B) YY1 and FER expression in YY1-OE + FER OE and YY1 shRNA + FER shRNA cells was measured by quantitative RT-PCR and Western blotting. (C) Schematic diagram of the luciferase reporter construct containing the human FER promoter (pFER) and the mutant YY1 construct (pFER-YY1-M) containing a FER promoter in which the presumed YY1 binding site was mutated. (D) Luciferase assays demonstrating the luciferase activity of pFER (WT or mutant form) in cells transfected with the YY1-overexpression lentiviruses or control lentiviruses. Each error bar indicates the variation between the means of three independent experiments. (E) EMSA and a supershift assay showing YY1 binding the FER promoter. The wild-type probe was incubated without (lane 1) or with (lane 2) nuclear proteins from BxPC-3-YY1 cells in the absence or presence of unlabeled probe (lanes 3–6). Lanes 3 and 4 contain the wild-type probe, and lanes 5 and 6 contain the mutant probe, with each at a 50- and 100-fold molar excess. A supershift assay was performed using an anti-YY1 antibody (lane 7), and the IgG antibody was used as a negative control (lane 8). (F) ChIP assays were performed in BxPC-3-YY1-OE and BxPC-3-vector cells. Lane 1, DNA marker; lane 2, input DNA; lane 3, DNA from BxPC-3-YY1-OE cells immunoprecipitated with normal rabbit IgG; lane 4, DNA from BxPC-3-YY1-OE cells immunoprecipitated with anti-YY1 antibody. (*represents $p < 0.05$, **represents $p < 0.01$, and *** represents $p < 0.001$ compared with the control group).

pancreatic cancer. FER was chosen as a potential target gene for YY1 involved in the migration and invasion of pancreatic cancer [9].

To clarify whether YY1 binds to the promoter region of FER then inactivates its expression, FER reporter gene plasmids and FER mutant plasmids were constructed for use in a luciferase activity assay (Fig. 5C). We found that YY1 overexpression significantly reduced the luciferase activity compared to that in the control group. The luciferase activity of FER promoter in YY1 overexpression cells was increased when the presumed YY1 binding site was mutated (Fig. 5D). These results indicated that the YY1 transcription factor is involved in the transcription of FER.

For the in vitro Electrophoretic mobility shift (EMSA), we designed and synthesized an oligonucleotide (472–453) near the promoter region. As shown in Fig. 5E, the complex formed by the reaction between

Table 1
Association of FER expression with clinicopathological features of PDAC.

Variable	Group	FER expression		*P* value
		Low	High	
Gender				
	Male	19	16	0.978
	Female	7	6	
Age (year)				
	≤60	5	10	0.051
	> 60	21	12	
TNM stage[a]				
	I-IIA	16	6	**0.008***
	IIB-IV	8	16	
Diameter (cm)				
	≤3	15	10	0.246
	> 3	9	12	
Location				
	Head	16	13	0.595
	Body/Tail	8	9	
T stage				
	T1	4	7	0.229
	T2 or T3	20	15	
N stage				
	Absent	16	6	**0.008***
	Present	8	16	
Histological grade				
	I–I/II	7	1	0.070
	II-III	17	21	
Serum CA19-9 (kU/L)				
	≤37	7	6	0.564
	> 37	11	14	
Serum CEA (ug/L)				
	≤5	8	11	0.516
	> 5	9	8	

TNM stage.
All the authors declare that they have no competing interests in this work.
*P < 0.05.
[a] Tumor-node-metastasis.

the labeled probe and the nucleo proteins of the BxPC-3-YY1-OE cells migrated slowly (lane 2). The addition of the unlabeled probes inhibited the formation of the complex (lanes 3–4). In contrast, the mutant probes reduced this inhibition, and the complex reappeared (lanes 5–6). Following the addition of the YY1 antibody, the specific DNA-protein complex was supershifted (lane 7), but this did not occur after the addition of the IgG antibody (lane 8). These results indicated that YY1 specifically binds to the promoter region of FER in vitro.

For the in vivo chromatin immunoprecipitation (ChIP) assay, DNA was extracted from the BxPC-3 cells and purified. The presence of a promoter-specific DNA region was confirmed by qRT-PCR using primers containing the YY1 binding site sequence prior to immunoprecipitation. As shown in Fig. 5F, the PCR product was observed in the YY1 immunoprecipitation groups, which is consistent with the EMSA results; YY1 and the promoter region of FER directly bound in vivo.

3.6. YY1 regulates FER and affects the migration and invasion of pancreatic cancer in vivo

To further investigate the roles of YY1 and FER in PDAC, stable cells were injected into the tail veins of BALB/cA-nu nude mice for the in vivo experiments. Stable cells (PANC-1-FER-OE, PANC-1-vector, PANC-1-YY1 shRNA, PANC-1-YY1 shRNA + FER shRNA and PANC-1-scramble shRNA) were prepared as described above. After four weeks, the mice were euthanized, and the lung and liver tissues were removed for future investigation (Fig. 6A). Metastases were observed in eight of ten mice injected with PANC-1-FER-OE, while only two of ten mice injected with PANC-1-vector exhibited metastasis (Fig. 6B). This finding indicated that FER might promote the metastasis of pancreatic cancer in vivo. Metastasis in the PANC-1-YY1 shRNA group was also significantly higher than that in the control group and was significantly lower in the YY1 shRNA + FER shRNA group (Fig. 6C). The results of the in vivo studies indicated that FER is a functional target of YY1 and that YY1 inhibits the migration and invasion of pancreatic cancer by downregulating FER in vivo.

3.7. YY1 regulates the STAT3/MMP2 signaling pathway via FER

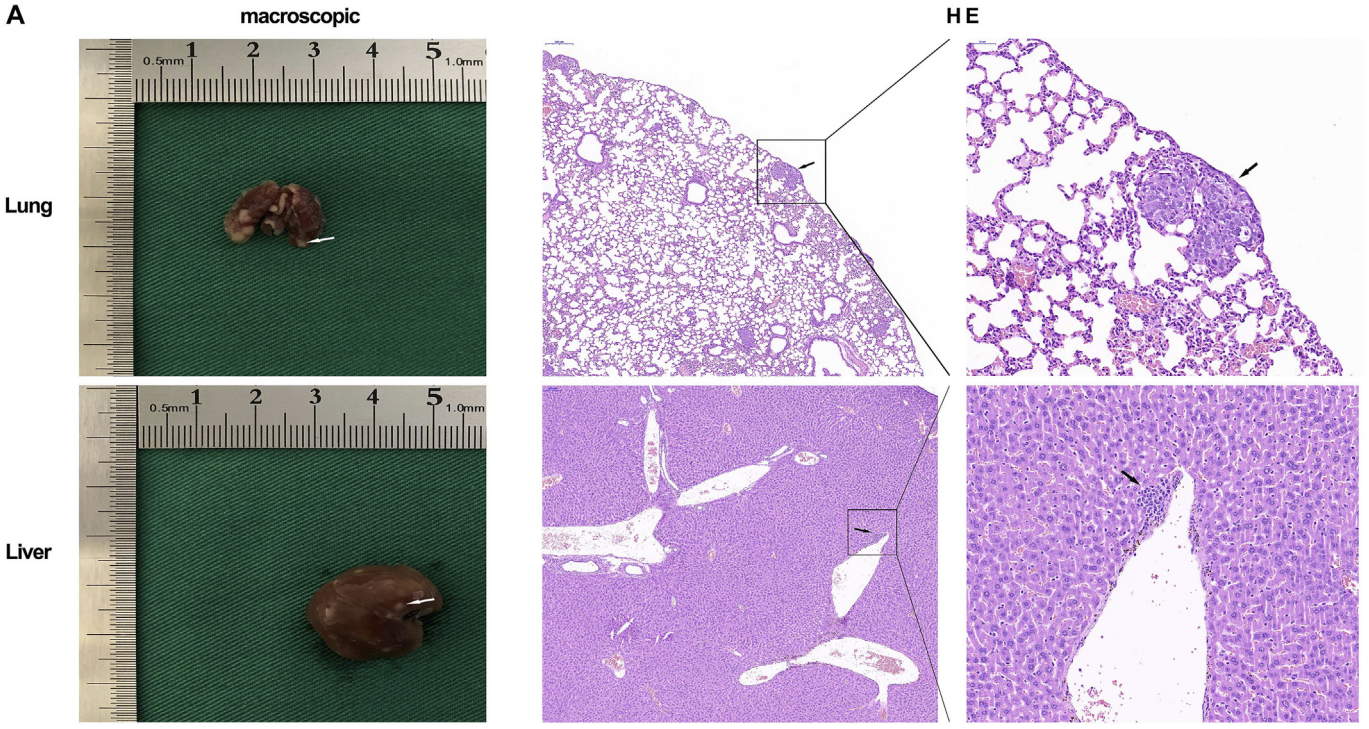
Western blotting was used to detect the specific mechanism of the regulation of pancreatic cancer invasion and metastasis by YY1. As shown in Fig. 6D, the total STAT3 level in the BxPC-3-YY1-OE and PANC-1-YY1-OE cells was unchanged compared with that in the control group, but the level of phosphorylated STAT3 decreased. The expression of the metastasis-associated protein MMP2 was decreased compared to the control group. However, when FER was upregulated in the YY1-overexpressing cells, STAT3 phosphorylation was increased, and MMP2 expression were also increased compared to those in the control group. These results indicated that overexpression of YY1 might inhibit the STAT3-MMP2 pathway by downregulating FER, thereby inhibiting the migration and invasion of pancreatic cancer cells. In contrast, the opposite results were obtained in the BxPC-3-YY1 shRNA and PANC-1-YY1 shRNA cells (Fig. 6E). As mentioned above, FER forms a complex with STAT3 to promote MMP2 expression. Altogether, YY1 inhibits the migration and invasion of pancreatic cancer through the FER/STAT3/MMP2 signaling pathway.

4. Discussion

YY1 is a ubiquitously expressed transcription factor which binds to hypomethylated DNA sequences through a C-terminal zinc finger region to form a homodimer, thereby modulating the interaction between the enhancer-promoter [36,37]. As indicated by its name, i.e., Yin-Yang, YY1 can both activate and inhibit cancer-related genes. YY1 positively regulates several oncogenes, such as c-Myc and VEGF, as well as several tumor suppressor genes, such as HJL1 and BRCA1 [38–41]. Previous studies have shown that high expression levels of YY1 are associated with prognosis in prostate cancer, gastric cancer, colon cancer and liver cancer [16,42–44]. In our previous study, YY1 was highly expressed in pancreatic cancer. However, YY1 acts as a tumor suppressor gene; the higher is its expression was, the better is the prognosis was [33]. In our previous ChIP-seq and DGE analysis, FER gene was a target of YY1 and was also upregulated following YY1 knockdown [9,33]. It means YY1 should directly bind to the promoter region of FER and regulate the transcription of FER, which might participate in the carcinogenesis and progression of PDAC.

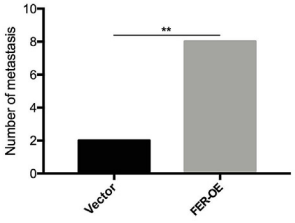
As an oncogene, FER is a nonreceptor tyrosine kinase with a molecular weight of 94 kDa and is overexpressed in many tumors, such as breast cancer, prostate cancer, ovarian cancer, and liver cancer, with a poor prognosis [45–48]. Studies have shown that the inhibition of FER and its phosphorylation activity can exert an effective anti-tumor effect [49].

According to CCK-8 assays, we found that FER has no effect on the proliferation of pancreatic cancer cells, so we focused on the effect of FER on the migration and invasion of PDAC. We found that FER is highly expressed in both pancreatic cancer tissues and cells and FER promotes the migration and invasion of pancreatic cancer cells. By performing in vivo experiments, we found that FER overexpression in mice promoted metastatic tumor formation compared to that in the control group. Moreover, FER overexpression significantly restored the ability of YY1 to inhibit the migration and invasion of pancreatic cancer cells, and FER knockdown yielded the same results. Our luciferase assay found that YY1 overexpression attenuated the activity of the FER promoter region. Furthermore, EMSA and ChIP assays showed that YY1 can directly bind to the predicted YY1 binding site in the promoter region of FER both in vitro and in vivo. These results further confirmed



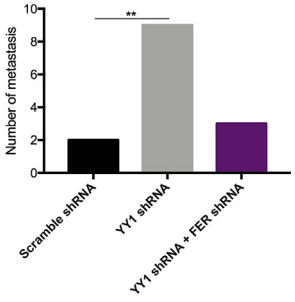
B

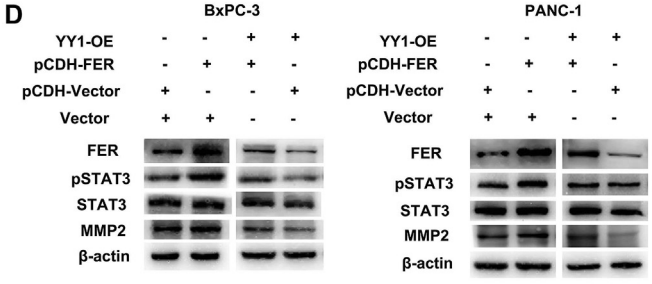
Number of animals	PANC-1-vector	PANC-1-FER-OE
Metastasis	2	8
No metastasis	8	2
Percentage	2/10	8/10
Lung metastasis	2	8
Liver metastasis	1	3

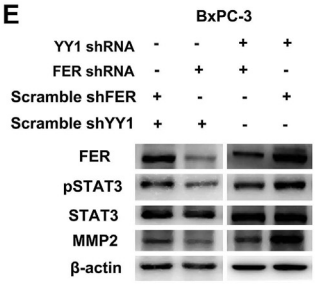


C

Number of animals	PANC-1-Scramble shRNA	PANC-1-YY1 shRNA	PANC-1-YY1 shRNA + FER shRNA
Metastasis	2	9	3
No metastasis	8	1	7
Percentage	2/10	9/10	3/10
Lung metastasis	2	8	3
Liver metastasis	0	4	1







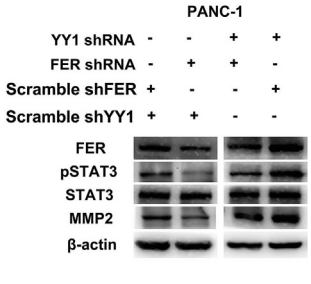


Fig. 6. YY1 regulates FER and affects the migration and invasion of pancreatic cancer in vivo. PANC-1-vector, PANC-1-FER-OE, PANC-1-Scramble shRNA, PANC-1-YY1 shRNA and PANC-1-YY1 shRNA + FER shRNA cells (1.5×10^6 cells/100 μl) were separately injected into the tail vein of each mouse. After four weeks, the lung and liver metastases were evaluated by macroscopic observation and by histomorphology under microscopy. (A) Representative pictures of lung and liver metastases are presented. Scale bar, 100 μm and 400 μm. The arrows indicate the metastases. (B and C) Table listing the incidence of metastases in the nude mice treated with vector or FER and Scramble shRNA, YY1 shRNA or YY1 shRNA + FER shRNA. (D and E) The effects of YY1 on the FER, STAT3 and MMP2 signaling pathways were examined in YY1-overexpressing cells and cells expressing a low level of YY1 or their corresponding control groups by Western blotting.

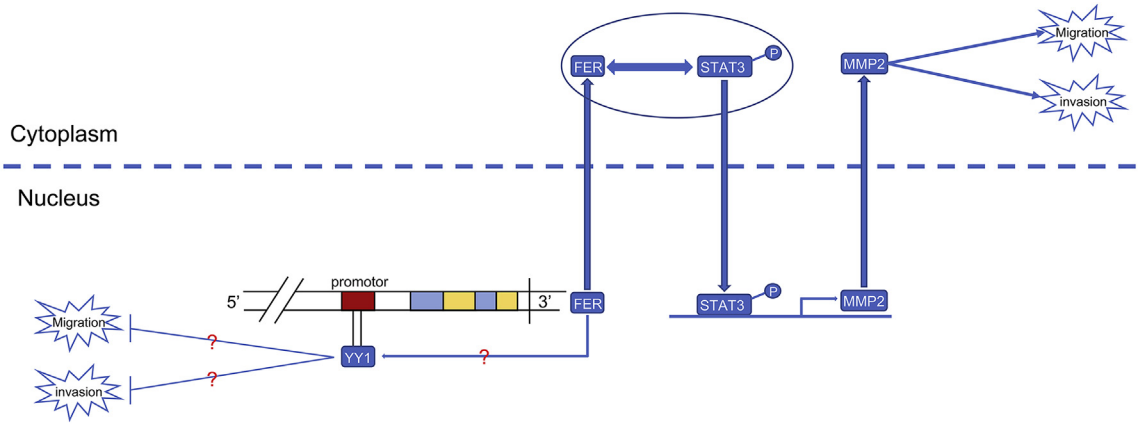


Fig. 7. Schematic representation of the roles of YY1 in the migrating and invasive properties of pancreatic cancer. YY1 suppresses invasion and metastasis by downregulating a FER/STAT3/MMP2-dependent mechanism. "?" represents other undiscovered mechanisms that might be involved.

that FER is an important gene involved in the regulation of pancreatic cancer migration and invasion by YY1.

As a downstream target of cytokines and oncogenes, STAT3 is activated by phosphorylation to drive various cancer-promoting effects [50]. FER and STAT3 are coexpressed in the cytoplasm and nucleus, and FER or the related Fes/Fps kinase can mediate STAT3 phosphorylation. P-STAT3 interacts with FER via the FERSH2 domain [51]. In our previous study, we found that YY1 can affect pancreatic cancer invasion and metastasis by regulating MMP10 expression [33]. MMP2 belongs to the MMP (matrix metalloproteinases) family [52]. STAT3 can directly bind to the promoter region of MMP2 and upregulate its expression [53]. In our study, YY1 overexpression did not result in significant changes in total STAT3 protein levels, but phosphorylated STAT3 levels were decreased and MMP2 expression was downregulated compared with those in the control group. Moreover, YY1 might play a potential role in tumor suppressor by adjusting FER phosphorylation. Interestingly, we found that FER overexpression or knockdown also affects the expression of YY1. This phenomenon suggested that FER may have a negative feedback mechanism responsible for YY1 regulation, i.e., FER may suppress FER inhibition by YY1. The specific mechanism will be addressed in the future study.

In summary, our study demonstrates that high expression levels of YY1 inhibit the migration and invasion of pancreatic cancer cells by downregulating the expression of FER. In addition, YY1 negatively regulates FER expression by directly binding to the promoter region of FER. As a member of the tyrosine kinase family, downregulation of FER inhibits STAT3 activation. Therefore, overexpression of YY1 exerts an inhibitory effect by downregulating MMP2 via the FER/STAT3 signaling pathway (Fig. 7). Although other undiscovered mechanisms may also be involved in the inhibition of tumor progression by YY1, our current study suggests that the YY1/FER/STAT3/MMP2 axis may serve as a novel therapeutic target for pancreatic cancer.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Affiliated Hospital with Nanjing Medical University. All animal experiments were performed in accordance with animal protocols approved by the Nanjing Medical University.

Author contributions

Qun Chen, Jing-Jing Zhang and Wan-Li Ge drafted the article;

Prof. Kui-Rong Jiang and Yi Miao revised it critically for important intellectual content;

All the authors contributed to acquisition of data, analysis and interpretation of data and approved the final version to be published.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.07.019.

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