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MiR-708-5p inhibits the progression of pancreatic ductal adenocarcinoma by targeting Sirt3

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ABSTRACT

Numbers of studies have indicated that miRNA-708 plays an important role in many types of cancer. However, the role of miRNA-708 in pancreatic ductal adenocarcinoma (PDAC) has yet to be fully elucidated. The present study aimed to investigate the role of miRNA-708-5p in the proliferation, invasion and metastasis of PDAC in vitro, as well as the underlying mechanism. We found that miRNA-708-5p was upregulated in PDAC tissues and cell lines, and high miRNA-708 expression indicated poor prognosis in PDAC patients. Besides, the CCK-8 assay, colony formation assay and transwell assay results suggested that miRNA-708-5p overexpression enhanced the ability of proliferation, invasion and migration in PDAC cell lines. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting and luciferase reporter assay demonstrated that SIRT3 was a direct target of miRNA-708-5p. Furthermore, a series of rescue experiments manifested that SIRT3 was involved in the oncogenic function of miRNA-708-5p in PDAC cells. Taken together, our study established a novel miRNA-708-5p/SIRT3 axis in the progression of pancreatic cancer and provided insight for pancreatic cancer treatment.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death. Pancreatic ductal adenocarcinoma (PDAC) is the most frequent type of pancreatic cancer, which comprises more than 85% of all pancreatic cancer, and presents the poor prognosis with less than 6% patients surviving more than 5 years [1]. The poor prognosis is attributed to its high propensity for locoregional invasion and early development of distant metastases [2]. In addition, lack of effective therapy for metastatic pancreatic cancer further aggravates the dismal outcome of pancreatic cancer patients. Thus, it is important to elucidate the underlying mechanisms that implicated in PDAC, which regulate its pathogenesis and progression, and provide useful information for the clinical management of pancreatic cancer.

MicroRNAs (miRNAs) are small (18–22 bases in length) and endogenous single-stranded RNA, which post-transcriptionally regulate target gene expression by degrading or inhibiting the 3′-untranslated region (3′-UTR) of mRNA [3]. Emerging evidences suggest that deviant levels of miRNAs are linked with disorders such as human disease and cancer [4–6]. Some miRNAs are tumor suppressors, whereas others are oncogenic, such as miRNA-505 inhibits proliferation, migration, and invasion in endometrial cancer by inhibiting TGF-a [7], miRNA-1908 promoted anchorage-independent growth and significantly increased the tumor forming potential in glioblastoma by suppressing PTEN tumor suppressor pathway [8]. Of interest, some miRNAs can either act as an oncogene or a tumor suppressor based on tumor types. MiRNA-708 inhibits cell proliferation and promotes cell apoptosis in osteosarcoma [9]; suppresses cell proliferation and invasion in gastric [10] and hepatocellular carcinomas [11]. As to the non-small cell lung cancer (NSCLC), forced miRNA-708 expression increased NSCLC cells proliferation, invasion and migration, and high miRNA-708 expression was strongly associated with poor survival in NSCLC patients [12]. However, studies suggesting the correlation between expression of miRNA-708 and PDAC along with specific mechanisms of miRNA-708 in development of PDAC have not been reported yet.

In the present research, we found that expression of miRNA-708-5p was significantly upregulated in PDAC cell lines and tissues. Our study first confirmed the oncogenic activity of miRNA-708-5p in PDAC via inhibiting the expression of sirt3. The outcomes of our study confirmed a new therapeutic target for PDAC.

2. Materials and methods

2.1. Cell culture and transfection

Human pancreatic cancer cell lines (Capan-1, CFPAC-1, BxPC-3,

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MIA PaCa-2, and Panc-1) and one human pancreatic duct epithelial cell line (HPDE6-C7) were purchased from Cell Bank of the Chinese Science Academy (Shanghai, China). HPDE6-C7 cells were grown in keratinocyte serum-free medium (ThermoFisher Scientific Inc. Cat#17005-042, Ref.). BxPC-3 cells were maintained in RPMI-1640 medium. Capan-1, CFPAC-1, MIA PaCa-2, and Panc-1 were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Both DMEM and RPMI1640 were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). All the cell lines were cultured in a humidified 5% CO2 atmosphere at 37 ˚C. Cells were seeded on the plate the day before transfection and then transfected with indicated miRNA mimics/inhibitors by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The miRNA-negative control, miRNA-708-5p mimic or miRNA-708-5p inhibitor were purchased from Genepharm company(Genepharm, Shanghai, China). The sequences were as follows: miRNA-708-5p inhibitor, 5′−CCCAGCUAGAUUGUAAGCUCCUU-3′; miRNA-708-5p mimics, 5′-AAGGAGCUUACAAUCUAGCUGGG-3′. 48 h after transfection, the cells were harvested for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

2.2. Clinical samples

Twenty-four pairs of pancreatic cancer tumor tissues and adjacent normal tissues were obtained from pancreatic cancer patients undergoing surgery at the First Affiliated Hospital of Nanchang University from April 2017 to May 2018. All patients had not received any treatment prior to surgery. The fresh tissues obtained during surgery were stored at liquid nitrogen for further analysis. The study was sanctioned by the Ethics Committee of the First Affiliated Hospital of Nanchang University. All patients were informed of the study and gave informed consent.

2.3. RT-PCR

Total RNA was extracted from tissues and all cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cDNA was converted using an miScript SYBR Green PCR kit (Invitrogen). RT-PCR was performed using a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with qPCR SuperMix (Invitrogen), according to the manufacturer's instructions. The primer sequences were as follows: SIRT3 forward, 5′-CAAGGAGCTGTACCCTGGAAA-3′ and reverse, 5′-CGACAC TCTCTCAAGCCCATC-3′; GAPDH forward: 5′-CAGGGCTGCTTTTAACT CTGGT-3′ and reverse: 5′-GATTTTGGAGGGATCTCGCT-3′; miRNA-708-5p forward: 5′−CCGCACGAAGGAGCTTACAAT-3′ and reverse: 5′-GTG CAGGGTCCGAGGTATTC-3′; U6 forward: 5′-CTCGCTTCGGCAGC ACA-3′ and U6 reverse: 5′-AACGCTTCACGAATTTGCGT-3′. GAPDH and U6 were used as endogenous controls for mRNA and miRNA, respectively. The qPCR was performed on an ABI7500 system (Applied Biosystems). The PCR conditions were as follows: Initialization at 95 ˚C for 5 min followed by 40 cycles of denaturation at 95 ˚C for 15 s and annealing/elongation at 60 ˚C for 30 s. The relative miRNA and mRNA expression were calculated using the comparative 2-ΔΔCq method [13].

2.4. Western blot

Proteins were extracted from cells and tissues. Equal quantities of protein (50 μg) were separated on 8% SDS-PAGE gels and then transferred to PVDF membranes (Millipore, USA). Membranes were then blocked with 5% skim milk at 4 ˚C overnight. After blocking, blots were incubated with primary antibody against SIRT3 (#2627; 1:1000, Cell Signaling Technology) and GAPDH (ab9485, 1: 2000, Abcam) 1 h at room temperature, followed by incubation with corresponding HRP-conjugated goat anti-rabbit secondary antibody (R4880, 1:1000, Sigma-Aldrich). The blots were detected using electrochemiluminescent (ECL) detection system (Thermo Scientific).

2.5. CCK-8 assay

Cell proliferation was measured using a CCK-8 kit (CCK-8; Beyotime Institute of Biotechnology, Haimen, China). The CFPAC-1 and BxPC-3 cells were transfected with miRNA-708-5p mimic or inhibitor, and seeded in 96-well tissue culture plates (2000 cells/well). After incubation for 12 h, 24 h, 48 h, 72 h, 96 h and 120 h, cell counting kit solution (10 μl) was added to each well, and incubated for another 4 h. The absorbance of each well was read using a Bio-Tek Instruments EL310 Microplate Autoreader at 450 nm.

2.6. Colony formation assay

Cells transfected with miRNA-708-5p mimic or inhibitor were seeded in six-well plate (200 cells/well) and cultured for two weeks. Cell colonies were fixed in 100% methanol and subsequently stained with 0.4% crystal violet. Clusters with cell count > 50 were counted as colonies.

2.7. Transwell invasion and migration assay

The transwell assay was conducted using an 8.0-μm pore-size cell culture insert (Costar, Corning, NY), that was either coated (to assess invasion) or non-coated (to assess migration) with 100 μl matrigel (BD Biosciences, San Jose, CA, USA). 800 μl of normal DMEM/RPMI1640 medium supplemented with 15% FBS was added to the lower chamber. The transfected cells (2×10^4 cells in 400ul of serum-free medium) were subjected to seeding in the upper chamber followed by incubation for 24 h at 37 ˚C. The cells on the bottom of the filter were fixed with methanol and stained with 0.4% crystal violet, and the cells were counted under a microscope.

2.8. Luciferase reporter assay

To validate whether SIRT3 was a target gene of miRNA-708-5p, a luciferase reporter assay was performed. The wild-type (WT) or mutant (MT) 3′-untranslated region (3′-UTR) of SIRT3 were cloned into a PsiCHECK2 vector (Promega, Madison, WI), and all constructs were verified via DNA sequencing. HEK-293 cells were co-transfected with PsiCHECK2 vector containing WT or MT SIRT3 3′-UTR, and with miRNA-708-5p mimics or miRNA-negative control for 48 h. Subsequently, the luciferase activity was determined using the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

2.9. Statistical analysis

Statistical analyses were performed using SPSS version 18.0 software (SPSS, Inc., Chicago, Il, USA). Data are presented as the mean ± standard deviation (SD). Differences between two groups were compared using a Student's *t*-test or Fisher's exact test. P-values < 0.05 was considered to indicate a statistically significant difference. All experiments were repeated three times in triplicate.

3. Results

3.1. Levels of miRNA-708-5p are increased in PDAC cell lines and tissues, and associated with poor survival in PDAC patients

The pancreatic ductal adenocarcinoma tissues and paired adjacent noncancerous tissues were obtained from PDAC patients, and the tissues were subjected to RT-PCR for comparing the expression of miRNA-708-5p. The results showed that the expression of miRNA-708-5p was significantly higher in tumor tissues compared to non-tumor adjacent

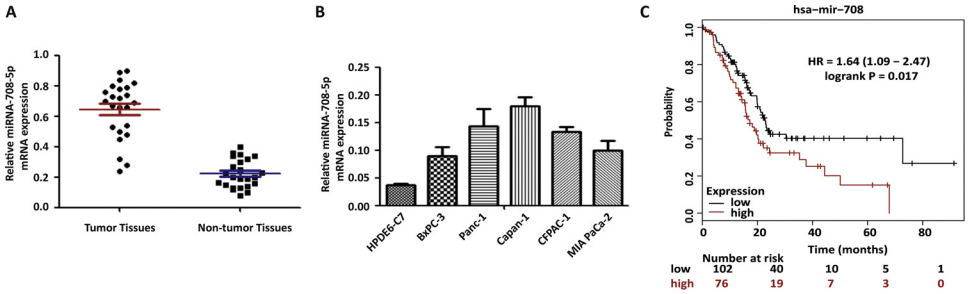

Fig. 1. Levels of miRNA-708-5p are increased in PDAC cell lines and tissues, and associated with poor survival in PDAC patients. (A) The miRNA-708-5p expression is significantly higher in PDAC carcinoma tissues than in paired normal tissues. (B) The relative expression of miRNA-708-5p in Human pancreatic cancer cell lines (Capan-1, CFPAC-1, BxPC-3, MIA PaCa-2, and Panc-1) and one human pancreatic duct epithelial cell line (HPDE6-C7). (C) Lower expression of miRNA-708-5p in PDAC patients indicated better prognosis.

tissues (Fig. 1A). We also examined miRNA-708-5p expression in a normal pancreatic ductal cell line (HPDE6-C7) and a series of PDAC cell lines by RT-PCR. MiRNA-708-5p was highly expressed in most of the PDAC cell lines compared with that in HPDE6-C7 cells (Fig. 1B). Furthermore, we found that PDAC patients with lower miRNA-708 expression had longer overall survival time than patients with high miRNA-708 expression based on the bioinformatic data collected in KM-plotter (http://kmplot.com/analysis/index.php?p=service&start=1) (Fig. 1C). All these results indicated an oncogenic role of miRNA-708-5p in PDAC.

3.2. MiRNA-708-5p promoted cell proliferation and colony formation ability of PDAC cells

Based on the relative mRNA expression levels of each pancreatic cancer cell lines, Capan-1 and BxPC-3 cell lines were chosen to investigate the biological function of miRNA-708-5p in PDAC. The Capan-1 cells were transfected with miRNA-708-5p inhibitors, while BxPC-3 cells were transfected with miRNA-708-5p mimics, and the cells viability were evaluated (Fig. 2A). The overexpression of miRNA-708-5p significantly promoted the cell proliferation ability of BxPC-3 cells and miRNA-708-5p inhibition dramatically hampered cell proliferation in Capan-1 cells as demonstrated by CCK-8 assay (Fig. 2B). We then assessed colony formation to determine the role of miRNA-708-5p in the long-term proliferation of PDAC cells. Enhanced miRNA-708-5p prominently promoted the clonogenic growth of PDAC cells compared with control cells, and vice versa (Fig. 2C).

3.3. MiRNA-708-5p promoted cell migration and invasion in PDAC cells

Transwell invasion and migration assays were conducted to analyze the role of miRNA-708-5p in regulating cell invasion and migration in PDAC cells (Fig. 3A and B). The results demonstrated that miRNA-708-5p overexpression boost the invasion and migration of BxPC-3 cells to 2.23 and 2.05 fold compared to control, respectively. And miRNA-708-5p inhibition suppressed the invasion and migration of Capan-1 cells by 61% and 55% compared to control.

3.4. Identification of sirt3 as a favorable target site of miRNA-708-5p

To explore the molecular mechanism behind miRNA-708-5p, potential target genes of miRNA-708-5p were predicted using online software. Targetscan (http://www.targetscan.org/), DIANA (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index), and miRDB4.0 (http://mirdb.org/miRDB/index.html) were fully searched. Among the numerous targets, SIRT3 is associated with cancers and acted as a tumor suppressor in PDAC [14]. Therefore, we selected SIRT3 as the candidate target gene for miRNA-708-5p for further analysis. The binding sites between miRNA-708-5p and SIRT3 are shown in Fig. 4A. To establish this prevision, luciferase reporter assays were performed to validate sirt3 as a target for miRNA-708-5p. It was found that up-regulation/down-regulation of miRNA-708-5p expression dramatically inhibited/enhanced luciferase activity induced by 3′-UTR of SIRT3, whereas miRNA-708-5p mut exert minimal effects on SIRT3 3′-UTR luciferase reporter gene (Fig. 4B). In addition, western blotting and RT-PCR indicated that the protein and mRNA levels of SIRT3 were significantly increased and decreased in response to miRNA-708-5p inhibition and overexpression, respectively (Fig. 4C and D). Collectively, these results indicated that SIRT3 is a direct target of miRNA-708-5p.

3.5. Involvement of sirt3 in miRNA-708-5p mediated promotion of cell progression in PDAC cells

Next, we investigated whether miRNA-708-5p promotes PDAC cells progression via inhibiting SIRT3. A series of rescue experiments were performed after co-transfected pcDNA3.1-SIRT3 plasmid and miRNA-708-5p mimics to BxPC-3 cells. CCK-8 and colony formation assays showed that SIRT3 overexpression partly reclaimed the promotion of cell proliferation caused by overexpression of miRNA-708-5p in PDAC cell lines (Fig. 5A and B). Transwell invasion and migration assays also revealed that the invasion and migration ability of miRNA-708-5p mimics treated cells can be rescued by SIRT3 overexpression (Fig. 5C), confirming the role of SIRT3 in reversing the consequences of miRNA-708-5p on the progression of PDAC cells.

4. Discussion

Emerging evidences have demonstrated that miRNAs play important roles in pancreatic cancer pathobiology, such as activating proliferation signaling, resisting cell apoptosis, evading tumor suppressor and accelerating invasion and migration [5,15]. MiRNA-130a and miRNA-454 are found to be upregulated in pancreatic cancer and to negatively regulate SMAD4 expression to promote tumor growth [16,17]. MiRNA-218 and miRNA-155, has been shown to be associated with lymphatic metastasis of pancreatic cancer [18,19]. Another study reported that upregulation of miR-146 and downregulation of miR-205 and let-7 in gemcitabine-resistant pancreatic cancer cell lines and clinical samples, suggesting their involvement in the development of chemoresistance [20]. Therefore, understanding the role of miRNAs in pancreatic cancer may aid in the diagnosis and treatment of pancreatic cancer.

In the present study, we studied the expression and the role of miRNA-708-5p in PDAC cells and its underlying molecular mechanisms involved. We found that the expression of miRNA-708-5p was upregulated in PDAC cell lines and tissues, and high miRNA-708-5p expression indicated poor prognosis in PDAC patients. Overexpression of miRNA-708-5p resulted in promotion of cell proliferation, colony formation, cell invasion and migration in PDAC cell lines. We also indentified that SIRT3 as a direct target of miRNA-708-5p, and partly involved in miRNA-708-5p-mediated PDAC cells progression.

SIRT3, an NAD-dependent deacetylase localized to mitochondria, regulates diverse cellular functions and has been extensively studied in tumorigenesis. SIRT3 has a dual role in cancer. In hepatocellular carcinoma and gastric cancer, SIRT3 functions as a tumor suppressor, as it could trigger cell death under stress conditions [21,22]. In breast

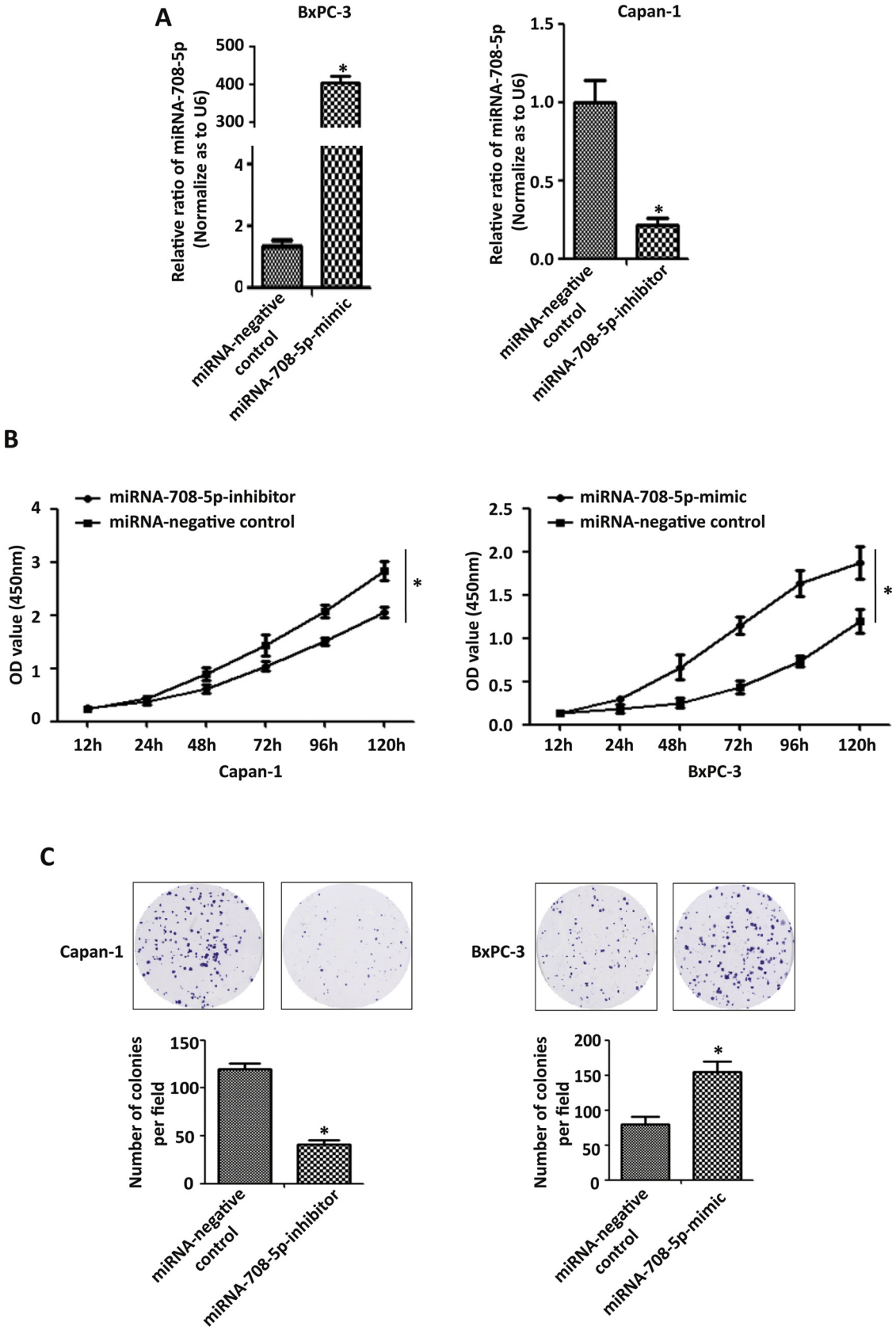

Fig. 2. MiRNA-708-5p promoted cell proliferation and colony formation ability of PDAC cells. (A) The efficiency of miRNA-708-5p mimics/inhibitors were verified by RT-PCR. (B–C) The proliferation ability of Capan-1 cells repressed after transfection with miRNA-708-5p inhibitor and the proliferation ability of BxPC-3 cells increased after transfection with miRNA-708-5p mimics, as observed by CCB-8 assay (B) and colony formation assay (C). Values are means of triplicate samples ± SD.; *p-value < 0.05 by two-tailed Student's *t*-test.

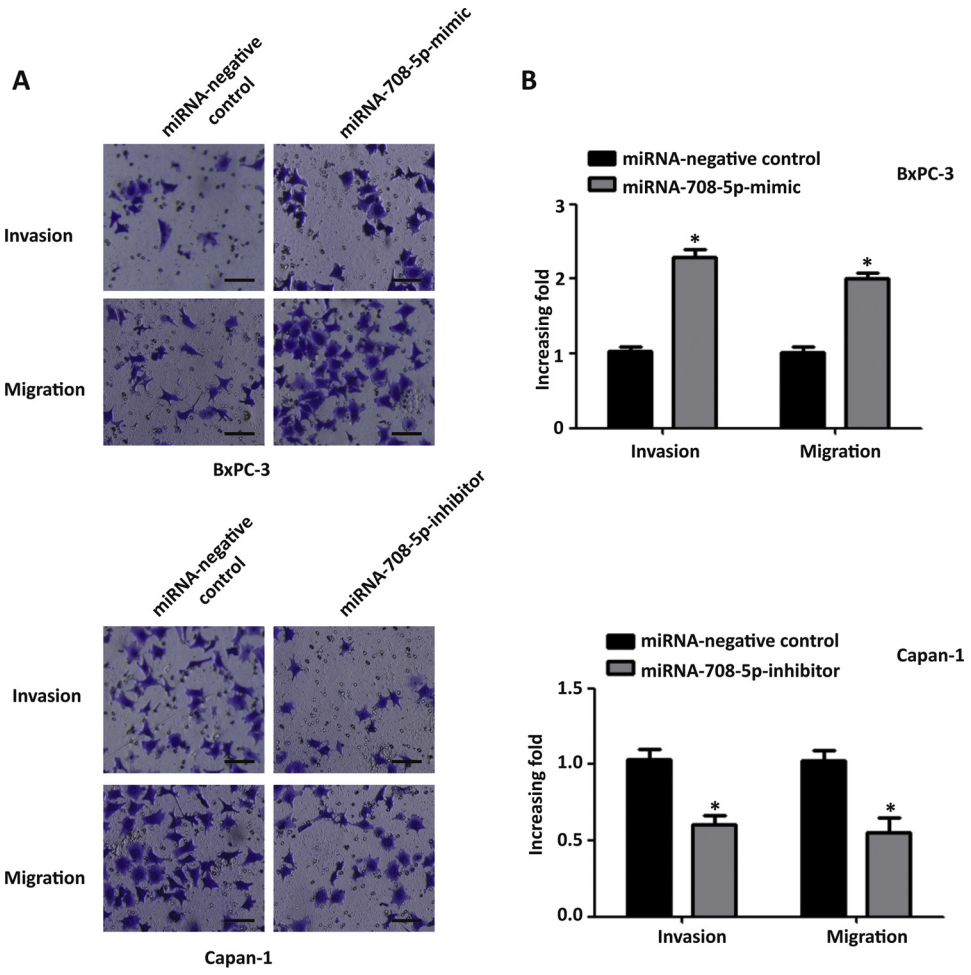

Fig. 3. MiRNA-708-5p promoted cell migration and invasion in PDAC cells. Transwell invasion and migration assays were performed in Capan-1 and BxPC-3 cells. After transfection with miRNA-708-5p mimics/inhibitors, cells were seeded onto Matrigel-coated membrane (for invasion assays) or transwell chamber (for migration assays) for 24 h. (A) Cells were fixed with methanol and stained with 0.4% crystal violet. (B) The relative cell invasion and migration were analyzed by comparison of the cell numbers of the treated groups over that of the untreated control. Values are means of triplicate samples ± SD.; *p-value < 0.05 by two-tailed Student's *t*-test.

cancer, lung cancer and bladder cancer, SIRT3 acts as an oncogene, since it keeps ROS levels under a certain threshold compatible with cell viability and proliferation [21,23]. As for the PDAC, both our previous study [14] and other researchers [24] reported that SIRT3 was downregulated in PDAC tumor tissues and low SIRT3 expression associated with dismal overall survival time in PDAC patients. Here, our data indicated that SIRT3 is a direct target of miRNA-708-5p, and the abnormal expression of SIRT3 ablated the stimulatory activity of

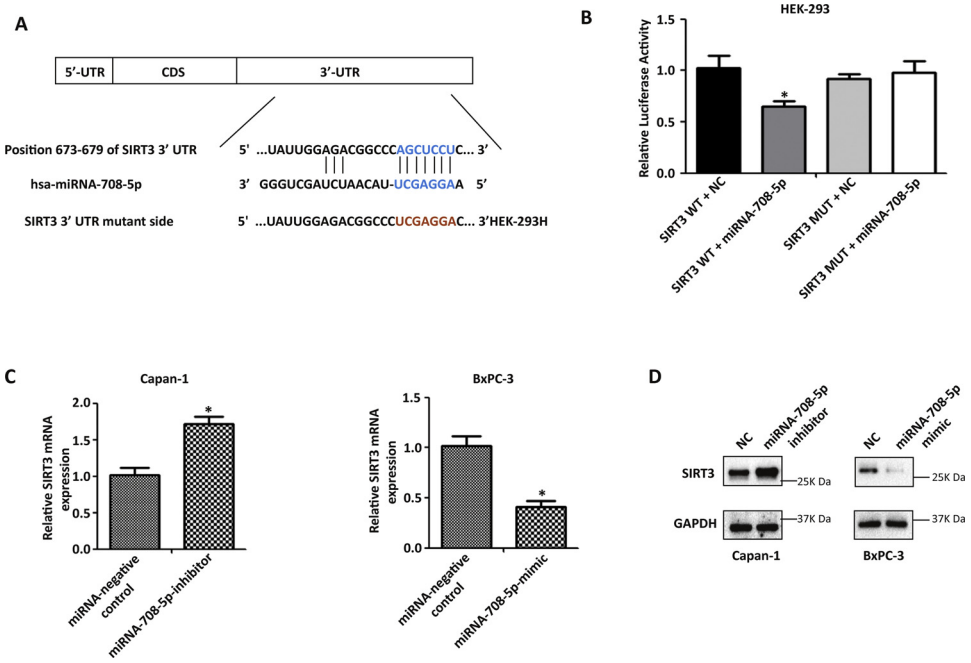

Fig. 4. SIRT3 is a direct target of miRNA-708-5p. (A) Schematic diagram of the putative binding sites of miRNA-708-5p in the wild-type SIRT3 3′-UTR (in blue). The miRNA-708-5p seed sequence matches in the SIRT3 3′-UTR are mutated at the positions as indicated (in orange). (B) Luciferase activity assays of WT and MUT SIRT3 3′-UTR luciferase reporters after cotransfection with miRNA-708-5p mimic and miRNA negative control (NC) in HEK-293 cells. (C–D) Expression of SIRT3 in PDAC cells after transfection with miRNA-708-5p mimics/inhibitors was assessed by RT-PCR (C) Western blot (D). Values are means of triplicate samples ± SD.; *p-value < 0.05 by two-tailed Student's *t*-test.

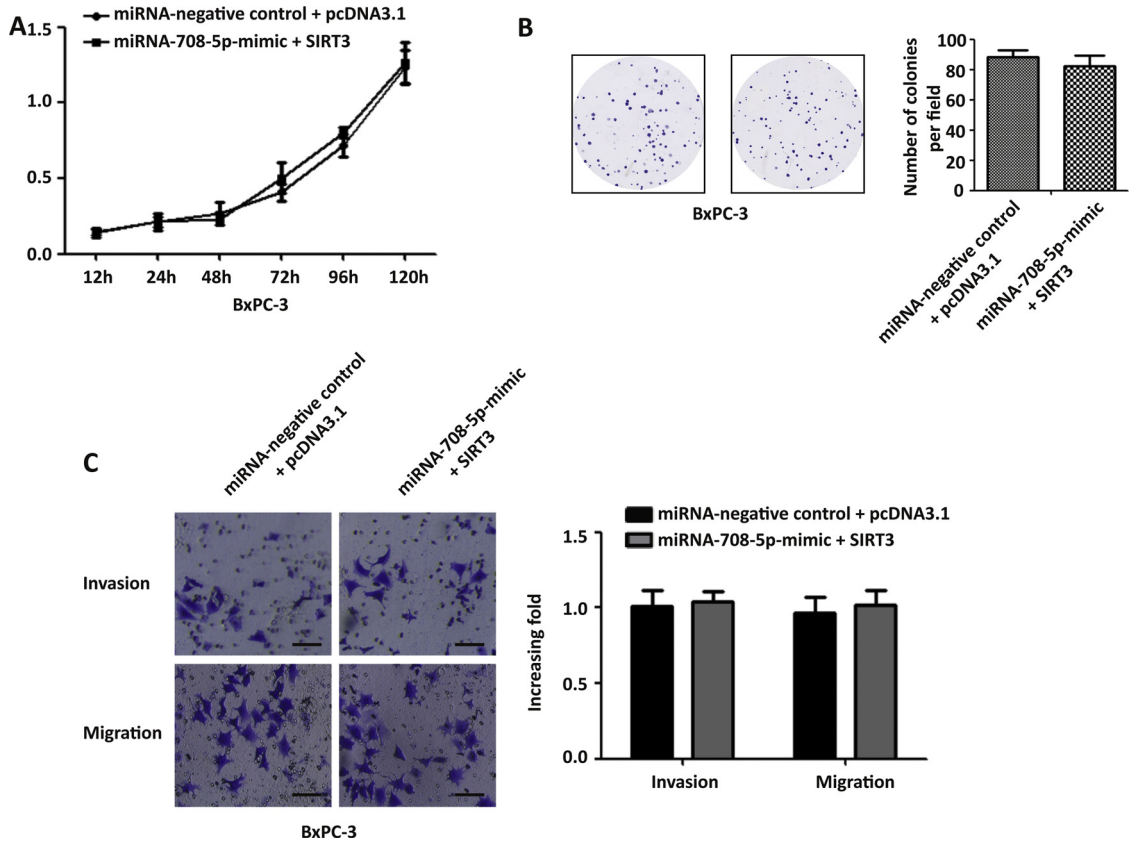

Fig. 5. Involvement of sirt3 in miRNA-708-5p mediated promotion of cell progression in PDAC cells. Co-transfected SIRT3 plasmid with miRNA-708-5p mimics into BxPC-3 cells abrogated the increased ability of cell proliferation, cell invasion and migration induced by miRNA-708-5p overexpression as measured by CCK-8 assay (A), colony formation assay (B) and transwell assay (C). Values are means of triplicate samples ± SD.; *p-value < 0.05 by two-tailed Student's *t*-test.

miRNA-708-5p on cell viability, invasion and migration. Our current results further confirmed that SIRT3 possess tumor suppressor properties in the context of PDAC.

To our knowledge, this study provides the first evidence that miRNA-708-5p effectively promote PDAC progression by negatively regulating SIRT3 expression, suggesting that miRNA-708-5p/SIRT3 may be an important regulatory mechanism in the progression of PDAC. Our study hence indicates a putative useful biomarker and provides a novel approach for the clinical treatment of PDAC.

Conflict of interest

The authors declare no competing financial interests.

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