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Positive feedback regulation of lncRNA TPT1-AS1 and ITGB3 promotes cell growth and metastasis in pancreatic cancer

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Abstract

Emerging evidence has indicated that long noncoding RNAs (lncRNAs) are potential biomarkers and play crucial roles in cancer development. However, the functions and underlying mechanisms of lncRNA TPT1-AS1 in pancreatic ductal adenocarcinoma (PDAC) remain elusive. RNAseq data of PDAC tissues and normal tissues were analyzed, and lncRNAs which were associated with PDAC prognosis were identified. The clinical relevance of TPT1-AS1 for PDAC patients was explored, and the effects of TPT1-AS1 in PDAC progression were investigated in vitro and in vivo. LncRNA TPT1-AS1 was highly expressed in PDAC, and high TPT1-AS1 levels predicted a poor prognosis. Moreover, functional experiments revealed that TPT1-AS1 promoted pancreatic cancer cell proliferation, migration, invasion, and epithelial-to-mesenchymal transition (EMT) process in vitro and in vivo. Mechanistically, TPT1-AS1 functioned as an endogenous sponge for miR-30a-5p, which increased integrin β3 (ITGB3) level in pancreatic cancer cells. Conversely, our data revealed that ITGB3 could activate the transcription factor signal transducer and activator of transcription 3 (STAT3), which in turn bound directly to the TPT1-AS1 promoter and affected the expression of TPT1-AS1, thus forming a positive feedback loop with TPT1-AS1. Taken together, our results uncovered a reciprocal loop of TPT1-AS1 and ITGB3 which contributed to pancreatic cancer growth and development, and indicated that TPT1-AS1 might serve as a novel potential diagnostic biomarker and therapeutic target for PDAC patients.

KEYWORDS
ITGB3, miR-30a-5p, pancreatic cancer, STAT3, TPT1-AS1

Abbreviations: cDNA, complementary DNA; ceRNA, competing endogenous RNA; EdU, 5-ethynyl-2-deoxyuridine; EMT, epithelial-mesenchymal transition; IHC, immunohistochemistry; ITGB3, integrin β3; lncRNAs, long noncoding RNAs; miRNAs, microRNAs; MUT, mutant-TPT1-AS1; PDAC, pancreatic ductal adenocarcinoma; qRT-PCR, real-time quantitative reverse-transcription polymerase chain reaction; si-NC, siRNA-negative control; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3.

Chundong Cheng, Danxi Liu, and Zonglin Liu contributed equally.

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1 | BACKGROUND

Pancreatic ductal adenocarcinoma (PDAC) accounts for 3% of all new cancer diagnoses and 7.5% of all cancer deaths in the United States. The 5-year overall survival rate for all types of cancers has exceeded 60%; however, PDAC is still one of the most aggressive and lethal malignancies. The 5-year survival rate is less than 9%.[1] The high mortality rate of PDAC patients is due to the absence of grave symptoms coupled with the unavailability of early prognostic and diagnostic markers and resistance to chemo- and radio-therapies.[2,3] Therefore, there is an urgent need to search for biomarkers which can effectively predict the prognosis of PDAC and develop efficient therapeutic approaches.

Accumulating evidence has shown that aberrant noncoding RNAs including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) play key roles in diverse biological processes, including proliferation, motility, immortality, viability, and angiogenesis.[4] lncRNAs exert their biological functions in regulating tumor progression through multiple mechanisms.[5–7] Recent studies have revealed that lncRNAs could act as competing endogenous RNA (ceRNAs) through their miRNAs' pairing sites, which restore the post-transcriptional repression of messenger RNAs (mRNAs) by competing with miRNAs.[8–10] Aberrant regulation of lncRNAs has been identified in gastric, colorectal, and hepatocellular carcinoma as well as PDAC, which affects cell proliferation, migration, and invasion through ceRNA mechanisms.[11–17] To the best of our knowledge, although some aberrant lncRNAs play key roles in the initiation and development of pancreatic cancer, more research is needed to elucidate the underlying mechanisms of lncRNAs in pancreatic cancer.

In this study, we found that the elevated expression of TPT1-AS1 led to a poor prognosis for pancreatic cancer patients. TPT1-AS1 promoted pancreatic cancer cell proliferation, migration, invasion, and EMT process in vitro and in vivo. Mechanistically, TPT1-AS1 promoted the expression of its target gene ITGB3 by sponging miR-30a-5p. Conversely, ITGB3 could promote TPT1-AS1 transcription by activating signal transducer and the activator of transcription 3 (STAT3) which bound to the TPT1-AS1 promoter region, thus forming a positive feedback circuit. In summary, we propose a positive feedback loop of TPT1-AS1/miR-30a-5p/ITGB3/STAT3 in pancreatic cancer, providing a new prognostic biomarker and therapeutic target for PDAC patients.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

Human PDAC specimens were collected from 69 patients with PDAC who underwent pancreatectomy in the Department of Pancreatic and Biliary Surgery (The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China) from January 2009 to January 2018 (Table 1). The study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. Every patient gave written informed consent before participating in this study. Fresh tissues were immediately frozen in liquid nitrogen and preserved at −80°C before use.

TABLE 1 Correlation between TPT1-AS1 expression and clinicopathological features of PDAC patients

Clinical characteristic	Total	TPT1-AS1 expression		*p* value
		Low (34)	High (35)	
Age (years)				
<60	40	17	23	0.191
≥60	29	17	12	
Gender				
Male	49	22	27	0.261
Female	20	12	8	
TNM stage				
I + IIa	40	16	24	0.016*
II_b + III	29	18	11	
Nodal metastasis				
Yes	27	17	10	0.070
No	42	17	25	
Pathologic stage				
G1 + G2	36	22	14	0.041*
G3	33	12	21	

*$p < 0.05$; $p < 0.05$ was considered significant.

2.2 | RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted and isolated from the cell lines and frozen tumor specimens using an AxyPrep Multisource Total RNA Miniprep Kit from Axygen (Coring), and the first strand of cDNA was synthesized using Rever TraAce qPCR RT Kit Master Mix with gDNA Remover (FSQ-101, Toyobo Co. Ltd.) according to the manufacturer instructions. Quantitative PCR assays were carried out on a 7500 FAST Real-Time PCR System (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 small nuclear RNA (snRNA) were used as endogenous controls for lncRNA, mRNA, and miRNA. The expression levels of RNA were calculated using the $2^{-\triangle\triangle T}$ method after normalization to the expression of the control. The primer sequences are described in Additional file 1: Table S1.

2.3 | Cell transfection and viral infection

siRNA for TPT1-AS1 and ITGB3 (si-TPT1-AS1, si-ITGB3), si-NC, miRNA mimics, control mimics, miRNA inhibitors, and control inhibitors were purchased from RiboBio. Human TPT1-AS1 and ITGB3 cDNA were cloned into a plasmid (GeneCopoeia) to overexpress TPT1-AS1 and ITGB3. Cell infection was performed using 50 nM siRNA or plasmids with Lipofectamine 2000 (Life Technologies Limited). To establish stable transfectants with knockdown or overexpression, lentiviruses with scrambled short hairpin RNA (shRNA) against TPT1-AS1 (shTPT1-AS1) and the shRNA control (shCtrl) were constructed in GV112 (GeneChem). Lentiviral vectors encoding human TPT1-AS1 (TPT1-AS1) and empty vectors were constructed in GV112 (GeneChem).

2.4 | Cell-counting kit (CCK-8) assay and colony assay

A CCK-8 (Dojindo) was used to assess cell proliferation according to the protocols of the manufacturer. Briefly, 2000 cells were seeded into 96-well plates. Ten microliters of CCK-8 solution was added to each well of cells. After 2 hours of incubation, the absorbance in each well was measured at 24, 48, 72, and 96 hours. In the colony formation experiment, 1000 cells transfected with pcDNA-TPT1-AS1, si-TPT1-AS1, and negative control were seeded in six-well plates. The colonies were fixed with methanol for 30 minutes and stained in 1% crystal violet after 2 weeks.

2.5 | EdU proliferation assay

EdU assay (RiboBio) was performed to measure cell proliferation. After transfection, 10,000 infected cells were seeded in 96-well plates, and then the cells were treated following the manufacturer's instructions.

2.6 | Luciferase reporter assay

Full-length MUT was amplified and cloned into the pmiR-Report luciferase vector (GenScript). According to the predicted binding site of TPT1-AS1 and miR-30a-5p, the sequence of the binding site was mutated to CACCCGT. Then, 50 nm miR-30a-5p mimic and 500 ng of luciferase constructs were cotransfected into PANC-1 cells, and 100 nm miR-30a-5p inhibitor and 500 ng of luciferase constructs were cotransfected into BxPC-3 cells according to the manufacturer's instructions. The luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) after 24 hours transfection using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific) according to the manufacturer's instructions. Firefly luciferase activity was used to normalize the transfection efficiency. For examining the binding between ITGB3 and miR-30a-5p, ITGB3-WT or ITGB3-MUT luciferase reporter was built to perform similar procedures. The plasmids that inserted four fragments of the promoter (P1, P2, P3, and P4) in front of the luciferase sequence were built to explore which fragments of the promoter could bind to STAT3.

2.7 | RNA-binding protein immunoprecipitation (RIP)

RNA-binding protein immunoprecipitation assays were performed to validate the interaction of TPT1-AS1 with miR-30a-5p. The Magna RIP RNA-binding Protein Immunoprecipitation Kit was used to conduct the RIP assay. Cells were lysed with 500 μl of lysis buffer for 20 minutes on ice. Cell lysates (500 μl) with 5 μg of isotype control IgG (Cell Signaling Technology) and anti-Ago2 antibody (Abcam) were incubated overnight at 4°C. Then, to digest the proteins, proteinase K was incubated in the samples. The supernatant was collected for RNA isolation using TRIzol Ls Reagent (Thermo Scientific) and was used for qRT-PCR analysis.

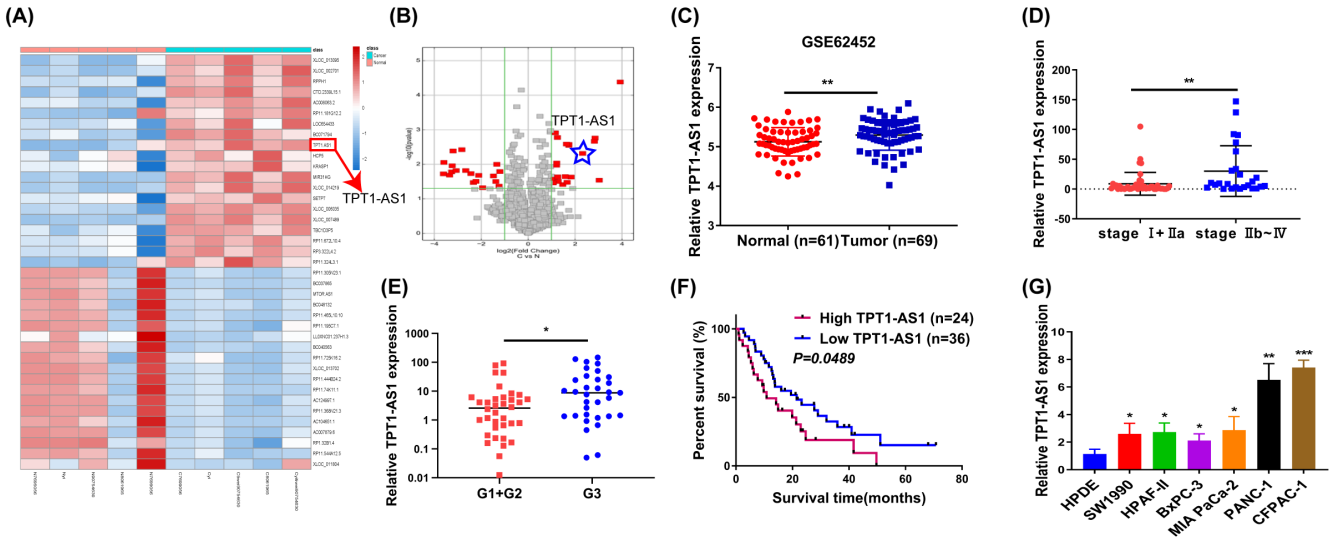


FIGURE 1 lncRNA TPT1-AS1 is overexpressed and associated with poor clinical outcome in pancreatic ductal adenocarcinoma (PDAC). (A, B) Heatmap and volcano plots showing the differentially expressed lncRNAs in five pairs of PDAC and normal pancreatic tissues (fold change ≥2, $p < 0.05$). (C) The relative expression of TPT1-AS1 in pancreatic cancer tissues and normal pancreatic tissues in GSE62452. (D, E) The relative expressions of TPT1-AS1 in PDAC with different TNM stages and pathological stages were detected by qRT-PCR. (F) Kaplan-Meier analysis of the survival rate of pancreatic cancer patients based on high TPT1-AS1 levels and low TPT1-AS1 levels in our cohort ($n = 69$, $p = 0.0096$). (G) Relative expression of TPT1-AS1 in pancreatic cancer cell lines were detected by qRT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

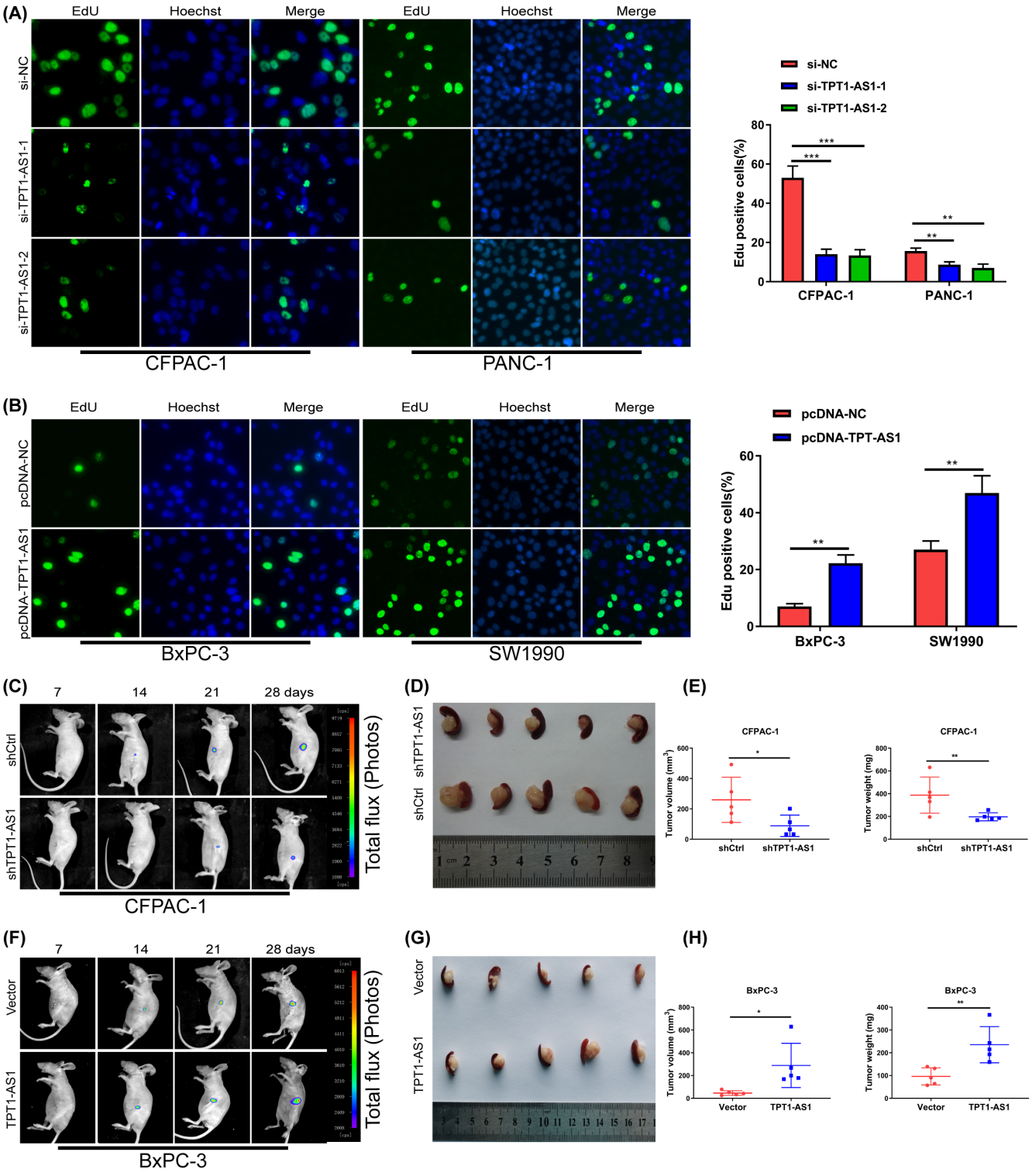


FIGURE 2 TPT1-AS1 promotes pancreatic cancer cell proliferation in vitro and in vivo. (A) The functions of TPT1-AS1 knockdown on proliferation were measured by EdU assays in CFPAC-1 and PANC-1 cells. (B) The functions of TPT1-AS1 overexpression on proliferation were measured by EdU assays in BxPC-3 and SW1990 cells (original magnification, 20×). (C–H) Representative bioluminescence imaging (following intraperitoneal injection of 0.1 mg/g luciferin) of mice on days 7, 14, 21, and 28. On day 28, all mice were sacrificed, the primary tumors were removed, and tumor volume and weight were evaluated. Statistical significance between different groups was calculated with Student's *t* test. Data are shown as mean ± SD of three replicates; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

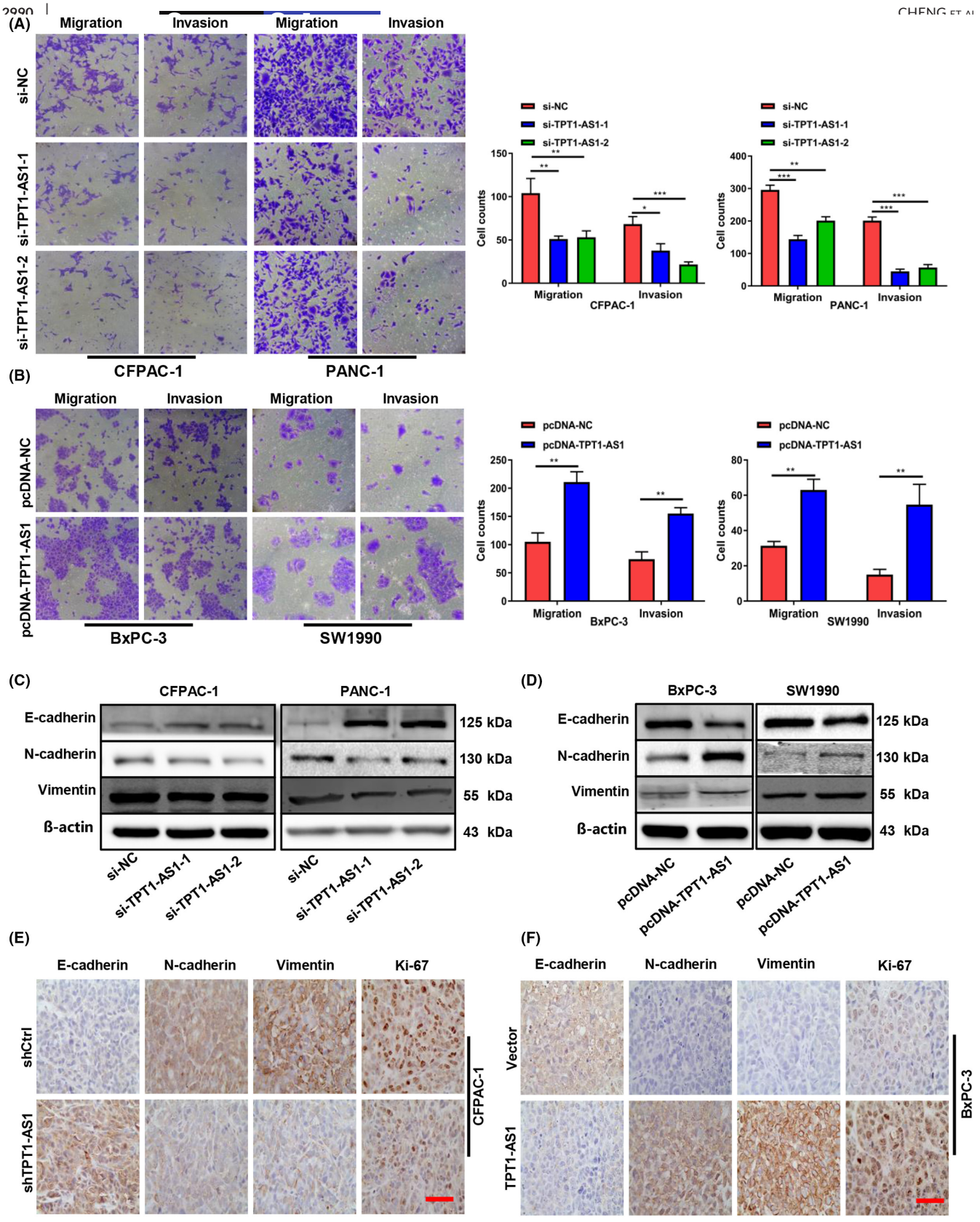


FIGURE 3 TPT1-AS1 promotes pancreatic cancer cell migration, invasion, and epithelial-mesenchymal transition (EMT). (A, B) The functions of TPT1-AS1 knockdown or overexpression on metastasis were assessed by Transwell assays (original magnification, 20×). (C, D) The expression of E-cadherin, N-cadherin, and vimentin were measured by Western blot assays after TPT1-AS1 knockdown or overexpression. (E, F) The expression of E-cadherin, N-cadherin, vimentin, and K-i67 were analyzed in paraffin-embedded tissue sections of orthotopic pancreatic cancer models from different groups by immunohistochemistry (original magnification, 20×; bar, 400 μm). Statistical significance between different groups was calculated with Student's *t* test. Data are shown as mean ± SD of three replicates; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

2.8 | Orthotopic tumor model

The animal studies were approved by the Institutional Review Board of the First Affiliated Hospital of Harbin Medical University. CFPAC-1-shTPT1-AS1, BxPC-3-TPT1-AS1, and their control cells expressing luciferase were injected into the flanks of nude mice. Then, 1-mm^3 pieces of tumor harvested from four mice were translocated into four groups of mouse pancreatic tails. The animals were imaged weekly using the Night OWLII LB983 imaging system in vivo (Berthold Technologies GmbH & Co. KG). The primary and metastatic pancreatic tumors were excised, weighed, and fixed in 4% paraformaldehyde.

2.9 | Immunohistochemical staining

The paraffin-embedded tissue sections were immunostained with anti-N-cadherin, anti-E-cadherin, vimentin, ITGB3, and anti-Ki-67. The number of positive cells was counted in five randomly selected microscopic fields (×20, Nikon). The primary antibodies for IHC are listed in Additional file 2: Table S2.

The materials and methods of cell culture, Western blot assay, Transwell assay, and wound healing assay are listed in Additional file: additional material and methods.

2.10 | Statistical analysis

Statistical analysis was performed with SPSS 19.0 software or GraphPad Prism 7.0 software. The data are shown as the mean ± SD. Pearson analysis, Kaplan-Meier survival analysis, one-way ANOVA, and Student's *t* test were used to evaluate the statistical significance.

3 | RESULTS

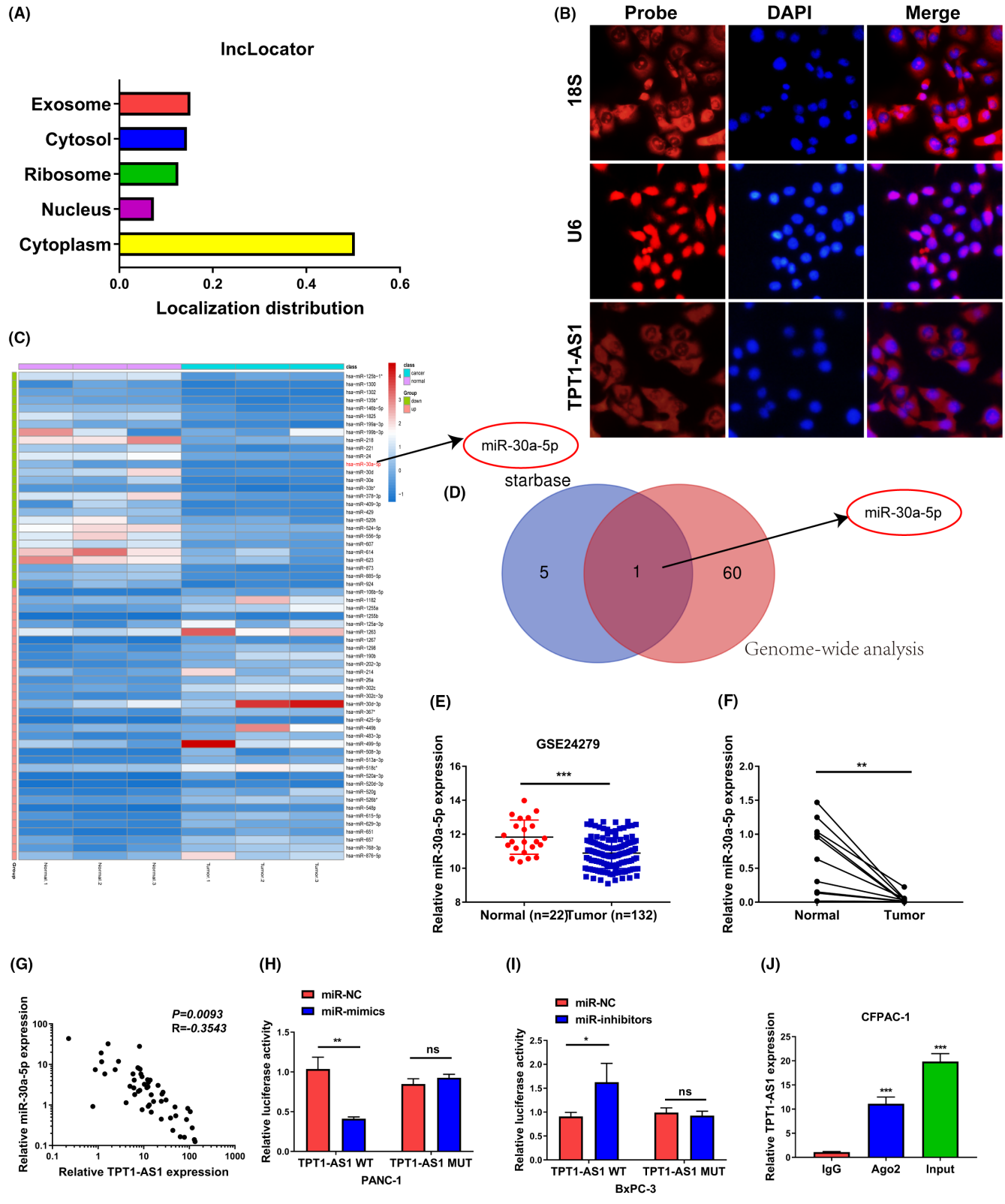
3.1 | High TPT1-AS1 levels are correlated with poor clinical outcomes

A genome-wide analysis of five pairs of PDAC tissues and normal pancreatic tissues was performed to explore the relationship between lncRNA expression and pancreatic cancer properties. We found 39 differentially expressed lncRNAs (fold change ≥2 and $p < 0.05$), including 20 upregulated and 19 downregulated genes, of which TPT1-AS1 was one of the top upregulated lncRNAs in PDAC (Figure 1A,B). TPT1-AS1 had been shown to promote tumor progression in different models, including, for example, colorectal cancer, ovarian cancer, and gastric cancer.[16,18,19] However, the specific role of TPT1-AS1 in PDAC remains elusive. Therefore, to investigate the roles of TPT1-AS1 in PDAC, we first compared the levels of TPT1-AS1 in normal pancreatic tissue and PDAC tissue in the GEO dataset (GSE62452) and found that TPT1-AS1 was highly expressed in pancreatic cancer tissues compared with normal tissues (Figure 1C). Furthermore, the expression of TPT1-AS1 in 69 PDAC tissues was evaluated. Our results showed that the level of TPT1-AS1 was significantly increased in advanced pancreatic cancer compared with that in early stage (Figure 1D). Meanwhile, we detected that the level of TPT1-AS1 in poorly differentiated pancreatic cancer tissues was significantly upregulated compared with that in highly differentiated tissues (Figure 1E). Furthermore, Kaplan-Meier analysis revealed that PDAC patients with high TPT1-AS1 levels had a poorer prognosis than those with low TPT1-AS1 levels (Figure 1F). We analyzed the relationship between TPT1-AS1 and clinical characteristics and discovered that the expression levels of TPT1-AS1 were significantly correlated with TNM stage ($p = 0.016$) and tumor differentiation ($p = 0.041$) (Table 1). Moreover, the expression levels of TPT1-AS1 were increased in pancreatic cancer cell lines compared with normal human pancreatic duct epithelial (HPDE) cell lines (Figure 1G). Taken together, these data demonstrated that TPT1-AS1 played an oncogenic role and was correlated with tumor progression in pancreatic cancer.

3.2 | TPT1-AS1 promotes pancreatic cancer proliferation

To explore the proliferative function of TPT1-AS1 in pancreatic cancer cells, siRNA (si-TPT1-AS1) and overexpression plasmids (pcDNA-TPT1-AS1) were transfected into pancreatic cancer cell lines. The efficiency of siRNA, plasmid, and lentiviruses were tested by qRT-PCR (Figure S1A–D). The results of CCK-8 assays (Figure S2A) and EdU assays (Figure 2A) indicated that TPT1-AS1 knockdown significantly inhibited cell proliferation and blocked

FIGURE 4 miR-30a-5p is a target of TPT1-AS1 in pancreatic cancer. (A) The localization of TPT1-AS1 in cells was predicted using a lncRNA subcellular localization predictor. (B) Fish assays were used to detect the subcellular localization of TPT1-AS1 in PANC-1 cells. 18S, U6, and TPT1-AS1 were stained red (Cy3), and nuclei were stained blue (DAPI) (original magnification, 20×; bars, 25 μm). (C) Heatmap showing differentially expressed miRNAs in three pairs of pancreatic ductal adenocarcinoma (PDAC) tissues and normal pancreatic tissues. (D) The potential target miRNAs of TPT1-AS1 were predicted by Starbase and genome-wide analysis. (E) Relative expression levels of miR-30a-5p in GSE24279 in normal pancreatic and pancreatic cancer tissues. (F) The expression levels of miR-30a-5p in 10 pairs of normal pancreatic tissues and pancreatic cancer tissues were detected by qRT-PCR. (G) Pearson correlation between miR-30a-5p and TPT1-AS1 expression in PDAC tissues was measured in pancreatic cancer tissues. (H, I) Relative luciferase activities of wild-type (WT) and mutated (MUT) TPT1-AS1 reporter plasmid in PANC-1 and BxPC-3 cells cotransfected with miR-30a-5p mimics or inhibitors. (J), RNA-binding protein immunoprecipitation (RIP) experiments were performed in CFPAC-1 cells, and the coprecipitated RNA was used to quantify TPT1-AS1 expression by qRT-PCR. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$



DNA replication in pancreatic cancer cells. Consistently, colony formation assays demonstrated that inhibition of TPT1-AS1 decreased pancreatic cancer cell colony formation (Figure S3A). In contrast, TPT1-AS1 overexpression had the opposite effects in pancreatic cancer cells (Figure 2B, Figures S2B and S3B).

To explore the role of TPT1-AS1 in vivo, a pancreatic orthotopic tumor model was used to assess the effect of TPT1-AS1 in tumor proliferation. As shown in Figure 2C–H, TPT1-AS1 overexpression increased tumor size, and TPT1-AS1 knockdown reduced tumor growth, which was consistent with the experimental data in vitro,

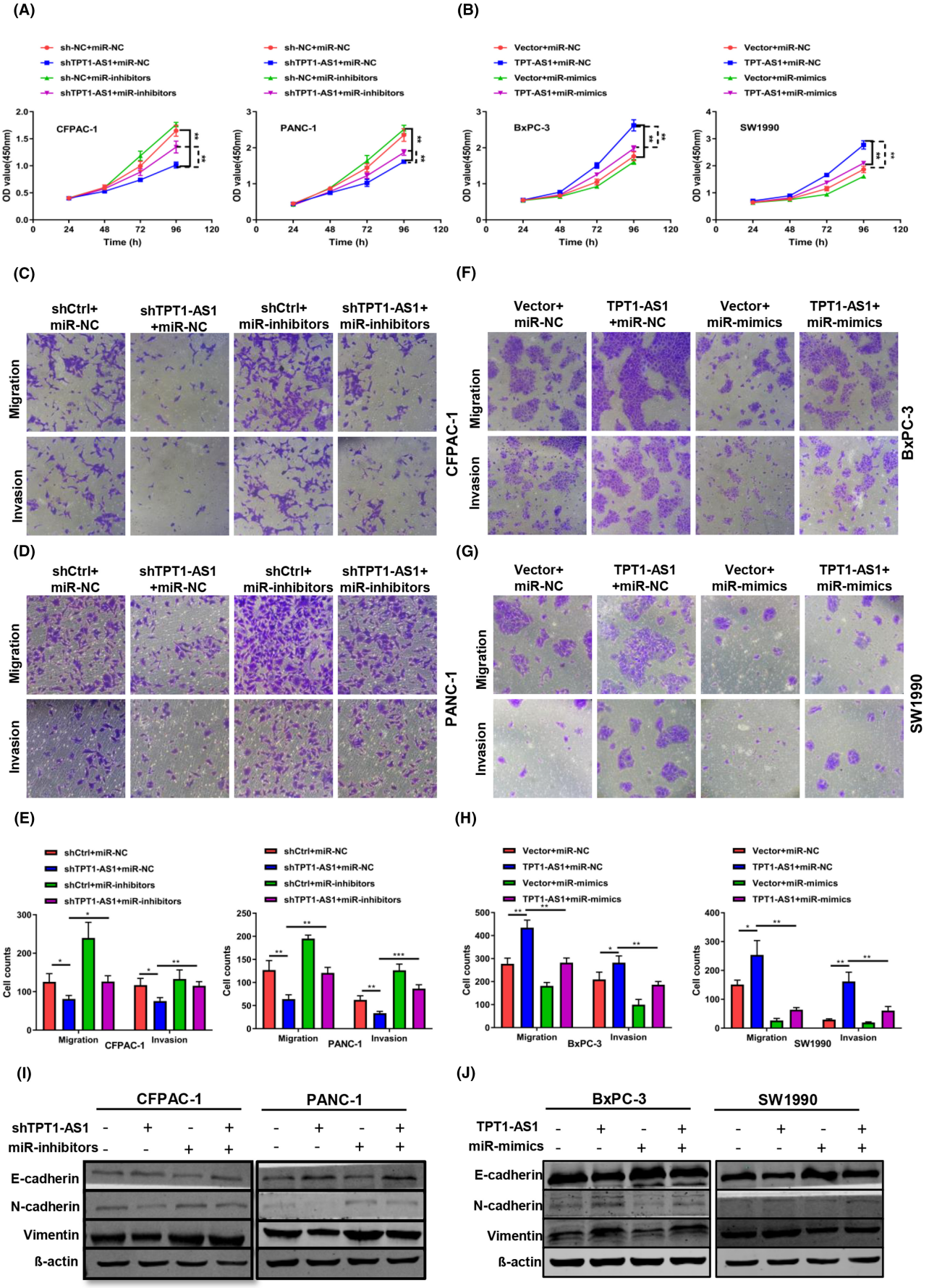

(A)
sh-NC+miR-NC
shTPT1-AS1+miR-NC
sh-NC+miR-inhibitors
shTPT1-AS1+miR-inhibitors
CFPAC-1
PANC-1
OD value(450nm)
Time (h)
(B)
Vector+miR-NC
TPT-AS1+miR-NC
Vector+miR-mimics
TPT-AS1+miR-mimics
BxPC-3
SW1990
(C)
shCtrl+ miR-NC
shTPT1-AS1 +miR-NC
shCtrl+ miR-inhibitors
shTPT1-AS1+ miR-inhibitors
Migration
Invasion
(D)
PANC-1
(F)
Vector+ miR-NC
TPT1-AS1 +miR-NC
Vector+ miR-mimics
TPT1-AS1+ miR-mimics
CFPAC-1
BxPC-3
(G)
SW1990
(E)
shCtrl+miR-NC
shTPT1-AS1+miR-NC
shCtrl+miR-inhibitors
shTPT1-AS1+miR-inhibitors
Cell counts
(H)
Vector+miR-NC
TPT-AS1+miR-NC
Vector+miR-mimics
TPT-AS1+miR-mimics
(I)
shTPT1-AS1
miR-inhibitors
E-cadherin
N-cadherin
Vimentin
ß-actin
(J)
TPT1-AS1
miR-mimics

FIGURE 5 TPT1-AS1 function is partially mediated by repressing miR-30a-5p. (A) Rescue effects of miR-30a-5p knockdown on TPT1-AS1 knockdown–mediated inhibition of cell growth in CFPAC-1 and PANC-1 cells determined by CCK-8 assays. (B) Rescue effects of miR-30a-5p overexpression on TPT1-AS1 overexpression–mediated promotion of cell growth in BxPC-3 and SW1990 cells determined by CCK-8 assays. (C–E) Rescue effects of miR-30a-5p knockdown on TPT1-AS1 knockdown–mediated inhibition of cell migration and invasion in CFPAC-1 and PANC-1 cells determined by Transwell assays. (F–H) Rescue effects of miR-30a-5p overexpression on TPT1-AS1 overexpression–mediated promotion of cell migration and invasion in BxPC-3 and SW1990 cells determined by Transwell assays. (I) Rescue effects of miR-30a-5p knockdown on TPT1-AS1 knockdown–mediated inhibition of cell epithelial-mesenchymal transition (EMT) process in CFPAC-1 and PANC-1 cells determined by Western blot. (J) Rescue effects of miR-30a-5p overexpression on TPT1-AS1 overexpression–mediated promotion of cell EMT process in BxPC-3 and SW1990 cells determined by Western blot. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

suggesting that TPT1-AS1 promoted pancreatic cancer cell proliferation in vitro and in vivo.

3.3 | TPT1-AS1 promotes migration, invasion, and EMT in pancreatic cancer

Next, to assess the effect of TPT1-AS1 on cancer cell migration and invasion, Transwell and wound-healing assays were conducted. Our results demonstrated that inhibition of TPT1-AS1 suppressed pancreatic cancer cell migration and invasion (Figure 3A, Figure S4A). Similarly, TPT1-AS1 overexpression had the opposite effects on migration and invasion (Figure 3B, Figure S4B). Epithelial-mesenchymal transition has been recognized as a key regulator of metastasis of cancers[20]; therefore, we then investigated the effects of TPT1-AS1 on the EMT process. Our data demonstrated that TPT1-AS1 overexpression decreased the expression of epithelial marker E-cadherin and increased the levels of the mesenchymal marker N-cadherin and vimentin (Figure 3D, Figure S5B). On the contrary, TPT1-AS1 knockdown showed opposite effects (Figure 3C, Figure S5A). Additionally, Ki-67, a key regulatory gene in cell proliferation, is a marker of proliferation to measure proliferation activity in tissues.[21] Immunohistochemical results of showed a higher rate of Ki67, N-cadherin, vimentin positivity, and E-cadherin negativity in pancreatic orthotopic tumors built with overexpressed TPT1-AS1 BxPC-3 cells (Figure 3F). The opposite results were displayed in tumors built with TPT1-AS1–knockdown CFPAC-1 cells (Figure 3E). Based on the results, we demonstrated that TPT1-AS1 promoted pancreatic cancer cell proliferation, migration, and invasion in vitro and in vivo.

3.4 | TPT1-AS1 acts as a sponge for miR-30a-5p in pancreatic cancer cells

lncRNAs have been shown to act as ceRNAs and sponge miRNAs to suppress target gene expression.[10,22–28] To determine the molecular mechanism by which TPT1-AS1 promotes proliferation, migration, invasion, and EMT process in pancreatic cancer, we first used the lncRNA subcellular localization predictor (lncLocator, http://www.csbio.sjtu.edu.cn/bioinf/lncLocator) to predict its location in pancreatic cancer cells (Figure 4A). Furthermore, FISH assays showed that TPT1-AS1 was predominantly located in the cytoplasm (Figure 4B). Both of the results indicated that TPT1-AS1 was mainly distributed in the cytoplasm, which suggested that TPT1-AS1 might regulate downstream protein expression via a post-transcriptional regulatory mechanism. To explore the ceRNA network induced by TPT1-AS1, differential miRNAs in pancreatic cancer tissues ($n = 3$) and normal pancreatic tissues ($n = 3$), including 34 upregulated and 27 downregulated genes, were identified (Figure 4C). Furthermore, StarBase v2.0 was used to predict the potential interaction between miRNA and TPT1-AS1. We found that miR-30a-5p, which was predicted through Venn diagram, was the only intersected gene (Figure 4D, Figure S6A). In our study, we found that miR-30a-5p expression in PDAC tissues was lower than that in normal tissues in the GEO database (GSE24279) (Figure 4E). Our results showed that miR-30a-5p was substantially downregulated in PDAC patients (Figure 4F), and an obvious negative association between TPT1-AS1 and miR-30a-5p expression was observed in PDAC tissues (Figure 4G). To examine the relationship between TPT1-AS1 and miR-30a-5p, we examined miR-30a-5p expression after altering TPT1-AS1 expression. TPT1-AS1 knockdown increased the expression of miR-30a-5p (Figure S6B), whereas TPT1-AS1 overexpression decreased miR-30a-5p expression (Figure S6C). Additionally, the interrelationship between miR-30a-5p and TPT1-AS1 was verified by luciferase assays, indicating that miR-30a-5p overexpression significantly inhibited luciferase activity (Figure 4H) and miR-30a-5p knockdown promoted luciferase activity. (Figure 4I). It is widely accepted that Ago2, a main component of the RNA-induced silencing complex, plays an important role in the miRNA-mediated repression of mRNA. The results of RIP assays confirmed that TPT1-AS1 could bind to the Ago2 protein (Figure 4J). In order to investigate the direct interaction between TPT1-AS1, miR-30a-5p, and Ago2, miR-mimics were transfected into CFPAC-1 cells. RNA-binding protein immunoprecipitation assay showed that higher abundance of TPT1-AS1 was precipitated with Ago2 when tumor cells were transfected with miR-mimics (Figure S6D). These results indicate that TPT1-AS1 acts as a sponge for miR-30a-5p in pancreatic cancer cells.

3.5 | TPT1-AS1 facilitates tumorigenesis through inhibiting miR-30a-5p

To investigate the roles of miR-30a-5p in PDAC, miR-30a-5p mimics or inhibitors were transfected into pancreatic cancer cells (Figure S6E,F). Our data indicated that miR-30a-5p overexpression significantly inhibited cell proliferation (Figure S7A,C,E) migration,

invasion (Figure S8A,C), and EMT process (Figure S9A). In contrast, miR-30a-5p knockdown increased pancreatic cancer cell proliferation (Figure S7B,D,F) migration, invasion (Figure S8B,D), and EMT process (Figure S9B). To investigate the roles of miR-30a-5p on TPT1-AS1–induced tumor progression, miR-30a-5p inhibitors and mimics were transfected into pancreatic cancer cells with TPT1-AS1 depletion (Figure S10A,B). Our results showed that miR-30a-5p knockdown was found to restore the suppressive effects of TPT1-AS1 knockdown on pancreatic cancer cell proliferation (Figure 5A, Figure S10C,D, Figure S11A), migration, invasion (Figure 5C,D,E,

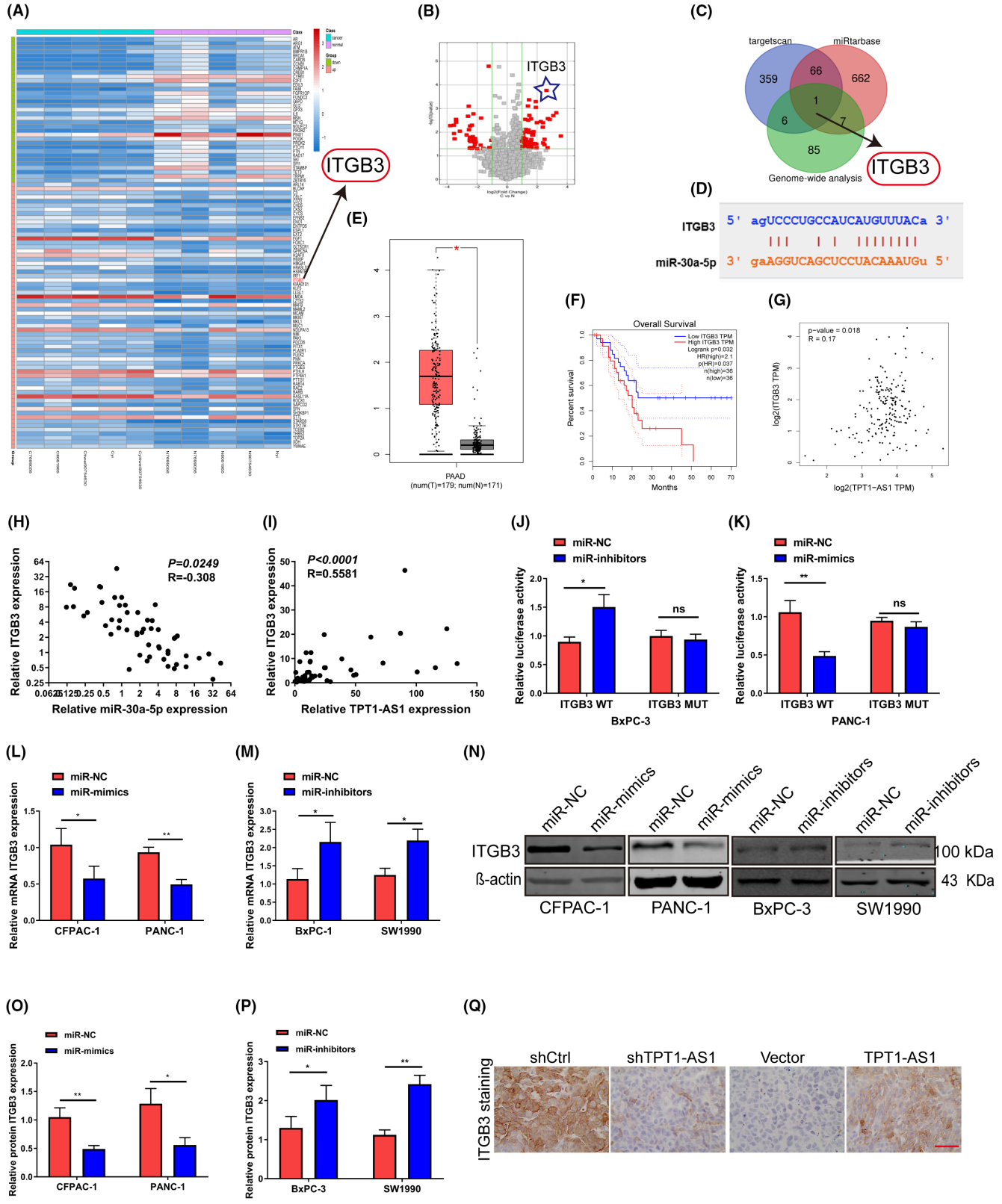


FIGURE 6 miR-30a-5p directly targets ITGB3 and is regulated by TPT1-AS1 in pancreatic cancer cells. (A, B) Heatmap and volcano plots showing differentially expressed mRNAs in five pairs of pancreatic ductal adenocarcinoma (PDAC) and normal pancreatic tissues. (C) Venn diagram showing overlapping mRNAs from the results of mRNA microarray analysis and two online miRNA-mRNA interaction databases' (miRtarbase and TargetScan) prediction. (D) The predicted binding sites of miR-30a-5p in the 3′-UTR of ITGB3 via the Starbase database. (E) Relative expression of TPT1-AS1 in pancreatic cancer tissues and adjacent normal tissues from the GEPIA cohort. (F) Kaplan-Meier curves of overall survival from the GEPIA database. (G) The relationship between TPT1-AS1 and ITGB3 from the GEPIA database. (H) Pearson's correlation analysis determined the relationship between ITGB3 and miR-30a-5p expression in pancreatic cancer tissues. (I) Pearson's correlation analysis determined the relationship between ITGB3 and TPT1-AS1 expression in pancreatic cancer tissues. (J, K) Relative luciferase activities of wild-type (WT) and mutated (MUT) ITGB3 reporter plasmid in PANC-1 and BxPC-3 cells cotransfected with miR-33a-5p mimics or inhibitors. The expression of ITGB3 was measured in CFPAC-1 and PANC-1 cells transfected with miR-mimics and BxPC-3 and SW1990 cells transfected with miR-inhibitors by qRT-PCR (L, M) and Western blot (N–P). (Q) The expression of ITGB3 in TPT1-AS1 high-expressing tumors and low-expressing tumors was measured by immunohistochemistry (IHC) (original magnification, 20×; bar, 400 μm). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Figure S12A), and EMT process (Figure 5I, Figure S13A) In addition, miR-30a-5p overexpression inhibited the effects of TPT1-AS1 on pancreatic cancer cell proliferation (Figure 5B, Figure S10E,F, Figure S11B), migration, invasion (Figure 5F,G,H, Figure S12B), and EMT process (Figure 5J, Figure S13B). The results suggested that TPT1-AS1 promoted pancreatic cancer cell proliferation, migration, invasion, and EMT process by competitively binding with miR-30a-5p.

3.6 | Silencing ITGB3 reverses the effect of promoting carcinogenesis induced by TPT1-AS1

It is well known that miRNAs induce mRNA degradation by binding to the 3′ untranslated region (3′ UTR). To explore the candidate target for miR-30a-5p, differential mRNAs in PDAC tissues ($n = 5$) and normal pancreatic tissues ($n = 5$) were identified (Figure 6A,B). TargetScan, miRtarbase, and differential mRNAs from genome-wide analysis were analyzed through Venn diagram (Figure 6C). According to Starbase v3.0 (http://starbase.sysu.edu.cn/index.php), ITGB3 has a presumed binding site for the seed sequence of miR-30a-5p (Figure 6D). According to the GEPIA program, ITGB3 was highly expressed in pancreatic cancer tissues (Figure 6E) and was strongly associated with patient survival (Figure 6F) and disease-free survival (Figure S14A). We also found that TPT1-AS1 was positively correlated with ITGB3 (Figure 6G). In our cohort research, we found a negative correlation between miR-30a-5p and ITGB3 (Figure 6H) and a positive correlation between TPT1-AS1 and ITGB3 (Figure 6I). Our data showed that miR-30a-5p inhibitors significantly increased the luciferase activity of ITGB3-WT (Figure 6J) and miR-30a-5p mimics inhibited the luciferase activity of ITGB3-WT (Figure 6K), while the reporter containing mutant sites did not respond to either miR-30a-5p overexpression or knockdown. These findings indicated that ITGB3 was a downstream target of miR-30a-5p. Moreover, according to the results of qRT-PCR assays (Figure S14B) and Western blot (Figure S14C,D), TPT1-AS1 overexpression increased the expression of ITGB3, and TPT1-AS1 knockdown decreased the expression of ITGB3. In addition, the mRNA and protein expression levels of ITGB3 were significantly decreased in the miR-30a-5p mimics group and increased in the miR-30a-5p inhibitors group (Figure 6L–P), which indicated that miR-30a-5p could target ITGB3 and inhibited its expression. Furthermore, IHC analysis showed that ITGB3 protein was higher in the high–TPT1-AS1 group (Figure 6Q). Moreover, transfection with miR-30a-5p inhibitors eliminated the decrease in ITGB3 expression levels in TPT1-AS1–knockdown pancreatic cancer cells (Figure S14E,G). Opposite results of Western blot were obtained in TPT1-AS1–overexpressed pancreatic cancer cells (Figure S14F,H). We then explored whether TPT1-AS1 increased carcinogenesis through elevating ITGB3. The results showed that ITGB3 overexpression reversed the inhibitory effects on proliferation (Figure 7A, Figure S15A,B, Figure S16A), migration, invasion (Figure 7C,D,E, Figure S17A), and EMT process (Figure 7I, Figure S18A) induced by shTPT1-AS1. The same results of cell proliferation (Figure 7B, Figures S15C,D and Figure S16B), migration, invasion (Figure 7F,G,H, Figure S17B), and EMT process (Figure 7J, Figure S18B) were obtained in BxPC-3 and SW1990. Taken together, our results revealed that TPT1-AS1 might act as a ceRNA to regulate ITGB3 expression through sponging miR-30a-5P and plays an oncogenic role in pancreatic cancer.

3.7 | ITGB3 elevated TPT1-AS1 expression through enhancing STAT3 in pancreatic cancer cells

Transcription factors have been reported to be engaged in regulating lncRNA expression.[29] In our study, we found that ITGB3 knockdown decreased the expression of TPT1-AS1. In contrast, overexpressing ITGB3 increased the expression of TPT1-AS1 (Figure 8A). Previous studies have reported that ITGB3 can induce STAT3 activation.[30] We then detected the expression of STAT3 and phosphorylation of STAT3 (p-STAT3) in ITGB3-knockdown or -overexpression pancreatic cancer cells. Our results demonstrated that ITGB3 enhanced p-STAT3 (Figure 8B, Figure S19A). STAT3 is a transcription factor which could participate in modulating lncRNAs expression.[15,31,32] The online bioinformatics tool JASPAR (http://jaspar.genereg.net) was used to explore the relationship between STAT3 and TPT1-AS1 (Figure 8C). Four major binding sites on the promoter region of TPT1-AS1 were predicted

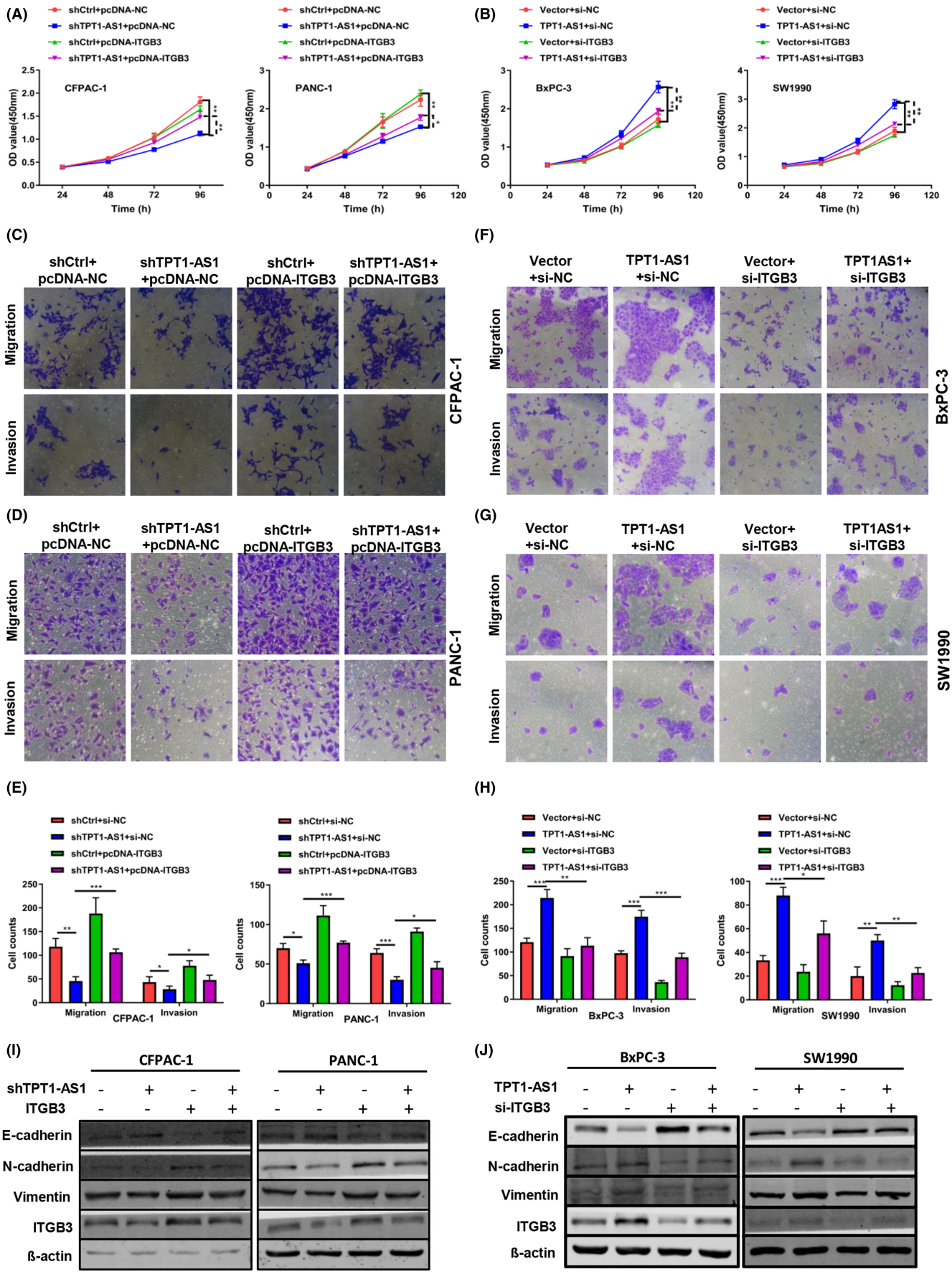

(A)
shCtrl+pcDNA-NC
shTPT1-AS1+pcDNA-NC
shCtrl+pcDNA-ITGB3
shTPT1-AS1+pcDNA-ITGB3
CFPAC-1
PANC-1
OD value(450nm)
Time (h)
(B)
Vector+si-NC
TPT1-AS1+si-NC
Vector+si-ITGB3
TPT1-AS1+si-ITGB3
BxPC-3
SW1990
(C)
shCtrl+ pcDNA-NC
shTPT1-AS1 +pcDNA-NC
shCtrl+ pcDNA-ITGB3
shTPT1-AS1+ pcDNA-ITGB3
Migration
Invasion
CFPAC-1
(F)
Vector +si-NC
TPT1-AS1 +si-NC
Vector+ si-ITGB3
TPT1AS1+ si-ITGB3
BxPC-3
(D)
PANC-1
(G)
SW1990
(E)
shCtrl+si-NC
shTPT1-AS1+si-NC
shCtrl+pcDNA-ITGB3
shTPT1-AS1+pcDNA-ITGB3
Cell counts
(H)
Vector+si-NC
TPT1-AS1+si-NC
Vector+si-ITGB3
TPT1-AS1+si-ITGB3
(I)
CFPAC-1
PANC-1
shTPT1-AS1
ITGB3
E-cadherin
N-cadherin
Vimentin
ITGB3
ß-actin
(J)
BxPC-3
SW1990
TPT1-AS1
si-ITGB3

FIGURE 7 Silencing ITGB3 reverses the functions of promoting carcinogenesis in pancreatic cancer cell lines induced by TPT1-AS1 overexpression. (A) Rescue effects of ITGB3 overexpression on TPT1-AS1 knockdown–mediated inhibition of cell proliferation in CFPAC-1 and PANC-1 cells determined by CCK-8 assays. (B) Rescue effects of ITGB3 knockdown on TPT1-AS1 overexpression–mediated promotion of cell proliferation in BxPC-3 and SW1990 cells determined by CCK-8 assays. (C–E) Rescue effects of ITGB3 overexpression on TPT1-AS1 knockdown–mediated inhibition of cell migration and invasion in CFPAC-1 and PANC-1 cells determined by Transwell assays. (F–H) Rescue effects of ITGB3 knockdown on TPT1-AS1 overexpression–mediated promotion of cell migration and invasion in BxPC-3 and SW1990 cells determined by Transwell assays. (I) Rescue effects of ITGB3 overexpression on TPT1-AS1 knockdown–mediated inhibition of cell epithelial-mesenchymal transition (EMT) process in CFPAC-1 and PANC-1 cells determined by Western blot. (J) Rescue effects of ITGB3 knockdown on TPT1-AS1 overexpression–mediated promotion of cell EMT process in BxPC-3 and SW1990 cells determined by Western blot. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

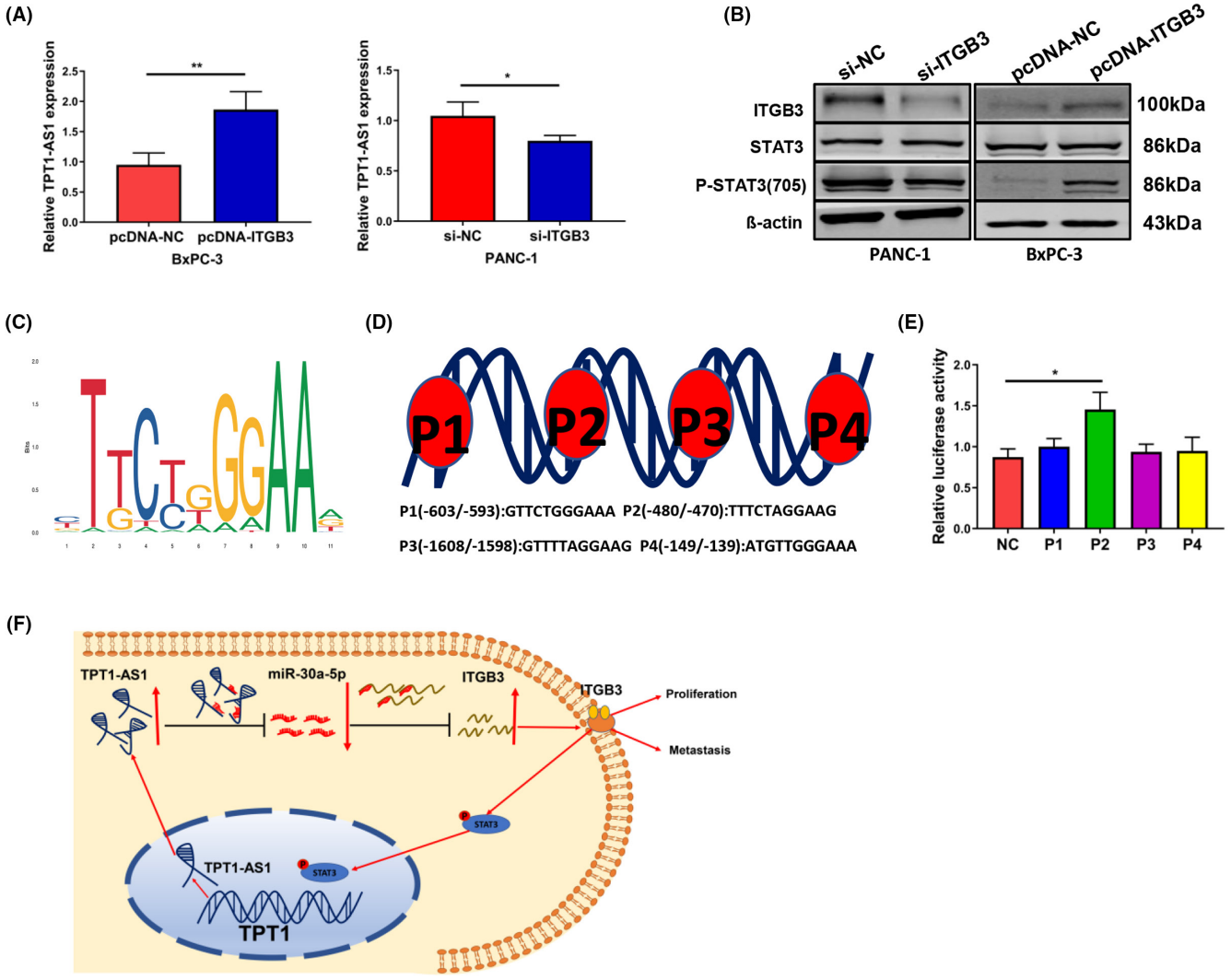


FIGURE 8 ITGB3 elevated TPT1-AS1 expression in pancreatic cancer cells. (A) qRT-PCR assays were used to detect the expression of TPT1-AS1 in PANC-1 and BxPC-3 cells with ITGB3 knockdown or overexpression. (B) Western blot assays were used to detect the expression of STAT3 and p-STAT3(705) in PANC-1 and BxPC-3 cells with ITGB3 knockdown or overexpression. (C) The STAT3 motif was predicted from the JASPAR database. (D) JASPAR (http://jaspar.genereg.net/) was used to predict the binding sites of transcription factor STAT3 on the TPT1-AS1 promoter region. (E) Luciferase reporter assays were performed in BxPC-3 after transfection with wild-type (WT) promoter or mutated (MUT)-promoter and NC or IL-6. (F) Schematic diagram of the mechanisms of TPT1-AS1 forming a positive feedback loop with ITGB3 in pancreatic ductal adenocarcinoma (PDAC). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

(Figure 8D), and the plasmids that inserted four fragments of the promoter (P1, P2, P3, and P4) in the front of the luciferase sequence were built to explore which fragments of the promoter could bind to STAT3. The dual luciferase assays showed that luciferase activity was increased when we transfected the plasmid containing the P2 fragment (Figure 8E). Previous literature

reported that IL-6 could promote tumor progression by activating STAT3; consistently, we found that upregulation of IL-6–activated STAT3 increased TPT1-AS1 expression in pancreatic cancer cells (Figure S19B). In summary, ITGB3 upregulated the transcription of TPT1-AS1 by activating STAT3, which could form a positive feedback loop to enhance the expression of TPT1-AS1.

4 | DISCUSSION

Numerous lncRNAs have been identified by human transcriptome sequencing, and an increasing number of studies have shown that plenty of cancer-associated lncRNAs play important roles in regulating tumorigenesis and progression.[33–38] Therefore, lncRNAs can serve as a valuable class of biomarkers and potential therapeutic targets. In this study, we identified a PDAC-related lncRNA, TPT1-AS1, by genome-wide analysis, which was significantly overexpressed in PDAC tissues and cell lines compared with normal pancreatic tissues and normal pancreatic ductal epithelial cell lines, respectively. Besides, high expression of TPT1-AS1 was positively correlated with advanced TNM stage and poor differentiation, indicating a poor clinical prognosis of PDAC patients. In addition, previous research showed that TPT1-AS1 functioned as an oncogene in gastric cancer, colorectal cancer, ovarian cancer, cervical cancer, among others.[16–19,39,40] Moreover, functional experiments revealed that TPT1-AS1 could promote pancreatic cancer cell proliferation, invasion, migration, and EMT process in vitro and in vivo. These findings were consistent with those of previous studies and suggested that TPT1-AS1 acted as an oncogenic lncRNA in PDAC.

In previous studies, tumor protein, translationally controlled tumor protein 1 (TPT1), located at the opposite strand of TPT1-AS1, is a regulator of cellular growth and proliferation. TPT1 is known as a tumor oncogene in many cancers, such as ovarian cancer and pancreatic cancer.[17,41] In our study, we found that TPT1-AS1 positively regulates the expression of TPT1.

Increasing researches has demonstrated that lncRNAs can function as ceRNAs, which can promote mRNA expression by competitively binding to miRNAs.[31,42,43] The biological functions performed by lncRNAs are mainly dependent on their subcellular localization, and several studies have revealed that lncRNAs located in the cytoplasm could participate in gene regulation at the post-transcriptional level, including but not limited to act as ceRNAs to protect the mRNAs from repression. By using bioinformatics databases and FISH assays, we demonstrated that TPT1-AS1 mainly located in the cytoplasm, indicating that it had the potential to function as a miRNA sponge. Our results showed that miR-30a-5p was substantially downregulated in PDAC patients, and a negative correlation between TPT1-AS1 and miR-30a-5p expression was confirmed in PDAC tissues in our cohort and GSE24279 database. Further, TPT1-AS1 knockdown increased the expression of miR-30a-5p, whereas TPT1-AS1 overexpression decreased miR-30a-5p expression. Importantly, the following luciferase reporter and RIP assays indicated that TPT1-AS1 acted as a sponge for miR-30a-5p by directly binding to a complementary sequence in pancreatic cancer cells. miR-30a-5p has been thoroughly shown to serve as a tumor suppressor in many cancers.[44–50] In our study, miR-30a-5p overexpression inhibited pancreatic cancer proliferation, migration, invasion, and EMT process. The rescue experiments revealed that miR-30a-5p overexpression partially reversed the TPT1-AS1 overexpression–mediated oncogenic effects. In contrast, miR-30a-5p knockdown led to the opposite effects of TPT1-AS1 knockdown in pancreatic cancer cells, which demonstrated that miR-30a-5p was a mediator of TPT1-AS1 in pancreatic cancer. These data showed that TPT1-AS1 exerted oncogenic functions partly by sponging miR-30a-5p in pancreatic cancer.

Generally, as a ceRNA, the biological behavior of lncRNAs depends on the miRNA targets. To further predict miR-30a-5p targets in PDAC, bioinformatics databases and microarray analysis were used, which might mediate the TPT1-AS1/miR-30a-5p axis, and we predicted that ITGB3 could be a potential target for miR-30a-5p, which was confirmed by luciferase reporter assays. ITGB3 is a transmembrane receptor comprising two subunits, α and β, which belongs to the integrin family, and it is an important class of cell surface adhesion molecules in regulation of proliferation, invasion, and migration in breast, colorectal, and nasopharyngeal cancer.[51–53] As expected, we found that ITGB3 expression was elevated in PDAC tissues compared with normal tissues, and high ITGB3 expression in PDAC patients was markedly associated with poor prognosis in PDAC patients. In addition, we found that ITGB3 levels in clinical samples were positively correlated with TPT1-AS1 and negatively correlated with miR-30a-5p. Notably, TPT1-AS1 positively regulated ITGB3 expression in pancreatic cancer cells, while miR-30a-5p showed an opposite regulatory effect. Finally, to investigate the roles of ITGB3 in TPT1-AS1, we performed rescue experiments, which demonstrated that ITGB3 knockdown could suppress the promotive effects on proliferation, migration, invasion, and EMT process induced by TPT1-AS1 overexpression, whereas restored ITGB3 eliminated the inhibitory effects induced by TPT1-AS1 knockdown. These findings suggested that ITGB3 mediated the promoting functions induced by TPT1-AS1/miR-30a-5p in PDAC.

It has also been shown that in tumor-repopulating cells, ITGB3 could mediate enhanced STAT3 activation, which was consistent with our previous findings[30] (Figure 8B, Figure S14A). An increasing number of studies have demonstrated STAT3 could participate in the modulation of abnormal expression of lncRNAs in human malignancies;[54] hence, we applied the JASPAR database (http://jaspar.genereg.net) and identified four major binding sites for STAT3 in the promoter region of TPT1-AS1. Previous studies have reported that IL-6 could promote tumor progression by activating STAT3; consistently, we found that upregulation of IL-6–activated STAT3 promoted high expression of TPT1-AS1 in pancreatic cancer cells. Finally, luciferase reporter assays demonstrated that upregulation of STAT3 led to an effective increase of TPT1-AS1 promoter activity. Overall, our results uncovered a reciprocal loop of TPT1-AS1 and ITGB3 which facilitated pancreatic cancer growth and development.

In summary, our study identified TPT1-AS1 as an oncogenic lncRNA in PDAC, which was significantly overexpressed in advanced pancreatic cancer and poorly differentiated pancreatic cancer. High TPT1-AS1 expression was associated with malignant clinical features and poor prognosis of PDAC patients. Functional experiments demonstrated that TPT1-AS1 could promote pancreatic cancer cell proliferation, invasion, migration, and EMT process in vitro and in vivo. Mechanistically, TPT1-AS1 directly bound to miR-30a-5p and served as a ceRNA to upregulate ITGB3 expression. In turn, ITGB3 could increase the expression of TPT1-AS1 via activating STAT3, thus forming a positive feedback loop. These findings demonstrated TPT1-AS1 could serve as an oncogene in pancreatic cancer, which might provide potential diagnostic and therapeutic targets for pancreatic cancer patients.

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DISCLOSURE

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by The First Affiliated Hospital of Harbin Medical University (Harbin, China), and written informed consent was obtained before any operation performed on patients.

CONSENT FOR PUBLICATION

We have obtained consents to publish this paper from all the participants of this study.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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