Original Article
MiR-27a-regulated FOXO1 promotes pancreatic ductal adenocarcinoma cell progression by enhancing Wnt/β-catenin signaling activity

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Abstract: FOXO1, also known as FKHR, is a member of the Forkhead transcription factor family. Our previous study revealed that FOXO1 expression is significantly downregulated in pancreatic ductal adenocarcinoma (PDAC). However, our knowledge on the clinical significance of FOXO1 and its biological roles and associated mechanisms in PDAC tumorigenesis remains limited. In this study, we confirmed that FOXO1 is commonly downregulated in PDAC tissues, at both the mRNA and protein levels, compared to adjacent tissues. Furthermore, FOXO1 inhibited cell proliferation and tumor formation both *in vitro* and *in vivo,* and promoted pancreatic cancer cell invasion. Downregulation of FOXO1 resulted in enhanced Wnt/β-catenin signaling activity, thereby promoting cell proliferation and epithelial-mesenchymal transition. The highly expressed miR-27a could potentially be used to target the 3'-UTR of FOXO1 in PDAC tissues to inhibit or at least slow down the invasion and proliferation of cancerous cells. Taken together, our findings suggest that the miR-27a/FOXO1/β-catenin axis may serve as a promising therapeutic target in PDAC progression.

Keywords: FOXO1, PDAC, proliferation, invasion, miR-27a, Wnt/β-catenin signaling

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignant tumor and is the fourth leading cause of cancer-associated mortality in the USA [1]. Patients with PDAC usually present with locally advanced, unresectable, or metastatic disease, and are often in a lot of pain. Despite developments in the detection and management of PDAC, the five-year relative survival rate has not improved [2, 3]. Thus, further investigation into the molecular mechanisms driving this largely incurable tumor is urgently needed.

The Forkhead transcription factor family member FOXO1 is a multifunctional transcription factor that regulates the transcription of a number of important genes involved in cell cycle arrest or apoptosis [4]. FOXO1 activation in transformed and non-transformed cells results in the upregulation of the cyclin-dependent kinase inhibitor p27^{KIP1} and downregulation of D-type cyclins, thereby arresting cells at the G1 phase [5]. Activated FOXO1 protein also triggers apoptosis in various cancer cell lines through the regulation of proapoptotic proteins, such as Fas ligand, TRAIL, and Bim [6]. Thus, it has been postulated that FOXO1 plays a pivotal role in cell transformation and tumorigenesis inhibition. Our previous study revealed that FOXO1 is significantly downregulated in PDAC tissues, and this downregulation correlates with cancer cell stemness in PDAC [7]. However, the detailed underlying mechanisms remain largely unknown.

The inhibitory function of FOXO1 in cell proliferation and survival is often disrupted by the overactivated phosphatidylinositol 3-kinase (PI3K) Akt pathway in cancer cells [8]. Activated Akt phosphorylates a wide range of downstream proapoptotic proteins, including FOXO1 [9]. Wnt/β-catenin signaling plays a pivotal role

Table 1. Sequence of primers

Name	Sequence (5'-3')
FOXO1	F: ACTCTGAGAAGTGCCTGATGT
	R: TGTGGCTGACAAGACTTAACTCAA
GAPDH	F: AGCCTCAAGATCATCAGCAATGCC
	R: TGTGGTCATGAGTCCTTCCACGAT
CCND1	F: TCCTCTCCAAAATGCCAGAG
	R: GGCGGATTGGAAATGAACTT
CCNE1	F: GAAATGGCCAAAATCGACAG
	R: TCTTTGTCAGGTGTGGGGA
MYC	F: CGTCCTCGGATTCTCTGCTC
	R: CTTCGCTTACCAGAGTCGCT
E-cadherin	F: CGAGAGCTACACGTTCACGG
	R: GGGTGTCGAGGGAAAAATAGG
Fibronectin	F: CGGTGGCTGTCAGTCAAAG
	R: AAACCTCGGCTTCCTCCATAA
ZEB1	F: GATGATGAATGCGAGTCAGATGC
	R: ACAGCAGTGTCTTGTTGTTGT

in maintaining cell stemness and crosstalks with the PI3K/AKT pathway [10, 11].

Epigenetic alterations are involved in the inhibition of tumor suppressors in pancreatic cancer cells [12-14]. MiR-27a has been found to be highly expressed in numerous types of cancers, including breast and ovarian cancers [15, 16]. However, the role of miR-27a/FOXO1 in PDAC remains unknown. This study aimed to further explore the function of FOXO1 and its epigenetic regulation in PDAC progression using *in-vitro* and *in-vivo* experiments and clinical specimens.

Materials and methods

Patient tissues and ethics statement

Fifty-seven fresh tumor and matched adjacent tissue samples were collected from patients with pathologically and clinically confirmed PDAC. Tissue microarrays (TMAs) containing 136 paired normal and adjacent PDAC tissues were obtained from Shanghai Renji Hospital (Shanghai, China). Written informed consent was obtained from patients prior to tissue sample collection. The study was conducted according to the Declaration of Helsinki. The Institutional Review Board of Shanghai General Hospital approved the use of tumor samples and animals in this study. All animal experiments were performed in accordance with The Animal Care

and Use Committee of Shanghai Jiao Tong University, School of Medicine.

Immunohistochemical (IHC) staining

The TMAs were probed with a FOXO1 monoclonal antibody (#2880, Cell Signaling Technology) overnight at 4°C. After incubation with a suitable secondary antibody, the TMAs were exposed to diaminobenzidine and counterstained with hematoxylin for detection. To quantify FOXO1 protein expression, all tissues were observed and photographed using a microscope (Carl Zeiss) and scored according to the ratio and intensity of positive cells: 0-5% scored 0; 6-35% scored 1; 36-70% scored 2; 70-100% scored 3. The final expression levels of FOXO1 were categorized as "low" or "high" as follows: score 0-1, low expression; 2-3, high expression. FOXO1 expression was quantified by two independent pathologists.

Cell culture

HPNE, BxPC3, and PANC1 cells were purchased from the American Type Culture Collection (ATCC). All cells were maintained under standard culture conditions (37°C, 5% CO_2) in culture medium recommended by the ATCC. All cells were characterized by DNA typing at the Shanghai Jiao Tong University Analysis Core.

RNA isolation and quantitative reverse-transcription (RT-q)PCR

Total RNA was purified from PDAC and adjacent tissues or cells using TRIzol (Invitrogen), following the manufacturer's protocol. RNA (1 µg) was reverse-transcribed using SuperScript Reverse Transcriptase III (Invitrogen). qPCRs were run using SYBR green Supermix (ABI) on an ABI 7300 instrument. *GAPDH* was used as a reference gene. Primers used in this study are listed in **Table 1**.

Western blotting

Tissues and cells were lysed in WB/IP lysis buffer (P0013; Beyotime) and nuclear proteins were extracted using lysis buffer (P0028; Beyotime), per the manufacturer's protocols. The cell lysates were boiled in 5× loading buffer for 10 min, resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. The fol-

lowing antibodies were used in this study: anti-FOXO1 (#2880) and anti-β-catenin (#8480) from Cell Signaling Technology, and anti-lamin A/C (10298-1-AP) and anti-GAPDH (60004-1-Ig) from Proteintech. Immunocomplexes were visualized using an ECL kit (Thermo Scientific).

Construction of stable cell lines

To generate cell lines with stable ectopic FOXO1 expression, a vector containing full-length *FOXO1* was purchased from GeneCopoeia. MiR-27a inhibitor and a negative control vector were purchased from GeneCopoeia. HEK293T cells were transfected with these vectors using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Virus-containing media were centrifuged to remove cellular contaminants, and the supernatant containing the viruses was used to infect the cells. Transformed cells were selected on puromycin (2 μg/ml) for 2 weeks. FOXO1 overexpression was confirmed by western blotting prior to further analysis.

Cell viability assays

Cells were seeded into a 96-well plate at 3×10^3 cells/well in 100 μl culture medium and incubated at 37°C in the presence of 5% CO_2. Ten microliters of CCK8 solution (Dojindo) was added to each well. After 1.5 h of incubation, the absorbance at 450 nm was read using a Power Wave XS microplate reader (BioTek).

Flow-cytometric cell cycle assays

Cells were seeded in 6-well plates at 2×10^5 cells/well in 100 μl culture medium and incubated at 37°C in the presence of 5% CO_2. Cells were collected at 24, 48, and 72 h. The cells were washed twice with 1× PBS, resuspended, and fixed in 2 ml of 70% ethanol at -20°C. Then, the cells were stained with propidium iodide (BD), according to the manufacturer's instructions.

Transwell cell invasion assay

For invasion assays, we used 8-μm filter-insert chambers (Millipore). Cells (4×10^4) in 100 μl of serum-free medium were placed in the upper chamber, which had been coated with 100 μl of Matrigel (BD Biosciences), and 0.7 ml of medium containing 10% FBS was placed in the lower chamber. After incubation for 24 h, cells on the upper side of the filters were wiped off with cotton-tipped swabs, and the filters were wa-

shed with PBS. Then, the cells were fixed in 2.5% glutaraldehyde for 15 min and stained with 0.5% crystal violet for 15 min. Cells on the lower side of the filters were viewed and counted under a microscope.

Animal experiment

Cells stably overexpressing FOXO1 (1×10^6) or control cells were subcutaneously injected into the right flanks of 5 BALB/c (nu/nu) mice. Tumor size was measured once a week. After 4 weeks, the mice were sacrificed for tumor burden analysis. The tumor volume was calculated as volume = (length × width^2)/2. For intrahepatic metastasis assays, cells (3×10^6/mouse) were injected into the livers of nude mice.

Immunofluorescence

Coverslip-grown cells were fixed in 4% paraformaldehyde at room temperature for 15 min and rinsed with PBS three times. The cells were permeabilized in 0.3% Triton X-100/PBS for 15 min and then rinsed with PBS three times. Subsequently, the cells were blocked in 5% bovine serum albumin in PBS at 25°C for 1 h. Then, the cells were incubated overnight with primary antibodies at 1:100 dilution in staining buffer at 4°C in a humid chamber. The coverslips were subsequently washed three times. Then, the cells were incubated for 1 h with secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 595 (Invitrogen) at 1:200 dilution in staining buffer at 37°C in a humid chamber in the dark. Prior to mounting using Vectashield with DAPI (Vector Laboratories), the coverslips were washed in PBS three times. Immunofluorescence analysis was conducted using a Carl Zeiss LSM710 Laser Scanning Microscope.

Luciferase reporter assays

Indicated cells were seeded in 96-well plates at 2×10^3 cells/well and transfected with 100 ng TCF/β-catenin reporter plasmid (Wnt/β-catenin signaling) or mutated TCF/β-catenin reporter plasmid and 10 ng Renilla, or vectors containing FOXO1 3'-UTR, FOXO1 mutant 3'-UTR, and miR-27a using Lipofectamine 2000 per the manufacturer's protocol. After a 48-h incubation, firefly and Renilla luciferase activities in cell lysates were measured using the dual-luciferase reporter assay system (Promega, Madison, WI).
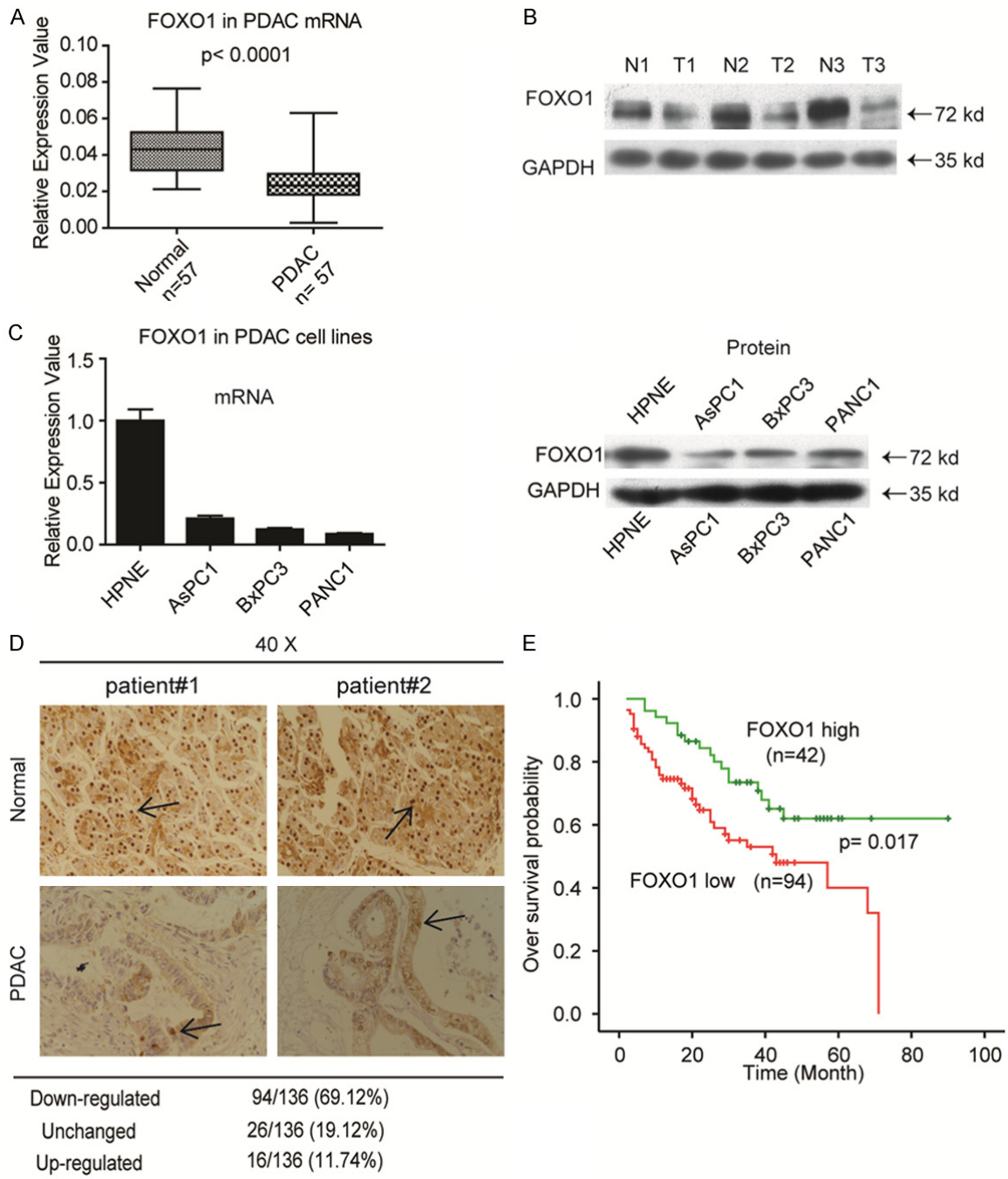
Figure 1. FOXO1 is downregulated and predicts poor patient prognosis in patients with PDAC. A: FOXO1 mRNA levels as detected by RT-qPCR in fresh PDAC tissues and normal tissues (n = 57). B: FOXO1 protein levels as detected by western blotting in three paired clinical normal and PDAC tissues. C: mRNA and protein levels of FOXO1 in the normal pancreatic ductal epithelial cell HPNE and in the indicated PDAC cell lines. D: IHC staining revealing FOXO1 protein expression in PDAC and adjacent tumor tissues of patients (scale bar, 50 μm). E: Patients with low FOXO1 expression have poor survival probability.

Statistics analysis

Data are expressed as the mean ± standard deviation. Correlations between FOXO1 expres-sion and clinicopathological parameters were evaluated using the Chi-squared test. Student's *t*-tests were used to compare group means. *P* < 0.05 was considered significant.

Table 2. Correlations between FOXO1 and key clinicopathological parameters

Variable		FOXO1 (n=136)		
		Low (94)	High (42)	P value (χ^2)
Age	≤ 65 years	35	22	0.098
	> 65 years	59	20	
Gender	Female	44	17	0.493
	Male	50	25	
Liver metastasis	Yes	68	23	0.044*
	no	26	19	
Tumor size	≤ 5 cm	45	31	0.004*
	> 5 cm	49	11	
Vascular invasion	yes	32	5	0.007*
	no	62	37	
TNM stage	T_1-T_2	74	24	0.001*
	T_3-T_4	27	18	

*P < 0.05.

Results

FOXO1 is downregulated in PDAC tissues and low FOXO1 expression correlates with clinical outcome

We first measured FOXO1 mRNA expression in 57 fresh PDAC tissues and FOXO1 protein levels in three paired PDAC samples. The data indicated that FOXO1 was remarkably downregulated in PDAC malignant tissues (**Figure 1A** and **1B**). Next, we evaluated FOXO1 mRNA and protein levels in the normal pancreatic ductal epithelial cell line HPNE and three PDAC cell lines, AsPC1, BxPC3, and PANC1. FOXO1 expression was significantly lower in PDAC malignant cell lines than in HPNE cells (**Figure 1C**). Furthermore, we evaluated FOXO1 expression by IHC staining using a TMA containing 136 paired PDAC and adjacent tissues, which revealed that FOXO1 was downregulated in most PDAC tissues (**Figure 1D**). Clinicopathological data analysis revealed that downregulation of FOXO1 was closely correlated with liver metastasis, tumor size, vascular invasion, and TNM stage (**Table 2**). Survival analysis indicated that patients with low FOXO1 expression have a poor prognosis (**Figure 1E**).

FOXO1 inhibits PDAC cell proliferation and invasion

The confirmation of the clinical significance of FOXO1 prompted us to determine its biological role in PDAC progression. We ectopically expressed FOXO1 in the cell lines BxPC3 and PANC1. FOXO1 mRNA and protein levels were significantly elevated in these cell lines (**Figure 2A** and **2B**). We then employed these stable cell lines in biological functional assays. CCK-8 cell viability assays showed that ectopic expression of FOXO1 significantly inhibited cell viability in both BxPC3 and PANC1 cell lines (**Figure 2C** and **2D**). Subcutaneous tumor formation assays revealed that FOXO1 overexpression impaired tumor formation *in vivo* (**Figure 2E** and **2G**), suggesting that FOXO1 inhibits the proliferation and growth of PDAC cells *in vivo*. IHC analysis revealed that the expression of FOXO1 was negatively correlated with that of PCNA, a well-accepted cell proliferation marker (**Figure 2H**). Together, these findings suggested that ectopic expression of FOXO1 inhibits PDAC cell proliferation and growth. As downregulation of FOXO1 was correlated with PDAC liver metastasis, we next performed Transwell invasion assays. FOXO1 overexpression remarkably inhibited the invasion ability of BxPC3 and PANC1 cells (**Figure 2I** and **2J**). Intrahepatic metastasis assays confirmed these findings (**Figure 2K** and **2L**).

Ectopic expression of FOXO1 in PDAC cells inhibits Wnt/β-catenin signaling

Next, we evaluated cell cycle alterations in FOXO1-expressing BxPC3 and PANC1 stable cell lines by flow cytometry. FOXO1 overexpression remarkably inhibited cell cycle transition from G0/G1 to S (**Figure 3A** and **3B**). Therefore, we examined the expression of CCND1 and CCNE1, which play essential roles in G0/G1-to-S transition. Consistently, FOXO1 overexpression significantly inhibited CCND1, but not CCNE1 expression in PDAC cell lines (**Figure 3C** and **3D**). To confirm these findings, we measured the expression of CCND1 in 19 fresh PDAC tissue samples. The data indicated that CCND1 expression was negatively correlated with FOXO1 expression (**Figure 3E**). Moreover, CCND1 protein levels were decreased in the FOXO1-expressing BxPC3 and PANC1 stable cell lines (**Figure 3F**). Collectively, these results indicated that FOXO1 overexpression inhibited PDAC cell cycle transition from G0/G1 to S by affecting the expression of CCND1.

Previous studies have verified that CCND1 is a target of Wnt/β-catenin signaling, which strongly suggests that FOXO1 overexpression might
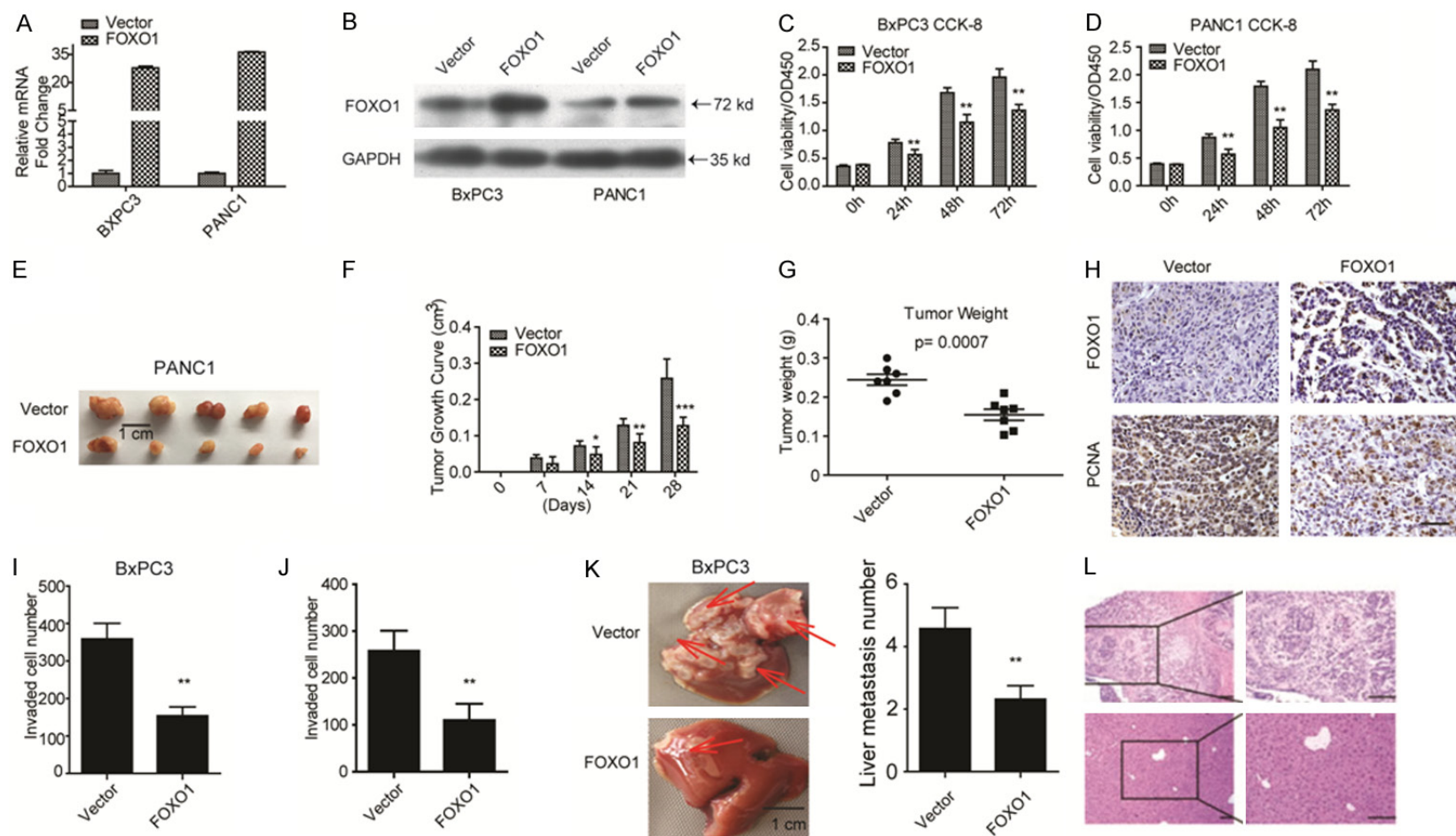
Figure 2. FOXO1 inhibits PDAC cell proliferation. (A, B) mRNA and protein levels of FOXO1 are significantly upregulated in FOXO1-overexpressing BXPC3 and PANC1 cell lines. (C, D) CCK-8 cell viability assays revealed that ectopic expression of FOXO1 significantly inhibits BxPC3 (C) and PANC1 (D) cell proliferation. (E) Xenograft tumor formation assays showed that overexpression of FOXO1 inhibits subcutaneous tumor formation capacity. (F, G) Tumor growth (F) and tumor weight (G) of xenografts of FOXO1-overexpressing PANC1 stable cells in nude mice. (H) IHC staining revealed that the expression of FOXO1 and PCNA in PANC1 stable cells induced tumor formation. (I, J) Matrigel-coated Transwell assays showed that overexpression of FOXO1 inhibits BxPC3 (I) and PANC1 (J) cell invasion. (K, L) Images showing intrahepatic metastasis (K) and morphological characters by HE staining (L), $**P < 0.01$. All assays were conducted in triplicate.
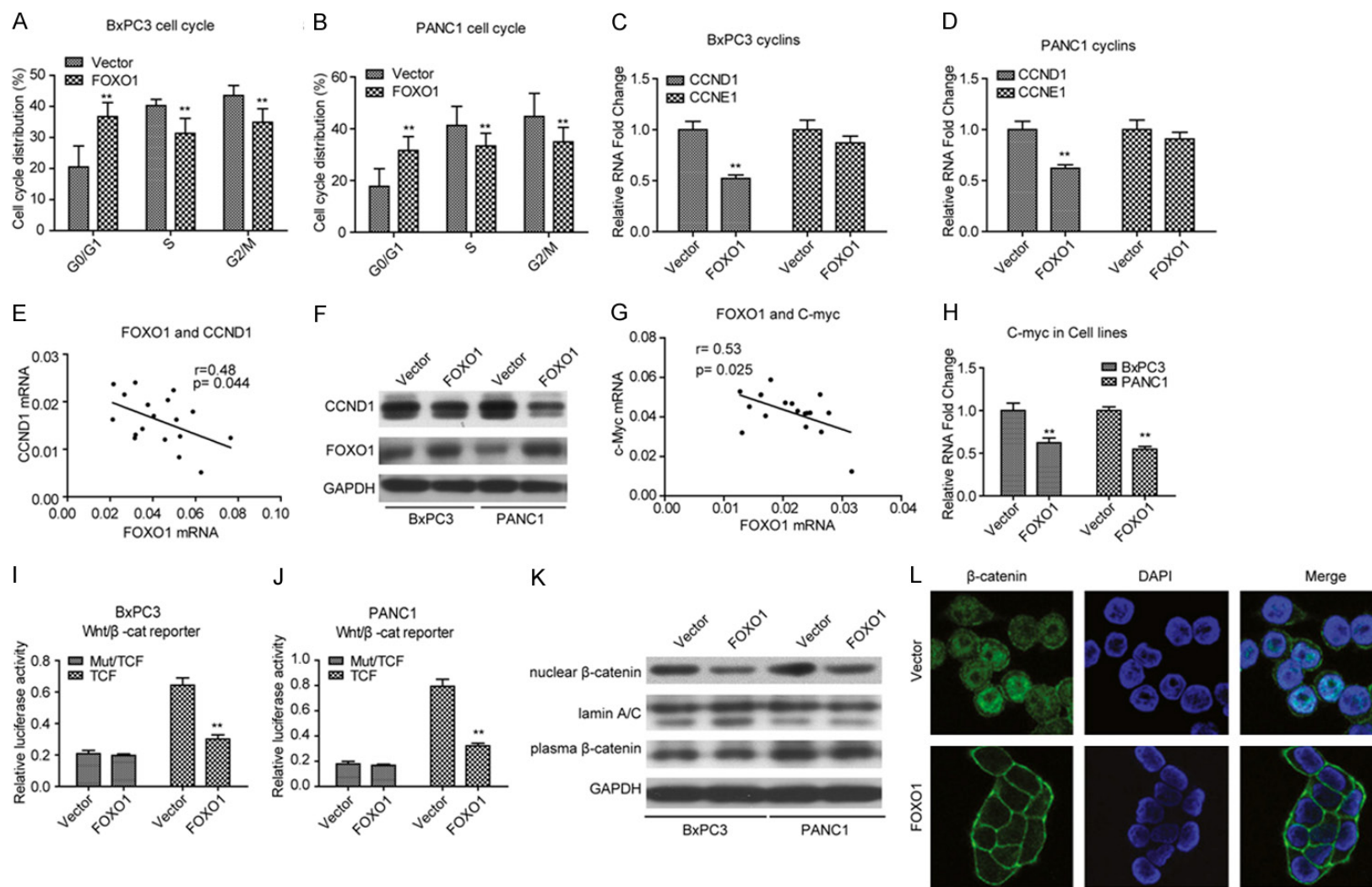
Figure 3. Ectopic expression of FOXO1 induces arrest of G0/G1-to-S transition in PDAC cell lines. (A, B) Cell cycle assay showed that overexpression of FOXO1 induces arrest of G0/G1-to-S transition BxPC3 (A) and PANC1 (B) cell lines. (C, D) mRNA levels of CCND1 and CCNE1 in FOXO1-overexpressing BxPC3 (C) and PANC1 (D) stable cell lines. (E) CCND1 expression is negatively correlated with FOXO1 expression in fresh PDAC tissues. (F) Protein levels of CCND1 in FOXO1-overexpressing BxPC3 and PANC1 cell lines. (G) The CCND1 mRNA level is negatively correlated with FOXO1 expression in fresh PDAC tissues. (H) mRNA level of MYC in FOXO1-overexpressing BxPC3 and PANC1 stable cell lines. (I, J) Dual luciferase reporter assays revealed that overexpression of FOXO1 inhibits Wnt/β-catenin signaling activity (TCF/β-catenin reporter) in BxPC3 (I) and PANC1 (J) cell lines, but not in cells carrying mutated TCF/β-catenin reporter plasmid. (K) Overexpression of FOXO1 impairs nuclear translocation of β-catenin in BxPC3 and PANC1 cell lines. (L) Immunofluorescence assays revealed that overexpression of FOXO1 impairs nuclear β-catenin translocation nuclear in BxPC3 cells. **$P < 0.01$. All assays were conducted in triplicate.
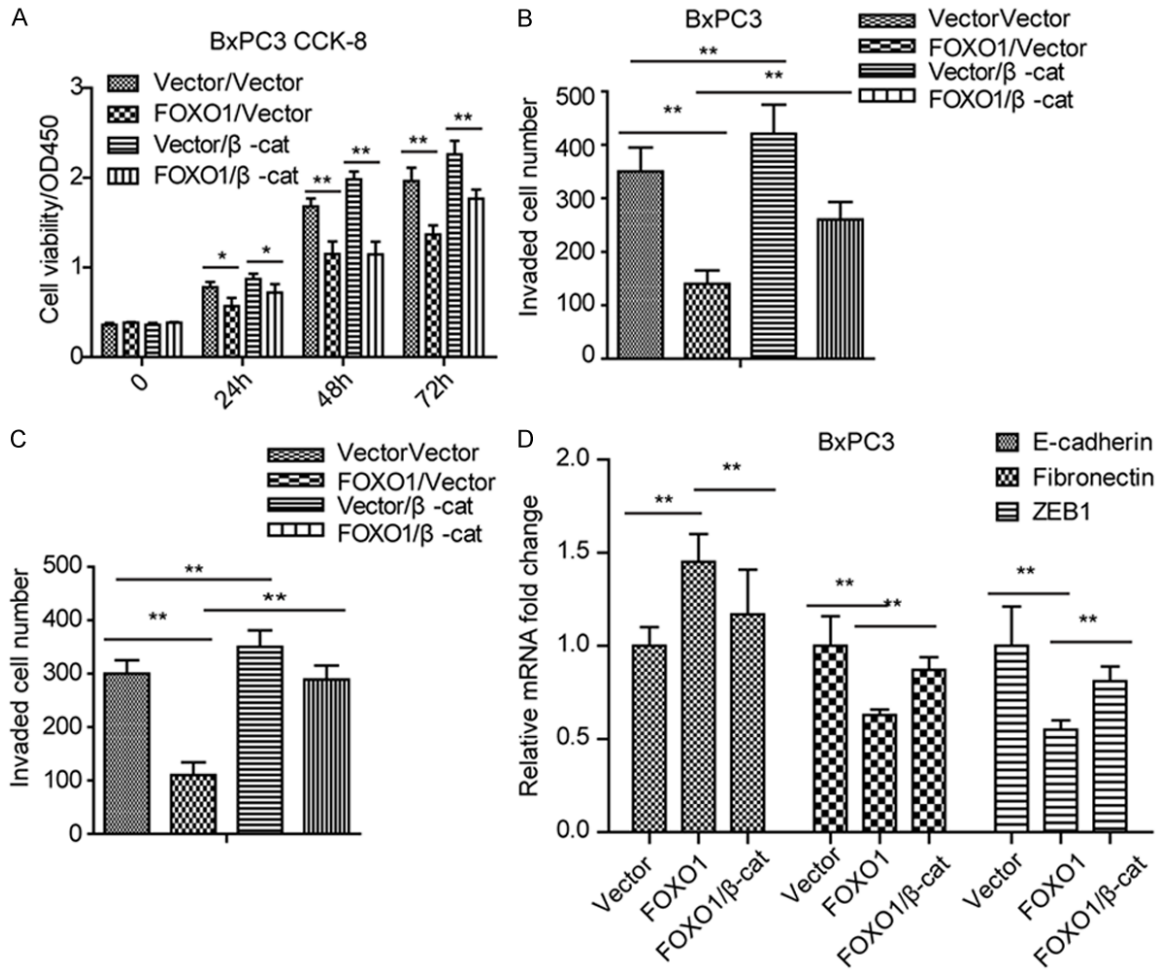
Figure 4. Overexpression of β-catenin signaling rescues cell proliferation and invasion under FOXO1 ectopic expression. (A) Overexpression of β-catenin rescues the inhibition of cell proliferation resulting from ectopic FOXO1 expression in BxPC3 cells. (B, C) Overexpression of β-catenin rescues the inhibition of cell invasion resulting from ectopic FOXO1 expression in BxPC3 (B) and PANC1 (C) cell lines. (D) Overexpression of β-catenin rescues the expression of EMT markers under ectopic FOXO1 expression in BxPC3 cells. *$P < 0.05$; **$P < 0.01$. All assays were conducted in triplicate.

result in inhibition of Wnt/β-catenin signaling activity. To investigate the relationship between FOXO1 and Wnt/β-catenin signaling, we examined another well-known target of Wnt/β-catenin signaling, MYC, in 19 fresh PDAC tissues. As shown in **Figure 3G**, the expression of MYC was similar to that of CCND1, which was negatively correlated with the expression of FOXO1. These findings prompted us to explore the correlation between FOXO1 and Wnt/β-catenin signaling. As shown in **Figure 3H**, MYC expression was remarkably suppressed in FOXO1-overexpressing BxPC3 and PANC1 cells. Dual luciferase reporter assays in the two stable cell lines revealed that elevated FOXO1 expression significantly inhibited Wnt/β-catenin signaling

activity (**Figure 3I** and **3J**). In addition, FOXO1 overexpression resulted in a robust decrease in nuclear β-catenin, but did not affect cytoplasmic β-catenin (**Figure 3K** and **3L**). These results suggested that FOXO1 inhibits nuclear translocation of β-catenin and thus inhibits Wnt/β-catenin signaling.

Ectopic expression of β-catenin rescues cell proliferation and invasion impaired by FOXO1 overexpression

To confirm that FOXO1 exerts its anti-tumor effects through Wnt/β-catenin signaling, we ectopically expressed β-catenin in stably FOXO1-overexpressing BxPC3 cells. As shown in **Figure 4A**, overexpression of β-catenin partly
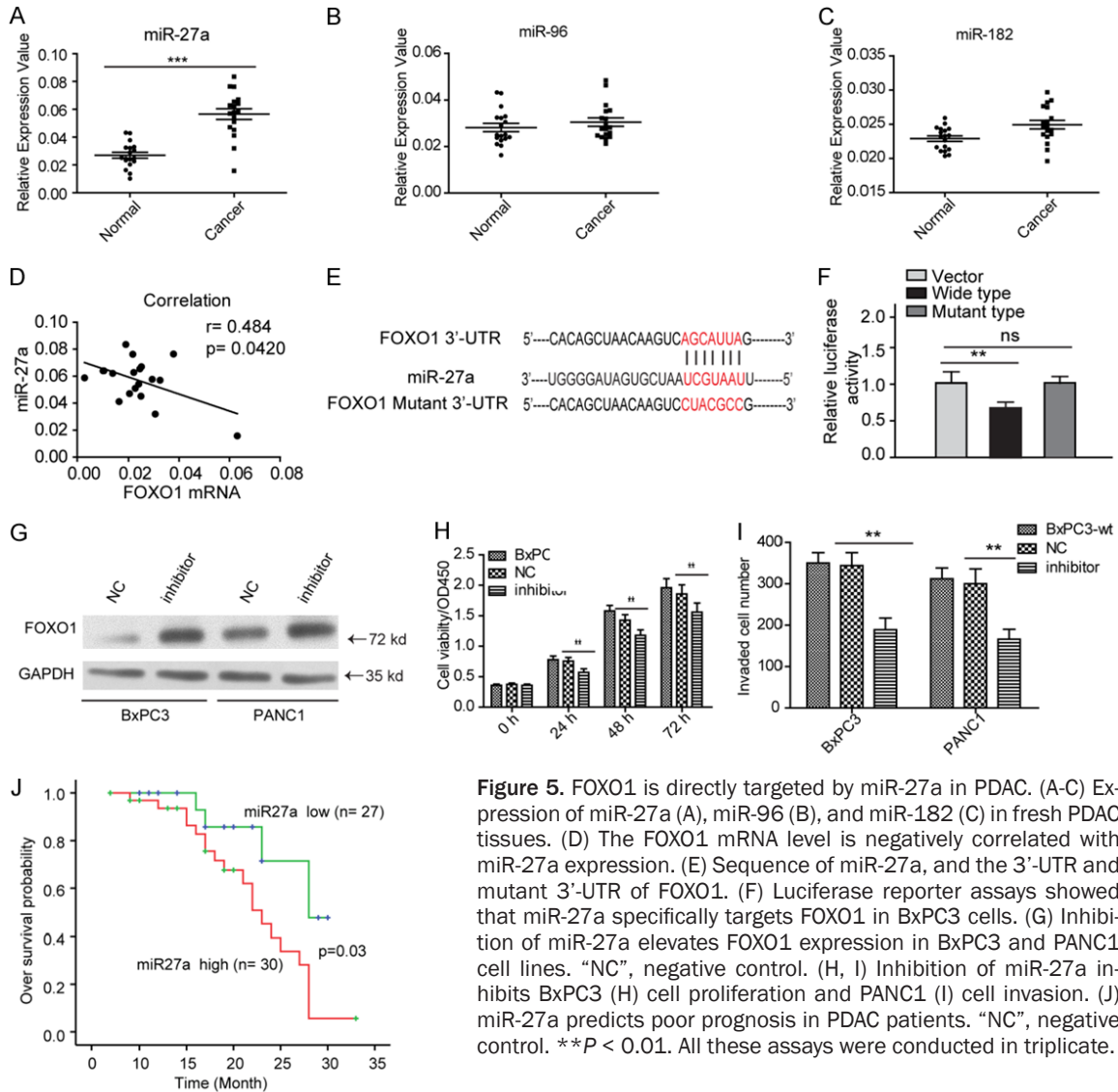
Figure 5. FOXO1 is directly targeted by miR-27a in PDAC. (A-C) Expression of miR-27a (A), miR-96 (B), and miR-182 (C) in fresh PDAC tissues. (D) The FOXO1 mRNA level is negatively correlated with miR-27a expression. (E) Sequence of miR-27a, and the 3'-UTR and mutant 3'-UTR of FOXO1. (F) Luciferase reporter assays showed that miR-27a specifically targets FOXO1 in BxPC3 cells. (G) Inhibition of miR-27a elevates FOXO1 expression in BxPC3 and PANC1 cell lines. "NC", negative control. (H, I) Inhibition of miR-27a inhibits BxPC3 (H) cell proliferation and PANC1 (I) cell invasion. (J) miR-27a predicts poor prognosis in PDAC patients. "NC", negative control. **P < 0.01. All these assays were conducted in triplicate.

rescued the inhibition of cell proliferation resulting from ectopic expression of FOXO1. Similar rescue effects were observed in both BxPC3 and PANC1 invasion assays (**Figure 4B and 4C**). We also found that the expression of the epithelial-mesenchymal transition (EMT) marker E-cadherin was reduced upon overexpression of β-catenin, whereas that of fibronectin and ZEB1 was increased (**Figure 4D**). These findings suggested that FOXO1 inhibits cell proliferation and invasion through Wnt/β-catenin signaling.

MiR-27a targets FOXO1 in PDAC tissues and cell lines

Next, we aimed to explore the mechanisms by which FOXO1 is downregulated in PDAC tissues.

DNA methylation and microRNAs can drive tumor suppressor inhibition in cancer development. We found no CpG islands in the *FOXO1* promoter region (data not shown). Thus, we used TargetScan and miRBase to screen for miRNAs potentially targeting FOXO1. We found that miR-27a, miR-96, and miR-182 were commonly predicted to target FOXO1 by these two databases. We examined the expression of these three miRNAs in the 57 fresh PDAC tissues. As shown in **Figure 5A-C**, miR-27a, but not miR-96 and miR-182, was significantly elevated in all 57 PDAC malignant tissues. Therefore, we analyzed the correlation between miR-27a and FOXO1 in tissues and we found that FOXO1 mRNA levels were negatively correlated with miR-27a levels (**Figure 5D**). To validate FOXO1 as a direct target of miR-27a, fragments from

the FOXO1 3'-UTR containing the putative miR-27a target sites were cloned into a luciferase reporter construct (**Figure 5E**). The results showed that miR-27a overexpression substantially suppressed the activity of the reporter carrying the wild-type 3'-UTR of FOXO1, but not that of the reporter carrying the mutant 3'-UTR (**Figure 5F**). To confirm this, we treated BxPC3 and PANC1 cells with miR-27a inhibitor and then measured FOXO1 expression. As shown in **Figure 5G**, inhibition of miR-27a rescued FOXO1 expression in both cell lines. Furthermore, miR-27a inhibitor significantly inhibited BxPC3 cell proliferation and PANC1 cell invasion (**Figure 5H** and **5I**). Finally, we analyzed the correlation between miR-27a and prognosis, and we found that high miR-27a expression predicted a dismal prognosis in PDAC patients (**Figure 5J**). Taken together, these findings indicated that highly expressed miR-27a inhibits FOXO1 expression in PDAC tissues, thereby promoting PDAC progression.

Discussion

PDAC is a globally prevalent and highly aggressive malignancy. Despite the tremendous efforts put in treating PDAC, clinical outcomes remain poor. Therefore, a better understanding of the underlying molecular mechanisms from multiple perspectives is highly desirable [17, 18]. Tumor initiation causes uncontrolled cell proliferation, and it is widely accepted that genetic and epigenetic alterations contribute to malignancy [18]. We previously found that FOXO1 is downregulated in PDAC tissues, and low FOXO1 expression correlates with the stemness of PDAC cells [7].

In this study, we first confirmed the expression pattern of FOXO1 in fresh PDAC tissues, and we found that FOXO1 is strongly depleted in malignant compared to adjacent tissues due to downregulation of its expression, which plays an important role in PDAC tumorigenesis. Furthermore, we found that downregulated FOXO1 not only promoted PDAC cell proliferation and growth both *in vitro* and *in vivo*, but also increased cell invasion. Aberrant cell cycle progression and invasion contribute to uncontrolled cell proliferation and metastasis [19-21]. Cancer cells employ various mechanisms, such as mutation of tumor suppressors p53 or PTEN or aberrant overexpression of cyclins, to evade cell cycle checkpoints and promote cell proliferation [22, 23]. In this study, we found

that CCND1 expression was inversely correlated with FOXO1 expression, suggesting that the downregulation of FOXO1 might, in part, promote cell proliferation by modulating cyclins.

Previous studies have demonstrated abnormal activation of Wnt/β-catenin signaling in numerous solid tumors, including PDAC [24, 25]. Moreover, CCND1 is a downstream target of Wnt/β-catenin signaling [26]. In this study, we found that Wnt/β-catenin signaling activity was negatively correlated with the FOXO1 expression level. Additionally, the level of nuclear β-catenin, a transcription factor activated by Wnt/β-catenin signaling, decreased when FOXO1 was ectopically expressed. These findings suggested that elevated FOXO1 could in some ways promote β-catenin entry into the nucleus to regulate CCND1 expression and thus promote cell proliferation and growth. Furthermore, we found that downregulation of FOXO1 promoted PDAC cell EMT, and overexpression of β-catenin rescued this effect. Wnt/β-catenin signaling not only plays a central role in cell physiological processes, it can also target various oncogenes, such as *TCF-1* and *MMP7*, all of which can contribute to tumor progression [27].

We found that FOXO1 expression in PDAC tissues was suppressed by highly expressed miR-27a. Moreover, miR-27a predicted poor prognosis of PDAC patients, suggesting that miR-27a targets FOXO1 and to exert aggressive effects.

In conclusion, we confirmed the expression and clinical significance of FOXO1 and explored its mechanism of action in PDAC. We found that highly expressed miR-27a targets FOXO1 to promote PDAC progression. The miR-27a/FOXO1/β-catenin axis might be a potential therapeutic target and might be useful to reduce PDAC progression in future clinical applications.

Disclosure of conflict of interest

None.

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