

MicroRNA-23a acts as an oncogene in pancreatic carcinoma by targeting TFPI-2

WEI WANG^{1*}, JIN-ZHUO NING^{2*}, ZHI-GANG TANG¹, YING HE³, LI-CHAO YAO¹, LIN YE¹ and LUN WU¹

Departments of ¹Pancreatic Surgery and ²Urology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060;

³School and Hospital of Stomatology, Wuhan University, Wuhan, Hubei 430079, P.R. China

Received August 8, 2019; Accepted June 10, 2020

DOI: 10.3892/etm.2020.9181

Abstract. Pancreatic carcinoma (PC) is a rapidly progressive, fatal malignant tumor with the poorest prognosis among all major carcinoma types. MicroRNAs (miRNAs/miRs) have been indicated to be key post-transcriptional regulatory factors, which are involved in cancer development. The present study was designed to investigate the effect of miR-23a on PC cell proliferation, metastasis and apoptosis. The expression of miR-23a was detected in a normal pancreatic ductal epithelial cell line and three PC cell lines, and miR-23a inhibitor or mimics were transfected into the Panc-1 and MiaPaCa2 PC cells. The association between miR-23a and tissue factor pathway inhibitor (TFPI)-2 was examined using a luciferase reporter assay. MTT and flow cytometry assays were used to assess cell viability and apoptosis, respectively. Furthermore, wound-healing, Transwell and Matrigel assays were used to evaluate cell migration and invasion abilities, and the protein expression level of TFPI-2 was determined using western blot analysis. The results of the present study revealed that miR-23a was upregulated in PC cells. Furthermore, TFPI-2 was identified as a downstream target of miR-23a, and TFPI-2 expression was found to be increased following miR-23a knockdown. In addition, functional assays revealed that downregulation of miR-23a decreased PC cell proliferation, migration and invasiveness and promoted cell apoptosis, while miR-23a overexpression exerted the opposite effects. Furthermore, TFPI-2 knockdown rescued the biological effects on PC cells, which were induced by miR-23a knockdown. The results of the present study indicated that miR-23a negatively modulated TFPI-2 expression *in vitro* and enhanced the malignant

phenotypes of PC cells. Therefore, miR-23a may be a potential marker and/or target for the diagnosis and treatment of PC.

Introduction

As pancreatic carcinoma (PC) is a rapidly progressive, fatal malignant tumor, patients with PC have been found to exhibit the poorest prognosis among all major carcinoma types (1). PC is the fourth leading cause of cancer-associated mortality with ~48,960 new cases and ~40,560 deaths estimated in 2015 (2,3) and it has been projected to be the second leading cause of death in the USA by 2030 (4). Considerable progress has been made in the diagnosis and treatment of PC in recent decades; however, no significant improvement in its high mortality rate has been observed, with a 5-year survival rate of only ~8% in patients with PC (5). Poor patient prognosis has been primarily attributed to the advanced stage at which the disease is diagnosed, with the majority of patients developing locally advanced and distant metastases and succumbing to cancer metastasis (6-8). Autopsy studies have revealed that ~90% of PC cases are complicated by distant metastases (9), which have also been associated with ~90% of cancer-related mortality (10). Therefore, the in-depth study of molecular interactions during the tumorigenesis and progression of PC may aid in the identification of therapeutic targets and diagnostic strategies.

MicroRNAs (miRNAs/miRs) are small non-protein coding RNAs that have been associated with tumorigenesis, cell cycle regulation, proliferation, apoptosis, invasiveness, metastasis and chemoresistance in multiple cancers, including PC (11). miRNAs modulate post-transcriptional gene expression by binding to the complementary regions in the 3'-untranslated (3'-UTR) region of their targets, and subsequently inducing degradation or inhibition of the translation of the target RNA (12). In the past decade, various miRNAs have been associated with numerous types of human cancer. For example, miR-373 has been found to enhance the proliferative and metastatic abilities of oral squamous cell carcinoma (13), while miR-146b-5p has been revealed to inhibit the progression of non-small cell lung carcinoma (14) and miR-495 has been reported to suppress the biological activities of gastric cancer cells by targeting Twist family bHLH transcription factor 1 (15). Certain miRNAs, including miR-381, miR-340 and miR-359, have also been associated with the progression

Correspondence to: Professor Zhi-Gang Tang, Department of Pancreatic Surgery, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan, Hubei 430060, P.R. China
E-mail: wdrn2017@163.com

*Contributed equally

Key words: pancreatic carcinoma, microRNA-23a, tissue factor pathway inhibitor 2, apoptosis, metastasis

of PC (16-18). Several studies have confirmed that the aberrant expression of miR-23a in numerous human malignancies. miR-23a expression has been previously found to be decreased in acute erythroid leukemia (19), whilst it was revealed increased in prostate cancer (20) and osteosarcoma (21). Furthermore, miRNA microarray analysis revealed that miR-23a expression was increased in PC compared with that in normal pancreatic tissues of patients (22). Additional studies have revealed that miR-23a modulated the biological functions of PC via targeting specific genes. For example, miR-23a has been found to promote the development of a tumor by targeting forkhead box P2 (FOXP2) gene in ductal adenocarcinoma (23). Furthermore, miR-23a has been revealed to promote PC cell proliferation by directly targeting apoptotic peptidase-activating factor 1 (APAF1) (24) and enhance the metastatic ability of PC cells by targeting epithelial splicing regulatory protein 1 (12).

Tissue factor pathway inhibitor (TFPI)-2, which is also known as placental protein, has been identified as a key molecule in angiogenesis, intravascular fibrinolysis, plasmin transfer, tumor invasion and trypsin-induced activation of matrix metalloproteinase zymogens (25). Previous studies have revealed that downregulated TFPI-2 and upregulated matrix metalloproteinase-2 protein expression was associated with angiogenesis, lymph node metastasis, perineural invasion and early postoperative recurrence of patients with PC (26,27). In the current study, a critical function of miR-23a in PC progression was identified, namely that miR-23a promoted PC progression by negatively targeting TFPI-2. In summary and to the best of our knowledge, this is the first study to report this function of miR-23a, which may provide novel strategies for the diagnosis and treatment of PC.

Materials and methods

Cell lines and culture. The hTERT-HPNE normal pancreatic ductal epithelial cell line and Panc-1, MiaPaCa2 and Aspc-1 PC cell lines were obtained from American Type Culture Collection. All cell lines were cultured in DMEM (PAN-Biotech GmbH) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with 5% CO₂.

Transfection. miR-23a mimics, negative control (NC) mimics, miR-23a inhibitor, NC inhibitor, small interfering RNA against TFPI-2 (si-TFPI-2) and the corresponding NC (TFPI-2 NC) were purchased from Shanghai GenePharma Co., Ltd. Panc-1 and MiaPaCa2 cells were transfected with the appropriate constructs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The transfection concentrations of the miR-23a mimics and inhibitor were 50 and 100 nmol/l, respectively, and the concentrations of si-TFPI-2 and TFPI-2 NC were 50 nmol/l. At 48 h post-transfection, cells were collected for subsequent experiments. The sequences were as follows: miR-23a mimics, 5'-AUCACAUUGCCAGGGAUUAUCC-3'; miR-23a inhibitor, 5'-GGAAAUCCUGGCAAUGUGAU-3'; mimics NC, 5'-CGUAAAGCAAUCAUGCCCUU-3'; inhibitor NC,

5'-AAUCUGAUACAUAUUGAGACC-3'; si-TFPI-2, 5'-GCCAAUGUGACUCGCUAUUAT-3' and TFPI-2 NC, 5'-AAUCCUACUGACUUAUCGCGU-3'.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from Panc-1 and MiaPaCa2 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA purity was determined using a DU 800 UV/Visible Spectrophotometer (Beckman Coulter, Inc.), and RNA was reverse transcribed into cDNA using ReverTra Ace-α® kit (Toyobo Life Science) according to manufacturer's instructions, using the following temperature protocol: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. qPCR was performed using the SYBR Green™ Real-Time PCR Master Mix (Toyobo Life Science) on an ABI 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific Inc.). U6 and GAPDH were used to normalize the relative expression of miR-23a and TFPI-2 mRNA, respectively. qPCR was conducted in a thermocycler using the following conditions: 94°C for 4 min, followed by 40 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 25 sec. The 2^{-ΔΔCq} method (28) was used to calculate the relative expression levels of miRNA or mRNA. The associated primers are presented in Table I.

Cell viability assay. MTT assay was used to determine the viability of Panc-1 cells following miR-23a knockdown and MiaPaCa2 cells following miR-23a overexpression. The cells were seeded into 96-well plates at a density of 5x10³ cells/well. Panc-1 and MiaPaCa2 cells were subsequently transfected with miR-23a inhibitor, NC inhibitor, miR-23a mimics or NC mimics using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Panc-1 and MiaPaCa2 cells were treated with or without Lipofectamine to observe the effects of Lipofectamine on cell viability. Panc-1 cells were treated with Lipofectamine and NC inhibitor in the NC group. MiaPaCa2 cells were treated with Lipofectamine and NC mimics in the NC group. MTT assays were conducted every 24 h for 3 days post-transfection, the purple formazan was dissolved by adding DMSO and the absorbance was determined at 570 nm using a microplate reader (Synergy™ H4 Hybrid; BioTek Instruments, Inc.).

Apoptosis assay. Panc-1 and MiaPaCa2 cells (1x10⁶ cells/ml) were seeded into a 6-well plate and transfected as aforementioned. The cells were washed with PBS at 24 h post-transfection and subsequently stained with Annexin V-FITC and propidium iodide (both Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. Apoptosis was evaluated using a flow cytometer BD FACSCalibur™ (BD Biosciences) and BD FACS™ software (v1.0.0.650; BD Biosciences).

Migration and invasion assays. Transwell plates (pore size, 8.0 μm) were used to assess the invasive and migratory abilities of PC cells. For the invasion assay, the membranes were pre-coated with Matrigel at 37°C for 4 h. Transwell plates and Matrigel were obtained from Corning Life Sciences. At 48 h post-transfection with miR-23a inhibitor, mimics or the corresponding NC, Panc-1 and MiaPaCa2 cells (3x10⁴ cells/well)

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Primer name	Primer sequences (5'-3')
TFPI-2, F	GAATTCTATGGACCCCGCTCGCCCC
TFPI-2, R	AGTCGACTTAAAATTGCTTCTTCCG
microRNA-23a, F	CAGGCGGGTAGTAGATG
microRNA-23a, R	AGGGACGGGCATGGAAAGG
U6 small nuclear RNA, F	TCGTCTATCGCAGCACATAGTCG
U6 small nuclear RNA, R	GCGATTACGAATTTGCCCCAG
GAPDH, F	CGACCAGCCGACGGGTGCAG
GAPDH, R	AGCTCGCCTACACCGAACGT

TFPI-2, tissue factor pathway inhibitor 2; F, forward; R, reverse.

were seeded into the upper chamber with serum-free DMEM medium in a 24-well plate, respectively, while the lower chamber was supplemented with medium containing 10% FBS. The plates were incubated for 24 (migration assay) and 48 h (invasion assay) at 37°C. The cells on the upper membrane were removed, and the invading cells on the lower membrane were fixed with 4% formaldehyde for 30 min at room temperature and stained with 0.2% crystal violet for 20 min, at room temperature. The cells in five random fields were counted using an inverted light microscope (magnification, x200; Olympus Corporation).

Wound healing assay. Panc-1 and MiaPaCa2 cells (2x10⁶ cells/well) were cultured in a six-well plate until ~100% confluence was achieved. The cell monolayers were scratched using a 200- μ l sterile pipette tip and then washed with PBS. The cells were subsequently cultured in serum-free DMEM and images were captured at 0 and 24 h time points, using an inverted light microscope (magnification, x200).

Target prediction. To investigate the association between miR-23a and TFPI-2, TargetScan (version 7.2; http://www.targetscan.org/vert_72/) and miRanda (august2010; <https://microrna.org>) online databases were used to predict the binding sites of miR-23a within the 3'-UTR of TFPI-2, according to the manufacturer's protocol.

Plasmid construction. TFPI-2 wild-type (TFPI-2-WT) or mutant (TFPI-2-MUT) reporter plasmids were constructed by synthesizing the putative miR-23a-WT target binding sequences within TFPI-2 and cloning these into a luciferase reporter plasmid. The overlap extension PCR method (29) was performed to obtain the TFPI-2-MUT 3'-UTR sequence. Subsequently, MUT and WT target sequences were inserted into the psiCHECK-2 vector, and Sanger sequencing was performed by Sangon Biotech Co., Ltd. to confirm the sequences. All vectors were purchased from Promega Corporation.

Dual-luciferase reporter assays. Panc-1 cells were seeded into a 24-well plate (1x10⁵ cells/well) and then co-transfected with 100 nM TFPI-2-WT or TFPI-2-MUT plasmids, with 50 nM miR-23a or NC mimics using Lipofectamine 2000

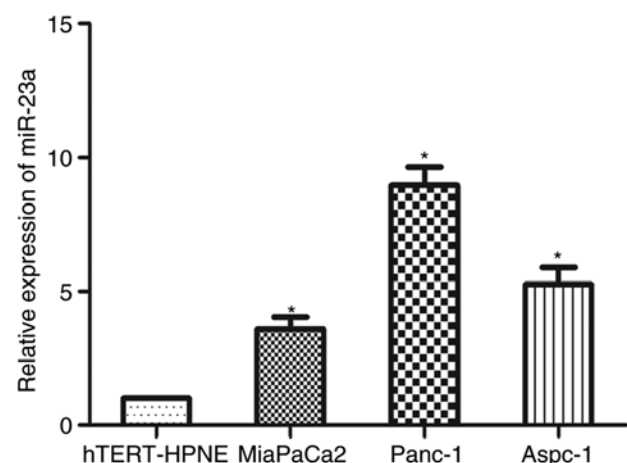


Figure 1. mRNA expression level of miR-23a in pancreatic carcinoma cell lines. miR-23a expression was evaluated using reverse transcription-quantitative PCR. *P<0.05 vs. expression in hTERT-HPNE cells. miR, microRNA.

(Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Cells were harvested after 48 h, and Dual-Luciferase[®] Reporter Assay system (Promega Corporation) was used to compare firefly luciferase and *Renilla* luciferase activities.

Western blot analysis. Total protein extraction from Panc-1 and MiaPaCa2 cells was performed using RIPA Buffer (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The protein concentrations were quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). SDS-PAGE (10%) was used to separate equal amounts of protein (30 μ g/sample), which were subsequently transferred to a PVDF membrane. The membranes were blocked with 5% skimmed milk for 60 min at room temperature, followed by incubation with anti-TFPI-2 antibody (dilution, 1:1,000; cat. no. ab86933; Abcam) or GAPDH (dilution, 1:1,000; cat. no. 5174S; CST Biological Reagents Co., Ltd.), which was used as the internal control, overnight at 4°C. The membranes were subsequently washed with TBS-Tween-20 buffer and incubated with the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (dilution, 1:5,000; cat. no. LK2001; Sungene Biotech Co., Ltd.)

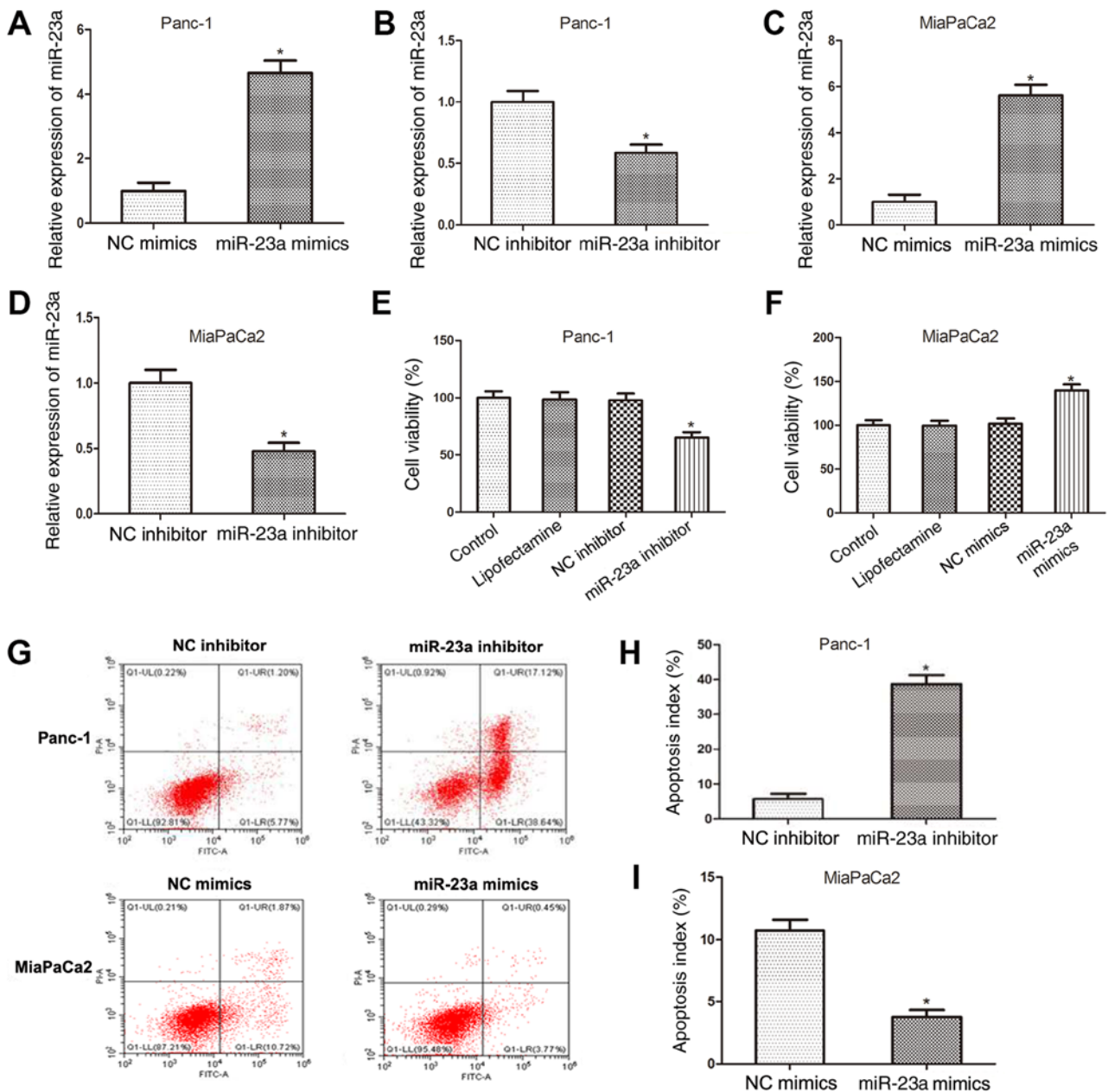


Figure 2. miR-23a promotes proliferation and suppresses apoptosis in pancreatic carcinoma cells. The transfection efficiency of (A) miR-23a mimics or (B) inhibitor in Panc-1 cells and (C) miR-23a mimics or (D) inhibitor in MiaPaCa2 cells was determined using reverse transcription-quantitative PCR. Cell viability was assessed using MTT assays following transfection of miR-23a inhibitor or mimics into (E) Panc-1 and (F) MiaPaCa2 cells, respectively. (G) Flow cytometry was performed to assess apoptosis following transfection of miR-23a inhibitor or mimics in Panc-1 and MiaPaCa2 cells, respectively and the results were quantified in (H) Panc-1 and (I) MiaPaCa2 cells. * $P < 0.05$ vs. the NC group. miR, microRNA; NC, negative control.

at 20°C for 60 min. The protein bands were visualized using an ECL kit [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] and ImageJ software (v1.8.0; National Institutes of Health) was used for densitometry analysis.

Statistical analysis. The data are presented as the mean \pm standard deviation and all experiments were performed three times in triplicate. SPSS v25.0 software (IBM Corp.) was used to analyze the experimental data. Statistical differences were analyzed using unpaired Student's t-test (for two groups) or one-way ANOVA followed by Tukey's or Dunnett's post hoc test (for ≥ 3 groups). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-23a expression is higher in PC cells compared with that in normal pancreatic cells. To determine whether miR-23a was associated with the development of PC, RT-qPCR was performed to quantify the mRNA expression levels of miR-23a in three PC cell lines (Panc-1, MiaPaCa2 and Aspc-1) and a normal pancreatic ductal epithelial cell line (hTERT-HPNE). Compared with that in the control, the results indicated that miR-23a expression level was significantly increased in the PC cell lines (Fig. 1). In the three PC cell lines, the expression level of miR-23a in Panc-1 was relatively high, whilst that in MiaPaCa2 was relatively low.

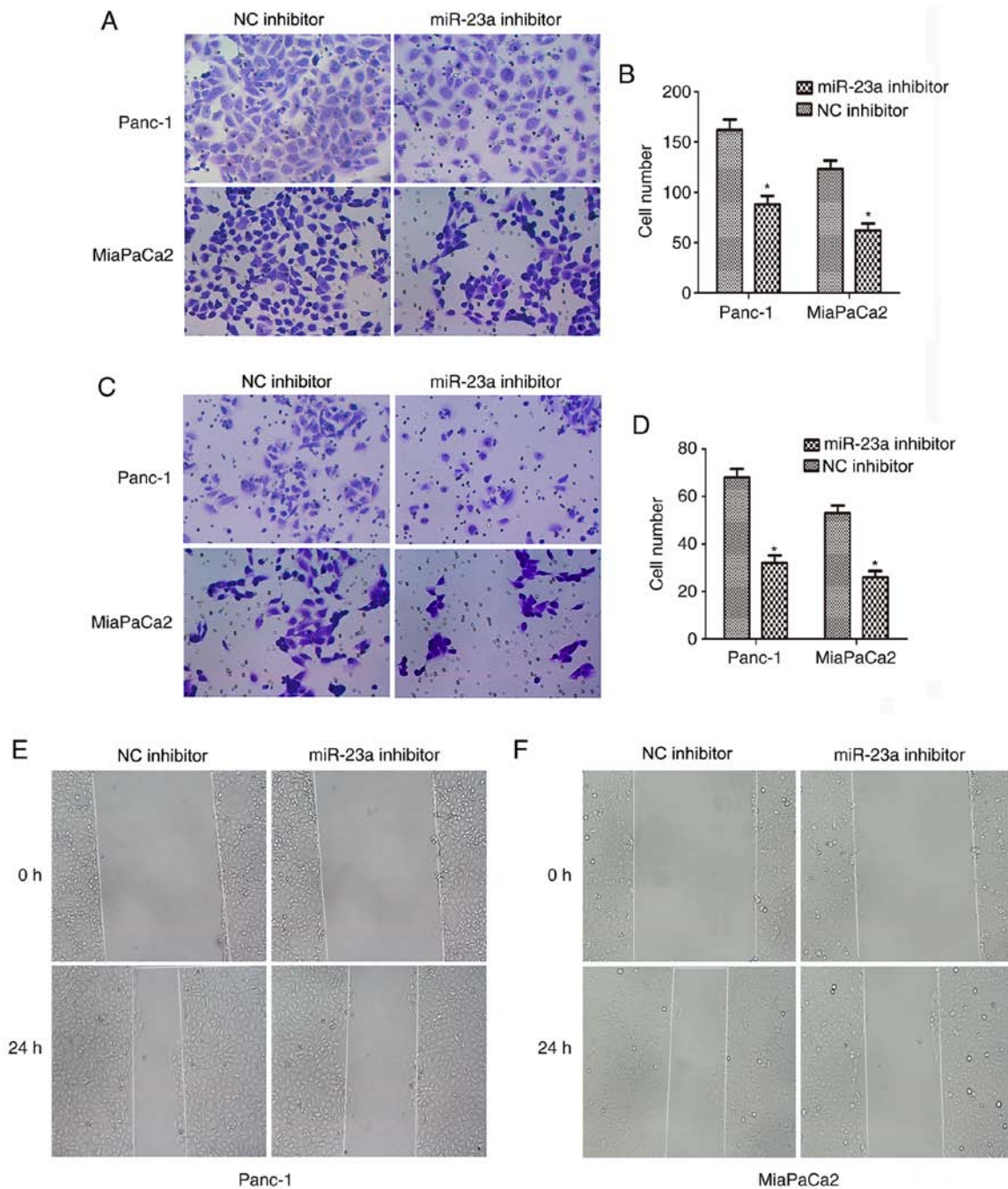


Figure 3. miR-23a induces PC cell migration and invasion. (A) Representative images from the Transwell assay showing migratory cells on the lower membrane, following transfection and (B) the quantitative analysis in each experimental group, indicating that miR-23a inhibitor suppressed the migration of PC cells. (C) Representative images from the Matrigel assay, following transfection showing invasive cells and (D) the quantitative analysis in each experimental group, indicating that miR-23a inhibitor suppressed the invasiveness of PC cells. Representative images from the wound-healing assay in (E) Panc-1 and (F) MiaPaCa2 cells, following transfection. *P<0.05 vs. NC. miR, microRNA; PC, pancreatic carcinoma; NC, negative control.

Panc-1 and MiaPaCa2 cells were therefore subsequently selected for further experimentation.

miR-23a promotes PC cell viability and inhibits apoptosis. To delineate the functions of miR-23a in PC progression, miR-23a mimics and inhibitor (and the respective NCs) were used to determine the biological effects of miR-23a in Panc-1 and MiaPaCa2 cells. RT-qPCR was performed to assess the transfection efficiency of miR-23a mimics and inhibitor and the results revealed that miR-23a expression was increased

in Panc-1 cells following transfection with miR-23a mimics (Fig. 2A), while it was downregulated following transfection with miR-23a inhibitor (Fig. 2B). Similar results were obtained in MiaPaCa2 cells transfected with miR-23a mimics or inhibitor (Fig. 2C and D, respectively). Subsequently, the viability of Panc-1 cells following miR-23a knockdown and MiaPaCa2 cells following miR-23a overexpression were determined using MTT assay. As presented in Fig. 2E and F, miR-23a inhibition significantly decreased Panc-1 cell viability compared with that in the respective NC, whilst

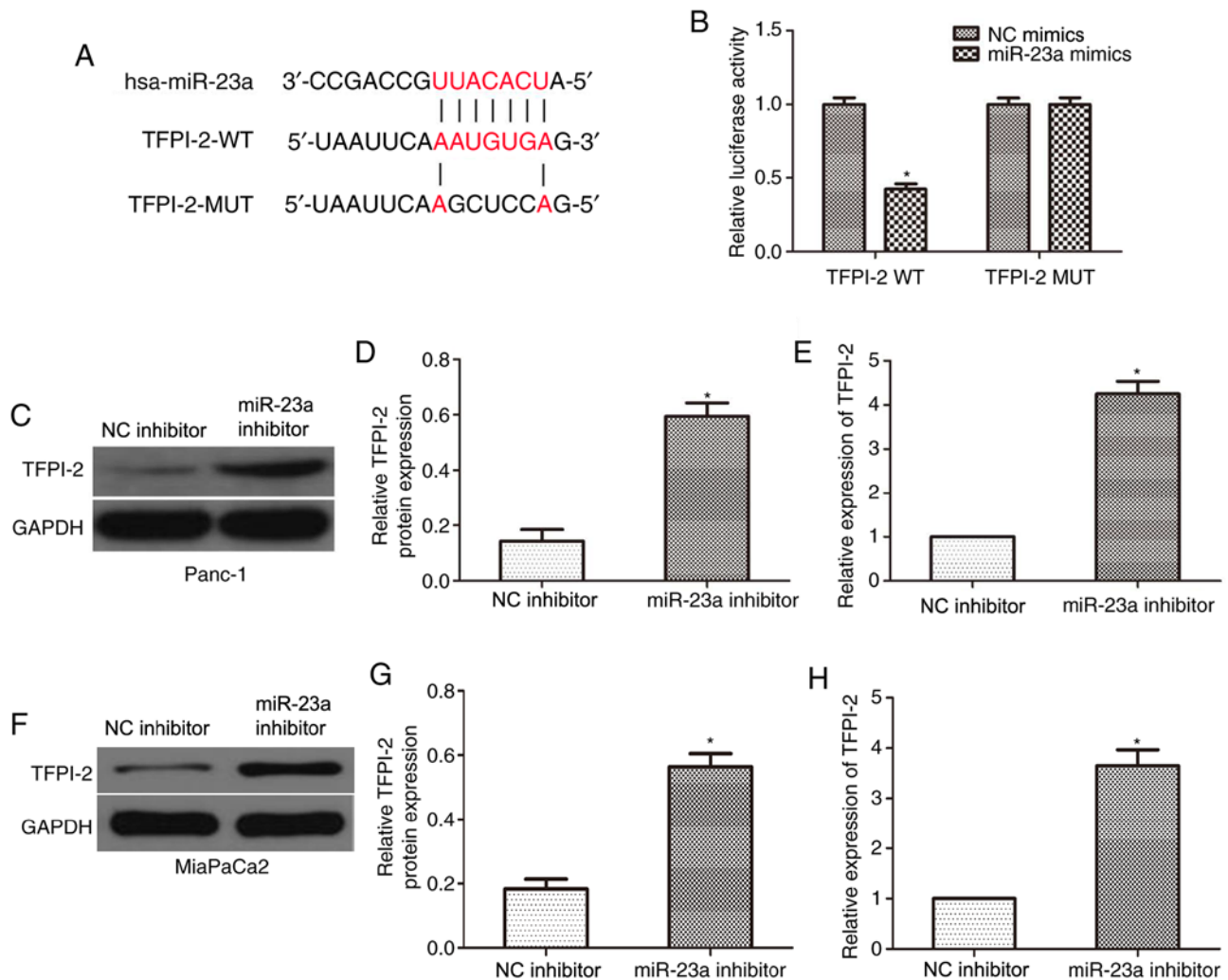


Figure 4. miR-23a directly targets and negatively modulates the expression of TFPI-2. (A) Prediction of the binding sequence between miR-23a and TFPI-2 and illustration of the mutated sequence of TFPI-2 that was used for the luciferase reporter assays. (B) Panc-1 cells co-transfected with miR-23a or NC mimics and reporter vectors containing TFPI-2-WT 3'-UTR or TFPI-2-MUT 3'-UTR were analyzed using luciferase reporter assays. (C) Representative western blots, (D) relative protein expression and (E) RT-qPCR analyses of TFPI-2 expression in Panc-1 cells following transfection with miR-23a inhibitor. (F) Representative western blots, (G) relative protein expression and (H) RT-qPCR analyses of TFPI-2 expression in MiaPaCa2 cells following transfection with miR-23a inhibitor. * $P < 0.05$ vs. NC. TFPI-2, tissue factor pathway inhibitor 2; WT, wild-type; MUT, mutant; miR, microRNA; NC, negative control; UTR, untranslated region; RT-qPCR, reverse transcription-quantitative PCR.

MiaPaCa2 viability was significantly increased by miR-23a overexpression. In addition, the flow cytometry data revealed that compared with that in the respective NC, the apoptotic rate of Panc-1 cells was significantly increased following transfection with miR-23a inhibitor, while that of MiaPaCa2 cells was significantly decreased following transfection with miR-23a mimics (Fig. 2G-I). These findings indicated that miR-23a promoted PC cell viability and inhibited apoptosis.

miR-23a inhibition suppresses PC cell migration and invasion. To further investigate the functions of miR-23a in PC metastasis, the migratory and invasive abilities of Panc-1 and MiaPaCa2 cells were evaluated using Transwell assays or without Matrigel pre-coating, respectively. The results indicated that miR-23a inhibition significantly suppressed the migration of Panc-1 and MiaPaCa2 cells (Fig. 3A and B). Furthermore, the invasive ability of Panc-1 and MiaPaCa2 cells was also inhibited following transfection with miR-23a inhibitor, compared with that

in cells transfected with NC (Fig. 3C and D). In addition, a wound-healing assay also confirmed the inhibitory effect of miR-23a inhibitor on the migration of Panc-1 and MiaPaCa2 cells (Fig. 3E and F). Taken together, these results indicated that miR-23a inhibition suppressed the migratory and invasive abilities of PC cells.

miR-23a directly targets TFPI-2 and negatively modulates the expression of TFPI-2. To determine the downstream targets of miR-23a, which may serve crucial roles in the development of PC, the TargetScan online tool predicted TFPI-2 to be the target gene of miR-23a (Fig. 4A). To confirm this, TFPI-2-WT and TFPI-2-MUT 3'-UTR plasmids were constructed and co-transfected with miR-23a mimics or NC into Panc-1 cells, and the reporter activities were subsequently evaluated using a dual-luciferase reporter assay. As presented in Fig. 4B, miR-23a mimics significantly inhibited the luciferase activity of TFPI-2-WT 3'-UTR in Panc-1 cells compared with that in the NC group; however, no significant difference was observed

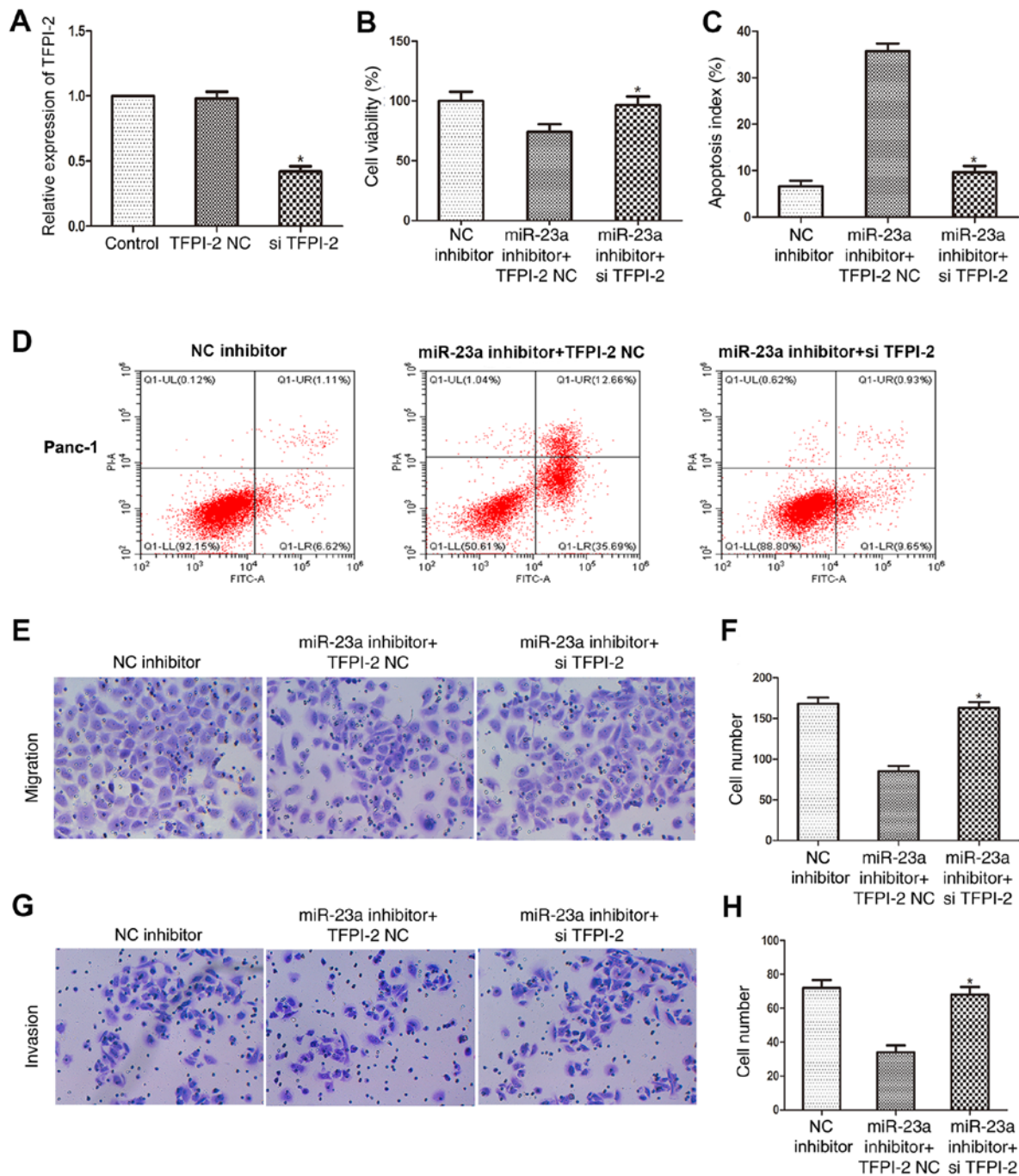


Figure 5. TFPI-2 knockdown restores the alterations in Panc-1 cell apoptosis, proliferation, migration and invasion induced by miR-23a inhibition. (A) The transfection efficiency of si-TFPI-2 in Panc-1 cells was determined using reverse transcription-quantitative PCR. (B) MTT assay results indicated that TFPI-2 knockdown increased Panc-1 cell proliferation following transfection with miR-23a inhibitor. (C) Quantification of (D) flow cytometry results revealed that TFPI-2 knockdown reduced apoptosis in Panc-1 cells transfected with miR-23a inhibitor. (E) Migration assays and (F) quantification of the migrated cells suggested that TFPI-2 knockdown promoted the migration of Panc-1 cells transfected with miR-23a inhibitor. (G) Invasion assays and (H) quantification of the invaded cells indicated that TFPI-2 knockdown enhanced the invasiveness of Panc-1 cells transfected with miR-23a inhibitor. * $P < 0.05$ vs. miR-23a inhibitor + TFPI-2 NC. TFPI-2, tissue factor pathway inhibitor 2; miR, microRNA; NC, negative control; si, small interfering RNA.

in the TFPI-2-MUT group. Western blot and RT-qPCR analyses were also performed and revealed that miR-23a inhibition significantly increased the TFPI-2 protein (Fig. 4C and D) and mRNA (Fig. 4E) expression levels in Panc-1 cells, compared with that in the control groups, and similar results were observed in the MiaPaCa2 cell line (Fig. 4F-H). The present results indicated that miR-23a directly targeted TFPI-2 and negatively modulated its expression.

TFPI-2 knockdown rescues the effects on PC cell apoptosis, viability, migration and invasion, which are induced by miR-23a downregulation. Finally, rescue experiments were performed to determine whether miR-23a affects the apoptosis, proliferation, migratory and invasive abilities of PC cells by regulating the expression level of TFPI-2. RT-qPCR was used to determine the transfection efficiency of si-TFPI-2 and the results revealed that TFPI-2 mRNA expression level was

significantly downregulated in Panc-1 cells following si-TFPI-2 transfection (Fig. 5A). An MTT assay indicated that si-TFPI-2 restored the viability of Panc-1 cells transfected with miR-23a inhibitor (Fig. 5B). In addition, flow cytometry analysis found that si-TFPI-2 attenuated the increase in Panc-1 cell apoptosis following transfection with miR-23a inhibitor (Fig. 5C and D). Furthermore, the migratory (Fig. 5E and F) and invasive (Fig. 5G and H) abilities of Panc-1 cells transfected with miR-23a inhibitor were restored by TFPI-2 knockdown, as determined using Transwell and Matrigel assays. These data provided additional evidence that miR-23a inhibited apoptosis and promoted the proliferation, migration and invasiveness of PC cells by targeting TFPI-2.

Discussion

Previous studies on different types of cancer, including non-small cell lung carcinoma, liver cancer and glioma, have revealed that miRNAs may regulate the migratory and invasive ability of cells by targeting specific genes (30-32). miR-499a has been shown to target ADAM metalloproteinase domain 10 in non-small cell lung carcinoma, whilst miR-99a has been previously demonstrated to target homeobox A1 in liver cancer and miR-374b targets epidermal growth factor receptor in glioma (30-32). miR-23a has been reported to be a critical regulator of tumorigenesis and serve distinct roles in different types of human cancer. For example, several studies have reported that miR-23a promoted the progression of a variety of cancers, including gastric cancer, colorectal and liver carcinoma (33-35). By contrast, miR-23a was found to serve as a cancer suppressor in melanoma and osteosarcoma, as the overexpression of miR-23a inhibited osteosarcoma cell proliferation, migration and invasive ability (36), and suppressed melanoma cell proliferation, migration and invasion in mice (37). The results of the current study revealed that miR-23a mRNA expression level was upregulated in PC cell lines (MiaPaCa2, Panc-1 and Aspc-1) compared with that in a normal pancreatic ductal cell line (hTERT-HPNE), suggesting that miR-23a may be associated with the tumorigenesis and progression of PC. The results of the MTT assay also indicated that miR-23a knockdown inhibited PC cell proliferation. Furthermore, wound-healing, Transwell and Matrigel assays revealed that miR-23a knockdown decreased PC cell migration and invasion, while flow cytometry demonstrated the pro-apoptotic effects of miR-23a knockdown. These data support those of a previous study (24), indicating that miR-23a acts as a tumor-promoting factor in PC cells, and may be utilized as a biomarker for PC diagnosis and treatment.

Extracellular matrix (ECM) proteolysis has been revealed to serve a key role in tumor metastasis (38,39), therefore protecting the structural integrity of the ECM may be an important aspect to inhibit cancer cell metastasis. TFPI-2 has been considered as an effective cancer suppressor, which maintains ECM structural integrity in various types of cancer, including breast cancer, amelanotic melanoma and lung cancer (40-42). A previous study revealed that TFPI-2 protein expression was decreased in PC compared with that in normal pancreatic tissues, and that overexpression of TFPI-2 prevented a PC malignant phenotype both *in vitro*

and *in vivo* (25). Several studies have reported that TFPI-2 is regulated by miRNAs. For example, TFPI-2 expression was found to be negatively regulated by miR-616, thereby inducing the androgen-dependent proliferation of PC cells (43). Furthermore, miR-494 has been demonstrated to indirectly upregulate TFPI-2 by modulating the transcription factors, aryl hydrocarbon receptor and ETS-related transcription factor Elf-1 in breast cancer cells (44). However, in-depth studies on TFPI-2 have been rarely reported in PC, and the upstream and downstream regulators of this gene remain largely unknown, to the best of our knowledge. miR-23a has been reported to affect the progression of PC by regulating specific genes. For example, miR-23a was found to inhibit PC development by negatively regulating serine/threonine-protein kinase PLK1 (45), and miR-23a overexpression was found to promote PC progression by suppressing FOXP2 mRNA expression levels (23). miR-23a has also been reported to act as an oncogene in PC via downregulating APAF1 (24).

Numerous studies have demonstrated the important role of miR-23a in PC tumorigenesis; however, other potential molecular mechanisms such as the role of miR-23a in ECM proteolysis and the detailed function of miR-23a, remain unclear. In the present study, bioinformatics analysis was used to predict the interaction between miR-23a and TFPI-2, and this interaction was confirmed using a luciferase reporter assay. Upregulation of TFPI-2 expression was also evaluated in PC cells following miR-23a knockdown. The results indicated that miR-23a knockdown increased the protein and mRNA expression level of TFPI-2, and that miR-23a may be involved in PC progression by negatively modulating TFPI-2 expression. Furthermore, rescue experiments demonstrated the interdependent relationship between miR-23a and TFPI-2 in PC cells. These results confirmed that downregulating TFPI-2 rescued the effects on PC cell apoptosis, proliferation, migration and invasion, which were induced by the inhibition of miR-23a.

There are also limitations in the present study. For example, the endogenous mRNA expression levels of miR-23a in human or animal tissues were not examined. In addition, functional studies investigating the inhibition of miR-23a indicated the role of miR-23a in inducing cell migration and invasion; however, functional studies using miR-23a mimics are missing.

In summary, the results of the present study indicated that by directly targeting TFPI-2, miR-23a exerted an oncogenic effect in PC cells, promoting cell proliferation, migration and invasiveness and suppressing apoptosis. The aforementioned results suggested that miR-23a may potentially be utilized as a useful target and/or biomarker for the diagnosis and treatment of PC.

Acknowledgements

The authors would like to thank Dr Hongmin Sun (School of Public Health, Anhui Medical University; Hefei, China) for his assistance in statistical analysis.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WW and JZN were responsible for conception and design of the study, data collection and analysis and contributed to writing the manuscript. ZGT designed the study, performed critical revision and supervised all phases of the study. YH and LCY performed the invasion and migration assays and analyzed the data. LY and LW performed the cell viability and apoptosis assays and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Klein AP: Identifying people at a high risk of developing pancreatic cancer. *Nat Rev Cancer* 13: 66-74, 2013.
- Kamisawa T, Wood LD, Itoi T and Takaori K: Pancreatic cancer. *Lancet* 388: 73-85, 2016.
- Moutinho-Ribeiro P, Coelho R, Giovannini M and Macedo G: Pancreatic cancer screening: Still a delusion? *Pancreatol* 17: 754-765, 2017.
- Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM and Matrisian LM: Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 74: 2913-2921, 2014.
- Siegel RL, Miller KD and Jemal A: Cancer statistics, 2017. *CA Cancer J Clin* 67: 7-30, 2017.
- Talmadge JE and Fidler IJ: AACR centennial series: The biology of cancer metastasis: Historical perspective. *Cancer Res* 70: 5649-5669, 2010.
- Pang EJ, Yang R, Fu XB and Liu YF: Overexpression of long non-coding RNA MALAT1 is correlated with clinical progression and unfavorable prognosis in pancreatic cancer. *Tumor Biol* 36: 2403-2407, 2015.
- Li Y, Vandenboom TG II, Wang Z, Kong D, Ali S, Philip PA and Sarkar FH: miR-146a suppresses invasion of pancreatic cancer cells. *Cancer Res* 70: 1486-1495, 2010.
- Kamisawa T, Isawa T, Koike M, Tsuruta K and Okamoto A: Hematogenous metastases of pancreatic ductal carcinoma. *Pancreas* 11: 345-349, 1995.
- Chaffer CL and Weinberg RA: A perspective on cancer cell metastasis. *Science* 331: 1559-1564, 2011.
- Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297, 2004.
- Wu G, Li ZH, Jiang P, Zhang X, Xu YQ, Chen K and Li XW: MicroRNA-23a promotes pancreatic cancer metastasis by targeting epithelial splicing regulator protein 1. *Oncotarget* 8: 82854-82871, 2017.
- Zhang XJ, Jin Y, Song JL and Deng F: MiR-373 promotes proliferation and metastasis of oral squamous cell carcinoma by targeting SPOP. *Eur Rev Med Pharmacol Sci* 23: 5270-5276, 2019.
- Li Y, Zhang H, Dong Y, Fan Y, Li Y, Zhao C, Wang C, Liu J, Li X, Dong M, *et al*: MiR-146b-5p functions as a suppressor miRNA and prognosis predictor in non-small cell lung cancer. *J Cancer* 8: 1704-1716, 2017.
- Liu C, Jian M, Qi H and Mao WZ: MicroRNA-495 inhibits proliferation and metastasis and promotes apoptosis by targeting twist1 in gastric cancer cells. *Oncol Res* 27: 389-397, 2019.
- Qiao G, Li J, Wang J, Wang Z and Bian W: miR-381 functions as a tumor suppressor by targeting ETS1 in pancreatic cancer. *Int J Mol Med* 44: 593-607, 2019.
- Zhou Y, Li Z, Ding Y, Zhang P and Wang J: MicroRNA-340 suppresses pancreatic cancer growth by targeting BICD2. *Pancreatol* 19: 30556-30563, 2019.
- Yu H, Gao G, Cai J, Song H, Ma Z, Jin X, Ji W and Pan B: MiR-539 functions as a tumor suppressor in pancreatic cancer by targeting TWIST1. *Exp Mol Pathol* 108: 143-149, 2019.
- Su R, Dong L, Zou D, Zhao H, Ren Y, Li F, Yi P, Li L, Zhu Y, Ma Y, *et al*: MicroRNA-23a, -27a and -24 synergistically regulate JAK1/Stat3 cascade and serve as novel therapeutic targets in human acute erythroid leukemia. *Oncogene* 35: 6001-6014, 2016.
- Cai S, Chen R, Li X, Cai Y, Ye Z, Li S, Li J, Huang H, Peng S, Wang J, *et al*: Downregulation of microRNA-23a suppresses prostate cancer metastasis by targeting the PAK6-LIMK1 signaling pathway. *Oncotarget* 6: 3904-3917, 2015.
- Wang G, Li B, Fu Y, He M, Wang J, Shen P and Bai L: miR-23a suppresses proliferation of osteosarcoma cells by targeting SATB1. *Tumour Biol* 36: 4715-4721, 2015.
- Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C and Croce CM: MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* 297: 1901-1908, 2007.
- Diao H, Ye Z and Qin R: miR-23a acts as an oncogene in pancreatic carcinoma by targeting FOXF2. *J Invest Med* 66: 676-683, 2018.
- Liu N, Sun YY, Zhang XW, Chen S, Wang Y, Zhang ZX, Song SW, Qiu GB and Fu WN: Oncogenic miR-23a in pancreatic ductal adenocarcinogenesis via inhibiting APAF1. *Dig Dis Sci* 60: 2000-2008, 2015.
- Tang Z, Geng G, Huang Q, Xu G, Hu H, Chen J and Li J: Expression of tissue factor pathway inhibitor 2 in human pancreatic carcinoma and its effect on tumor growth, invasion, and migration in vitro and in vivo. *J Surg Res* 167: 62-69, 2011.
- Zhai LL, Wu Y, Huang DW and Tang ZG: Increased matrix metalloproteinase-2 and reduced tissue factor pathway inhibitor-2 expression correlate with angiogenesis and early postoperative recurrence of pancreatic carcinoma. *Am J Transl Res* 7: 2412-2422, 2015.
- Zhai LL, Wu Y, Cai CY and Tang ZG: Upregulated matrix metalloproteinase-2 and downregulated tissue factor pathway inhibitor-2 are risk factors for lymph node metastasis and perineural invasion in pancreatic carcinoma. *Oncotargets Ther* 8: 2827-2834, 2015.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2012.
- Hussain H and Chong NF: Combined overlap extension PCR method for improved site directed mutagenesis. *Biomed Res Int* 2016: 8041532, 2016.
- Meng H, Huang Q, Zhang X, Huang J, Shen R and Zhang B: MiR-449a regulates the cell migration and invasion of human non-small cell lung carcinoma by targeting ADAM10. *Oncotargets Ther* 12: 3829-3838, 2019.
- Tao C, Sun H, Sang W and Li S: miRNA-99a inhibits cell invasion and migration in liver cancer by directly targeting HOXA1. *Oncol Lett* 17: 5108-5114, 2019.
- Pan DS, Cao P, Li JJ, Fan D and Song ZQ: MicroRNA-374b inhibits migration and invasion of glioma cells by targeting EGFR. *Eur Rev Med Pharmacol Sci* 23: 4254-4263, 2019.
- Zhu LH, Liu T, Tang H, Tian RQ, Su C, Liu M and Li X: MicroRNA-23a promotes the growth of gastric adenocarcinoma cell line MGC803 and downregulates interleukin-6 receptor. *FEBS J* 277: 3726-3734, 2010.
- Wang Z, Wei W and Sarkar FH: miR-23a, a critical regulator of 'mig'ration and metastasis in colorectal cancer. *Cancer Discov* 2: 489-491, 2012.
- Huang S, He X, Ding J, Liang L, Zhao Y, Zhang Z, Yao X, Pan Z, Zhang P, Li J, *et al*: Upregulation of miR-23a approximately 27a approximately 24 decreases transforming growth factor-beta-induced tumor-suppressive activities in human hepatocellular carcinoma cells. *Int J Cancer* 123: 972-978, 2008.

36. He Y, Meng C, Shao Z, Wang H and Yang S: MiR-23a functions as a tumor suppressor in osteosarcoma. *Cell Physiol Biochem* 34: 1485-1496, 2014.
37. Lu S and Xu Q: MicroRNA-23a inhibits melanoma cell proliferation, migration, and invasion in mice through a negative feedback regulation of SDCBP and the MAPK/ERK signaling pathway. *IUBMB Life* 71: 587-600, 2019.
38. Eble JA and Niland S: The extracellular matrix in tumor progression and metastasis. *Clin Exp Metastasis* 36: 171-198, 2019.
39. Chang TT, Thakar D and Weaver VM: Force-dependent breaching of the basement membrane. *Matrix Biol* 57-58: 178-189, 2017.
40. Wang G, Zeng Y, Chen S, Li D, Li W, Zhou Y, Singer RH and Gu W: Localization of TFPI-2 in the nucleus modulates MMP-2 gene expression in breast cancer cells. *Sci Rep* 7: 13575, 2017.
41. Konduri SD, Tasiou A, Chandrasekar N, Nicolson GL and Rao JS: Role of tissue factor pathway inhibitor-2 (TFPI-2) in amelanotic melanoma (C-32) invasion. *Clin Exp Metastasis* 18: 303-308, 2000.
42. Iochmann S, Bléchet C, Chabot V, Saulnier A, Amini A, Gaud G, Gruel Y and Reverdiau P: Transient RNA silencing of tissue factor pathway inhibitor-2 modulates lung cancer cell invasion. *Clin Exp Metastasis* 26: 457-467, 2009.
43. Ma S, Chan YP, Kwan PS, Lee TK, Yan M, Tang KH, Ling MT, Vielkind JR, Guan XY and Chan KW: MicroRNA-616 induces androgen-independent growth of prostate cancer cells by suppressing expression of tissue factor pathway inhibitor TFPI-2. *Cancer Res* 71: 583-592, 2011.
44. Andresen MS, Stavik B, Sletten M, Tinholt M, Sandset PM, Iversen N and Skretting G: Indirect regulation of TFPI-2 expression by miR-494 in breast cancer cells. *Sci Rep* 10: 4036, 2020.
45. Chen B, Zhu A, Tian L, Xin Y, Liu X, Peng Y, Zhang J, Miao Y and Wei J: miR 23a suppresses pancreatic cancer cell progression by inhibiting PLK 1 expression. *Mol Med Rep* 18: 105-112, 2018.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.