

Original Article

miR-557 inhibits the proliferation and invasion of pancreatic cancer cells by targeting EGFR

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Abstract: Deregulation of microRNA has been suggested as a critical event in pancreatic cancer development and progression. Thus far, very little is known about the role of miR-557; therefore, the goal of this study was to investigate the potential role of miR-557 in pancreatic cancer. In the present study, we discovered that miR-557 expression was lowered in cancerous pancreatic tissue samples relative to non-cancerous adjacent controls, and when miR-557 was overexpressed we found that this promoted the apoptotic death of pancreatic cancer cells, suppressing their proliferation, invasion, and migration. Using western blotting and luciferase reporter assays, we further found evidence that this miRNA may directly suppress expression of the epidermal growth factor receptor via suppressing its translation through 3'-UTR binding. When EGFR was overexpressed in our pancreatic cancer cells, this was sufficient to reverse the effects of miR-557 inhibition. In summary, miR-557 acts as a tumor suppressor in pancreatic cancer cells, impairing their ability to grow and invade surrounding tissues due at least in part to EGFR inhibition. Harnessing this targeting of EGFR via this miRNA may therefore be a viable strategy useful for patient suffering from this deadly disease.

Keywords: Pancreatic cancer, miR-557, proliferation, invasion, apoptosis, EGFR

Introduction

Pancreatic cancer (PC) remains a cancer with very poor patient outcomes and median survival time following diagnosis is as low as six months [1]. The only means of curing this disease remains surgical resection, but because PC tumors are very aggressive and can rapidly metastasize, only about one in five patients is eligible for such surgery [2]. While chemotherapy can be used to provide palliative care to patients, it generally incurs serious side effects while offering minimal survival benefits [3]. Given this lack of treatment options, there is a clear and urgent need to develop novel strategies aimed at curing this deadly disease. The epidermal growth factor receptor (EGFR) is an essential receptor found on the membrane of cells, playing vital roles in mediating the survival, growth, and movement of cells in response to environmental stimuli [4-6]. EGFR overexpression is a common feature of PC and

other cancer types, and as such it is often highlighted as a possible target for PC therapy [7].

MicroRNAs are small RNA molecules (19-22 nucleotides) that are widely expressed and play regulatory roles by binding the 3'UTR of many mRNAs, resulting in their degradation or repression of their subsequent translation [8, 9]. miRNAs are well known to be important for regulating a wide array of cellular and disease-related processes [10]. Indeed, specific miRNAs act as oncogenes and tumor suppressors, allowing their expression to directly affect tumor development, progression, and invasion [11]. miR-557 is a miRNA found to be frequently downregulated in a range of tumor types, including liposarcomas and hepatocellular carcinomas, with low levels also found in breast and gastric cancer patients [12-15]. The specific importance of miR-557 in PC, however, is uncertain and little-studied. Therefore, in this study we assessed this importance, revealing this miRNA to be downregulated in PC tissues and to direct-

ly target EGFR, thereby interfering with the growth, migration, and invasion of PC cells.

Materials and methods

Specimens and ethics statement

Twelve PC and paired adjacent noncancerous specimens were obtained from patients undergoing surgical resection in the first hospital affiliated to Soochow University. There was no radiotherapy or chemotherapy prior to surgery. The dissected tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C . Use of clinical sample cohorts in this study was approved by the human ethics committee of The First Hospital Affiliated to Soochow University, and this study complied with the Declaration of Helsinki. All patients are informed and have declared written informed consent that their samples can be used for research.

RNA extraction and microRNA microarray expression profiling

Paired PC tissues and adjacent normal tissues from every subject were snap-frozen in liquid nitrogen immediately after resection and stored at -80°C until use. The mirVana miRNA isolation kit (Ambion, Austin, TX, USA) was used to extract total RNA from frozen samples, according to the manufacturer's protocols, which were then eluted with 100 μL of nuclease-free water. Total RNA was quantified with the NanoDrop ND-2000 (Thermo Scientific) and the RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies). The microarray profiling was conducted in the laboratory of the Novobio Biotechnology Company in Shanghai, People's Republic of China. The sample labeling, microarray hybridization and washing were performed based on the manufacturer's standard protocols. The labeled cRNAs were hybridized onto the Human GeneChip® Agilent miRNA 4.0 Array (Affymetrix). Differentially expressed miRNAs were then identified through fold-change as well as P values calculated with t -test. The threshold set for up- and down-regulated genes was fold change ≥ 2.0 and p value ≤ 0.05 .

Real-time quantitative reverse transcription-PCR

Total RNA was isolated using a mirVana miRNA isolation kit (Ambion, Austin, TX, USA) per the

manufacturer's instructions. A two-step reaction process was used for quantification reverse transcription (RT) and PCR. The RNA was reverse-transcribed into cDNA using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). Real-time quantitative PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using an ABI 7500 Sequence Detection System (Applied Biosystems) with the following amplification procedure: 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 30 s. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. All experiments were done in triplicate. The primer sequences used for real-time quantitative PCR were as follows: miR-557 5'-CTCAACTGGTGTCTGGA-GTCGGCAATTCAGTTGAGAGACAAGG-3' (stem-loop primer), 5'-ACACTCCAGCTGGGGTTTGCA-CGGGTGGGC-3' (forward) and 5'-TGGTGTCTGGAGTCG-3' (reverse); EGFR (forward, 5'-CAGATGGATGTGAACCCGA-3' and reverse, 5'-TTCTTACACTTGCAGACGCC-3'); U6 (forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-GAATTCCTGCTTGAGCCGC-3'); Actin (forward, 5'-GCTCAGGAGGAGCAATGATCTTG-3' and reverse, 5'-GTACGCCAACACAGTGCTGTC-3'). U6 served as the internal control for miR-557 expression, and Actin served as the internal control for mRNA expression. Relative expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

Cell lines and culture

The human PC cell line PANC1 was purchased from American Type Culture Collection (ATCC, Manassas, VA), and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics. Cells were cultured in a humidified incubator containing 5% CO_2 at 37°C .

Lentivirus transduction

Lentiviral miR-557 and EGFR expression constructs and the respective negative control lentiviruses were purchased from Novobio (Shanghai, China). PANC1 cells were infected with recombinant lentivirus transducing units in the presence of 8 mg/mL polybrene (Sigma-Aldrich, St Louis, MO) according to the manufacturer's protocol. The transfection rate was confirmed by real-time quantitative PCR.

Cell counting kit-8 (CCK-8) assay

Cells were seeded into a 96-well plate at a density of 1×10^4 cells/well and cultured overnight. Cells were transfected with miR-557 vector or negative control for 48 h. Then, 10 μ L of CCK-8 solution (Sigma, St. Louis, MO, USA) was added to each well. After incubation for 2 h at 37°C, the optical density (OD) at 490 nm was detected by an enzyme-linked immunoassay analyzer (BioTek Instruments, Inc. Winooski, VT, USA).

Wound healing assay

PANC1 cells after the transfection treatment like the aforementioned were seeded in 6-well plates and incubated overnight until the cells achieved to 95% confluency. The scratch wound was obtained by drawing the surface cells of plates with a pipette tip and the detached cells were washed off by PBS. Then the remaining attached cells in 6-well plates were incubated for 24 hours. The wound healing images at 0 hour and 24 hours were photographed from each well.

Transwell cell invasion assay

Cells were plated on a transwell 24-well chamber precoated with Matrigel Basement Membrane Matrix (BD Science, San Jose, CA, USA) according to the manufacturer's instructions. The upper chamber was filled with 200 μ L of serum-free DMEM containing (1×10^4 cells), and 500 μ L of DMEM containing 10% FBS was added to the lower chamber. After culture for 24 h, the cells on the upper side of the membrane were removed, and the cells on the lower side of the membrane were fixed with methanol and stained with 0.1% crystal violet. The cell numbers were counted under a microscope.

Apoptosis analysis

For flow cytometry, the treated cells were cultured in the medium supplemented with serum for 48 h and then collected and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) that were included in the Annexin V-FITC Apoptosis Detection kit. All samples were analyzed three times using a Becton-Dickinson flow cytometer (BD Bio-sciences, Franklin Lakes, NJ, USA).

Plasmid construction and luciferase reporter assay

The wild-type 3'-UTR sequence of EGFR containing the predicted miR-557 binding site was amplified by PCR in a total volume of 50 μ L using the Primer star kit (Takara) in accordance with the manufacturer's instructions. The primers used were 5'-AATTAAGGAAGCTAGATGCGACCCTCCGGA-3' (sense); 5'-TAAACCCAA-GGCGCGTCAGCTCAAACCTGTGATTTCTTTACG-3' (antisense). The mutant constructs were generated by mutation. Fragments were subcloned into the Xho I site in the 3'-UTR of Renilla luciferase of the psiCHECK-2 reporter vector. Wild-type or mutant luciferase reporter constructs were transfected into PANC1 cells infected with miR-557 or control lentivirus. After 48 h, cells were lysed and reporter activity was assessed using the Dual-luciferase reporter assay system (Promega, USA) in accordance with the manufacturer's protocols. Renilla luciferase activity was normalized to firefly luciferase activity.

Western blot

Total protein was extracted using cell lysis buffer for western blots, and the protein concentration was quantitated using the BCA protein assay kit (Beyotime Biotechnology, Haimen, China). Equivalent amounts of proteins (50 μ g) from each sample were separated by 12% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membranes (Beyotime, China). The membrane was blocked with 3% skim milk and then immunoblotted with primary antibodies for EGFR and β -actin (Abcam, Cambridge, UK). After incubation overnight at 4°C, the membrane was probed with horseradish peroxidase-linked secondary antibodies. Immunoreactive protein bands were detected using an Odyssey Scanning system.

Statistical analysis

The data were presented as the means \pm standard deviation. All statistical analyses were performed using SPSS 17.0 statistical software. Student's t-test was used to analyze the differences between two groups. *P*-value <0.05 indicates statistical significance.

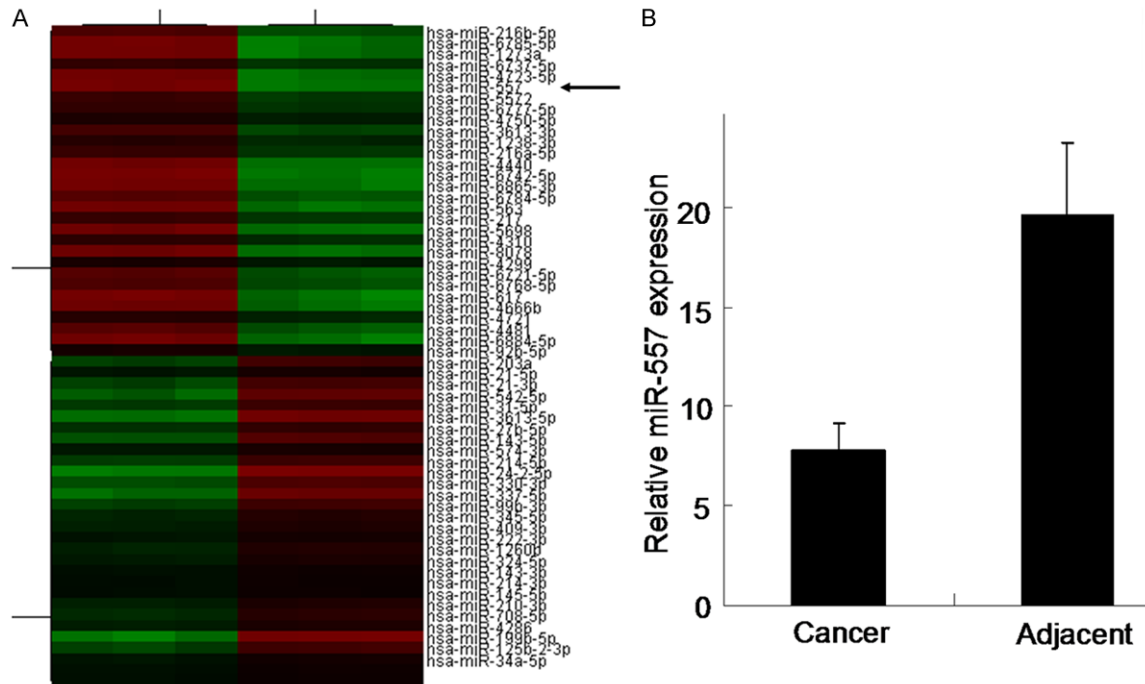


Figure 1. miR-557 expression in pancreatic cancer tissues. A. Hierarchical clustering and a heat map of the miRNA profile comparison between pancreatic cancer and adjacent normal tissues. B. Real-time PCR was used to determine relative expression of miR-557 in pancreatic cancer tissues and adjacent non-cancer tissues.

Results

Expression of miR-557 is decreased in human PC

We first performed miRNA profiling to identify any miRNAs differentially expressed in PC tissue samples relative to adjacent normal samples. In total, 558 such miRNAs were identified (213 upregulated, 345 downregulated), among which miR-557 was included (**Figure 1A**). To confirm the accuracy of these results, we measured miR-557 levels in 9 sample pairs via qPCR, confirming a significant decrease in miR-557 expression in PC samples relative to normal controls (2.6 fold, $P < 0.01$) (**Figure 1B**).

Overexpression of miR-557 inhibits PC proliferation and induces apoptosis

To better understand the influence of miR-557 on PC cells, we transduced cells of the PANC1 human pancreatic cancer cell line with a miR-557 expressing lentivirus (**Figure 2A**), allowing us to successfully overexpress this miRNA in these cells (**Figure 2B**). We then used a CCK-8 assay to explore how these cells proliferated, revealing a gradual decline in proliferation upon

miR-557 overexpression (**Figure 2C**). We further explored cell death by flow cytometry, revealing a significant increase in apoptosis in these cells upon miR-557 overexpression (**Figure 2D**). We next used a Transwell assay to assess how miR-557 affected the invasive abilities of PANC1 cells. We found that cells overexpressing this miRNA were significantly less invasive than those cells transduced with a negative control lentivirus (**Figure 2E**). Furthermore, in a wound healing assay wound healing was reduced when cells overexpressed miR-557, indicating reduced cellular migration and proliferation (**Figure 2F**). Together these results indicate miR-557 can suppress the proliferation of PC cells, suggesting it serves a tumor suppressor function.

MiR-557 regulates EGFR expression in PC cells

We next sought to determine what genes are targeted by miR-557 in order to mediate the observed anti-proliferative effects of this miRNA, using miRanda and TargetScan to predict possible targets. Of the identified putative targets, a binding site in the EGFR 3'-UTR was of immediate interest given the relevance of this gene to cancers. To confirm that this binding

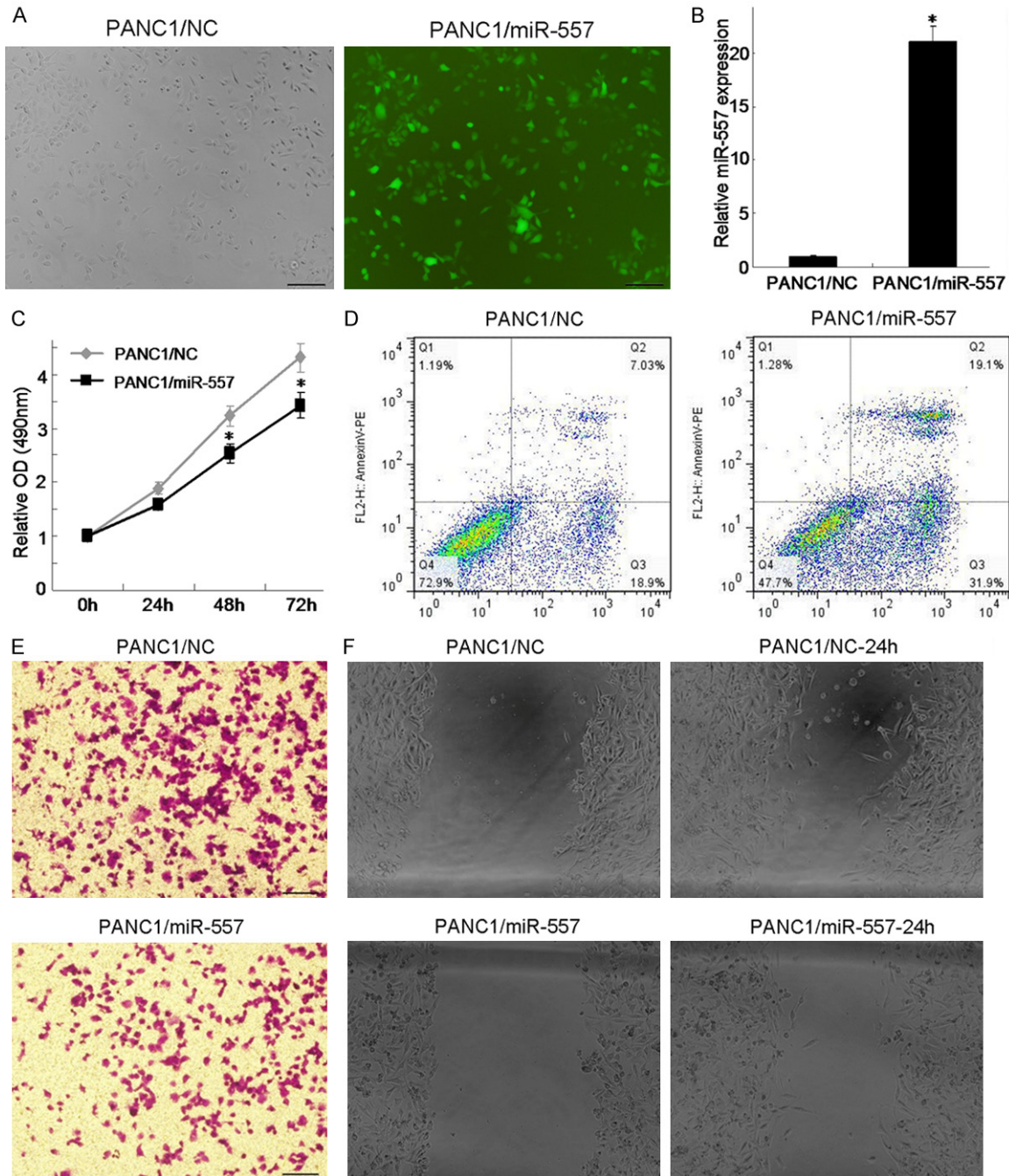


Figure 2. Overexpression of miR-557 inhibited growth of pancreatic cancer cells. A. PANC1 cells were infected with a scramble lentivirus (Control) or over-expression lentivirus of miR-557. B. Real-time PCR was used to measure the relative expression level of miR-557 in PANC1 cells infected with indicated lentivirus. C. The proliferation rate of PANC1 infected with indicated lentivirus was measured by CCK-8 assay. D. Apoptosis was measured via flow cytometry. E. Matrigel invasion chambers were used to determine the invasiveness of PANC1 cells. F. Cell migration was determined at 0 h and 24 h in PANC1 cells by the wound healing assay after treatment indicated lentivirus.

site was valid, we generated luciferase reporter vectors encoding wild-type (psiCHECK-2-EGFR-3'-UTR) and mutated (psiCHECK-2-EGFR-3'-UTR MUT) versions of the putative binding region of

the EGFR 3'-UTR. These vectors were then transfected into PANC1 cells to assess binding of miR-557 to these constructs (**Figure 3A**). We observed a significant decrease in luciferase

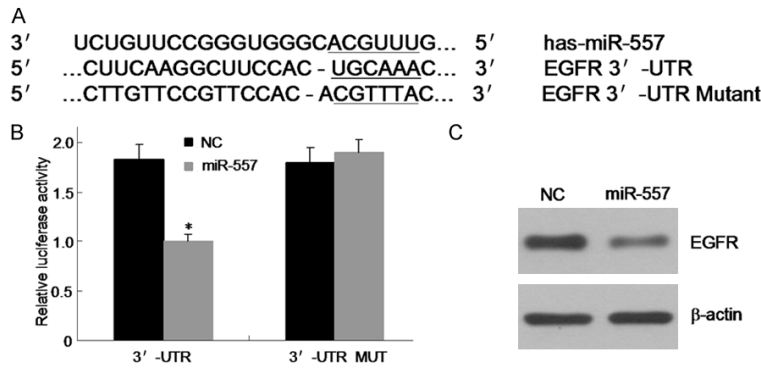


Figure 3. EGFR is a direct downstream target of miR-557. **A.** A fragment of EGFR 3'-UTR containing the wild-type miR-557 or mutant binding site was cloned downstream of the reporter gene vector. **B.** Luciferase activity of wild-type or mutant EGFR 3'-UTR luciferase vectors in the presence of miR-557. **C.** Levels of EGFR protein were measured using western blot in response to overexpression of miR-557 in PANC1 cells.

activity when psiCHECK-2-EGFR 3'-UTR and miR-557 were co-transfected into cells, whereas no change was observed when negative control miRNA or mutated EGFR 3'-UTR were instead added to cells (**Figure 3B**). We additionally assessed how miR-557 transfection affected the expression of endogenous EGFR by western blotting, revealing a decrease in EGFR expression in cells transfected with this miRNA relative to negative control cells (**Figure 3C**). Together these results indicate that miR-557 directly binds to the 3'-UTR of EGFR in PC cells.

EGFR overexpression reverses inhibitory effects of miR-557

To formally confirm the functional relationship of miR-557 and EGFR, PANC1 cells were co-transfected with plasmids for miR-557 and EGFR expression. EGFR expression was significantly higher in cells transfected with both constructs relative to those transfected with only miR-557, and this EGFR overexpression enhanced PANC1 invasion and proliferation (**Figure 4A** and **4B**). Similarly, this overexpression of EGFR rescued cells from the apoptosis induced by miR-557 (**Figure 4C**), and overcame migratory inhibition in these same cells (**Figure 4D**).

Discussion

miRNAs have been increasingly found to be dysregulated in the context of pancreatic cancer [16], suggesting that they regulate many target genes important for the regulation of this

disease. Indeed, profiling miRNA expression in PC patients has been suggested to be an ideal strategy for diagnosis the disease and predicting its prognosis [17, 18]. Several miRNAs dysregulated in PC, including miR-7, miR-23a, miR-1271, and miR-1297, are known to act as either oncogenes or tumor suppressors, thereby regulating cancer development and progression [19-22]. EGFR is a receptor of the ErbB family, and its expression is commonly increased in solid tumors, making it an ideal target for the treatment of a wide variety of

cancers [23-25]. EGFR overexpression is closely associated with metastasis, and PC is well known to be an extremely aggressive form of cancer in part due to EGFR overexpression. Identifying miRNAs that target this receptor may thus allow for therapeutic downregulation of EGFR in tumor cells, thereby potentially improving patient outcomes and arresting disease progression. For example, miR-146a has been identified as a miRNA that functions in PC cells to suppress cellular invasion [26, 27].

Previous studies have shown miR-557 to regulate a range of genes of the insulin-like growth factor 1 (IGF1) pathway in Ewing's sarcoma [28]. Similarly, this miRNA acts as a tumor suppressor in lung cancer cells, suppressing lymphocyte enhancement factor 1 (LEF1) expression [29]. miR-557 expression has also been shown to be markedly decreased in triple-negative breast cancer, wherein it targets S6K1, a component of the mTOR signaling pathway [30]. miR-557 overexpression has been shown to suppress the growth, migration, and tube formation activities of human umbilical vein endothelial cells (HUVECs), with 165 identified miR-557 targets being linked to the positive regulation of angiogenesis [31].

We conducted miRNA profiling in PC tissue samples, revealing a significant decrease in miR-557 expression in tumor tissues relative to normal controls, and this result was confirmed by qPCR. We additionally identified miR-557 as a miRNA likely to play key regulatory roles in PC

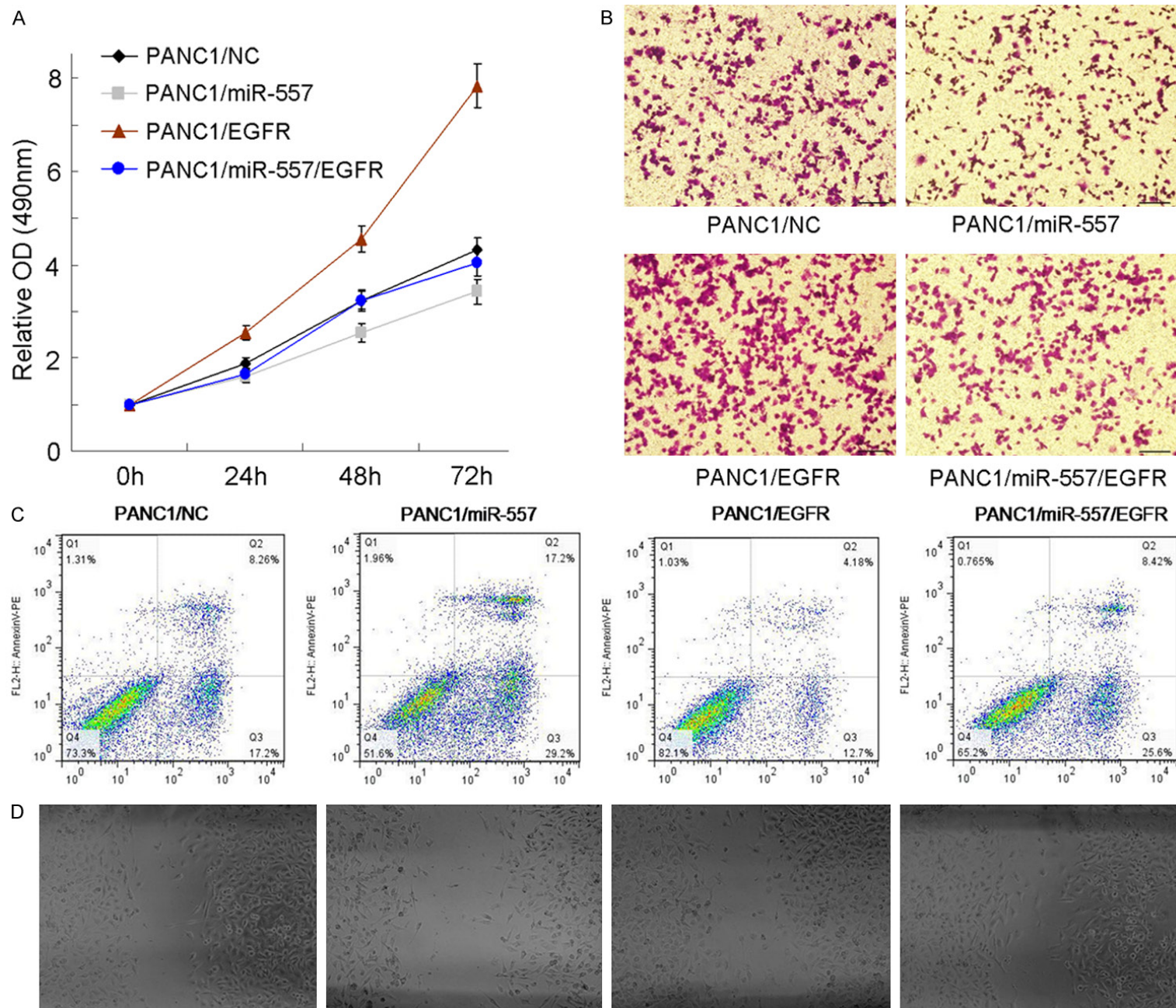


Figure 4. Overexpression of EGFR rescues growth and invasion inhibitory effect of over-expression of miR-557. A. The proliferation rate of PANC1 from each treatment group was measured by CCK-8 assay. B. The invading PANC1 cells were photographed. C and D. Apoptosis and wound healing assay were measured in each group 24 h later.

based on subnetwork topological and functional analyses. When miR-557 was overexpressed, PANC1 cell invasion, proliferation, and migration were impaired indicating that this miRNA can suppress the growth of PC cells. In addition, following predictions indicating that EGFR was a target of this miRNA, a luciferase reporter assay confirmed that miR-557 directly binds the EGFR 3'-UTR, thereby suppressing the expression of this gene at the post-transcriptional level. Critically, when EGFR was overexpressed in cells, this was sufficient to overcome the inhibitory effects of miR-557 on PC cells. Together, these findings clearly demonstrated that EGFR is a miR-557 target and that this pathway is at least partially responsible for the observed suppression of PC cell growth and motility upon miR-557 overexpression.

In summary, we found that miR-557 represents a novel miRNA downregulated in PC patient samples. The overexpression of this miRNA impairs PC cell proliferation and invasion, thus highlighting the role of this miRNA as a tumor suppressor. This is at least in part due to the ability of miR-557 to suppress EGFR expression by targeting the 3'-UTR of the EGFR mRNA. Based on these results, miR-557 may represent a novel target useful for the therapeutic treatment of PC patients.

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Disclosure of conflict of interest

None.

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