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Aberrant Expression miR-196a Is Associated With Abnormal Apoptosis, Invasion, and Proliferation of Pancreatic Cancer Cells

Minghao Liu, BS, Yiqi Du, MD, Jun Gao, MD, Jianqiang Liu, MD, Xiangyu Kong, MD, Yanfang Gong, BS, Zhaoshen Li, MD, Hongyu Wu, BS, and Hui Chen, BS

Objectives: MiR-196a levels inversely correlated with survival in pancreatic adenocarcinoma patients. However, the functional contributions of miR-196a to pancreatic cancer remain unclear.

Methods: Three lentiviral vectors encoding microRNA miR-196a precursor, inhibitor, and scrambled microRNA oligomer were transfected into Panc-1 cells, respectively. Then we explored the regulation of inhibitor of growth 5 (ING5) expression by miR-196a and its impact on apoptosis, invasion, and growth of pancreatic cancer cells. The lentiviral transfected Panc-1 cells were surgically implanted into the pancreas of mice. In vivo tumor growth and ING5 expression were measured.

Results: Down-regulation of ING5 expression was detected in cells transfected with miR-196a precursor ($P < 0.01$), accompanied by less apoptosis, increased invasion, and proliferation compared with control cells ($P < 0.05$). Cells transfected with miR-196a inhibitor revealed an opposite trend. Smaller detectable tumors were found in only 60% of mice after implantation of Lenti.miR-196a inhibitor–transfected Panc-1 cells compared with controls (360.7 ± 303.6 mm^3 vs 511.58 ± 365.9 mm^3 in controls; $P < 0.01$).

Conclusion: Our results provide experimental evidence to support aberrant expression of miR-196a is associated with abnormal apoptosis, invasion, and proliferation of pancreatic cancer cells.

Key Words: microRNA, miR-196a, ING5, pancreatic cancer

(*Pancreas* 2013;42: 1169–1181)

Pancreatic cancer is the fourth leading cause of cancer-related deaths in both men and women in the United States.[1] Pancreatic cancer is a devastating disease with poor prognosis in humans because it is prone to aggressively invade surrounding tissues, leading to rapid and extensive metastasis. The malignant transformation of pancreatic tissue is a genetically complex process that involves chromosomal deletion, mutation, or hypermethylation, which can lead to loss or inactivation of tumor suppressor genes (TSGs) causing immortality of cancer cells. It has, however, provided clues to the identification of genes critical for initiation, promotion, and development of tumors, such as the inhibitor of growth (ING) family of genes.[2]

From the Department of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai, China.
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Reprints: Zhaoshen Li, MD, or Yiqi Du, MD, Department of Gastroenterology, Changhai Hospital, The Second Military Medical University, 168 Changhai Rd, Shanghai, China (e-mail: zhaoshen-li@hotmail.com; duyiqi@hotmail.com).
Dr Gao is co–first author.
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Inhibitor of growth proteins consist of 5 isoforms. Inhibitor of growth 5 (ING5) is localized in human chromosome 2q37.3, contains 8 exons and 7 introns, and encodes a 5233–base pair (bp) cDNA, whose 1068 nucleotides are translated into 28-kd protein of 240 amino acids.[2] Inhibitor of growth 5 can induce p53-dependent apoptosis in response to DNA damage in multiple cancer cell lines via mechanisms that involve increasing p53 acetylation via recruiting p300 or activation of the cyclin-dependent kinase inhibitor p21/waf1 promoter to induce $p21^{WAF1/CIP1}$ expression.[3] This leads to the inhibition of Mdm2-mediated degradation of p53 and enhancement of the expression of p53-responsive genes at both the transcriptional and posttranslational levels.[4,5] Inhibitor of growth 5 encoding product, a tumor suppressor protein, can inhibit cell growth and induce apoptosis,[5,6] so we hypothesized that aberrant ING5 expression might be involved in the carcinogenesis and subsequent progression of tumor growth.

MicroRNAs (miRNAs) are a class of short, endogenously initiated, noncoding RNAs that posttranscriptionally control gene expression via either translational repression or mRNA degradation.[7,8] This modification of gene expression by miRNAs may play an important role in tumor development and malignant transformation. MicroRNAs control the target expression by base pairing to sequence motifs in the 3′ untranslated region (UTR) of mRNAs with perfect or near-perfect complementarities. For example, in *Drosophila*, a large number of miRNAs have been identified to perfectly complement the 3′ UTR of mRNAs, which are validated to mediate negative posttranscriptional regulation.[9,10] Of these, miR-196a has been suggested to influence growth, invasion, and metastasis in different cancer cell lines.[11] Recent studies have demonstrated that miR-196a levels allowed discrimination of normal pancreas from chronic pancreatitis and adenocarcinoma and that miR-196a levels inversely correlated with survival in pancreatic adenocarcinoma patients.[12] The miR-196a miRNAs have complementary sites on the 3′ UTRs of HOX genes representing each cluster.[13] Hornstein et al[14] described that miR-196a acts upstream of HoxB8 in vivo during limb development. Therefore, the potential function of miR-196a in pancreatic cancer needs to be explored by identifying relative target genes. In Panc-1 cells, miR-196a expression level was higher than other pancreatic cancer cells,[15] so we focused on Panc-1 cells in this study.

According to the prediction of TargetScan software, the ING5 mRNA 3′ UTR contains a sequence motif (position 80–86), which is nearly complementary to miR-196a sequence and may be a putative binding site for miR-196a. So we hypothesized that miR-196a regulates ING5 expression, thereby affecting pancreatic cancer cell proliferation, apoptosis, and invasion. Such data have not been presented in pancreatic cancer cells, and whether miR-196a affects ING5 expression has yet to be discovered.

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MATERIALS AND METHODS

Cell Culture

Human pancreatic adenocarcinoma cells (Panc-1) were obtained from the Chinese Academy of Sciences Institute (CRL-1469). Cells were maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2 humidified atmosphere.

Construction and Transfection of Lentiviral Vectors With miR-196a

The lentiviral vectors expressing human pre-miR-196a gene, the miR-196a inhibitor (antagomir), and scrambled sequences were purchased from GeneCopoeia (Guangzhou, China) (Fig. 1A). Briefly, the miRNA inhibitor expressing clone (pEZX-AMR-196a) was based on Cherry-tagged vector pEZX-AM03. A sequence expressing antagonist against miR-196a was inserted under the control of the H1 promoter. The transcription of the hairpin precursor following the coding sequence of enhanced red fluorescent protein (eRFP) gene was driven by an H1 promoter. The precursor miRNA expression clone (pEZX-miR-196a) was constructed using the vector pEZX-AM04. The pEZX-AM04 plasmid with a scrambled sequence (CmiR0001-MR04) was used as a negative control vector. The transcription of the hairpin precursor following the coding sequence of enhanced green fluorescent protein (eGFP) gene was driven by a cytomegalovirus promoter. This vector allows for processing of precursor miRNAs (approximately 150 nucleotides in length) to generate mature miRNAs using the enzymes involved in the RNAi machinery. Lentivirus packaging and titer determination followed the standard instructions described previously.[16,17]

The lentiviral vectors expressed eGFP and eRFP, which allowed for titrating and measuring their infection efficiency in transfected cells (Fig. 1B). The viral titer was determined by counting GFP- and RFP-positive cells after transfection. The lentiviral vectors were transfected into the Panc-1 cells with a multiplicity of infection from 30 to 50 in the presence of polybrene (6 μg/mL).

Reverse Transcriptase–Polymerase Chain Reaction and Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA from Panc-1 was extracted using the Trizol (Invitrogen, Osaka, Japan) reagent method. A TaqMan miRNA real-time reverse transcriptase–polymerase chain reaction (RT-PCR) kit (Applied Biosystems, New York, NY) was used to detect and quantify the MiR-196a amounts in total RNA. Briefly, 2 μL of total RNA was reverse transcribed by TaqMan miRNA RT kit. Negative controls were included with every real-time RT-PCR assay, and no amplification of the signal was detected when nuclease-free water was added instead of RNA or cDNA sample. Data were analyzed with 7500 software v.2.0.1. (Applied Biosystems), with the automatic Ct setting for adapting baseline and threshold for Ct determination. Reverse transcriptase–PCR assays were performed in triplicate on each cDNA sample. Data obtained were translated into $2^{-\Delta\Delta CT}$ (relative quantification).

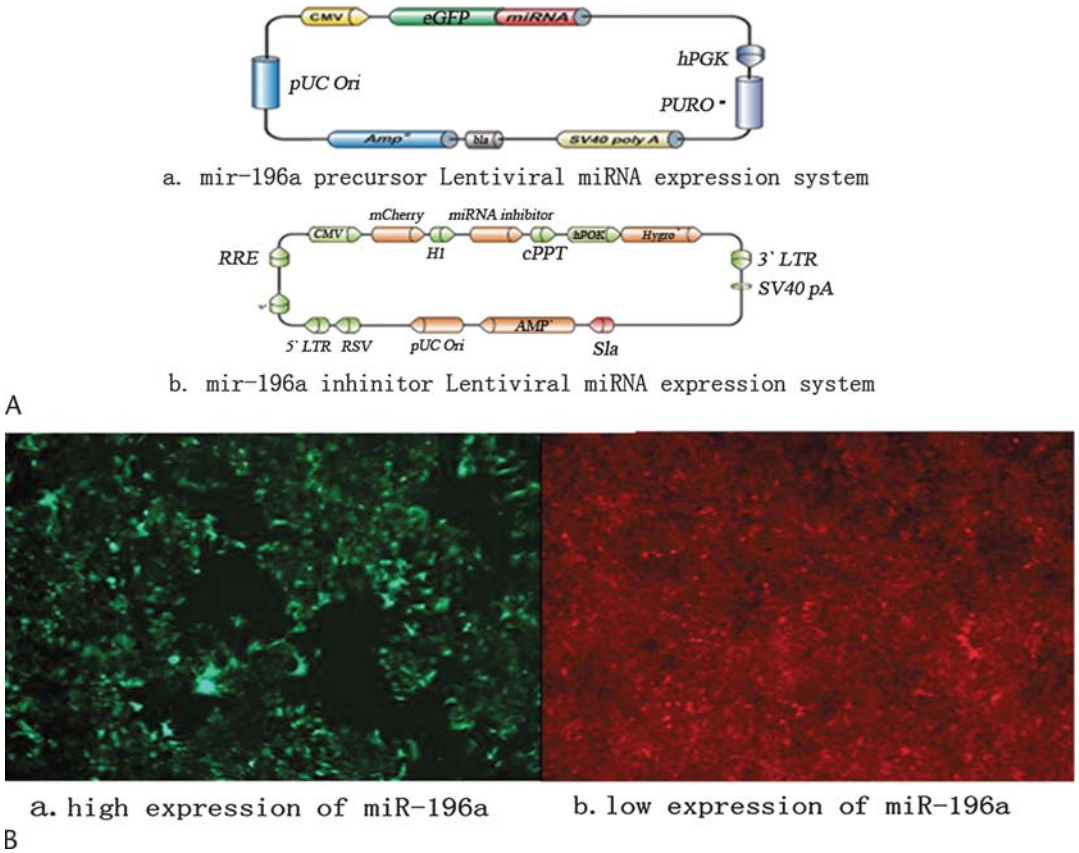

FIGURE 1. Construction of lentiviral-mediated miRNA. A, Lentiviral miRNA expression system. B, Lentiviral vectors express eGFP and RFP, which allowed for titrating and measuring their infection efficiency in transfected cells.

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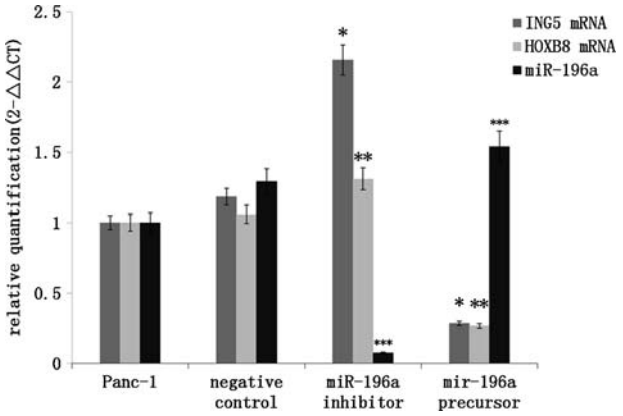

FIGURE 2. Expression of miR-196a in Panc-1 cells with miR-196a precursor, inhibitor, and scrambled RNA oligomer transfection. The figure demonstrates an inverse relationship between ING5 gene expression and miR-196a levels in Panc-1 pancreatic cancer cells. At 48 hours after transfection with miR-196a precursor, inhibitor, and negative control, total RNAs were isolated from Panc-1 cells, and comparative real-time quantitative PCR was performed to quantify the expression of ING5 and HOXB8 genes using human GAPDH as control. *Y* axis represents $2^{-\Delta\Delta CT}$ values. For relative quantitation, mRNA expression level in control cells (Panc-1 cells) was set at 1. Data represent mean ± SD (n = 3). $^{*}P < 0.005$, $^{**}P < 0.01$, $^{***}P < 0.005$ (control vs transfection).

First-strand cDNA was synthesized in a volume of 20 μL using 1 μg of total RNA and TaqMan reverse transcription reagents (Applied Biosystems). We carried out real-time RT-PCR using SYBR Premix Ex Taq II kit (TAKARA, Shiga, Japan). According to the Genbank (NM_032329.4), oligonucleotide primers for ING5 were designed as follows: forward: 5′-GGGAGATGATTGGCTGTG-3′ and reverse: 5′-CCTTTGGGTTTCGTGGTA-3′ (614–759, 146 bp). The primers for the internal control, GAPDH, were forward: 5′-CAATGACCCCTTCATTGACC-3′ and reverse: 5′-TGGA AGATGGTGATGGGATT-3′ (201–335, 135 bp; NM_002046.3). The primers for the positive control, HOXB8, were forward: 5′-AATTTCTACGGCTACGACCC-3′ and reverse: 5′- TGGAACCAGATTTTGACCTG-3′ (485–819, 335 bp; NM_024016.3). Polymerase chain reaction amplification of cDNA was performed in 20 μL mixtures containing 10 μL SYBR Premix Ex Taq (×2) with 0.08 μL of each primer, 0.4 μL of ROX Reference Dye, and 2 μL of template cDNA (100 μg/μL). The protocol included the following parameters: an initial 30 seconds of incubation at 95°C followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing at 60°C for 34 seconds.

Western Blot Analysis on ING5 Protein Expression

Cells were digested with trypsin solution and lysed with RIPA buffer. Total proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, Mass). After blocking with 5% nonfat dry milk, the membrane was incubated overnight at 4°C with primary antibodies (rabbit anti–human ING5 polyclonal antibodies [1:1000; Proteintech Group Inc, Chicago, Ill]). The membrane was then incubated with a horseradish peroxidase–conjugated secondary antibody

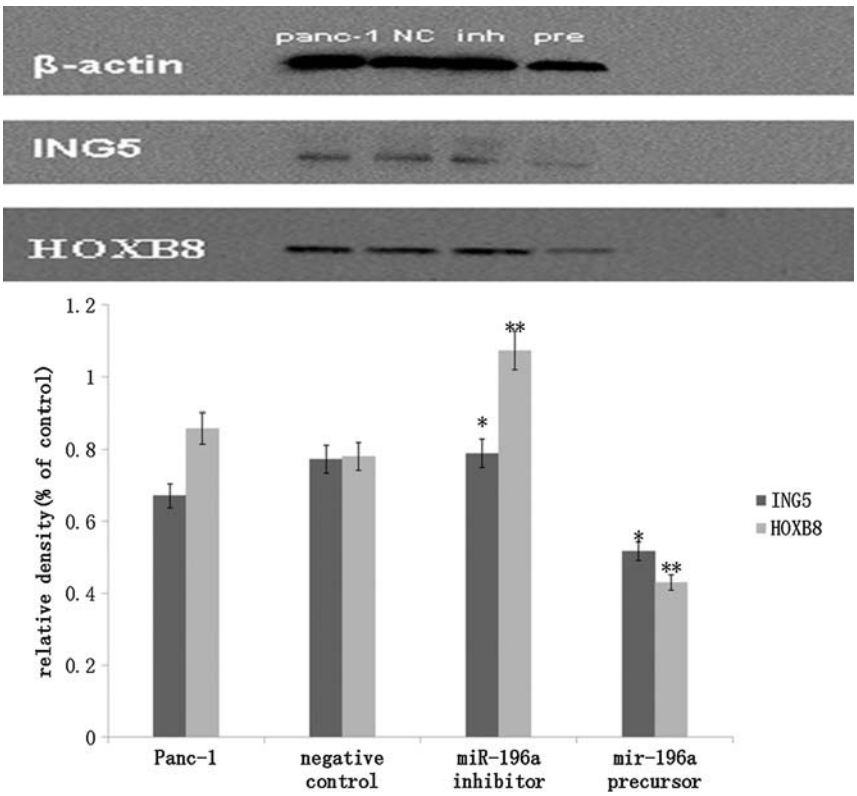

FIGURE 3. Expression of ING5 and HOXB8 in Panc-1 cells transfected with miR-196a precursor, inhibitor, and negative control. The signals were quantitated using ImageJ for Windows software (Wayne Rasband, National Institutes of Health, Bethesda, Md). Data represent mean ± SD (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ (control vs transfection).

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(M21002; 1:2000; Abmart, Shanghai, China) at room temperature. Protein bands were visualized by Supersignal West Pico Trial Kit (Thermo, Waltham, Mass). β-Actin was detected by using rabbit anti–human β-actin polyclonal antibody (P30002; Abmart), used as the internal control. HOXB8, a promising native target gene of miR-196a, was also detected by polyclonal antibody (ab85745; 1:2000; Abcam, Cambridge, UK) as a positive control.

Luciferase Activity Assay

The portion of the 3′ UTR region (677 bp) of human ING5 gene containing the miR-196a binding site was amplified by PCR using the following primers: ING5-3′ UTR-F: 5′-atcg-GAATTCacctgttaaggaggaacgcctg-3′ and ING5-3′ UTR-R: 5′-gcgtGTCGACCACAGGAAAGCTACGCGAGG-3′. This portion was amplified and cloned into the *Eco*RI and *Sal*I site of the pMIR (three dimension high throughput screening). For the luciferase reporter assay, the HEK 293Tcell lines were cotransfected with luciferase reporter vectors (10 ng/well) and miR-196a mimics (50 nM) using Lipofectamine 2000 (Invitrogen). A 2-ng pRL-TK Renilla luciferase construct was used for normalization. After 48 hours, luciferase activity was analyzed by the Dual-Luciferase Reporter Assay System according to the manufacturer's protocols (Promega, Madison, Wis).

Measurement of Cell Apoptosis by Flow Cytometry

Cells were fixed in 70% cold ethanol and stored at −20°C overnight. Fixed cells were washed twice with phosphate-buffered solution (PBS) and stained in annexin V–fluorescein isothiocyanate (FITC) (20 μg/mL, 15 minutes)/propidium iodide (50 μg/mL, 2 minutes) (eBioscience, San Diego, Calif) solutions. Flow cytometry was then performed using BD Calibur cytometer to examine cell apoptosis. Gating was selected using untreated cells to separate most double-negative cells from minor populations of propidium iodide or annexin V–FITC–positive cells. Cells that were $FITC^-/PI^-$ were considered viable, $FITC+/PI^-$ cells were considered early apoptotic, and FITC+/PI+ cells were considered nonviable.

Cell Proliferation Assay

Cell proliferation was determined using a cell count kit (CCK-8; Dojindo, Kumamoto, Japan). Briefly, cells were seeded into 96-well plates at 5×10^4 cells/well and then were transfected

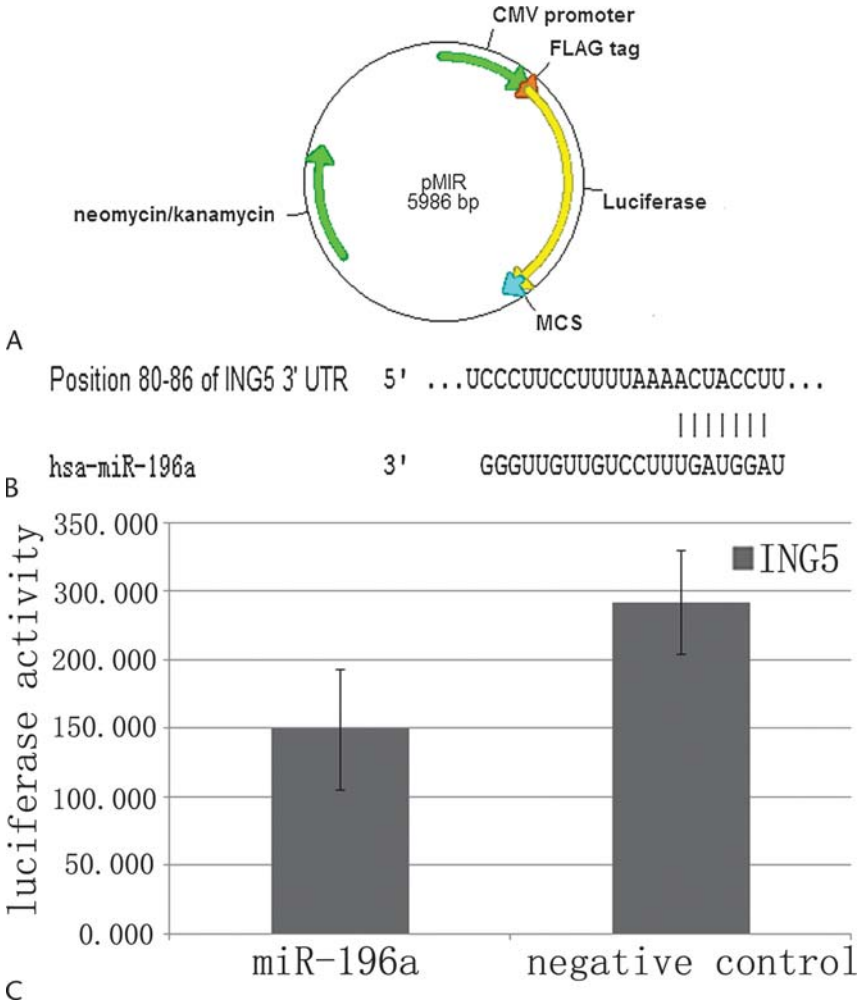

FIGURE 4. MiR-196a binds to 3′ UTR of ING5 mRNA in Panc-1 cells. A, ING5 luciferase-3′ UTR constructs. B, A nearly complementary sequence to miR-196a in ING5 3′ UTR (80-86). C, Luciferase activity in cells cotransfected with miR-196a mimics and ING5 luciferase–3′ UTR constructs, miR-196a can suppress the luciferase expression (*$P < 0.05$).

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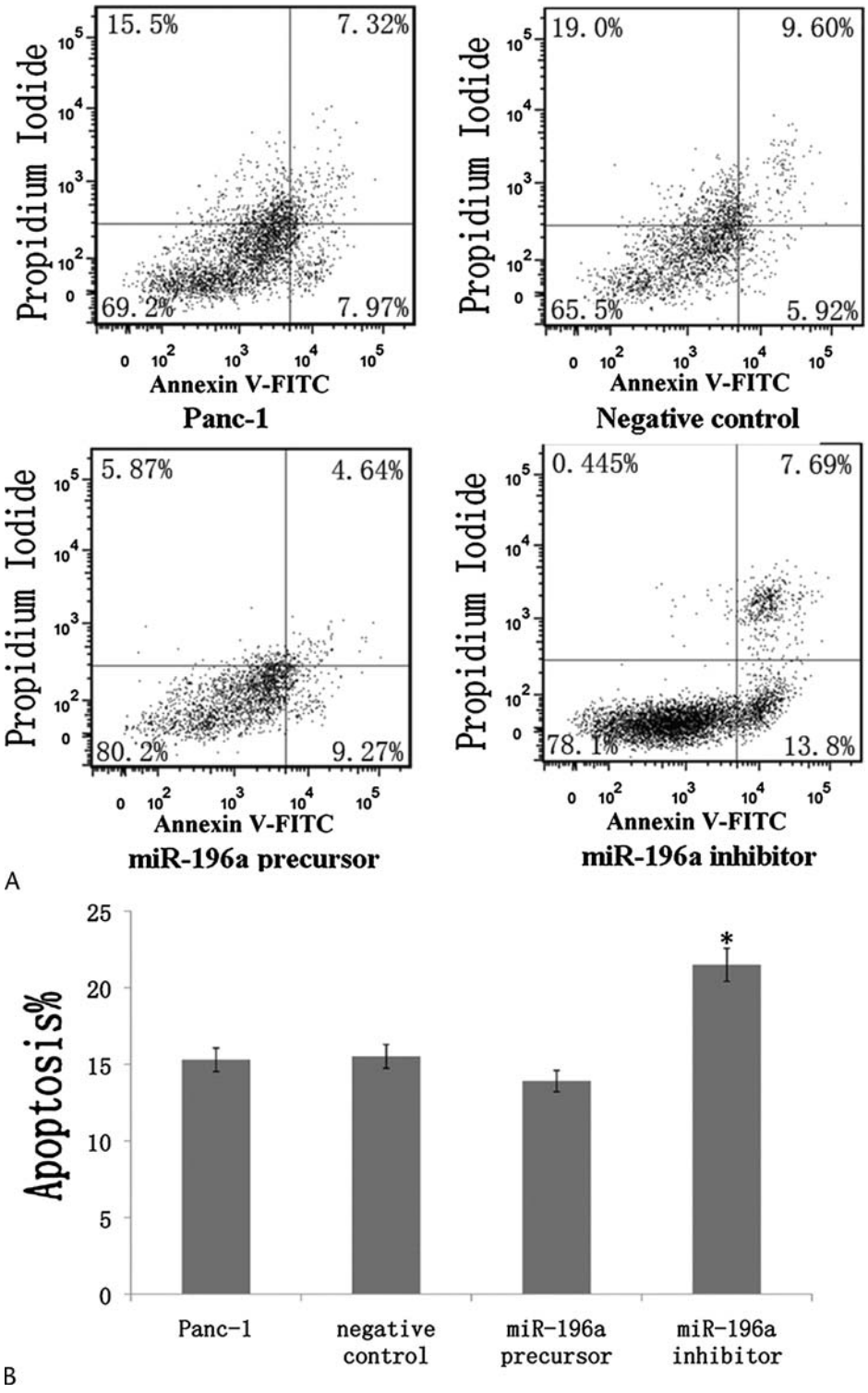

FIGURE 5. Apoptosis of cells transfected with miR-196a mimics, inhibitor, and negative control, measured by flow cytometry. No significant differences in the rate of apoptosis were observed in the cells transfected with miR-196a precursor compared with the negative control cells (13.91% ± 0.69% vs 15.52% ± 0.78%, $P > 0.05$), whereas cells transfected with miR-196a inhibitor showed a significant increase in the rate of apoptosis when compared with the controls (21.49% ± 1.05% vs 15.52% ± 0.78%, $*P < 0.05$).

with various concentrations of miR-196a precursor, miR-196a inhibitor, or negative control (Ambion, New York, NY). CCK-8 assay was performed at various time points ranging from 0, 24, 48, 72, and 96 hours. After 4-hour incubation with cell culture medium containing CCK-8 reagent (10 μL), at each time point, absorbance at 450 nm was detected using a microplate enzyme-linked

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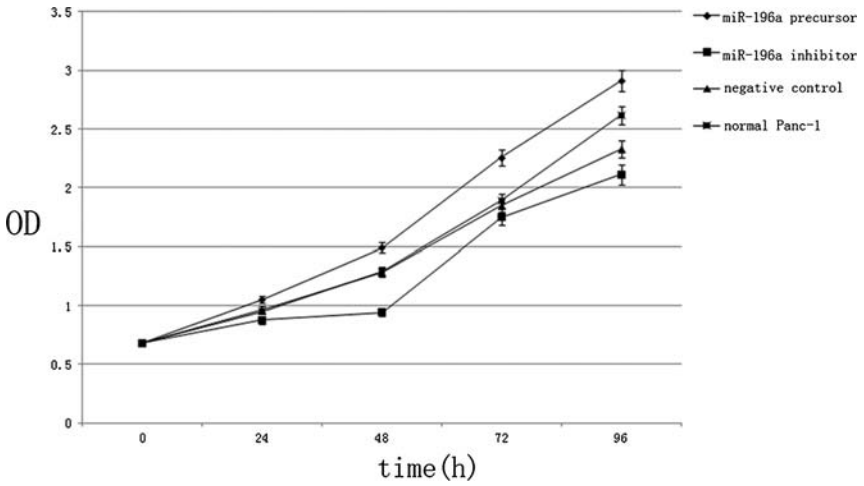

FIGURE 6. Proliferation of Panc-1 cells measured by CCK-8 assay. The cells showed significantly extensive proliferation 48 hours after transfection in CCK-8 assay (OD 1.49 ± 0.07 vs 1.05 ± 0.05, $P < 0.05$; Fig. 5), whereas cells transfected with miR-196a inhibitor exhibited decreased proliferation, at 48 hours after transfection (OD 0.94 ± 0.04 vs 1.05 ± 0.05, $P < 0.05$).

immunosorbent assay reader (MULTISKAN MK3; Thermo/Labsystems, Vantaa, Finland).

Transwell Invasion Assay

The ability of Panc-1 cells to pass through filters coated with Matrigel (BD Biosciences, San Diego, Calif) was measured by Transwell invasion assay. Matrigel was diluted to 200 μg/mL with cold filtered distilled water and applied to the top of the 8-μm-pore polycarbonate filter. Briefly, Panc-1 cells (4×10^5 cells/mL) were treated with miR-196a precursor, miR-196a inhibitor, or negative control (Ambion). After 48 hours, cells were detached using trypsin and resuspended in serum-free medium. Medium containing 10% fetal bovine serum was applied to the lower chamber as a chemoattractant, and then cells were seeded on the upper chamber at a density of 1×10^5 cells/well in 50 μL of serum-free medium. The chamber was incubated for 8 hours at 37°C. At the end of incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab. Cells invading across the Matrigel to the lower surface of the membrane were fixed with methanol and stained with 5% hematoxylin solution. The invading cells on the lower surface of the membrane filter were counted with a light microscope. The data are presented as the average number of cells attached to the bottom surface from randomly chosen fields. Each experiment was carried out in triplicate.

In Vivo Animal Model of Pancreatic Cancer

The animals used in this study received humane care in compliance with the Guide to the Care and Use of Experimental Animals formulated by the Medical Ethical Committee on animal experiments of the Second Military Medical University. Eighteen 4-week-old nude mice weighing 18 to 20 g were separated into 3 groups: miR-196a precursor, miR-196a inhibitor, and control group. The nude mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). In a mini laparotomy, the recipient nude mice pancreas was exposed, and a small stab wound made in the pancreas parenchyma with a knife blade. The lentiviral-transfected Panc-1 cell suspension (1×10^5 cells/mL, 0.2 mL) was inoculated under the parenchyma of the pancreatic tail. Any leakage of the cell suspension into abdominal cavity was carefully removed with 75% ethanol to avoid peritoneal metastasis.

Measurement of Tumor Volume by Ultrasonic Imaging

Ultrasonic inspection was performed by using a GF-UCT240-AL5 (Olympus Co Ltd, Tokyo, Japan) endoscopic ultrasound 4 weeks after implantation. After anesthetizing the animal by intraperitoneal injection of sodium pentobarbital (50 mg/kg), the mouse abdomen was soaked with sterile deionized water. The ultrasonic images were acquired using the endoscopic ultrasound probe with a water bag and by direct contact method. The long (a) and short (b) diameters were measured from the ultrasonic images. Volumes of the tumors were calculated according to the following formula: $a \times b^2 / 2$. The mice were killed after measurement of tumor size.

TUNEL Analysis

The sections of pancreatic cancer were incubated with proteinase K for 15 minutes at room temperature. After rinsing in PBS–Tween 20 for 2 minutes twice, sections were incubated in 3% H_2O_2 in PBS for 10 minutes to block endogenous peroxidase activity. After washing with PBS twice, sections were labeled (60 minutes; 37°C) with fluorescein TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) reagent mixture according to the manufacturer's instructions.[18] After rinsing with PBS thrice, these sections were visualized under an Olympus (Olympus Co Ltd) inverted microscope, and representative images were captured with a camera.

Immunohistochemical Staining

Formalin-fixed tissues were dehydrated, embedded in paraffin, and sectioned. Tissue sections were deparaffinized, rehydrated, and incubated for 30 minutes in 0.3% hydrogen peroxide in methanol and then for 10 minutes with 1% goat serum in Tris-buffered saline (TBS). Then the sections were incubated with rabbit anti–human anti-ING5 antibody (1:800; Proteintech Group Inc) at room temperature overnight. After washing 3 times in TBS, the sections were incubated with biotinylated mouse anti–rabbit immunoglobulin G (1:5000; Abcam) for 30 minutes and followed by three 5-minute wash in TBS. The final incubation was for 30 minutes with horseradish peroxidase–avidin D at 37°C. The peroxidase was detected with 0.05% 3,3-diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin and mounted in neutral

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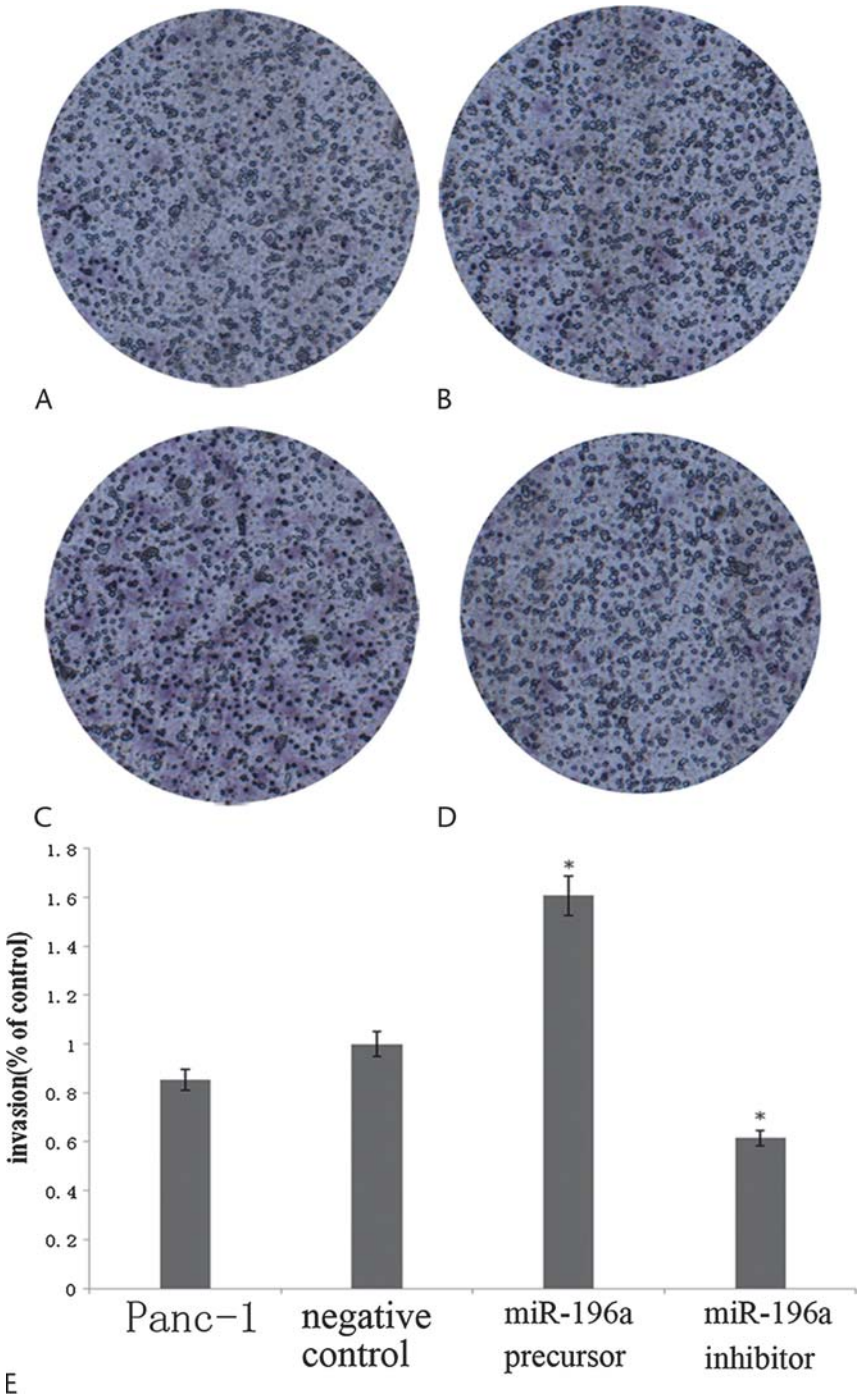

FIGURE 7. Effect of miR-196a on invasion of Panc-1 cells. Panc-1 cells (A) untreated then subjected to analyses for Transwell invasion assay as described in Materials and Methods; Panc-1 cells were treated with scrambled RNA oligomer (B), miR-196a pre-miR precursor (C), and miR-196a anti-miR miRNA inhibitors (D) for 48 hours, then cell invasion was measured by Transwell migration for 8 hours; polycarbonate filters (pore size, 8 μm) were precoated with Matrigel. Cells on the bottom of filter were fixed, stained, and counted. *$P < 0.05$(vs control, E).

gum medium for light microscopy. Positive protein expression was visualized as nuclear localization of granular brown-yellow precipitate. The counts were performed in 3 high-power fields of vision under a high magnification (×400) for each section. In the proliferation (Ki67) assay with the same methods as described above, primary antibody is Ki67 (Sc-7846; 1:100; Santa Cruz, Dallas, Tex), Secondary Antibody is peroxidase-conjugated Affinipure Rabbit anti–goat immunoglobulin G (67164; 1:1000; Jackson Immunoresearch, West Grove, Penn). The percentage of positive cells was calculated as the ratio of positive cells to the total number of cells. The scoring scale for the percentage of positive cells was as follows: 0 = less than 1%; 1 = 1% to 24%; 2 = 25% to 50%; 3 = 51% to 75%; 4 = more than 75%. The scoring scale for staining intensity was as follows: 0 = no color;

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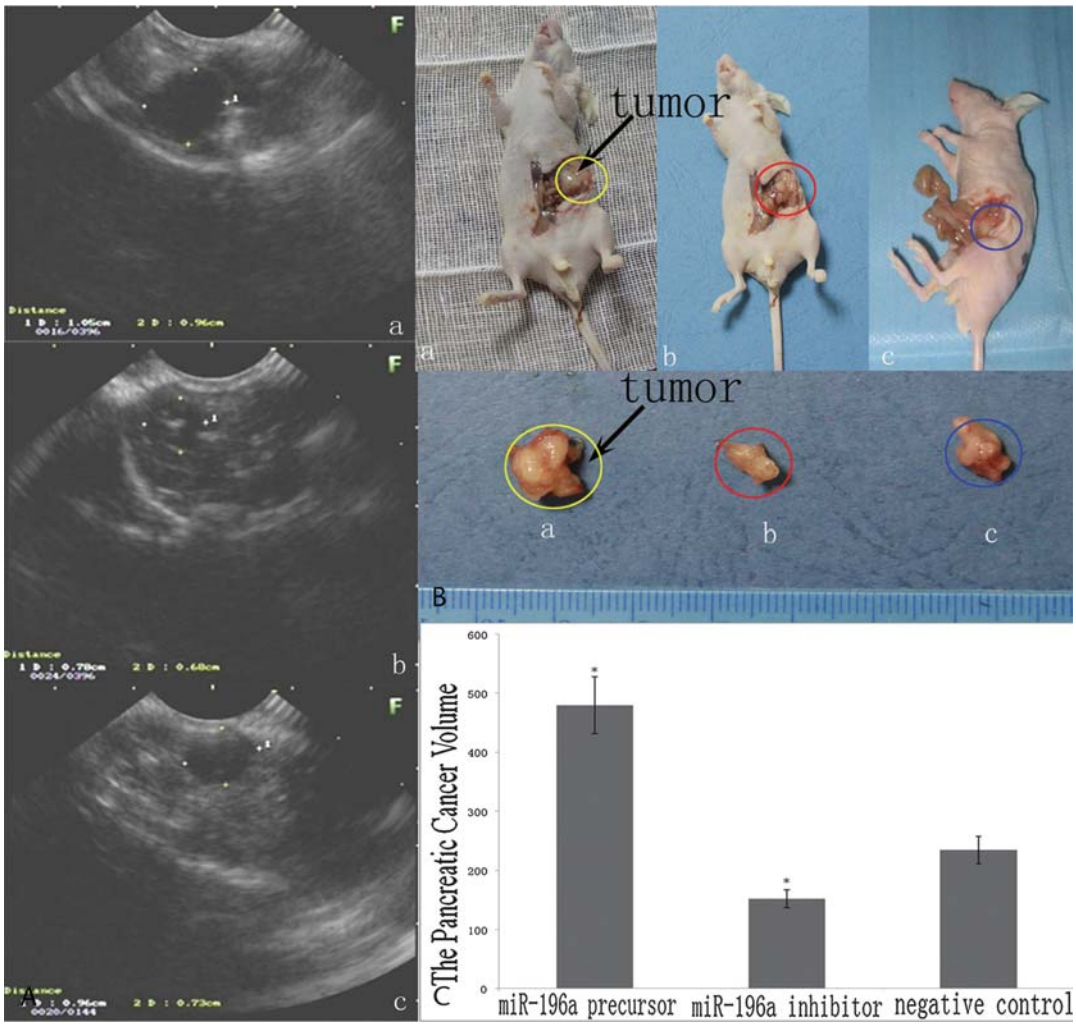

FIGURE 8. Tumor volume of pancreatic cancer after implantation. A, A representative ultrasound image. B, Photographs of tumors and (C) histogram show in vivo tumor growth in miR-196a inhibitor–transfected mice was significantly less compared with miR-196a precursor–transfected mice and negative controls.

1 = bright yellow; 2 = yellow; 3 = brown yellow; 4 = brown. The final score was obtained by multiplying the percentage of positive cells by the staining intensity score.

Statistical Analyses

All experiments were repeated 3 times, and data presented as mean ± SD. Student *t* test was used to calculate statistical significance between groups; $P < 0.05$ was considered statistically significant.

RESULTS

MiR-196a Down-regulates ING5 Expression in Panc-1 Cells

Reverse transcriptase–polymerase chain reaction of miR-196a precursor-transfected cells showed high expression of miR-196a when compared with normal control cells (relative quantity [RQ] = 1.54, $P < 0.01$; Fig. 2), and cells transfected with miR-196a inhibitor showed low expression of miR-196a when compared with control cells (RQ = 0.08, $P < 0.01$; Fig. 2). Reverse transcriptase–PCR showed decreased ING5 expression in miR-196a precursor-transfected cells compared with those in the negative control, whereas an opposite trend was found in the cells transfected with miR-196a inhibitor. Inhibitor of growth 5 mRNA levels were indeed suppressed in miR-196a precursor-transfected cells compared with the negative and normal control cells (RQ = 0.29, $P < 0.05$; Fig. 2). Western blot analysis revealed that ING5 protein levels were also suppressed in miR-196a precursor-transfected cells compared with the negative control cells ($P < 0.05$; Fig. 3). The decrease in the ING5 mRNA levels was accompanied by a decrease in ING5 protein. On the whole, these experiments demonstrated that increasing the level of miR-196a resulted in concomitant suppression of the mRNA as well as protein levels of ING5 in Panc-1 cell lines. This finding provides evidence that ING5 is probably a target of miR-196a.

MiR-196a Down-regulates ING5 by Binding Its 3′ UTR in Panc-1 Pancreatic Cancer Cells

The experimental results of Luciferase Reporter Gene Activity Assay show that miR-196a can suppress the luciferase expression with 38% inhibition rate (Fig. 4). Besides, this suppression relates to 3′ UTR of ING5, through which miR-196a was possible to suppress the luciferase expression. Based on

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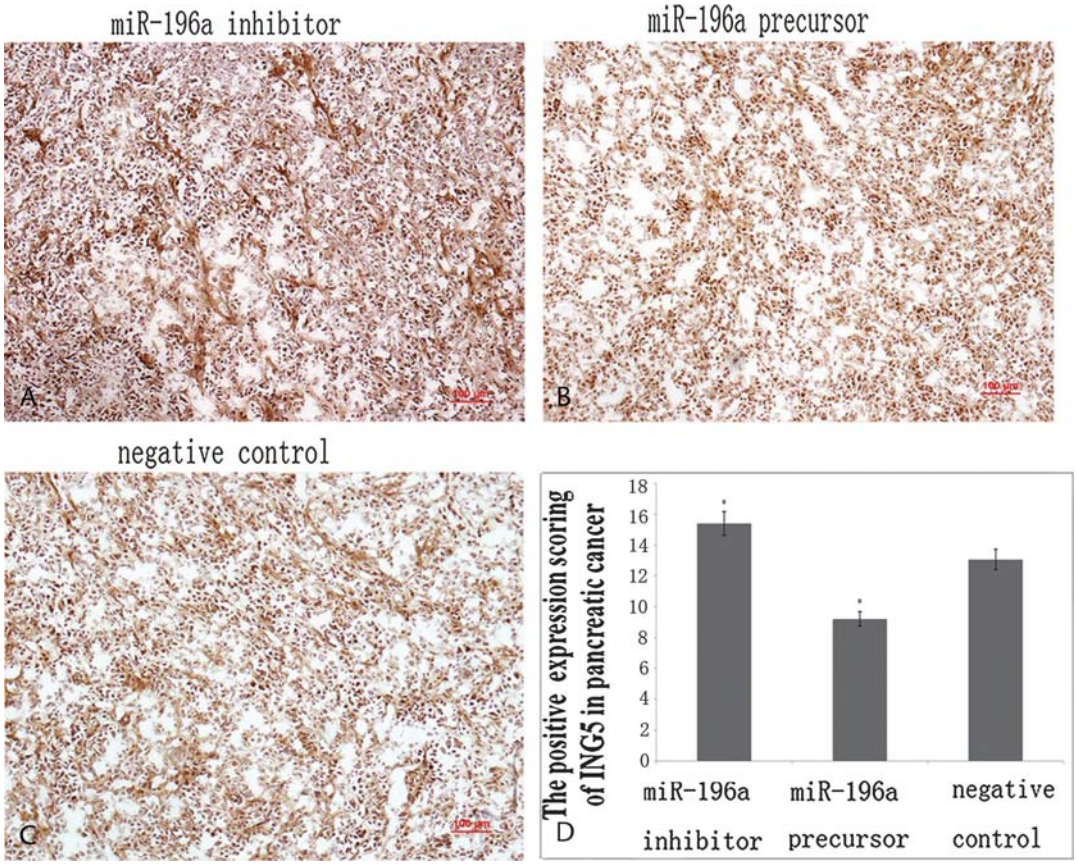

FIGURE 9. Immunohistochemical staining for ING5 in pancreatic cancer. Representative staining sections for ING5 were prepared as described in Materials and Methods. The brownish yellow spots represent positive staining for ING5. Scale bars represent 100 μm.

that, we also tested the influence of miR-196a on the ING5 protein expression in pancreatic cancer cells. As a result, Western blot indicates that miR-196a is able to suppress ING5 expression (Fig. 3).

MiR-196a Inhibits Apoptosis of Panc-1 Cells

Flow cytometery was used to examine Panc-1 cell apoptosis (Fig. 5). Following transfection, no significant differences in the rate of apoptosis were observed in the cells transfected with miR-196a precursor compared with the negative control cells (13.91% ± 0.69% vs 15.52% ± 0.78%, $P > 0.05$), whereas cells transfected with miR-196a inhibitor showed a significant increase in the rate of apoptosis when compared with the controls (21.49% ± 1.05% vs 15.52% ± 0.78%, $P < 0.05$). These results show that the inhibition miR-196a expression can promote Panc-1 cell apoptosis, but overexpression of miR-196a had no effect on apoptosis.

MiR-196a Promotes Proliferation of Panc-1 Cell

After transfection with miR-196a precursor, we examined the proliferation of Panc-1 cells at different time points (0, 24, 48, 72, 96 hours), and the cells showed significantly extensive proliferation 48 hours after transfection in CCK-8 assay (optical density [OD] 1.49 ± 0.07 vs 1.05 ± 0.05, $P < 0.05$; Fig. 6), whereas cells transfected with miR-196a inhibitor exhibited decreased proliferation, at 48 hours after transfection (OD 0.94 ± 0.04 vs 1.05 ± 0.05, $P < 0.05$). At the 72- and 96-hour time points, transfection with miR-196a precursor led to significantly increased proliferation of Panc-1 cells, but there was no significant difference in proliferation between the cells transfected with miR-196a inhibitor and negative control. At 24 hours, the proliferation of the transfected and control cells was not significantly different.

miR-196a Promoted Invasion in Panc-1 Cells

To investigate whether miR-196a could affect invasive properties of Panc-1 cancer cells, Transwell invasion assays were used. Figure 7 shows that cells transfected with miR-196a inhibitor exhibited significantly less invasion with cell numbers of 58.5% ± 1.9% compared with 99.1% ± 4.9% (% of control, $P < 0.05$) as seen in the negative control. Panc-1 cells transfected with miR-196a precursor, on the other hand, showed a significant increase in Transwell invasion when compared with the controls (160.7% of control, $P < 0.05$). These results demonstrate that miR-196a could significantly affect the invasive ability of pancreatic cancer cells.

Tumor Volume of Pancreatic Cancer at 4 Weeks After Implantation

Representative ultrasonic images at 4 weeks after implantation are shown in Figure 7. The tumor volume in the miR-196a precursor–transfected Panc-1 cell group (479.73 ± 58.50 v/mm^3, Fig. 8A) was significantly larger than the control group (234.45 ± 20.81, $P < 0.01$; Fig. 8B). Cells transfected with miR-196a inhibitor revealed an opposite trend showing a significant reduction in tumor volume (152.0 ± 32.1, $P < 0.01$; Fig. 8C), when compared with the controls. These results suggest that high expression of miR-196a promotes tumor growth as evidenced

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TABLE 1. Positive Expression Scoring of ING5 Protein in Pancreatic Cancers After Implantation

	miR-196a Inhibitor	miR-196a Precursor	Negative Control
ING5	15.39 ± 4.15	9.21 ± 3.54	13.07 ± 3.98

by the increased tumor volume, whereas decrease in miR-196a expression leads to a reduction in tumor volume.

Immunohistochemistrical Staining for ING5 in Pancreatic Cancer After Implantation

Inhibitor of growth 5 protein expression was detected as brownish yellow spots by immunohistochemical staining (Fig. 9). The brownish yellow staining for ING5 was observed in every group. However, ING5 staining was significantly weaker in miR-196a precursor–transfected Panc-1 cells compared with the miR-196a inhibitor–transfected Panc-1 cells. Table 1 shows the quantitation of positive ING5 protein expression. MiR-196a inhibitor–transfected Panc-1 cells had an ING5 protein expression scoring of 15.3 ± 4.15 that was significantly higher than that in the miR-196a precursor–transfected Panc-1 cells (9.21 ± 3.54, $P < 0.05$). The expression changes in the ING5 protein expression in the animal model were in agreement with those observed in in vitro cells transfected with miR-196a precursor or inhibitor. These results suggest that regulation of miR-196a can alter the expression of ING5 in pancreatic cancer.

MiR-196a Promotes Proliferation and Inhibits Apoptosis in Pancreatic Cancer After Implantation

Cell apoptosis in pancreatic cancer tissues after implantation was studied using the TUNEL method (n = 24). Animals in the control group, which did not receive MCA ligation, showed almost no TUNEL-positive cells in their pancreatic cancer tissues. Animals with miR-196a precursor and negative control group contained fewer TUNEL-positive cells than those in the miR-196a inhibitor group (Fig. 10). The results were consisted with the in vitro apoptosis experiments. MiR-196a precursor (15.78 ± 4.12) showed significantly extensive proliferation than miR-196a inhibitor (7.13 ± 2.16) and negative control (8.34 ± 2.79) ($P < 0.05$) in pancreatic cancer after implantation (Fig. 11).

DISCUSSION

As is the case with most malignant tumors, metastasis in pancreatic carcinomas is associated with an increased mortality rate.[19] Pancreatic cancer has long been the leading cause of

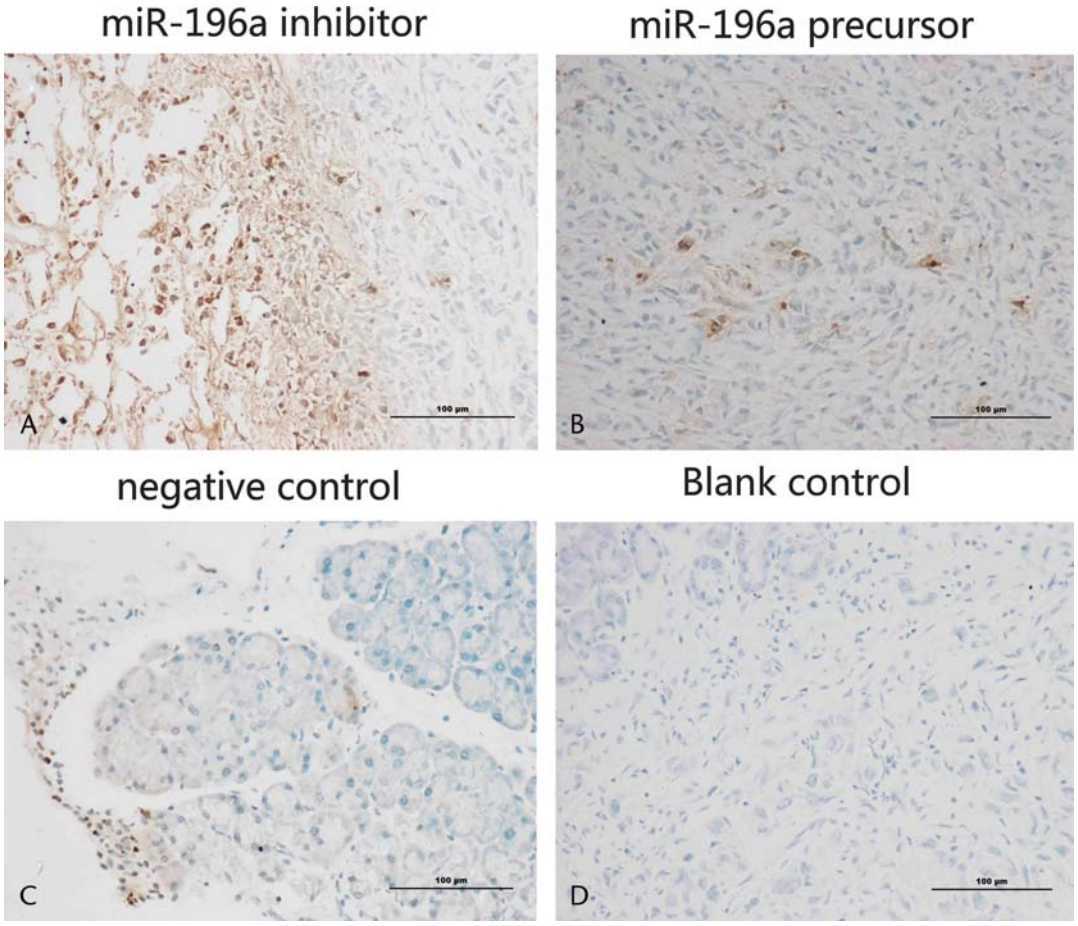

FIGURE 10. TUNEL analysis. MiR-196a inhibits apoptosis in pancreatic cancer after implantation. Animals with miR-196a precursor and negative control group contained fewer TUNEL-positive cells than those in the miR-196a inhibitor group.

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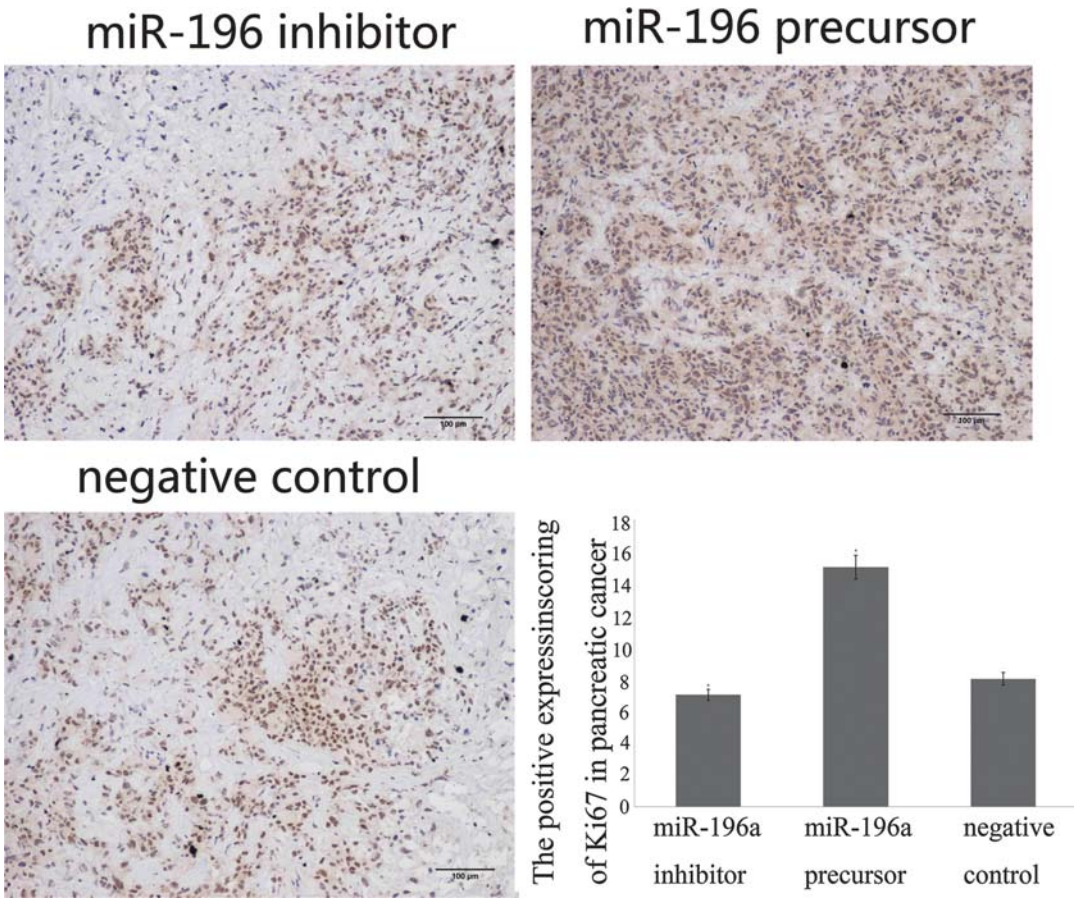

FIGURE 11. Immunohistochemical staining for Ki67 in pancreatic cancer. Representative staining sections for Ki67 were prepared as described in Materials and Methods. The brownish yellow spots represent positive staining for Ki67 (*$P < 0.05$). Scale bars represent 100 μm.

cancer-related deaths in both developed and developing countries. Unfortunately, there are limited treatment options available for this disease because chemotherapy and radiation therapy are largely ineffective, and metastasis occurs even after potentially curative surgery.[20–23] Expression patterns of miRNAs are systematically altered in pancreatic cancer as described by Bloomston et al[12] and Szafranska et al.[24] In particular, Bloomston et al[12] looked at the pattern of miRNA expression with respect to long-term (~24 months) survival, and they found that miR-196a levels were inversely correlated with survival in pancreatic adenocarcinoma patients. Also, our previous data showed that the plasma levels of miR-196a have been found to be significantly higher in patients with pancreatic adenocarcinoma than in healthy controls.[15] MiR-196a has always been overexpressed in pancreatic cancer and is related to the poor prognosis of these patients.

Our present study reveals the impact of miR-196a on proliferation, invasion, and apoptosis of Panc-1 pancreatic cancer cells. We observed that miR-196a could regulate apoptosis in Panc-1 cells. Using flow cytometry, cells transfected with miR-196a inhibitor showed a significant promotion of apoptosis when compared with control cells, suggesting the inhibitory effect of miR-196a in the apoptosis of Panc-1 cells. Transfection with miR-196a precursor did not affect the apoptosis of Panc-1 cells, and this may be due to the overexpression of miR-196a in these cells,[17] which in turn leads to a maximal inhibition of apoptosis genes, and further increases in miR-196a levels, by transfection, have no effect on Panc-1 cells apoptosis. Meanwhile, inhibition of miR-196a expression can lead to the up-regulation of apoptosis genes, thereby leading to an increase in Panc-1 cell death. Schimanski et al[25] reported high levels of miR-196a promoted cancer cell detachment, migration, and invasion but had no effect on the proliferation or apoptosis of colorectal cancer cells. This further illustrates that miR-196a may be tissue and cell type specific in its regulation of apoptosis. Moreover, our results warrant further studies to confirm the relationship between miR-196a and apoptosis.

In the CCK-8 proliferation assay, cells showed significantly high proliferation at 48 hours following transfection with miR-196a precursor, whereas cells transfected with lentiviral vectors of miR-196a inhibitor exhibited significant reduction in proliferation, at 48 hours. These results strongly indicate that miR-196a can promote proliferation of Panc-1 pancreatic cancer cells. To further illustrate the relationship between miR-196a and prognosis in pancreatic cancer patients, we performed a Transwell invasion assay. The results showed that down-regulation of miR-196a inhibited invasion in Panc-1 cells. Zhang et al[26] also reported that miR-196a can promote pancreatic cancer cell invasion. Our in vivo experiments showed that mice transplanted with miR-196a inhibitor–transfected Panc-1 cells developed significantly smaller tumors compared with the controls, whereas the mice that received miR-196a

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precursor–transfected cells developed significantly bigger tumors when compared with controls. These results were consistent with our in vitro studies, further indicating the influence of miR-196a in pancreatic tumor growth. These findings and concepts disclosed here provide an important basis for further exploration to understand the role of miR-196a and its effect in tumor metastasis and prognosis in pancreatic carcinomas.

However, there has been no previous report about the influence of miR-196a in pancreatic cancer or its mechanism of action in tumor growth and metastasis. Our study provides an insight into the possible role of miR-196a and its influence on ING5 expression in pancreatic carcinomas. Further comprehensive studies, particularly as to miR-196a's exact targets and its role in tumor metastasis, remain to be explored. There is, however, evidence that some tumor-associated genes are regulated by miR-196a in normal cells as well as other cancers.

Hornstein and colleagues[14] described HOXB8 as a restriction target of miR-196a and identified HOXA7, HOXC8, and HOXD8 as additional restriction targets in humans. HOX genes are known to be master regulators of embryogenesis and oncogenesis.[27] High-mobility group A2 gene product was identified as a putative target of miR-196a-2.[28] Luthra et al[29] reported annexin A1 as another target gene of miR-196a. Annexin A1, also known as lipocortin or p35, is a well-characterized member of the calcium- and phospholipid-binding protein family of annexins. A potential miR-196a target recognition site is present at the annexin A1 mRNA 3′ UTR, and the conserved sequence is observed in 3 species. Several other genes have been identified to be target molecules of miR-196a, including S100 calcium-binding protein A9, small proline-rich protein 2C, keratin 5, CLCA family member 2 (achloride channel regulator), cytochrome P450 (family 4, subfamily B, polypeptide 1), keratin 4, LDOC1, leukotriene A4 hydrolase, pleiotrophin, T-cell differentiation protein, tumor protein D52-like1, visinin-like 1, and v-ETS erythroblastosis virus E26 oncogene homolog (ERG).[30]

These above data clearly reveal that miR-196a is involved in the regulation of multiple tumor-related genes. In this study, we aimed to identify other miR-196a target genes. It has been reported that ING5 expression may suppress growth, invasion, and metastasis of gastric carcinogenesis.[31] So we hypothesized that miR-196a could influence apoptosis and invasion capabilities of pancreatic cancer by regulating ING5. It was reported that ING5 was involved in tumor genesis and tumor differentiation in head and neck squamous cell carcinomas.[32] Nuclear ING5 may modulate the transactivation of target genes and may promote apoptosis and cell cycle arrest by interacting with the p300 and p21 proteins. Inhibitor of growth 5 may function as a TSG or oncogene tightly linked with p53 status and may play an important role in the prognosis of head and neck squamous cell carcinoma patients.[32] Cengiz et al[33] reported tumor-specific mutation and down-regulation of ING5 mRNA, suggesting that it is a TSG in oral squamous cell carcinoma. The nuclear ING5 loss and its cytoplasmic overexpression were closely linked to the aggressive behaviors of colorectal carcinomas.[34] So, ING5 has a synergistic effect with P53, and therefore miR-196a inhibition of the expression of ING5 may affect P53 functions. P53 mutations are relatively common and occur in up to 70% of cases with pancreatic adenocarcinoma and are most commonly seen in poorly differentiated tumors.[35,36] MiR-196a may have indirect effects on the P53 gene, and this further clarifies the mechanism of action of miR-196a in affecting the prognosis of pancreatic cancer patients. Inhibitor of growth 5 is closely related to tumor development, and MiR-196a may execute part of its function by regulating ING5. Although this study helps in understanding the possible mechanism by which miR-196a regulates the function of ING5, thereby inhibiting pancreatic tumor growth and improved the prognosis, further studies are warranted to better understand the pathways involved in ING5 regulation by miR-196a.

In summary, our data reveal for the first time that miR-196a regulates ING5 expression in pancreatic cell carcinoma. Therefore, ING5 may serve as one of the new targets of miR-196a in pancreatic cancers. The mechanism may be associated with phenotypes including proliferation, apoptosis, and invasion of pancreatic cancer.

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