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Original Article

Downregulation of Notch3 links TIMP3 inhibition to suppress aggressive phenotypes of pancreatic ductal adenocarcinoma

Tai-Jan Chiu[1,2,3], Yi-Ju Chen[4], Jui Lan[5], Yen-Yang Chen[1,2], Yueh-Chiu Chen[1,2], Hsiao-Wu Lin[1,2], Hsin-Ting Tsai[8], Yu-Sheng Lin[6], Chang-Chun Hsiao[3,7], Chang-Han Chen[8,9]

[1]Division of Hematology-Oncology, Department of Internal Medicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan; [2]Kaohsiung Chang Gung Cholangiocarcinoma and Pancreatic Cancer Group, Cancer Center, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung 83301, Taiwan; [3]Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan; [4]Department of Anatomic Pathology, E-Da Hospital, I-Shou University, Kaohsiung 84001, Taiwan; [5]Department of Pathology, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan; [6]State Key Laboratory of Optoelectronic Materials and Technologies, School of Electronics and Information Technology, Sun Yat-sen University, Guangzhou 510060, China; [7]Division of Pulmonary and Critical Care Medicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan; [8]Institute of Medicine, Chung Shan Medical University, Taichung 40201, Taiwan; [9]Department of Medical Research, Chung Shan Medical University Hospital, Taichung 40201, Taiwan

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Abstract: Pancreatic ductal adenocarcinoma (PDAC), one of the most deadly digestive cancers, has a poor 5-year survival rate and is resistant to chemotherapeutic agents, such as gemcitabine. Notch3 plays an important role in cancer progression, and its expression facilitates chemoresistance in cancers. This study examined the clinical significance of Notch3 and explored the mechanisms through which it may affect disease progression in PDAC. We found Notch3 to be upregulated in PDAC patients in whom it correlated with lymph node stage and poor survival. *In vitro* and *in vivo,* functional assays indicated that silencing Notch3 could suppress the growth, migration, invasion of PDAC cells and sensitize PDAC cells to gemcitabine. QPCR array, which was performed to elucidate the Notch3-regulated pathway, revealed that inhibition of Notch3 decreased the transcription and secretion of TIMP3 in PDAC cells. Overexpression of TIMP3 reversed the impaired growth, migration, invasion, and chemosensitivity induced by Notch3 silencing. We also found a positive correlation between Notch3 mRNA expression and TIMP3 expression in patients with PDAC. We concluded that blocking Notch3/TIMP3 pathway could considered a potentially new therapeutic strategy for treating PDAC.

Keywords: Pancreatic ductal adenocarcinoma, Notch3, TIMP3, gemcitabine, motility

Introduction

Pancreatic ductal adenocarcinoma (PDAC), a highly aggressive human digestive systemic malignancy, is the fourth cause of cancer-related deaths worldwide [1]. Early-stage pancreatic ductal adenocarcinoma is often asymptomatic. More than 80% of these patients are reported to have unresectable advanced stage or metastatic disease at initial diagnosis. The median survival time of these inoperable patients ranges between four and fourteen months [2]. Although considerable progress has been made in its diagnosis and treatment of pancreatic ductal adenocarcinoma over the past decades, its prognosis remains dismal. Complete tumor resection continues to be the primary curative treatment for these patients. Even after extended pancreatectomy with dissection of lymph nodes, these patients have a high rate of local recurrence and distant metastases. Several pathological factors can predict the survival of patients with pancreatic cancer after pancreatectomy, among them lymph node metastasis

and tumor stage [3]. Specific molecules known to be associated with cancer metastasis might also serve as markers for the early detection of recurrence and metastasis and serve as prognostic factors to consider when making surgical decisions.

Four transmembrane receptors (Notch1 to 4) and five cell-bound ligands (Jagged 1 and 2, Delta-like 1, 3, and 4) constitute the Notch family [4-6]. The Notch signaling pathway can be activated when Notch ligands bind to Notch receptors. This pathway plays an essential role in cell proliferation, differentiation, and survival [7-9], and is often considered a critical diagnostic marker and therapeutic target for the treatment of several diseases [10]. This signaling pathway has also been found to regulate cell proliferation, differentiation, apoptosis, invasion, and metastases in pancreatic ductal adenocarcinoma [11]. Although a few studies have found a high expression of Notch1 or Notch3 to be predictive of poor survival in patients with pancreatic ductal adenocarcinoma [12-16], the relationship between Notch3 expression and prognosis in resectable pancreatic ductal adenocarcinoma remains unclear.

In the present study, we examined the clinical significance of Notch3 on survival in PDAC and performed *in vitro* functional assays and array studies examining the effect of overexpressing and silencing Notch3 on growth, migration, invasion of PDAC cells and sensitize PDAC and the pathway through which it regulates these processes in PDAC.

Materials and methods

Patient and tissue specimens

The study retrospectively enrolled 80 patients with primary pancreatic ductal adenocarcinoma (PDAC) who had received tumor resections between January 2000 and December 2013 at Kaohsiung Chang Gung Memorial Hospital, a medical center located in southern Taiwan. We also performed immunohistochemical studies of Notch3 in those patients for whom we had pancreatic ductal adenocarcinoma tissue samples. We retrospectively reviewed the medical records of patients with PDAC to collect personal history, related comorbidities, laboratory data, tumor markers, serum hepatitis viral markers, and pathological results following their operations. When data were available, we collected lab results on various tumor markers including pre- and post-resection serum AFP, carbohydrate antigen 19-9, and carcinoembryonic antigen levels. Seropositivities for HBsAg and anti-HCV Ab for more than six months were interpreted as indicating chronic hepatitis B and C infection, respectively. Tumor staging was performed in accordance with the American Joint Committee on Cancer staging system seventh edition. We calculated the cumulative recurrence rate, median disease-free survival, and median overall survival and collected data on suspected prognostic factors, including AFP, CEA, and CA-199 levels, seropositivity for HBsAg or anti-HCV Ab, sex, and pathological tumor stage. The protocol for this study was approved by the Institutional Review Boards of Chang-Gung Medical Center (Taiwan) (IRB 102-4567B) and conformed to the ethical guidelines set forth in the 2004 Declaration of Helsinki. Informed consent was obtained from study participants.

Cell lines, cell culture, and transfection

Human pancreatic/duct cancer cell lines BxPC3 (RRID:CVCL_0186) and Panc-1 (RRID:CVCL_0480) were obtained from ATCC (American Type Culture Collection) and cultured in DMEM (Life Technologies, Inc., Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc., Carlsbad, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, 1% non-essential amino acid and 1% sodium pyruvate (Life Technologies, Inc., Carlsbad, USA). Cells were maintained at 37°C, 5% CO_2 in a humidified atmosphere. All cell lines were authenticated using STR profiling within the previous three years. Cells with fewer than 15 passages were used for each experiment and kept in culture for no more than one month after thawing. All experiments were performed using mycoplasma-free cells. Transfections of cells were carried out using Lipofectamine™ 3000 Transfection Reagent (Invitrogen) according to manufacturer's instructions. Cells were harvested after 24 h transfection for subsequent treatments. The human Notch3-mediated siRNA sequences were siNotch3-1: 5'-CACCUAUAACUGCCAGUGC-3' and siNotch3-2: 5'-AACUGCGAAGUGAACAUUG-3'.

RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (QIAGEN, Hilden, GERMANY) according to manufacturer's instructions. Reverse-transcription

to cDNA was performed using a random primer and a QuantiNova™ Reverse Transcription Kit (QIAGEN, Hilden, GERMANY). For QPCR, cDNAs were amplified in RT2 SYBR® Green qPCR Master mix (QIAGEN, Hilden, GERMANY) and detected using an Applied Biosystems StepOnePlus™ Q-PCR detection system. Expression genes were normalized to GAPDH and calculated using the $2^{(-\Delta\Delta CT)}$ method. The primer sequences were as follows: Notch3, forward: 5'-ACACAGGGCCACTAT GTGAGAA-3', reverse: 5'-CGTCCACGTTCACTTCACAATT-3', GAPDH, forward: 5'-CCACCCATGGCAAATTCC-3', reverse: 5'-CAGCATCGCCCCACTTG-3', HES1, forward: 5'-TCAACACGACACCGGATAAA-3', reverse: 5'-TCAGCTGG CTCAGACTTTCA-3', HEY1, forward: 5'-TGGATCACCTGAAAATGCTG-3', reverse: 5'-CGAAATCCCAAACTCCGATA-3', JAG1, forward: 5'-GTCCATGC AGAACGTGAACG-3', reverse: 5'-GCGGGACTGATACTCCTTGA-3'.

PCR array

Human Tumor Metastasis PCR array was obtained from Life Technologies Corporation (California, USA) and performed according to manufacturer's instructions. The amplification reaction and the results were analyzed using RQ manager software.

Western blotting

Cells were harvested and lysed with RIPA buffer (Thermo SCIENTIFIC, Rockford, USA), protease inhibitor cocktail set III (EMD Millipore Corp, USA) and phosphatase inhibitor cocktail set V (EMD Millipore Corp, USA) on ice for 30 minutes. The clear lysate was harvested by centrifugation at 13000 rpm for 30 minutes at 4°C. The concentration of total protein was measured and equal amounts of protein were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. For blocking, membranes were incubated with PBST (NaCl 137 μM, KCl 2.7 μM, Na_2HPO_4 10 μM and KH_2PO_4 1.8 μM, pH 7.4) containing 5% nonfat milk for 60 minutes. Antibodies to detect anti-Notch (Cell Signaling #5276s), TIMP3 (Abcam #39184), and β-actin (Sigma #A5441) were added and the membranes were then incubated overnight at 4°C. The next day horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were added to the membranes and left for 1 hour at room temperature. Signals were visualized using chemiluminescence reagents (Perkin-Elmer, Inc., Waltham, USA).

Immunohistochemical staining for Notch3

Experienced pathologists reviewed the macroscopic and microscopic pathological findings to confirm that the tumor samples contained an adequate number of cancer cells and that no cancer cells had contaminated the non-cancerous tissues. Hematoxylin and Eosin (H&E) and immunohistochemical staining were performed. All specimens were fixed in 10% formalin, embedded in paraffin, and decalcified in 10% EDTA solution. Representative blocks of the formalin-fixed, paraffin-embedded tissues were cut to 4-μm serial sections. Xylene was used to deparaffinize the sections, and a graded alcohol series was used to dehydrate them prior to blocking endogenous peroxidase activity using 0.5% H_2O_2 in methanol for 10 min. Slides were incubated with phosphate-buffered saline (PBS) for 1 hour at room temperature and treated with 3% H_2O_2 for 30 minutes to block endogenous peroxidase activity. After antigen retrieval, the sections were incubated with diluted anti-Notch3 antibody (monoclonal; 8F1; Thermo scientific, Fremont, CA, USA; 1:50) at 4°C overnight in a humidified chamber. Horseradish peroxidase/Fab polymer conjugate (PicTure™-Plus kit; Zymed, South San Francisco, CA, USA) was then applied to the sections for 30 mins followed by washing with PBS. Finally, the sections were incubated with diaminobenzidine for 5 min to develop signals.

Evaluation of Notch3 expression

Notch3 staining was independently accessed by two pathologists under double-blinded conditions without prior knowledge of the clinical status of patients whose specimens they were to examine. The pathologists recorded whether tumor or stromal cells expressed Notch3. An immunoreactivity score (IRS) system was assigned. To do this, the proportion of positive stained cells was first calculated and scored 0 to 4: 0 indicating <5%, 1 indicating 6-25%, 2 indicating 26-50%, 3 indicating 51-75% and 4 indicating >75%. Stain intensity was then scored 0 to 3: 0 being colorless, 1 pallide-flavens, 2 yellow, and 3 brown. The final staining score was obtained by multiplying proportion and intensity and stratified as - (0 scores, absent), + (1-4 score, weak), ++ (5-8 score, moderate), and +++ (9-12 score, strong). Based

on this staining score, all PDAC specimens were divided either a negative expression group (score 0-4) or the positive expression group (score 5-12).

MTT assay

BxPC3 and Panc-1 cells were plated in a 96-well plate (3000 cells/well) and treated with or without gemcitabine at indicated dosages for 72 h. After treatment, 100 µl of 0.5 mg/ml MTT were added to each well and incubated at 37°C for 3 h. The medium was then removed and 100 µl DMSO was added to each well to lyse cells. Plates were measured at 595 nm using Umax Kinetic Microplate Reader (Molecular Devices, Celifomie, USA).

Migration and invasion assay

Transwell inserts (pore size: 8 µm) coated with or without Matrigel (BD Biosciences) were used to evaluate the migratory and invasive abilities of PDAC cells. For migration assays, 1.5×10^4 BxPC3 and Panc-1 cells in 300 µl of DMEM medium in the upper chamber and added 500 µl of DMEM medium to the lower chamber. For invasion assays, 3×10^4 BxPC3 and Panc-1 cells in 300 µl of DMEM medium in the upper chamber and added 500 µl of DMEM medium to the lower chamber. After the cells were incubated for 20 hours, they were fixed and stained with crystal violet for 15 minutes. The number of migratory and invasive cells were counted in five fields under a microscope. All groups of experiments were conducted in triplicate.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using Notch3 antibody. Briefly, cells were crosslinked with 1% formaldehyde and DNA/protein complex was extracted by using EZ-CHIP KIT (Millipore). Protein and DNA were de-crosslinked with 5 M NaCl and proteinase K. The precipitated DNA was analyzed by qPCR. IgG antibody was used for negative control.

Tumor xenograft model

The animal study was approved by the Animal Ethics Committee of Chang Gung Memorial Hospital. A total of 10 male BALB/c nude mice were purchased from BioLASCO Taiwan Co., Ltd. For tumor growth study, 5×10^6 Notch3-depleted or shControl cells were subcutaneously injected into the right flank of mice. Tumor volumes were recorded twice every week. All the mice were sacrificed at 6 weeks postimplantation. Removed tumor tissues were embedded with paraffin and subjected to IHC staining.

Statistical analysis

Patients' clinical characteristics and pathological factors were analyzed by Notch-3 expression and compared using either Pearson's chi-square test or Fisher's exact test (if cell numbers were less than five). Overall and disease-free survivals were calculated and compared using a Log-rank test. All statistical analyses were performed using GraphPad Prism 8 and SPSS 22. All data are presented as the mean ± SD unless otherwise stated.

Results

Clinicopathologic factors of PDAC patients with Notch3 expression

To investigate whether the expression of Notch3 was associated with various prognostic factors, we classified the eighty PDAC patients into two groups based on their immunohistochemical results: a positive-expression group (n=44) and a negative-expression group (n=36). Representative immunohistochemical stainings were pictured in **Figure 1A**. A summary of the correlations of Notch3 immunostaining in the cancer cells and pancreatic patients' characteristics were presented in Table S1. There was a significant association between Notch3 expression and lymph node metastases but no correlation between age, gender, AJCC tumor stage, T stage, viral hepatitis, comorbidities, or tumor markers and this outcome. Additionally, the results of an analysis of a cohort consisting of 179 patients with PDAC tumor tissues and 171 normal groups in GEPIA database showed mRNA expression levels of Notch3 in PDAC tumor tissues to be higher than in normal tissues (**Figure 1B**). These results suggest that Notch3 might play an oncogenic role in PDAC.

Survival analysis of PDAC after tumor resection

The median follow-up in this study was 23.0 months (1.2-91.4 months). After surgery, 61

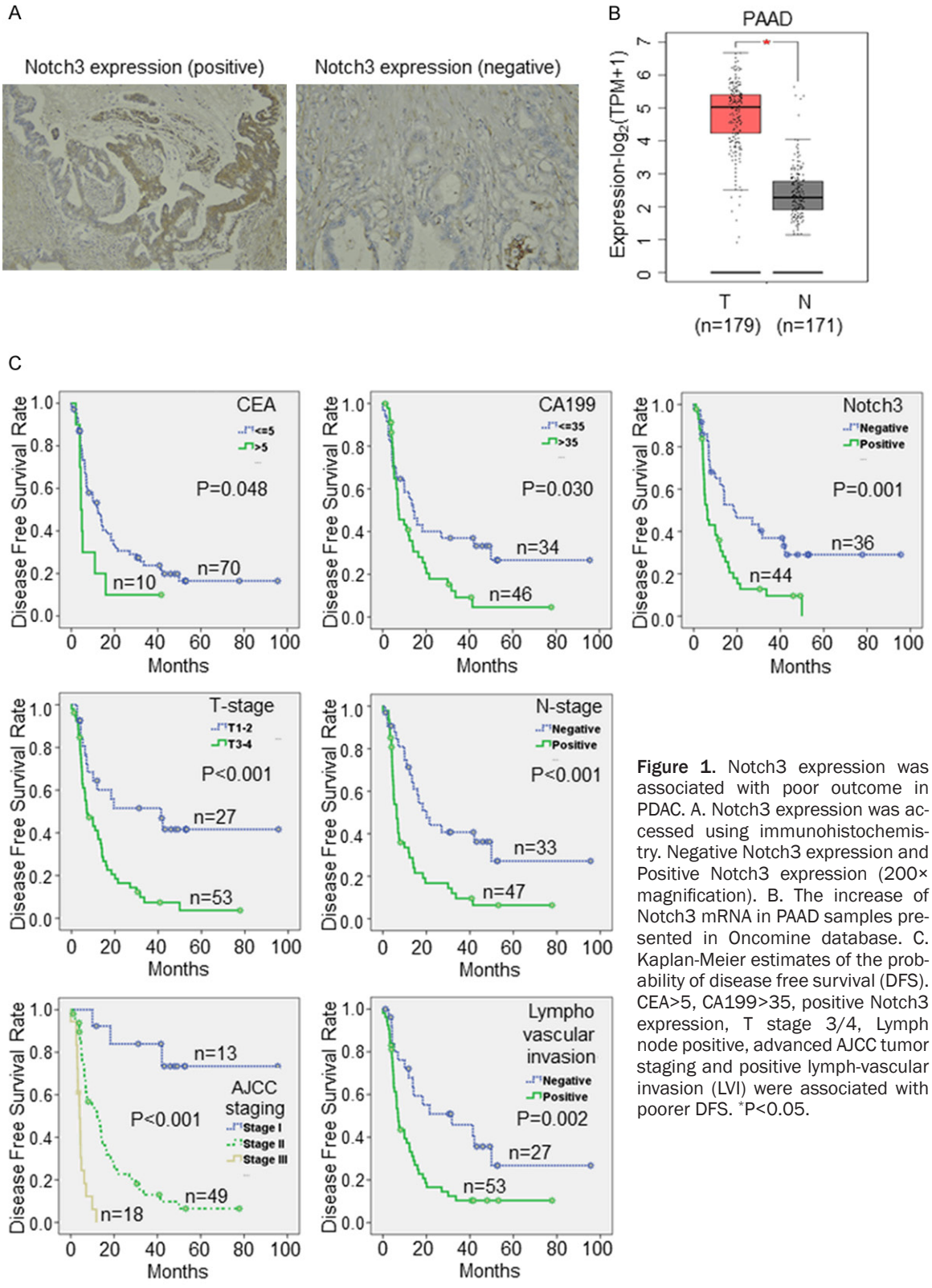


Figure 1. Notch3 expression was associated with poor outcome in PDAC. A. Notch3 expression was accessed using immunohistochemistry. Negative Notch3 expression and Positive Notch3 expression (200× magnification). B. The increase of Notch3 mRNA in PAAD samples presented in Oncomine database. C. Kaplan-Meier estimates of the probability of disease free survival (DFS). CEA>5, CA199>35, positive Notch3 expression, T stage 3/4, Lymph node positive, advanced AJCC tumor staging and positive lymph-vascular invasion (LVI) were associated with poorer DFS. *P<0.05.

patients had tumor recurrences and 55 patients died during this period. Median disease-free survival was 17.3 months, two-year disease-free survival 27.9%, and three-year

overall survival 33.5%. Kaplan-Meier was used to correlate the disease-free survival and overall survival rates of patients with PDAC and the expression of Notch3. As shown in **Table 1** and **Figure 1C**, PDCA patients with positive high CEA, CA199 expression, lymph-vascular invasion, T stage III/IV, positive lymph node metastases, advanced AJCC tumor staging, and positive Notch3 expression had significantly lower disease-free survival. Overall survival was also significantly lower in patients with high CA199, lymph-vascular invasion, T3/4 stage, positive N stage, advanced AJCC tumor staging, and positive Notch3 expression (Table S2 and Figure S1). Multivariable analysis revealed T3/4 stage, positive lymph nodes metastases, advanced AJCC tumor stage, and positive Notch3 expression to be independent prognostic factors for disease-free survival after resection (**Table 1**). T3/4 stage and advanced AJCC tumor stage were found to be independent prognostic factors predicting poor overall survival.

Inhibition of Notch3 suppressed PC cell growth and motility

We first surveyed the protein expression levels of the Notch family by Western blotting in four pancreatic cancer (PC) cell lines which had been preserved in our laboratory. Notch1-4 proteins were expressed in all PC cell lines. Both BxPC3 and Panc-1 with low but detectable Notch3 protein expressions were used in this study of the role of Notch3 in PC cell lines (**Figure 2A**). These two cell lines were prepared for transfection with Notch3 siRNA or siControl and analyzed by Q-PCR and Western blotting (**Figures 2B** and S2A). Subsequent MTT assay showed that Notch3 depletion significantly suppressed the proliferation of BxPC3 and Panc-1 cells compared to the siControls (**Figures 2C** and S2B). We also performed an *in vivo* experiment to find out whether Notch3 expression participated in the malignant progression of PC. To do this, we generated Panc-1 cells with Notch3 knocked down using Notch3-mediated shRNAs (Figure S2C). Our results revealed that tumor growth was inhibited in mice bearing stable Notch3-depleted Panc-1 xenograft tumor (Figure S2D and S2E). To further explore the biological function of Notch3 in PC cells, we also performed wound healing, migration, and invasion assays in Notch3-suppressed BxPC3 and Panc-1 cells. As shown in **Figures 2D**, **2E**, S2F and S2G, knockdown of Notch3 significantly inhibited cell migration and invasion, compared to controls. We then examined the immediate effects of Notch3 knockdown on downstream signaling in PC cells. Q-PCR revealed that HES1 and HEY1 but not JAG1 mRNA expression levels were dramatically reduced in BxPC3- and Panc-1-depleted cells, compared to their corresponding controls (**Figures 2F** and S2H). Collectively, these results showed that Notch3 expression modulated cell growth, migration, and invasion in PC cells.

Notch3 inhibition sensitized PC cells to gemcitabine

Gemcitabine is used as chemotherapeutic agent in the treatment of PC, though most patients with PC exhibit decreased sensitivity to this drug. Therefore, we wanted to investigate whether Notch3 expression is involved in PC cell chemosensitivity. BxPC3 and Panc-1 cells were first transfected with siNotch3 and siControl and then treated with gemcitabine. We found that endogenous protein expression level of Notch3 dramatically decreased in siNotch3 PC cells also treated with gemcitabine but not in cells treated siNotch3 alone (**Figures 3A** and S3A). MTT assay revealed that BxPC3 and Panc-1 cells in which Notch3 had been knocked down by Notch3-mediated siRNA had become more sensitive to gemcitabine than siControl group respectively and this change in sensitivity was dose-dependent (**Figures 3B** and S3B). Notably, BxPC3 cells transfected with siNotch3 plus gemcitabine exhibited less migration and invasion than siNotch3-BcPC3 not treated with gemcitabine (**Figure 3C**). Similar results were also found in Panc-1 cells (Figure S3C). Considered together, these results suggest that inhibition of Notch3 may render PC cells more sensitive to gemcitabine.

TIMP3 is one of the downstream targets of Notch3 in PC cells

To further investigate the mechanisms underlying Notch3's effect, we used a commercially available transcriptome tumor metastasis PCR array to identify potential regulators mediated by Notch3. We compared the gene expression profiles of siNotch3 and siControl groups. As

Table 1. Correlation between the clinicopathological features and 2-year progression-free survival in resectable pancreatic adenocarcinoma

Variables	No. of patients	Cumulative 2-year progression-free survival rate	P	HR (95% CI)	*P*
Age					
<60	42	27.7%	0.973		
≥60	38	28.6%			
Gender					
Male	41	29.6%	0.479		
female	39	26.7%			
Hepatitis B					
Negative	75	28.5%	0.704		
Positive	5	20.0%			
Hepatitis C					
Negative	76	26.6%	0.298		
Positive	4	50.0%			
Liver cirrhosis					
No cirrhosis	79	28.3%	0.296	11.596 (1.146-117.299)	*0.038*
Cirrhosis	1	0%			
DM					
Negative	57	25.9%	0.797		
Positive	23	33.0%			
Intrahepatic stone					
Negative	79	28.3%	0.006		
Positive	1	0%			
CEA					
≤5	70	30.7%	*0.048*		
>5	10	10.0%			
CA-199					
≤35	34	40.1%	*0.030*		
>35	46	17.9%			
Histology grade					
Well or Moderate	55	27.9%	0.468		
Poorly	25	27.3%			
LVI					
Negative	27	50.9%	*0.002**		
Positive	53	14.5%			
PNI					
Negative	56	42.1%	0.086	2.488 (1.127-5.495)	*0.024*
Positive	24	21.8%			
T stage					
TI-II	27	51.5%	*<0.001**	2.488 (1.066-5.804)	*0.035**
TIII-IV	53	16.4%			
N stage					
Negative	33	44.1%	*<0.001**	2.324 (1.234-4.379)	*0.009**
Positive	47	16.7%			
AJCC staging					
I	13	83.9%	*<0.001**	4.757 (2.303-9.826)	
II	49	22.7%			*<0.001**
III	18	0%			
Notch-3 expression					
Negative	36	46.5%	*0.001**	2.515 (1.388-4.555)	*0.002**
Positive	44	12.8%			

AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, Hazard ratio; LVI, lympho-vascular invasion; PNI, perineural invasion. *: Significant.

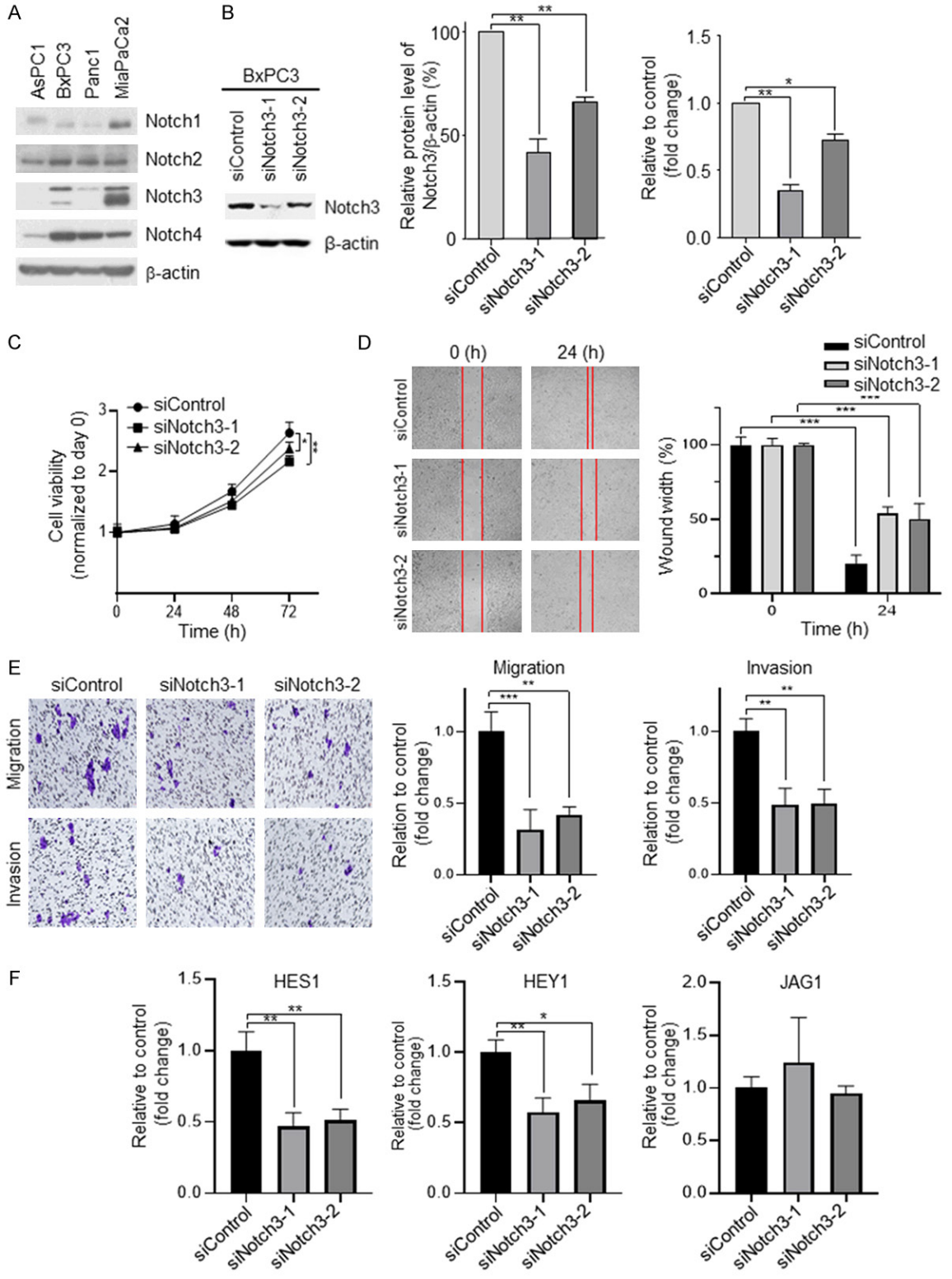


Figure 2. Suppression of Notch3 reduced BxPC3 cell proliferation, migration and invasion. A. Western blots showing Notch1-4 protein expressions in human PC cell lines. B. The protein (left) and mRNA (right) levels of Notch3 were assessed in BxPC3 cells transfected with Notch3 siRNAs and siControl by Western blotting and QPCR. C-E. MTT, wound healing, and Transwell assays were performed to estimate the cell proliferation and motility of BxPC3 cells transfected with siNotch3 and siControl. F. Notch3 downstream targets were analyzed by QPCR in BxPC3 cells transfected with siNotch3 or siControl. Data are presented as the mean ± SD. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

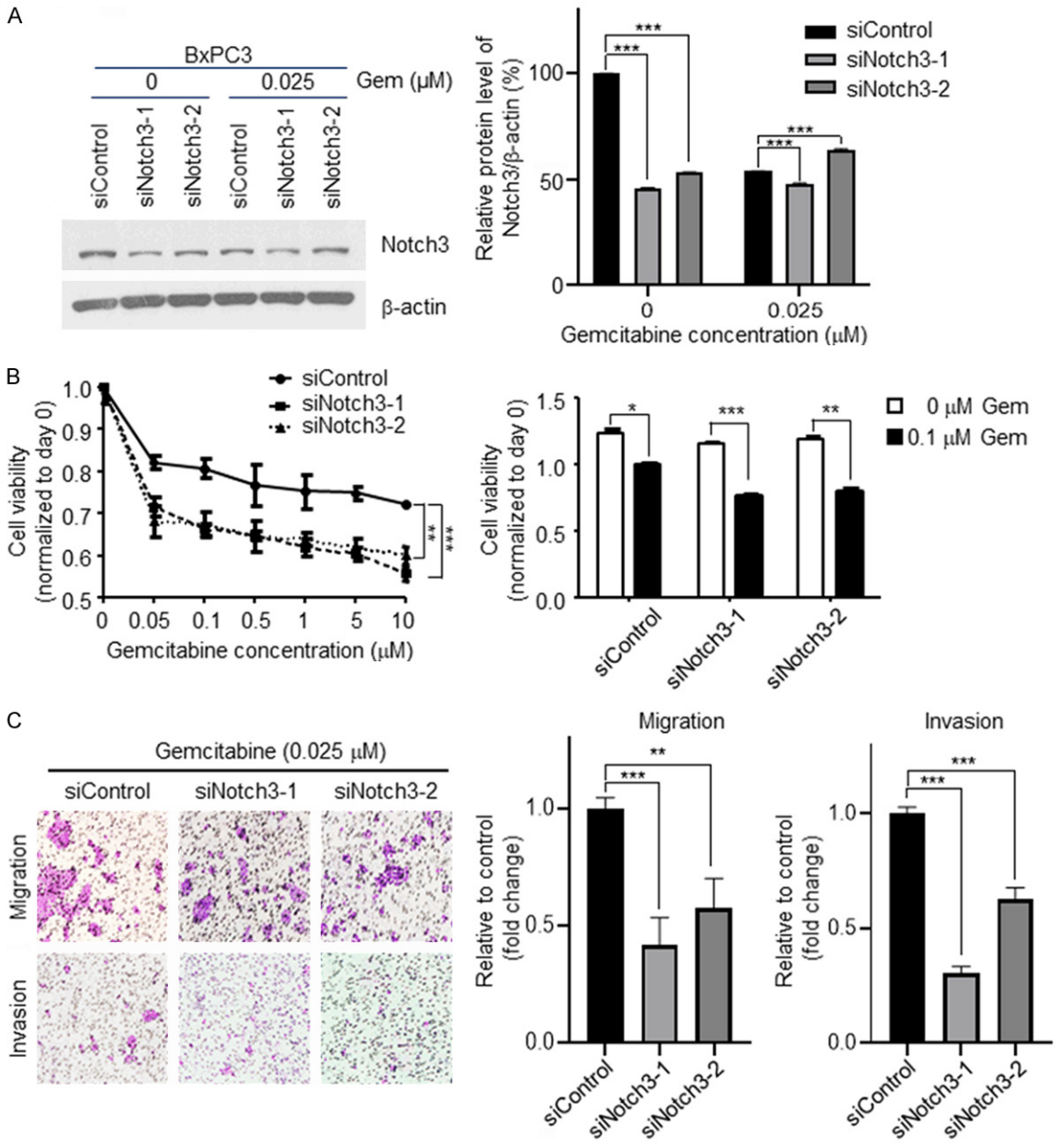


Figure 3. Knocking down Notch3 resulted in increased gemcitabine sensitivity in BxPC3 cells. A. Protein expression level of Notch3 in BxPC3 cells transfected with siNotch3 or siControl in the absence or presence of gemcitabine. B. MTT assay was performed to examine cell viability following gemcitabine treatment in siNotch3 and siControl cells. C. The migration and invasion assays were performed by Transwell in siNotch3 and siControl cells after gemcitabine (0.025 μM) treatment for 24 h. Data are presented as means ± SD. **P<0.01; ***P<0.001.

shown in Table S3A, S3B, a noticeable number of genes had their expressions altered (fold change >2 or <0.5) by Notch3 in BxPC3 and Panc-1. BxPC3 and Panc-1 were found respectively to have 20 and 17 differentially expressed genes that responded to Notch3 regulation. In the down-regulated gene groups, TIMP3 was substantially down-regulated in both BxPC3 and Panc-1 cells transfected with siNotch3 (**Figure 4A**), further validating our QPCR array results and confirming our Western blot findings indicating that the knockdown of Notch3 significantly suppressed the protein expression of TIMP3 in both BxPC3 and Panc-1 cell lines (**Figures 4B** and S4A). Importantly, the protein level of TIMP3 in the culture medium containing

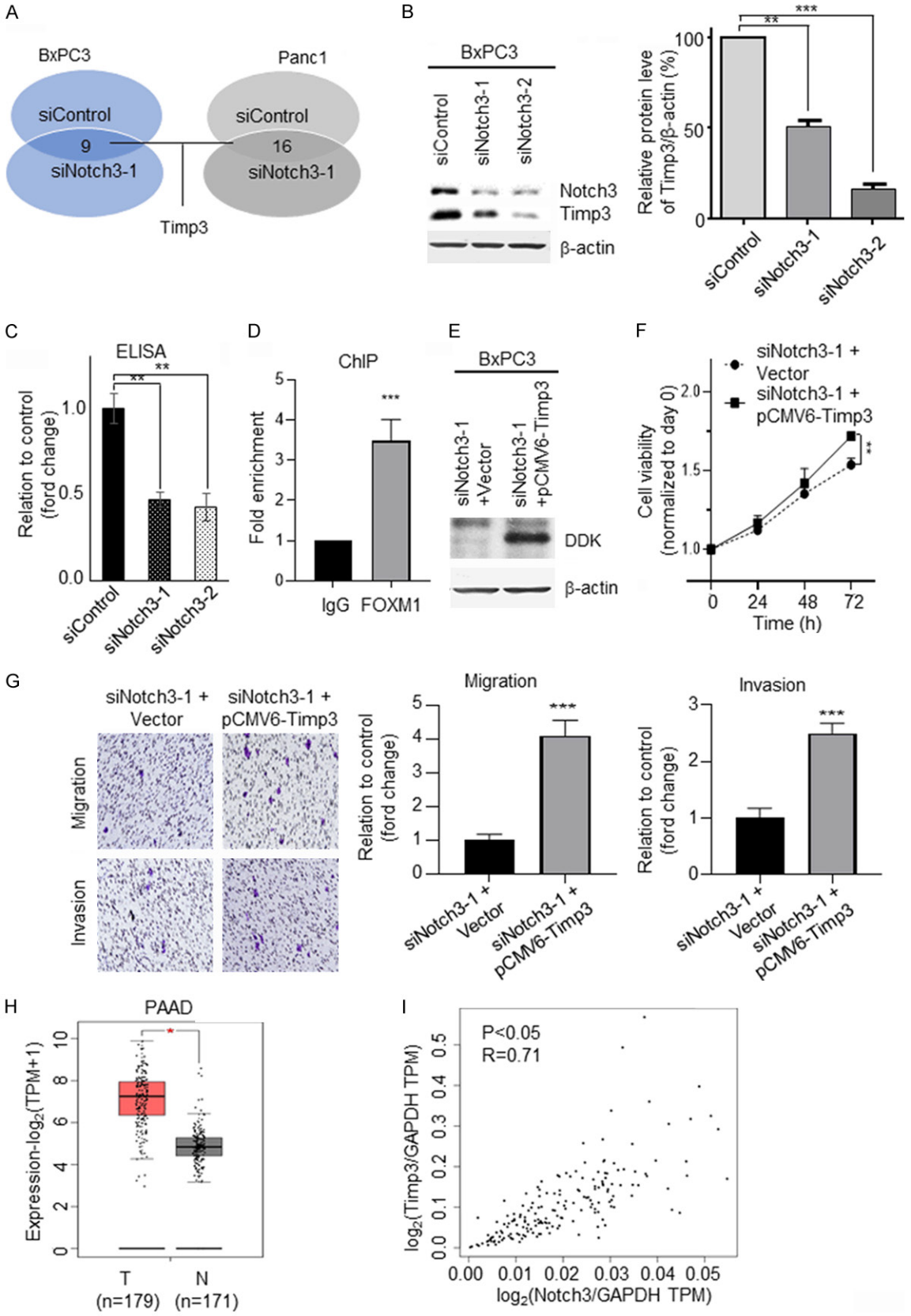


Figure 4. TIMP3 was a key effector of the Notch3-raised aggressive phenotypes in BxPC3 cells. A. Venn diagrams comparing genes that were up or down-regulated in BxPC3 and Panc-1 cells transfected with siNotch3 or siControl.

B. The protein expression level of TIMP3 was examined in siNotch3- and siControl-BxPC3 cells. C. The expression level of extracellular matrix TIMP3 was assessed in siNotch3-BxPC3 and siContrtol-BxPC3 cells by ELISA. D. ChIP assays were performed to determine the binding of Notch3 to the TIMP3 promoter in BxPC3 cells using an anti-Notch3 antibody. Isotype IgGs were used as a negative control. E. Western blot analysis of siNotch3 cells transfected with Flag-TIMP3 or vector alone. F and G. Cell proliferation, migration and invasion of siNotch3/vector and siNotch3/TIMP3 were estimated. H. The increase of TIMP3 mRNA in PAAD samples were found in the Oncomine database. I. A positive correlation between Notch3 mRNA and TIMP3 mRNA was found in the GEPIA cohort. Data are presented as the means ± SD. **P<0.01; ***P<0.001.

Notch3-depleted cells was also lower than it was in the siControl group (**Figures 4C** and S4B). We performed a ChIP assay revealing that Notch3 could bind to the promoter region of TIMP3 in BxPC3 cells (**Figure 4D**). Based on these results, we suggest that TIMP3 is a downstream target of Notch3 and that TIMP3 may play the role in Notch3's effect on PC cell cancer progression. TIMP3 was overexpressed in Notch3-depleted PC cells (**Figures 4E** and S4C) and conducted a series of MTT, migration, and invasion assays. We found that the overexpression of TIMP3 very clearly reversed siNotch3-suppressed cell proliferation, migration, and invasion (**Figures 4F**, **4G**, S4D and S4E). In clinical samples of PDAC, we found more overexpression of TIMP3 mRNA in tumor tissues than in normal tissues obtained from a previous cohort in the GEPIA database (**Figure 4H**). Furthermore, TIMP3 expression was found to be highly correlated with the expression of Notch3 mRNA in patients with PDAC (**Figure 4I**). Collectively, these findings provide evidence that TIMP3's importance in Notch3-elicited malignant phenotypes of PC.

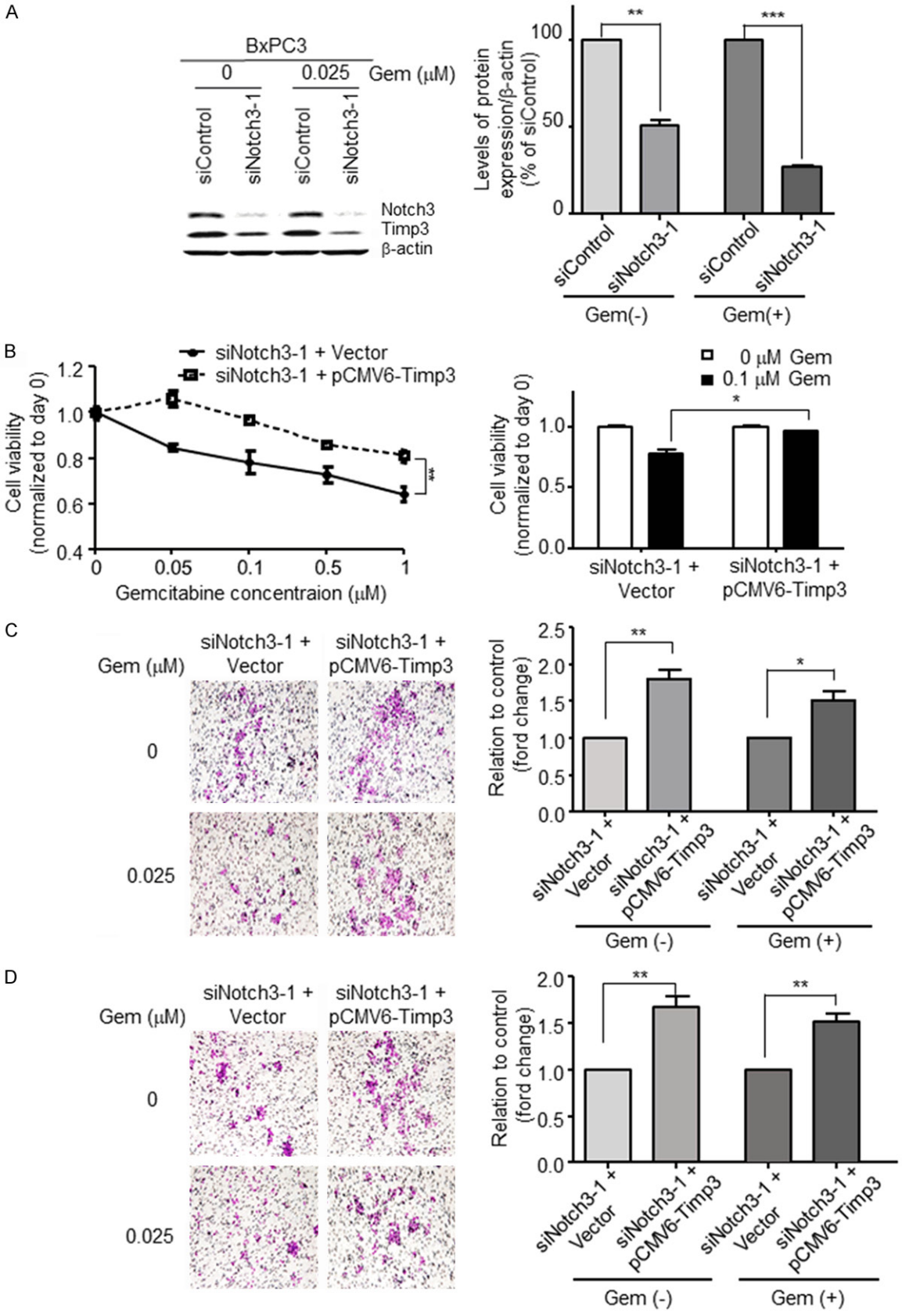
Overexpression of TIMP3 prevented Notch3-depleted PC cell gemcitabine chemoresistance

Because we found that Notch3 depletion mediated sensitivity to gemcitabine and modulated TIMP3 expression in PC cells, we wanted to find out whether TIMP3 expression would influence the effect of Notch3 had on sensitivity to gemcitabine. To do this, we first determined the protein expression levels of TIMP3 in siNotch3 cells treating with gemcitabine. We found a greater decrease in protein expression of TIMP3 in siNotch3 PC cells treated with gemcitabine than siNotch3 cells not treated with this drug (**Figures 5A** and S5A). We then performed MTT asssay to measure cell viability in Notch3-inhibited PC cells transfected with vector or TIMP3 following gemcitabine treatment. As shown in **Figures 5B** and S5B, in the presence of gemcitabine, gain-of-function of TIMP3 in siNotch3 PC cells had faster growth than in siNotch3 cells. In addition, Transwell assays confirmed the effect of TIMP3 on siNotch3 mediated PC cell migration and invasion under gemcitabine treatment, results indicating that when TIMP3 was overexpressed, cell motility was increased (**Figure 5C**, **5D**, S5C and S5D). These findings suggest that Notch3 regulation of TIMP3 may play an important role PC cell resistance to gemcitabine.

Discussion

In the current study, we identified a possibly new mechanism underlying chemoresistance in PDAC. Notch3 was upregulated in PDAC tissues and its expression was significantly correlated with poor survival. Silencing Notch3 in PDAC cells decreased their growth, migration, and invasion and it sensitized them to gemcitabine. Mechanically, Notch3 inhibition prevented TIMP3 transcription, translation, and secretion. More importantly, overexpression of TIMP3 significantly reversed sensitivity to gemcitabine in Notch3-depleted PDAC cells and increased cell growth, migration, and invasion. These findings suggest that the Notch3/TIMP3 pathway could potentially be targeted to reduce drug resistance in PDAC.

Notch, a transmembrane protein, plays a vital role in cell fate, growth, and survival [17-21]. Notch3, a member of the Notch family, is composed of 2321 amino acids and encodes a 243 kDa protein. There is increasing evidence that Notch3 expression might be involved in pathophysiological processes of human cancers. For example, Notch3 is overexpressed in many human cancers, including hemagioma, Kaposl's sarcoma, T-All, T-cell lymphoma, NPC, SCC, breast cancer, ovarian cancer, colorectal cancer, HCC, PDAC, prostatic adenocarcinoma, and NSCLC where it functions as an oncogene in the regulation of cancer growth and development [22-35]. Conversely, in neuroendocrine tumors, such as medullary thyroid carcinoma


A
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0.025
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siNotch3-1
Notch3
Timp3
β-actin
Levels of protein expression/β-actin (% of siControl)
100
50
0
**
***
siControl
siNotch3-1
siControl
siNotch3-1
Gem(-)
Gem(+)
B
siNotch3-1 + Vector
siNotch3-1 + pCMV6-Timp3
Cell viability (normalized to day 0)
1.2
1.0
0.8
0.6
0.4
0
0.05
0.1
0.5
1
Gemcitabine concentraion (μM)
**
0 μM Gem
0.1 μM Gem
*
Cell viability (normalized to day 0)
1.0
0.5
0.0
siNotch3-1 + Vector
siNotch3-1 + pCMV6-Timp3
C
Gem (μM)
siNotch3-1 + Vector
siNotch3-1 + pCMV6-Timp3
0
0.025
Relation to control (ford change)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
siNotch3-1 + Vector
siNotch3-1 + pCMV6-Timp3
siNotch3-1 + Vector
siNotch3-1 + pCMV6-Timp3
Gem (-)
Gem (+)
D
Gem (μM)
siNotch3-1 + Vector
siNotch3-1 + pCMV6-Timp3
0
0.025
Relation to control (ford change)
2.0
1.5
1.0
0.5
0.0
**
**
siNotch3-1 + Vector
siNotch3-1 + pCMV6-Timp3
siNotch3-1 + Vector
siNotch3-1 + pCMV6-Timp3
Gem (-)
Gem (+)

Figure 5. TIMP3 activation reversed siNotch3-increased sensitivity to gemcitabine in BxPC3 cells. A. Protein expression levels of Notch3 and TIMP3 in siNotch3 and siControl BxPC3 cells in the absence or presence of gemcitabine. B. Cell viability was accessed in siNotch3-BxPC3 cells transfected with vector or pCMV6-TIMP3 in a gemcitabine. C and D. Cell migratory and invasive abilities of indicated cells treated with gemcitabine. Data are presented as means ± SD. *P<0.05; **P<0.01; ***P<0.001.

(MTC), Notch3 acts as a strong negative regulator suppressing tumor cell proliferation and inducing cell apoptosis [36]. The findings of our current study are consistent with those of a previous study reporting a significant correlation between highly expressed Notch3 and disease progression and poor survival in PDAC [16]. Our *in vitro* study showed that inhibition of endogenous Notch3 in PDAC cells interrupted their ability to grow, migrate, and invade and increased their sensitivity to gemcitabine, results suggesting that Notch3 might serve as a valuable target for patients with PDAC.

To further understand the role of Notch3, we also analyzed the mRNA expression of Notch3 in human cancers in the TCGA database. We found Notch3 to be overexpressed in cholangio carcinoma (P<0.05), large B-cell lymphoma (P<0.05), glioblastoma multiforme (P<0.05), kidney renal clear cell carcinoma (P<0.05), pheochromocytoma and paraganglioma (P< 0.05), stomach adenocarcinoma (P<0.05), testicular germ cell tumors (P<0.05), and thymoma (P<0.05). Considered together, these results suggest that Notch3 plays a crucial role in the progression of cancer in humans.

TIMP3 is a member of the TIMP family where it is approximately a 24 kDa secreted glycoprotein [37]. In human cancers, TIMP3 controls cell viability, metastasis, angiogenesis, and inflammation [38]. While many clinical studies have reported that TIMP3 plays a tumor-suppressive role in human cancers, including brain, gastric, esophageal, clear cell renal cell carcinoma, and meningiomas [39-42], another study has reported an association between a high TIMP3 mRNA levels in the stroma of head and neck cancers and poor patient survival [43]. Thus, it may be that TIMP3 acts differently depending on the particular human cancer context, though this would require further study to explore. Using QPCR gene signature array, we identified TIMP3 to be a downstream target of Notch3 in PDAC cells. Knocking down endogenous Notch3 with Notch3-mediated siRNA led to the down regulation of TIMP3 mRNA and protein in both BxPC3 and Panc-1 cells. Additionally, we found TIMP3 mRNA to be more highly expressed in the tumor tissues of PDAC than that in normal tissues in the TCGA database. High Notch3 mRNA expression in PDAC tumor tissues was positively correlated with TIMP3 mRNA expression. These results suggest that TIMP3 plays an oncogenic role in PDAC and cooperates with Notch3 to promote the development of cancer.

Gemcitabine, an anti-metabolic deoxypyrimidine analog, was first used in antiviral therapy and has been approved as an anti-cancer drug for the treatment of human cancers, including PDAC [44, 45]. However, gemcitabine resistance is a major obstacle when treating PDAC patients and leads to a poor prognosis. Hence, it is important to explore the mechanism underlying gemcitabine resistance in PDAC. More importantly, knocking down Notch3 prevented tumor growth and motility and increased sensitivity to gemcitabine. In this study, siNotch3 inhibition of tumor development was abrogated by overexpressing TIMP3 in PDAC cells, suggesting Notch3-induced increases in PDAC aggression requires the activation of TIMP3.

Conclusion

This study not only found that Notch3 to be a valuable prognostic biomarker but also found that its expression enhanced PDAC cell growth, migration, invasion, and gemcitabine resistance, an effect it achieves via its effect of on TIMP3. Enforced expression of TIMP3 in Notch3-depleted PDAC cells reversed the siNotch3-attenuated aggressiveness. We concluded that Notch3/TIMP3 signaling plays a critical role in the progression of PDAC and its chemoresistance. This pathway could potentially be used as a valuable target for the treatment of PDAC.

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Disclosure of conflict of interest

None.

Abbreviations

PDAC, Pancreatic ductal adenocarcinoma; TIMP3, Tissue inhibitor of metalloproteinases 3; SCC, Squamous cell carcinoma.

Address correspondence to: Dr. Chang-Han Chen, Institute of Medicine, Chung Shan Medical University, 110 Jianguo Road, Taichung 40201, Taiwan. Tel: +886-24730022 #12189; E-mail: changhan@csmu.edu.tw; Dr. Chang-Chun Hsiao, Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, 259 Wenhua Road, Kushan Township, Taoyuan 33302, Taiwan. Tel: +886-7317123; E-mail: cchsiao@mail.cgu.edu.tw

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Table S1. Relationships between Notch3 expression and clinicopathological factors

Notch-3	Negative expression	Positive expression	P
Age			
<60	20 (47.6%)	22 (52.4%)	0.621
≥60	16 (42.1%)	22 (57.9%)	
Gender			
male	19 (40.5%)	22 (59.5%)	0.805
female	17 (46.7%)	22 (53.3%)	
Hepatitis B			
negative	34 (45.3%)	41(54.7%)	0.816
positive	2 (40.0%)	3 (60.0%)	
Hepatitis C			
negative	33 (43.4%)	43 (56.6%)	0.236
positive	3 (75.0%)	1 (25.0%)	
Liver cirrhosis			
No cirrhosis	36 (45.6%)	43 (54.4%)	1.00
cirrhosis	0 (0%)	1 (100%)	
DM			
negative	23 (40.4%)	34 (59.6%)	0.188
Positive	13 (56.5%)	10 (43.5%)	
Cigarette smoking			
negative	24 (41.4%)	34 (58.6%)	0.219
positive	12 (54.5%)	10 (45.5%)	
Alcohol drinking			
negative	26 (44.1%)	33 (55.9%)	0.779
positive	10 (47.6%)	11 (52.4%)	
CEA			
≤5	33 (47.1%)	37 (52.9%)	0.311
>5	3 (30.0%)	7 (70.0%)	
CA-199			
≤35	16 (47.1%)	18 (52.9%)	0.750
>35	20 (43.5%)	26 (56.5%)	
Histology grade			
well and moderately	27 (49.1%)	28 (50.9%)	0.753
poorly	9 (35.7%)	16 (64.3%)	
LVI			
negative	13 (48.1%)	14 (51.9%)	0.686
positive	23 (43.4%)	30 (56.6%)	
PNI			
negative	12 (50.0%)	12 (50.0%)	0.556
positive	24 (42.9%)	32 (57.1%)	
T stage			
T1-T2	16 (33.3%)	11 (66.7%)	0.067
T3-T4	20 (80.0%)	33 (20.0%)	
N stage			
negative	10 (30.3%)	23 (69.7%)	*0.027***
positive	26 (55.3%)	21 (44.7%)	
AJCC staging			
I-II	30 (48.4%)	32 (51.6%)	0.389
III	6 (33.3%)	12 (66.7%)	

AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, Hazard ratio; LVI, lympho-vascular invasion; PNI, perineural invasion. **: Significant.

Table S2. Correlation between the clinicopathological features and 3-year overall survival in resectable pancreatic adenocarcinoma

Variables	No. of patients	Cumulative 3-year Overall survival rate	P	HR (95% CI)	*P*
Age					
<60	42	33.7%	0.768		
≥60	38	33.2%			
Gender					
male	41	38.1%	0.579		
female	39	28.5%			
Hepatitis B					
Negative	75	34.6%	0.758		
Positive	5	20.0%			
Hepatitis C					
Negative	76	32.5%	0.581		
Positive	4	50.0%			
Liver cirrhosis					
No cirrhosis	79	34.0%	0.088	16.133 (1.908-136.387)	*0.011**
Cirrhosis	1	0%			
DM					
negative	57	29.9%	0.487		
positive	23	42.5%			
CEA					
≤5	70	35.6%	0.207		
>5	10	20.0%			
CA-199					
≤35	34	43.7%	*0.036**		
>35	46	25.2%			
Histology grade					
Well or Moderate	55	36.9%	0.182		
Poorly	25	26.2%			
LVI					
Negative	27	51.1%	*0.005**		
Positive	53	24.6%			
PNI					
Negative	24	42.6%	0.173		
Positive	56	29.4%			
T stage					
TI-II	27	54.1%	*<0.004**	2.267 (1.364-5.243)	*0.004**
TIII-IV	53	23.7%			
N stage					
negative	33	51.5%	*0.006**		
positive	47	20.6%			
AJCC staging					
I	13	83.3%	*<0.001**	3.356 (1.774-6.346)	*<0.001**
II	49	30.5%			
III	18	0			
Notch3 expression					
negative	36	46.7%	*0.025**		
positive	44	22.1%			

*: Significant.

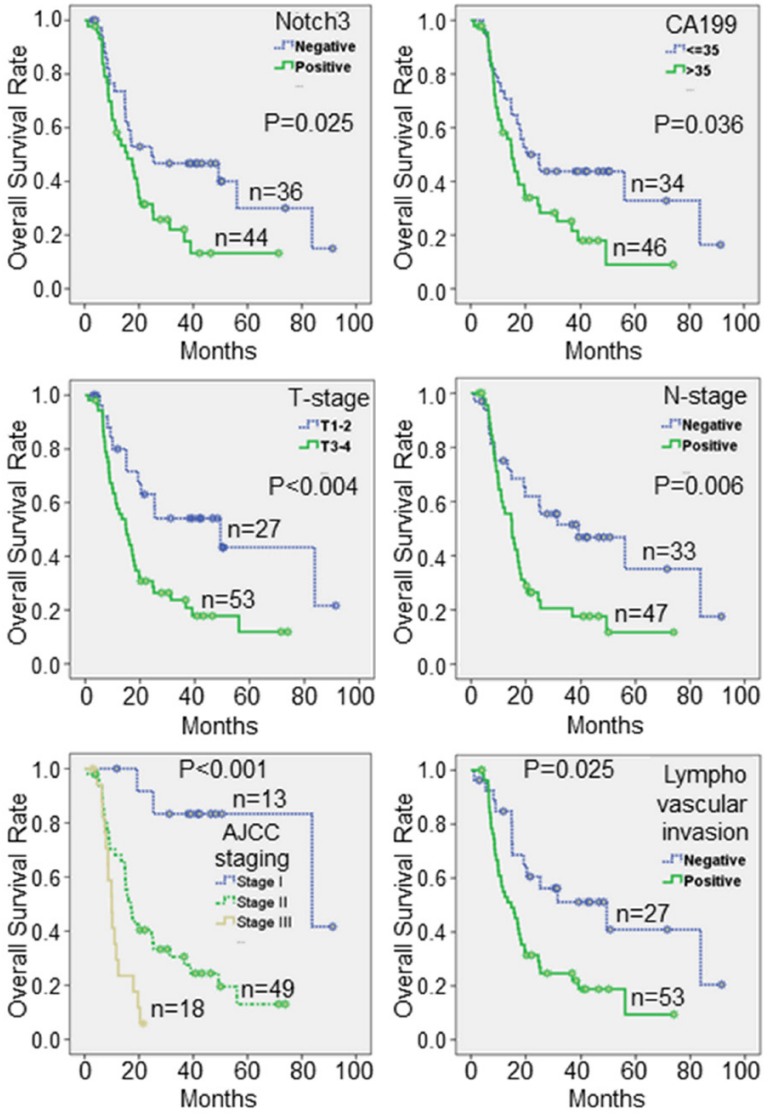
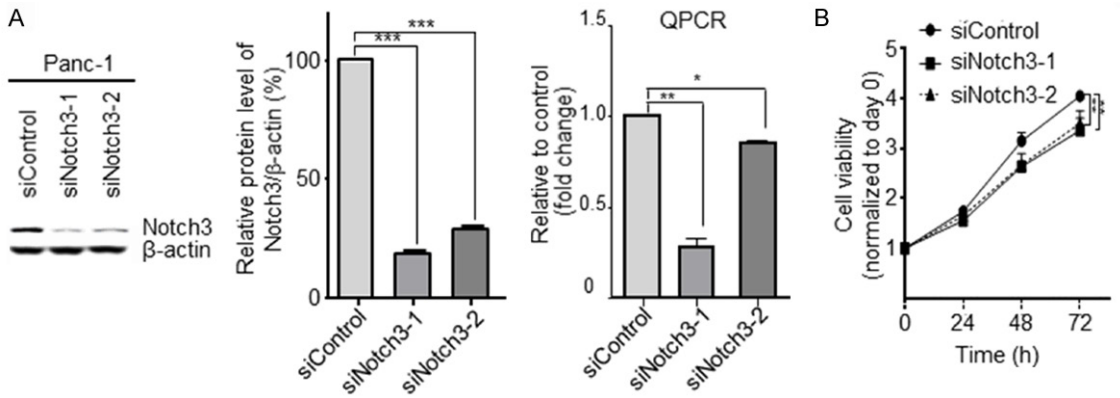


Figure S1. Kaplan-Meier estimates of the probability of overall survival (OS). Positive Notch3 expression, CA199>35, T stage 3/4, Lymph node positive, advanced AJCC tumor staging and positive lymph-vascular invasion (LVI) were associated with poor overall survival.



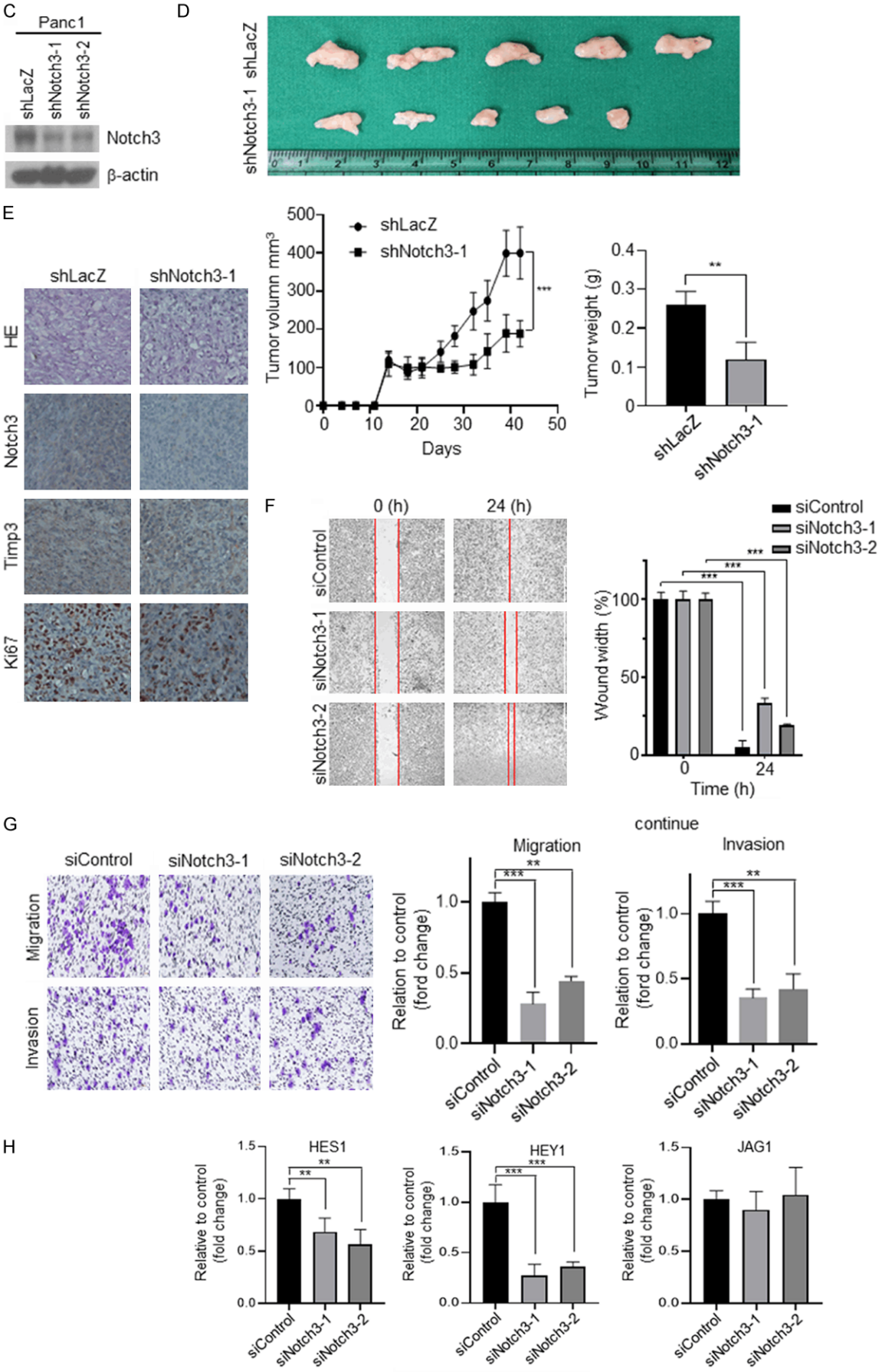

C
Panc1
shLacZ
shNotch3-1
shNotch3-2
Notch3
β-actin
D
shLacZ
shNotch3-1
E
shLacZ
shNotch3-1
HE
Notch3
Timp3
Ki67
Tumor volumn mm3
Days
Tumor weight (g)
F
0 (h)
24 (h)
siControl
siNotch3-1
siNotch3-2
Wound width (%)
Time (h)
G
Migration
Invasion
Relation to control (ford change)
continue
H
HES1
HEY1
JAG1
Relative to control (fold change)

Figure S2. Suppression of Notch3 reduced Panc-1 cell proliferation, migration and invasion. A. The protein (left) and mRNA (right) levels of Notch3 were assessed in Panc-1 cells transfected with Notch3 siRNAs and siControl by Western blotting and QPCR. B. MTT was performed to estimate the cell proliferation of Panc-1 cell with transfection siNotch3 and siControl. C. The protein level of Notch3 was determined in Panc-1 cells transfected with Notch3-mediated shRNAs shcontrol by Western blotting. D and E. *In vivo* tumor growth assay of stable Notch3-knockdown Panc-1 cells. Tumor growth rate and tumor weight were shown in shControl and shNotch3. The protein expressions profiles of Notch3, TIMP3 and Ki67 in tumor tissues were determined by IHC. F and G. Wound healing and Transwell assays were performed to estimate the cell motility of Panc-1 cells transfected with siNotch3 and siControl. H. Notch3 downstream targets were analyzed by QPCR in Panc-1 cells transfected with siNotch3 or siControl. Data are presented as means ± SD. *P<0.05; **P<0.01; ***P<0.001.

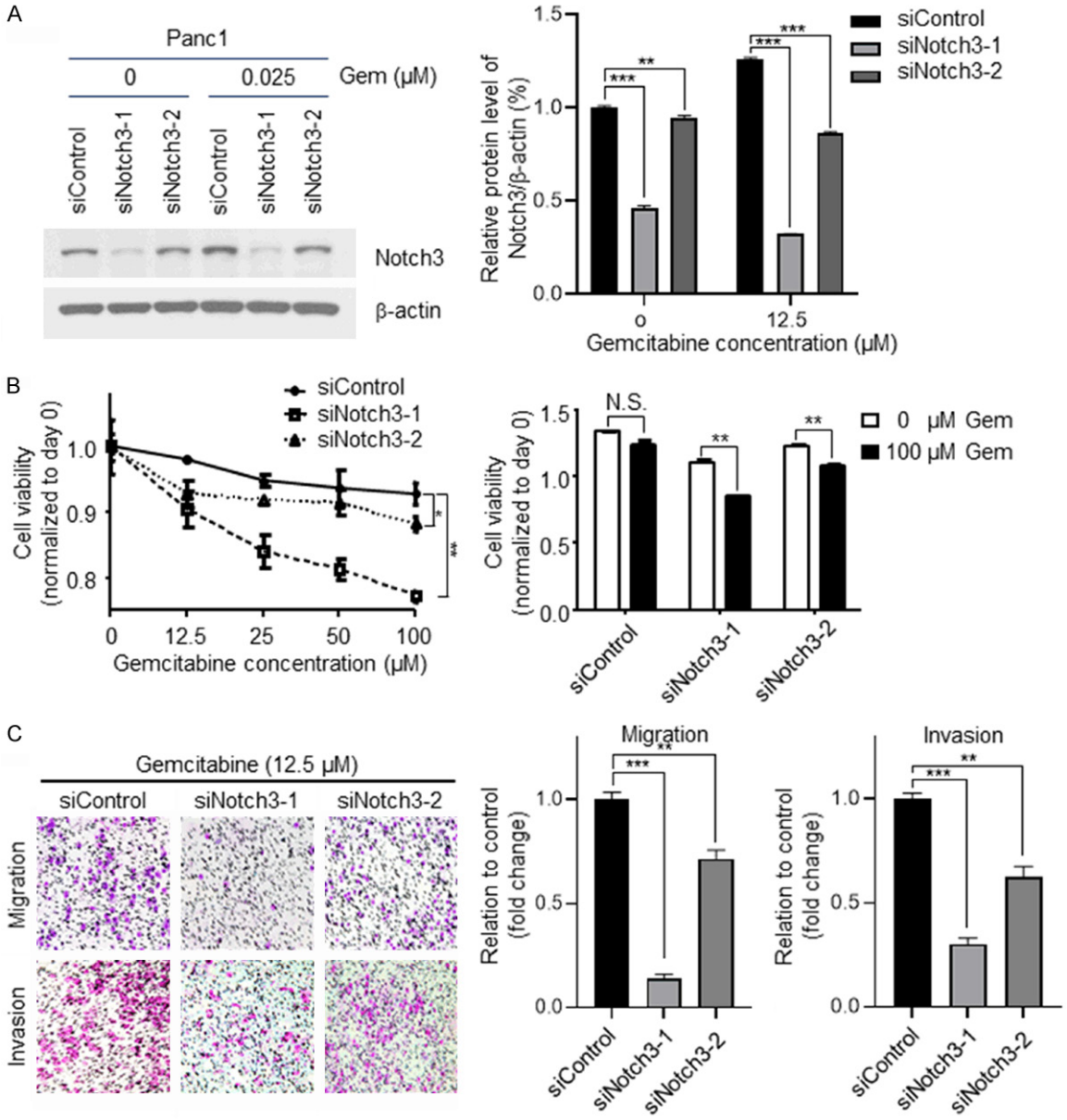


Figure S3. Knocking down Notch3 resulted in increased sensitivity to gemcitabine in Panc-1 cells. A. Protein expression level of Notch3 in Panc-1 cells transfected with siNotch3 or siControl in the absence or presence of gemcitabine. B. MTT assay was performed to examine cell viability following gemcitabine treatment in siNotch3 and siControl cells. C. The migration and invasion were performed by Transwell assays in siNotch3 and siControl cells after gemcitabine (12.5 μM) treatment for 24 h. Data are presented as means ± SD. *P<0.05; **P<0.01; ***P<0.001.

Table S3A. The gene differential expression in siNotch3 vs siControl in Panc-1 cells

	Gene Symbol	Description	Fold Regulation
upregulation	TGFB1	Transforming growth factor, beta 1	2.2992
downregulation	CCL7	Chemokine (C-C motif) ligand 7	-2.5392
	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	-4.4367
	CHD4	Chromodomain helicase DNA binding protein 4	-2.6621
	CST7	Cystatin F (leukocystatin)	-3.3561
	CTBP1	C-terminal binding protein 1	-4.1505
	CTSK	Cathepsin K	-3.0389
	CXCR4	Chemokine (C-X-C motif) receptor 4	-3.2172
	DENR	Density-regulated protein	-80.6134
	FAT1	FAT tumor suppressor homolog 1 (Drosophila)	-4.0019
	KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	-15.507
	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagen)	-2.3876
	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagen)	-4.7056
	MYCL	V-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	-9.5242
	PNN	Pinin, desmosome associated protein	-5.8738
	TIMP3	TIMP metallopeptidase inhibitor 3	-4.4932
	RPLP0	Ribosomal protein, large, P0	-3.7952

Table S3B. The gene differential expression in siNotch3 vs siControl in BxPC3 cells

	Gene Symbol	Description	Fold Regulation
upregulation	CTBP1	C-terminal binding protein 1	2.8619
	DENR	Density-regulated protein	7.1892
	METAP2	Methionyl aminopeptidase 2	11.6512
	MGAT5	Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase	4.0811
	MMP10	Matrix metallopeptidase 10 (stromelysin 2)	2.3334
	MMP13	Matrix metallopeptidase 13 (collagenase 3)	7.672
	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagen)	2.3571
	NF2	Neurofibromin 2 (merlin)	2.4327
	NR4A3	Nuclear receptor subfamily 4, group A, member 3	3.3528
	SET	SET nuclear oncogene	4.9018
	TP53	Tumor protein p53	3.8724
downregulation	ETV4	Ets variant 4	-2.2337
	HPSE	Heparanase	-2.0714
	KISS1	KiSS-1 metastasis-suppressor	-9.7508
	KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	-2.2317
	MTSS1	Metastasis suppressor 1	-5.9125
	SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	-5.7693
	TGFB1	Transforming growth factor, beta 1	-2.7758
	TIMP2	TIMP metallopeptidase inhibitor 2	-2.0676
	TIMP3	TIMP metallopeptidase inhibitor 3	-3.2013

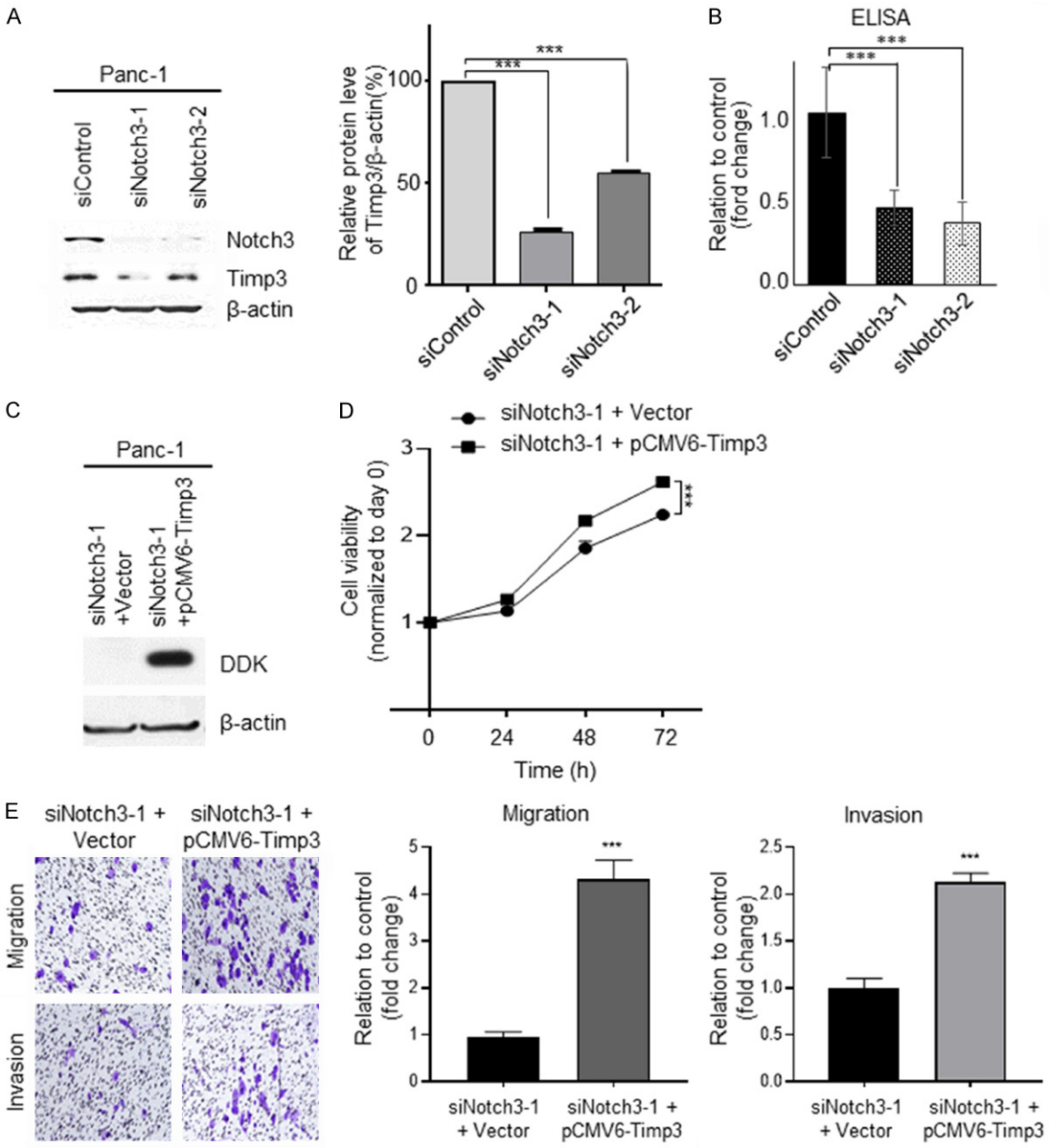


Figure S4. TIMP3 expression played a role in Notch3-elicited cell growth and motility in Panc-1. A. The protein expression level of TIMP3 was assessed in siNotch3- and siControl-Panc-1 cells. B. The expression level of extracellular matrix TIMP3 was assessed in siNotch3-Panc-1 and siContrtol-Panc-1 cells by ELISA. C. Western blot analysis of siNotch3 cells transfected with pCMV6-TIMP3. D and E. Cell proliferation, migration and invasion in siNotch3/vector and siNotch3/TIMP3. Data are presented as means ± SD. ***P<0.001.

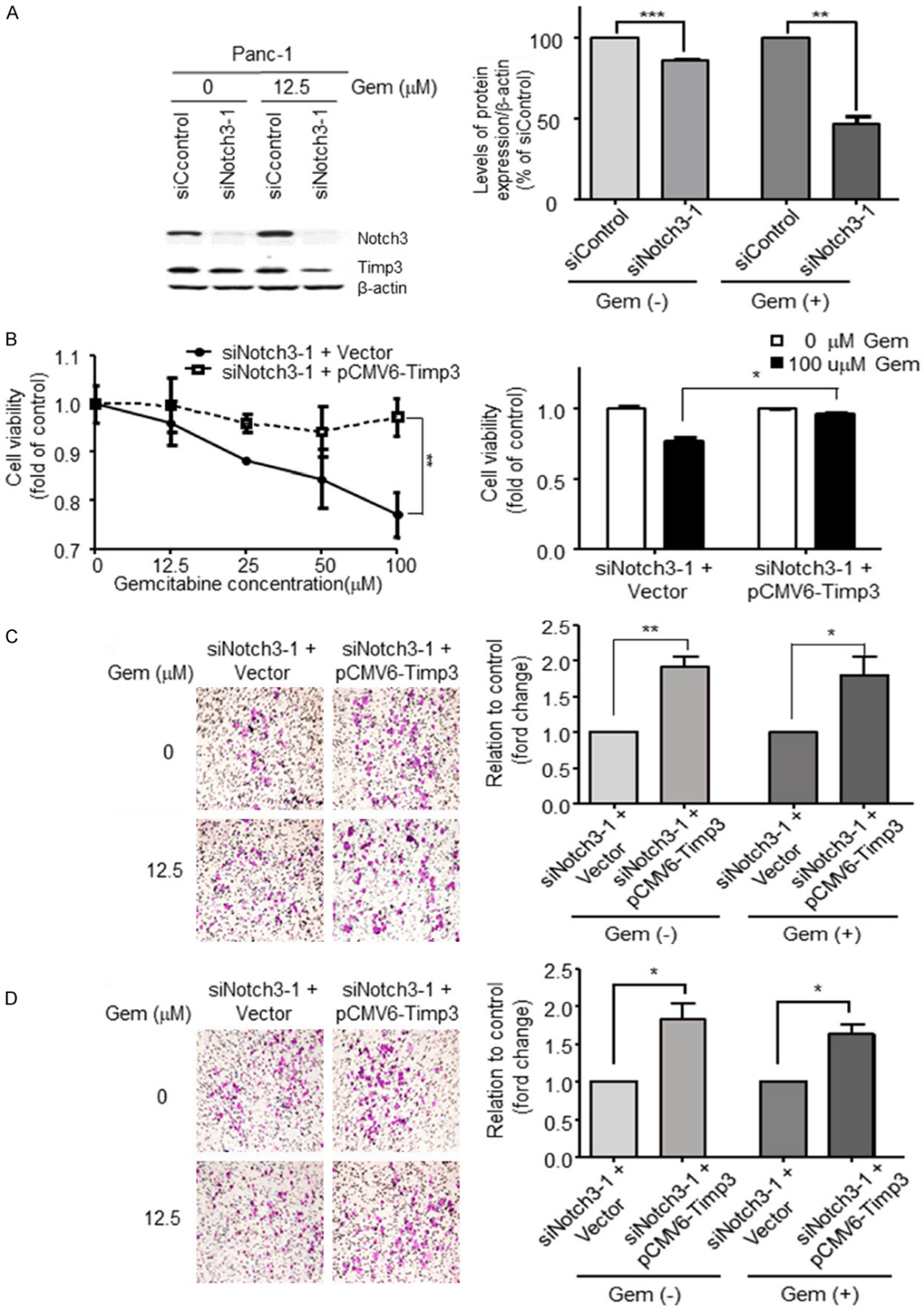


Figure S5. TIMP3 activation reversed siNotch3-increased sensitivity to gemcitabine in Panc-1 cell. A. Protein expression levels of Notch3 and TIMP3 in siNotch3 and siControl Panc-1 cells in the absence or presence of gemcitabine. B. Cell viability was determined in siNotch3-Panc-1 cells transfected with vector or pCMV6-TIMP3 in a gemcitabine dose-dependent manner. C and D. Cell migration and invasion of cells treated with gemcitabine. Data are presented as means ± SD. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.