miRNA-141, Downregulated in Pancreatic Cancer, Inhibits Cell Proliferation and Invasion by Directly Targeting MAP4K4

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Abstract

miRNAs are associated with various types of cancer due to their ability to affect expression of genes that modulate tumorigenesis. In this study, we explored the role of miR-141 in pancreatic cancer. The analysis of clinical characteristics showed that miR-141 was significantly downregulated in tissues and cell lines of pancreatic cancer. Moreover, the decreased miR-141 level was significantly associated with tumor size and TNM stage, as well as lymph node and distant metastasis. Meanwhile, both Kaplan–Meier and multivariate survival analysis showed decreased miR-141 were associated with overall survival. Overexpression of miR-141 in pancreatic cancer cells inhibited cell proliferation, clonogenicity, and invasion; induced G1 arrest and apoptosis; and enhanced chemosensitivity. To understand how miR-141 mediates the phenotype of pancreatic cancer cells, a bioinformatics tool was used to identify MAP4K4 as a potential target of miR-141. The Dual-Luciferase reporter gene assay showed that miR-141 binds directly to the 3'-untranslated region (3'UTR) of MAP4K4 to inhibit MAP4K4 expression. Western blot and quantitative real-time PCR (qRT-PCR) analyses revealed that MAP4K4 expression was inversely correlated with miR-141 expression both in pancreatic cancer samples and cell lines. Knockdown of MAP4K4 inhibited cell proliferation, clonogenicity, and invasion, induced G1 arrest and apoptosis, and enhanced chemosensitivity. In a nude mouse xenograft model, both overexpression of miR-141 and knockdown of MAP4K4 significantly repressed pancreatic cancer cell growth. Therefore, we conclude that miR-141 targets MAP4K4, acts as a tumor suppressor in pancreatic cancer cells, and may serve as a novel therapeutic agent for miRNA-based pancreatic cancer therapy.

Introduction

miRNAs are small, endogenous, noncoding RNA molecules comprising 18 to 24 nucleotides that cause degradation of mRNA or inhibit translation by binding with complementary sequences in the 3'-untranslated region (3'UTR) of their target mRNAs (1, 2). miRNA regulation of gene expression plays an important role in tissue differentiation, cell proliferation, and apoptosis (3–5). Evidence clearly shows miRNAs are diversely expressed in different types of tissues and play crucial roles in tumorigenesis (6–8). Recently, miRNAs that may function as oncogene or tumor suppressor genes have been identified. Given the impact of miRNAs on gene expression, it is inevitable that miRNAs may be implicated in cancer, which is a complex genetic disease caused by deregulation of gene expression.

Pancreatic cancer is one of the most lethal of all human cancers, and traditional diagnosis and treatments have little effect on the disease course (9). High priority must be given to understanding the mechanisms of occurrence and development of pancreatic cancer and identifying novel diagnostics and effective therapeutics (10, 11). MiR-141 is a member of the miR-200 family that mainly affects the epithelial–mesenchymal transition (EMT) process (12–18). In addition, MiR-141 may repress hepatitis B virus (HBV) replication by targeting peroxisome proliferator-activated receptor α (PPARA; ref. 19). Recent data have shown that miR-141 and miR-200c play important roles in tumorigenesis. For example, miR-141 increases in ovarian cancer (20) and colon cancer (21) and acts as an oncogene. Other researches show miR-141 is downregulated in renal cell carcinoma (22), breast cancer (23), and pancreatic cancer (24), and can inhibit cell growth in gastric cancer (25). However, the exact role of miR-141 and its mechanisms in pancreatic cancer remain to be studied.
in pancreatic cancer has not yet been elucidated. Therefore, our present study compared miR-141 expression in human pancreatic cancer tissues and cell lines with noncancerous pancreatic tissue and human pancreatic duct epithelial cell lines (HPDE). Furthermore, the correlations between miR-141 and clinical characteristics were analyzed.

Because a single miRNA can target tens of hundreds of mRNAs, TargetScan—an online software for prediction of miRNA targets—was applied to predict the downstream target genes of miR-141. The results show that mitogen-activated protein kinase isoform 4 (MAP4K4) is a potential target of miR-141. MAP4K4 belongs to the mammalian STE20/MAP4K family. Recent studies have shown that MAP4K4 is associated with cell motility, rearrangement of the cytoskeleton, and cell growth (26–28). Downregulation of MAP4K4 prevents TNF-α-induced insulin resistance in human skeletal muscle (29), and suppresses systemic inflammation by targeting macrophages (30). Moreover, MAP4K4 is overexpressed in diverse types of human cancer (31). Silencing of MAP4K4 by siRNA inhibits cancer cell invasion and migration of breast cancer, prostate cancer, ovarian cancer, and malignant melanoma (28). In addition, knockdown of MAP4K4 expression in hepatocellular carcinoma reduces cell proliferation, blocks the cell cycle, and increases apoptosis (32). Furthermore, the overexpression of MAP4K4 is associated with worse prognosis in patients with stage II pancreatic ductal adenocarcinoma (33). These findings suggest that MAP4K4 may act as a promoter in pancreatic cancer. However, the functions of MAP4K4 in pancreatic cancer have not yet been fully clarified. Thus, the present study investigated the hypothesis that downregulated miR-141 inhibition of MAP4K4, overexpression, and inhibition of miR-141

The MAP4K4 interference sequence was obtained as previously described (32). Cells were seeded into plate wells, incubated overnight, and then transfected with 50 nmol/L MAP4K4 siRNA, miR-141 mimic, miR-141 inhibitor, and their matched negative controls (NC; siRNA NC, mimic NC, and miRNA inhibitor NC; Ribobio Co.). Lipofectamine 2000 (Invitrogen) was used for cell transfection according to the manufacturer’s instructions. Cells were collected for further assays or for RNA/protein extraction after an additional 72 hours.

**RNA isolation and quantitative real-time RT-PCR**

Total RNA, containing miRNA, was extracted from either tissue samples or transfected cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The reverse transcription was conducted by using a reverse transcription kit (Takara). The expression of mature miRNAs and potential target genes were measured by quantitative real-time PCR (qRT-PCR) with SYBR Green PCR Kit (Takara) on the Applied Biosystems StepOne-Plus Real-Time PCR System. For evaluating miRNA expression, human U6 RNA was amplified as an internal control. For the detection of target gene mRNA, human GAPDH RNA was used as a control. The mRNA or miRNA levels were calculated according to $2^{-\Delta\Delta C_{t}}$. Primer sequences were shown in Supplementary Table S1. The reverse primers of U6 and miR-141 were the universal primer provided by Takara. The miR-141 expression was determined to be high when the expression level was equal to or more than the median of the cohort, and low when the level was below the median of the cohort (10).

**Western blot analysis**

Total protein was extracted from cells after 72-hour transfection with lysis buffer (50 mmol/L Tris–HCl, pH 7.4; 150 mmol/L NaCl; 1% NP-40, and 0.5% sodium deoxycholate with proteinase inhibitors (Roche Diagnostics). Lysates were denatured with SDS sample buffer at 100°C for 10 minutes, and separated in 10% polyacrylamide gels, then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% non-fat milk powder in Tris-buffered saline containing 0.1% Tween 20 (TBST) and probed with primary antibodies overnight at 4°C (primary antibody is...
shown in Supplementary Table S2). After washing with TBST, membranes were incubated with secondary antibodies. Band signals were visualized using ECL (Pierce), exposed to a Kodak X-OMAT film (Sigma-Aldrich), and the band density was evaluated by Bio-Rad Quantity One software.

**Immunohistochemistry**

Tissue samples were embedded in paraffin. And then, the paraffin sections (4 μm) were deparaffinized with xylene, rehydrated, permeabilized in 0.1% Triton X-100, and incubated overnight at 4°C with mouse monoclonal anti-MAP4K4 (1:50, HGK, sc-100445; Santa Cruz Biotechnology). The next day, sections were incubated with biotinylated goat anti-mouse secondary antibody (Boster) for 1 hour at room temperature. Then, 3, 3-diaminobenzidine was used to visualize the antigen–antibody complex, and hematoxylin was used for counter staining. Negative control sections were incubated in PBS instead of primary antibody.

The staining results were blindly evaluated by two experienced pathologists without known the information of the patients. Using the median value of 10% as a cutoff, for MAP4K4, positive expression was defined as cytoplasmic staining of more than 10% of the cells (34).

**Cell proliferation assay**

Cells (3,000/well) were collected and seeded in 96-well plates and incubated at 37°C after transfection. After incubation for 1 to 5 days, to the media of each well was added 20 μL of MTT solution (5 mg/mL), and the plates were further incubated for another 4 hours at 37°C. Then, the media was replaced with 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich), and the absorbance was measured at 570 nm using a microplate reader (Sunrise). The MTT assay was repeated 3 times with six replicates.

**Colony-formation assay**

At 24 hours posttransfection with miR-141 mimic, MAP4K4 siRNA, or the matched NC, 500 cells were seeded into each well of a 6-well plate and incubated for 4 days. Media was replaced with fresh on day 5. Following incubation, cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet. The colony-formation assay was repeated 3 times with duplicate wells.

**Cell invasion assay**

Cell invasion detection was evaluated using Transwell migration chambers (8 μm pore size; Costar) precoated with a layer of Matrigel (ECM gel; Sigma) diluted with medium containing no FBS. Cells were seeded in the upper chamber (3 × 10^4 cells/well). The lower chamber contained 700 μL medium with 30% FBS as chemotaxin. After 48 hours of incubation at 37°C, noninvaded cells on the top of the membrane were removed with suitable cotton swabs, and the invaded cells were washed with PBS, fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and then counted using light microscopy. This invasion detection was repeated 3 times with duplicate wells.

**Cell-cycle and cell apoptosis analysis**

For the cell-cycle assay, at 72-hours posttransfection, cells were collected, fixed in 70% ethanol at 4°C overnight, and treated with 5 μg/mL RNase A at 37°C for 30 minutes. After staining with 50 μmol/L propidium iodide, the cells were detected by flow cytometry (BD Biosciences). For cell apoptosis analysis, at 72-hours posttransfection, cells were collected and stained with the Annexin V-FITC/Propidium Iodide Kit (KeyGen Biotech. Co. Ltd) according to the manufacturer's instructions. Data were analyzed with FlowJo V7 software (Tree Star).

**Cell viability assay**

Cell chemosensitivity was assessed with the MTT assay. At 72 hours posttransfection, cells were harvested, and 5,000 cells per well were seeded in 96-well plates for 24 hours. Subsequently, media were replaced with various concentrations of 5-FU (Sigma-Aldrich) or gemcitabine (Eli Lilly Co. Ltd) diluted with serum-free media. Cells were cultivated for a further 48 hours, and cell viability was measured with the MTT assay. The survival rate was calculated as: cell viability = OD_{exp}/OD_{ctrl} × 100%. This assay was repeated 3 times with six replicate wells for each concentration.

**Dual-Luciferase assay**

To confirm whether MAP4K4 was a target of miR-141, the pMIR-REPORT assay was applied. Briefly, PANC-1 cells were seeded in 96-well plates (5,000 cells per well) and cotransfected with 100 ng pMIR-REPORT Luciferase vector (Ribobio Co.) containing MAP4K43'UTR (3441 bp) or mutated forms with or without 50 nmol/L miR-141 mimic or mimic NC. After incubation for 48 hours, Luciferase activity was determined using the Dual-Luciferase reporter assay system (Promega). Relative Luciferase activity was normalized to that of Firefly Luciferase.

**Tumor formation study in vivo**

Tumor formation was studied by establishing a xenograft model. Lentiviral vectors (GeneChem Co. Ltd) containing miR-141 (LV-miR-141) or MAP4K4 (LV-MAP4K4-RNAi) RNA interference sequences were used to infect PANC-1 cells and MiaPaCa-2 cells according to the manufacturer's instructions. BALB/c female nude mice (4 weeks old) were purchased from Beijing HFK Biotechnology Co., Ltd. The animal experiments in this study were approved and reviewed by the Animal Research Committee of the Academic Medical Center at Huazhong University of Science and Technology. Care and handling of the animals were in accordance with the guidelines for Institutional and Animal Care and Use Committees.
Mice were randomly divided into 4 groups with 4 mice in each group. Infected PANC-1 cells (1 × 10⁷ cells/mouse) suspended in PBS solution were injected subcutaneously into the mice. Tumor volumes were measured every 4 days using calipers along two major axes, and calculated according to the formula $V = 0.5 \times L \times W^2$ (width). At 32 days after cell inoculation, mice were sacrificed. Excised tumors were evaluated for volume and weight. The expression of MAP4K4 in tumors was detected by qRT-PCR and Western blot.

**Statistical analysis**

Unless otherwise stated, data were analyzed using the SPSS 13.0 software (SPSS, Chicago, IL) and expressed as mean ± SD. Differences between groups were assessed using the Student $t$ test and Fisher exact test. The relationship between the expression of MAP4K4 and miR-141 in tissues or cell lines was analyzed with Pearson correlation. The relationship between miR-141 expression and clinicopathologic features of pancreatic cancer was analyzed using the Pearson $\chi^2$ test. Kaplan–Meier method was used to calculate overall survival of 2 patient groups, and differences were analyzed by log-rank test. The survival data were evaluated using a multivariate Cox regression analysis. $P < 0.05$ was considered statistically significant.

**Results**

**MiR-141 is downregulated in pancreatic cancer tissues and cell lines**

Quantitative RT-PCR was used to examine miR-141 expression in 40 pancreatic cancer tissues and their paired adjacent noncancerous tissues. The expression of miR-141 was significantly lower in pancreatic cancer tissues than in noncancerous pancreatic tissues (Fig. 1A; $0.61 \pm 0.68$ vs. $1.52 \pm 0.7\%$, $P < 0.01$). In addition, compared with the HPDE cells, miR-141 expression in five human pancreatic cancer cell lines (AsPC-1, BxPC-3, PANC-1, MiaPaCa-2, and SW1990) was significantly decreased (Fig. 1B; 1.0 vs. $0.48 \pm 0.09$, $0.62 \pm 0.05$, $0.26 \pm 0.06$, $0.21 \pm 0.05$, $0.57 \pm 0.11\%$, $P < 0.01$). Because PANC-1 and MiaPaCa-2 cells were the two cell lines with the lowest expression of miR-141, the two cell lines were used throughout the rest of the study.

**Decreased miR-141 is associated with poorer prognosis in patients with pancreatic cancer**

To investigate whether the expression level of miR-141 was related to clinical characteristics and overall survival of patients, a follow-up study was conducted. The results showed that low miR-141 expression was significantly associated with large tumor size ($P = 0.009$), advanced TNM stage ($P = 0.037$), lymph node metastasis ($P = 0.028$), and distant metastasis ($P = 0.038$), but not with patients’ age ($P = 0.773$), gender ($P = 0.602$), and tumor differentiation ($P = 0.292$; Table 1). Furthermore, patients of pancreatic cancer with low levels of miR-141 expression had a significantly shorter median survival (14.8 vs. 38.2 months, $P = 0.04–0.05$) than those with high levels of miR-141 expression (Fig. 1C). Meanwhile, multivariate survival analysis showed that TNM stage, metastasis, and miR-141 were associated with overall survival (Table 2). These results indicate that decreased miR-141 expression predicts poorer prognosis in patients of pancreatic cancer.

**Overexpression of miR-141 results in inhibition of cell proliferation, colony formation, and invasion; induces $G_1$ phase arrest and apoptosis; and enhances chemosensitivity**

To explore the role of miR-141 in pancreatic cancer cells, we transfected PANC-1 and MiaPaCa-2 with miR-141 mimic to upregulate miR-141 expression. After transfection with miR-141 mimic, a significant increase in miR-141 expression was confirmed using qRT-PCR (Fig. 2A). MTT assay showed that the proliferation rate of PANC-1 and MiaPaCa-2 cells was significantly repressed after

![Figure 1](image-url)
overexpression of miR141 (Fig. 2B); furthermore, the ability of colony formation (Fig. 2C) and cell invasion were both notably weakened (Fig. 2D).

Upregulation of miR-141 resulted in an accumulation of both PANC-1 (41.19 ± 3.53% vs. 51.98 ± 3.46%, P = 0.02; Fig. 3A) and MiaPaCa-2 (44.07 ± 2.28% vs. 34.31 ± 4.17%, P = 0.02) cells in the G1 phase of the cell cycle (Supplementary Fig. S1A). The cell apoptosis analysis revealed that the percentage of apoptotic cells was significantly increased by upregulation of miR-141 in both PANC-1 (3.63 ± 0.35 vs. 7.63 ± 0.55%, P < 0.01; Fig. 3B) and MiaPaCa-2 (1.27 ± 0.35 vs. 4.67 ± 0.61%, P < 0.01) cells (Supplementary Fig. S1B). Given chemoresistance is a characteristic of pancreatic cancer cells, chemosensitivity was also evaluated. After transfection with miR-141 mimic, the IC50 of PANC-1 cells was significantly decreased to both 5-FU (7.97 vs. 20.78 μg/mL, P < 0.01) and gemcitabine (7.42 vs. 23.3 μg/mL in PANC-1, P < 0.01; Fig. 3C). MiR-141-mimic-transfected MiaPaCa-2 cells also showed a lower IC50 to 5-FU (7.96 vs. 17.06 μg/mL, P < 0.01) and gemcitabine (10.4 vs. 26.45 μg/mL, P < 0.01; Supplementary Fig. S1C). These results imply that overexpression of miR-141 could increase the chemosensitivity of pancreatic cancer cells. The expression of associated protein, cyclinD1, Bcl-2, MMP-2, and MMP-9 were significantly decreased in cells transfected with miR-141 mimic, whereas the expression of Bax was increased in both PANC-1 (Fig. 3D) and MiaPaCa-2 cells (Supplementary Fig. S1D).

**Inhibition of miR-141 promotes cell growth and invasion in PANC-1 cells**

To further clarify the functions of miR-141 in pancreatic cancer, we assessed the biologic characteristics of PANC-1 cells treated with miR-141 inhibitor. The results showed that inhibition of miR-141 significantly promoted cell proliferation, colony formation and invasion in PANC-1 cells. Enhanced invasion was associated with increased MMP-2 and MMP-9 expression (Supplementary Fig. S2).

**The correlation between miR-141 and MAP4K4 expression in pancreatic cancer**

We analyzed the correlation of miR-141 and MAP4K4 expression in pancreatic cancer samples. Immunohistochemical staining showed stronger MAP4K4 expression in pancreatic cancer tissues than in paired adjacent noncancerous tissues (Fig. 4A; Supplementary Table S3, χ2 = 20.05, P < 0.01). To further identify the aberrant expression of MAP4K4 in pancreatic cancer tissues, qRT-PCR was used. In accordance with our immunohistochemical analyses, the expression of MAP4K4 was much higher in pancreatic cancer tissues than in noncancerous tissues (Fig. 4B). Interestingly, Pearson correlation showed that the expression of miR-141 was inversely related to that of MAP4K4 in pancreatic tissues (Fig. 4C), which further suggests that MiR-141 targets MAP4K4.

**MAP4K4 is a direct target of miR-141**

To verify that MAP4K4 was a direct target of miR-141, the 3’UTR of MAP4K4 with wild-type or mutant seed-sequence–recognizing sites was cloned to a Dual-Luciferase reporter (Fig. 5A). After cotransfection of miR-141 mimic or NC mimic with MAP4K4-UTR-WT or MAP4K4-UTR-MUT plasmid to PANC-1 cells, Luciferase activity was analyzed. The results showed that the relative Luciferase activity of the plasmid carrying MAP4K4-UTR-WT

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**Table 1.** Clinicopathologic correlations of miR-141 expression in pancreatic cancer

<table>
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<th>Low (n = 24)</th>
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<td>16</td>
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*aStatistically significant (P < 0.05)."
was significantly suppressed in the presence of miR-141 mimic. This effect was not detected in the plasmid carrying MAP4K4-UTR-MUT (Fig. 5B). We further evaluated the expression level of MAP4K4 mRNA and protein after modulation of miR-141 expression. The results showed that overexpression of miR-141 significantly decreased MAP4K4 expression at the mRNA level and protein level (Fig. 5C and D). The miR-141 inhibitor significantly increased MAP4K4 protein expression without obvious increase of MAP4K4 mRNA (Fig. 5E and F). These data indicate that miR-141 can directly target its predicted MAP4K4 seed region.

Figure 2. MiR-141 inhibits tumorigenesis of PANC-1 and MiaPaCa-2 cells transfected with miR-141. A, the expression levels of miR-141 after transfection of miR-141 mimic with Lipofectamine 2000 (examined by qRT-PCR). Average miRNA expression from noncancerous pancreatic (NC) cells was designated a value of 1. B, assessment of cell proliferation by MTT assay. The proliferation of PANC-1 and MiaPaCa-2 cells was significantly inhibited by overexpression of miR-141. C, overexpression of miR-141 reduced cell colony formation. D, overexpression of miR-141 markedly decreased invasion of PANC-1 and MiaPaCa-2 cells. This assay was conducted using a Transwell migration chamber with an 8-μm pore membrane precoated with Matrigel.
Knockdown of MAP4K4 inhibits the biologic behavior of pancreatic cancer cells

To investigate the role of MAP4K4 in pancreatic cancer cells, we used RNA interference (RNAi) to knock down MAP4K4 expression. MAP4K4 expression was greatly decreased in PANC-1 and MiaPaCa-2 after transfection of siRNA-MAP4K4 compared with siRNA-NC. Moreover, similar to miR-141 mimic, knockdown of MAP4K4 significantly inhibited cell proliferation, colony formation, and invasion, and promoted G1 arrest, apoptosis, and chemosensitivity of cells to 5-FU and gemcitabine (Supplementary Fig. S3).
Both overexpression of miR-141 and knockdown of MAP4K4 repress xenograft tumor growth in vivo

To gain further insight into the effect of miR-141 on pancreatic cancer, we established a xenograft tumor model in nude mice with LV-miR-141 and LV-MAP4K4-RNAi–infected PANC-1 cells. LV-miR-141- and LV-MAP4K4-RNAi–infected PANC-1 cells showed favorable infectivity (Fig. 6A). The results showed both LV-miR-141 and LV-MAP4K4-RNAi significantly reduced xenograft tumor growth (Fig. 6B–D). Quantitative RT-PCR (Fig. 7A) and Western blot analyses showed that MAP4K4 expression was decreased in the LV-miR-141 and LV-MAP4K4-RNAi xenograft tumor (Fig. 7B and C).

Discussion

Aberrant upregulation or downregulation of miRNAs is closely associated with tumorigenesis. MiR-141 is a miRNA that is implicated in multiple types of cancers, but its function in pancreatic cancer is still not clarified. Our study showed that miR-141 expression was significantly downregulated in pancreatic cancer tissues. These results are in accordance with the microarray analyses of Szafranska AE (24), and strongly indicate that miR-141 is involved in pancreatic tumorigenesis. In addition, our data show, for the first time, that a reduced expression of miR-141 is correlated with poor prognosis in patients with pancreatic cancer. These observations imply that miR-141 may be a useful prognostic predictor in pancreatic cancer.

We showed that overexpression of miR-141 significantly suppressed pancreatic cancer cell proliferation, colony formation, and invasion, and induced G1 arrest and apoptosis. Inhibition of miR-141 promoted the proliferation, colony formation, and invasion of pancreatic cancer cells.
Furthermore, our in vivo study revealed that overexpression of miR-141 could suppress pancreatic xenograft tumor growth in nude mice. These results intensively imply that miR-141 acts as an inhibitor of pancreatic cancer tumorigenesis. Our data are in accordance with those of Du and colleagues (25) who showed miR-141 inhibited the proliferation of gastric cancer cells, but is in contrast to other studies that suggest miR-141 acts as an oncogene in ovarian (20) and colon cancers (21). These observations suggest that miR-141 has a heterogeneous role in tumorigenesis that may be highly dependent on its targets in different cells.

It is well known that pancreatic cancer is a highly aggressive malignancy, which is partly attributable to the chemotherapy-resistant characteristics. Therefore, the identification of biomarkers beneficial for chemoresistance and understanding the related mechanisms will display advisable strategies to overcome this problem. Interestingly, our study showed that miR-141 mimic transfection partially enhanced chemosensitivity of PANC-1 and MiaPaCa-2 cells to 5-FU and gemcitabine. Therefore, therapeutic approaches to introduce miR-141 into pancreatic cancer cells might be potentially feasible, not only for prohibiting the development of tumorigenesis but also in sensitizing cancer cells to chemotherapeutic drugs.

TargetScan software predicts that MAP4K4 is one of the downstream targets of miR-141. Previous studies have shown that MAP4K4 was significantly upregulated in many different types of carcinomas, including...
hepatocellular carcinoma (32), lung adenocarcinoma (35), and even pancreatic ductal adenocarcinoma (33). The reported expression level of MAP4K4 was closely related to the overall survival of these patients with cancer, especially those diagnosed with pancreatic cancer. In our study, we clarify that knockdown of MAP4K4 repressed cell growth in vitro and in vivo, induced G1 arrest and apoptosis, and improved the

Figure 6. Infection with LV-miR-141 and LV-MAP4K4-RNAi resulted in inhibition of xenograft tumor growth in vivo. A, fluorescent field microscopy displayed the effective infection of PANC-1 cells with GFP-labeled lentivirus containing either miR-141 or MAP4K4 interference sequences. Cells in the same area are also shown under bright field. (Magnification, ×100). B, pictures of the nude mice and their tumors after 32 days inoculation was shown in two subgroups. C, tumor volumes of each group during the tumor growth process. D, tumor weight measured from the xenograft model. Overexpression of miR-141 and knockdown of MAP4K4 significantly inhibited tumor growth in vivo.
Our study is the first to report that MAP4K4 acts as a tumor promoter and plays a key role in progression of pancreatic cancer. We further investigated whether MAP4K4 is a target of miR-141 in pancreatic cancer cells. The Luciferase assay showed that miR-141 could inhibit the expression of MAP4K4 by combining directly to the 3′ UTR of MAP4K4, whereas inhibition of miR-141 expression resulted in upregulation of MAP4K4. Similar to the results obtained with miR-141 mimic, knockdown of MAP4K4 significantly prohibited the proliferation of pancreatic cancer cells, both in vitro and in vivo. Furthermore, our analyses revealed an inverse correlation between miR-141 and MAP4K4 expression in pancreatic cancer tissues. These results show that MAP4K4 is involved in the inhibitory function of miR-141 in pancreatic cancer. The orthotopic model of tumor with fluorescence is more useful for observation in time. As the present study showed that miR-141 expression is closely associated with overall survival of patients who have pancreatic cancer. Ectopic expression of miR-141 and knockdown of MAP4K4 inhibited tumorigenesis of pancreatic cancer cells. Therefore, results from this study imply that miR-141 acts as inhibitor in pancreatic cancer by targeting MAP4K4, and may serve as a novel therapeutic agent for miRNA-based therapy in pancreatic cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed by the other authors.

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Conception and design: G. Zhao, B. Wang, C-Y. Wang
Development of methodology: G. Zhao, B. Wang, J-G. Zhang, S-C. Deng, Q. Qin, K. Tian, Y. Niu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Zhao, Y. Liu, J-G. Zhang, S. Zhu, Q. Gong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Wang, X. Li
Writing, review, and/or revision of the manuscript: G. Zhao, B. Wang, Q. Gong, C-Y. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Zhao, Y. Liu, K. Tian
Study supervision: G. Zhao, C-Y. Wang

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Figure 7. MiR-141 and MAP4K4 expression were measured in tissues from tumors. A, both LV-miR-141 and LV-MAP4K4-RNAi significantly decreased MAP4K4 mRNA expression. B, both LV-miR-141 and LV-MAP4K4-RNAi significantly decreased MAP4K4 protein expression. The average gene expression from LV-miR-NC group was designated a value of 1. C, representative images of MAP4K4 expression at the protein level by Western blot in tumor tissues. Compared with the respective negative control groups, both the LV-miR-141 and LV-MAP4K4 RNAi group showed significantly lower MAP4K4 expression. *P < 0.05; **P < 0.01.
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References


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