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# Downregulation of miR-132 by promoter methylation contributes to pancreatic cancer development

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**MicroRNAs (miRNAs), which regulate gene expression by partial complementarity to the 3′ untranslated region of their target genes, have been implicated in cancer initiation and progression. However, the molecular mechanism underlying the regulation of miRNA expression during pancreatic tumorigenesis has not been extensively reported. In this study, we first compared the miRNA expression in human pancreatic cancers and adjacent normal tissues by miRNA array and identified 12 differentially expressed miRNAs. miR-132, which is downregulated in tumors, was further studied in greater detail. Decreased expression of miR-132 was confirmed in 16 of 20 pancreatic carcinomas ($P < 0.0001$), compared with their respective benign tissues by TaqMan miRNA assays. miR-132 expression was remarkably influenced by promoter methylation in PANC1 and SW1990 cells. Promoter hypermethylation was observed in tumor samples but not in the normal counterparts, and the expression of miR-132 negatively correlated with its methylation status ($P = 0.013$). miR-132 was transcribed by RNA polymerase II, and Sp1 played a major role in miR-132 transcription. The expression of *Sp1* correlated with that of miR-132 in tissues. Moreover, cancerous tissues showed significantly lower Sp1-binding affinity to the miR-132 promoter, relative to non-tumor samples. Proliferation and colony formation of pancreatic cancer cells were suppressed in cells transfected with miR-132 mimics and enhanced in cells transfected with miR-132 inhibitor by negatively regulating the Akt-signaling pathway. Our present findings illustrate the mechanism driving miR-132 downregulation and the important role of miR-132 in pancreatic cancer development.**

## Introduction

Pancreatic cancer is an aggressive malignancy with one of the worst mortality. It is the sixth leading cause of death from malignant disease in China and the fourth leading cause of cancer related death in the USA (1,2). The majority of patients have developed an aggressive form of the disease by the time of diagnosis, limiting the potential for therapeutic intervention (3). At this stage, several genetic and epigenetic changes have taken place, resulting in the silencing of tumor suppressors, overexpression of oncogenes and ultimately tumor progression (4). In recent years, there have been important advances in understanding the molecular biology of pancreatic cancer and genetic analyses have shown that the basis of this dismal disease is extremely complex and heterogenous (5).

MicroRNAs (miRNAs) are small non-coding regulatory RNAs that suppress gene expression through partial complementary elements in the 3′ untranslated regions of their target messenger RNAs (mRNAs) (6). Most animal miRNAs are evolutionarily conserved and are often found in clusters (7). Primary miRNAs with the stem-loop structure are transcribed by RNA polymerases (RNA Pols) and are processed both in the nucleus and cytoplasm by Drosha and Dicer to generate mature miRNAs (8,9). The mature miRNA is assembled into a miRNA-induced silencing complex, which then directs its binding to the cognate sequence in the 3′ untranslated region of the target mRNAs. miRNAs are implicated in a wide variety of biological processes including cell proliferation, apoptosis, metabolism, cell differentiation and tumor initiation and promotion (10), and their identification may provide new insights in the elucidation of cancer progression (11). Over the past several years, advanced technologies have been used to quantify miRNAs in pancreatic tumor tissues and several deregulated miRNAs have recently been revealed including miR-21, miR-146, miR-221 and miR-196a (12).

These findings suggest the involvement of miRNAs in the pathogenesis of pancreatic cancer. The deregulation of miRNAs might be attributed to disorders in transcription and subsequent procession. Therefore, transcriptional regulation of miRNAs may represent an underexplored etiology of pancreatic cancer. However, few studies have demonstrated the mechanism of miRNA transcription during the development of pancreatic cancer. Furthermore, the regulatory role of only a few miRNAs has been elucidated in pancreatic cancer initiation and progression. More extensive investigations of miRNA function are warranted.

In this study, we first analyzed miRNA expression between normal and cancerous pancreatic tissues and identified 12 preferentially expressed miRNAs. We further investigated the underlying mechanism of miR-132 transcription and the consequence of its deregulation. *Cis*-modulating elements and *trans*-factors were identified in the miR-132 promoter. Downregulation of miR-132 in tumors was caused by promoter hypermethylation and decreased Sp1-binding affinity to its promoter. Moreover, miR-132 attenuates proliferation and colony formation of pancreatic cancer cells via the Akt-signaling pathway.

**Abbreviations:** CREB, cyclic adenosine 3′,5′-monophosphate response element-binding; ChIP, chromatin immunoprecipitation; ERK, external signal-regulated kinase; mRNA, messenger RNA; miRNA, microRNA; OD, optical density; PCR, polymerase chain reaction.

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## Materials and methods

### *Tissue samples*

Twenty pancreatic cancer tissue samples and matched normal adjacent pancreatic tissue samples were obtained postoperatively in 2009 from the Department of General Surgery, Changhai Hospital, Second Military Medical University (Shanghai, China). All patients gave signed, informed consent for their tissues to be used for scientific research. Ethical approval for the study was obtained from the Department of General Surgery, Changhai Hospital, Second Military Medical University (Shanghai, China). All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by a senior pathologist according to the WHO classification criteria. Tissues were obtained before chemotherapy and radiation therapy and were immediately frozen and stored at −80°C prior to the microarray, real-time polymerase chain reaction (PCR) and methylation analyses.

*miRNA microarray and real-time PCR analyses*

Total RNA was extracted from cells or tissues with Trizol reagent (Invitrogen, Carlsbad, CA). Microarray-based miRNA expression profiling was performed using the miRCURY LNA human microRNA Array (Exiqon, Vedbaek, Denmark). The microarrays contained ~1200 assay probes corresponding to all of the annotated human and non-human primate miRNA sequences (miRBase, version 12, 2008; the Wellcome Trust Sanger Institute, Cambridgeshire, UK). Total RNA labeling and hybridization were performed using standard conditions according to manufacturer instructions.

The expression levels of mature miR-132/212 and their precursor were quantified by quantitative real-time PCR using TaqMan MicroRNA assays according to the manufacturer's protocols (Applied Biosystems, Foster City, CA). Briefly, 5 ng of small RNA or total RNA were reverse transcribed using specific stem-loop RT primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes. U6 snRNA (RNU6B; Applied Biosystems) served as an internal normalized reference. Thermocycler conditions included an initial step at 95°C for 2 min followed by 35 cycles at 95°C for 15 s and 55°C for 1 min. The quantitative real-time PCR results were analyzed and expressed as relative miRNA or mRNA levels based on the $C_t$ (cycle threshold) value, which was then converted to fold change.

*Cell culture and demethylation experiment*

The human pancreatic cancer cell lines SW1990 and PANC1 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G Sodium Salt and 100 U/ml streptomycin sulfate; Gibco, Grand Island, NY). The cells were grown in a 37°C incubator with 5% $CO_2$.

To block DNA methylation, SW1990 and PANC1 cells were treated with 10 μM 5-aza-2′-deoxycytidine (5-Aza; Sigma–Aldrich, St Louis, MO) for 72 h. The cells were then collected for mRNA analysis.

*DNA extraction and bisulfite sequencing*

Genomic DNA was extracted from 20 pairs of pancreatic tissues using the sodium dodecyl sulfate and proteinase K methods and then subjected to sodium bisulfite treatment (13). We amplified and sequenced the proximal promoter DNA from −1425 to −1161 (relative to the transcription start site). This region contains 14 CpG sites. These regions were amplified using the primers (forward) 5′-GGTTTTTAGTTTTTGGGTGGTATTT-3′; (reverse) 5′-CCCTCCTCAATTCCTAAACCTAA-3′. The initial denaturation was for 2 min at 96°C, followed by 32 cycles for 10 s at 94°C, 5 s at 54°C and 10 s at 72°C and a final elongation for 7 min at 72°C. The PCR products were separated by 2% agarose gel electrophoresis, extracted and then cloned into the T-easy vector (Tiangen, China). After bacterial amplification of the cloned PCR fragments by standard procedures, 10 clones were subjected to DNA sequencing.

*Construction of promoter reporter plasmids*

To explore the transcriptional regulation of miR-132, a fragment of the miR-132 gene promoter (−2668 to −74 relative to the transcription start site of pri-miR-212/132) was amplified by PCR using the following primers: (forward) 5′-TACTCGAGTTCTGTAAGGGAGGGTCTCACA-3′ and (reverse) 5′-CTAAGCTTGCTCGCGACCAGGCACG-3′ (underlined nucleotide sequence means restriction enzyme cutting site). The amplified fragment was inserted into the pGL3-basic vector (Promega, Madison, WI) between the XhoI and HindIII sites. This pGL3-2668 vector was used as a template for the cloning of serially deleted miR-132 promoter reporter vectors with the same reverse primer and the forward primers described in Supplementary Table 1, available at *Carcinogenesis* Online. These recombination plasmids were then sequenced for confirmation. The pcDNA3.1-Sp1, pcDNA3.1-Oct-1 and pcDNA3.1-cyclic adenosine 3′,5′-monophosphate response element-binding (CREB) vectors, which can the overexpress corresponding transcription factor, were gifts from Dr Wenzhang Wang (Fudan University, China).

*In vitro* DNA methylation of constructs

The pGL3-2668 construct was methylated *in vitro* using the bacterial methylase SssI (New England Biolabs, Ipswich, MA). Briefly, each reporter gene construct (4 μg) was incubated at 37°C for 4 h with 40 U of SssI supplemented with 160 μM *S*-adenosylmethionine. After methylation, the plasmid was purified and quantified.

*Cell transfection and luciferase assays*

PANC1 and SW1990 cells were transfected by Lipofectamine 2000 (Invitrogen) with 1 μg of each constructed vector, either methylated or unmethylated. To confirm the participation of transcription factors, 200 ng of the transcription factor expression vector or control vector (pcDNA3.1) was cotransfected with 800 ng of the constructed reporter vector. In each transfection, 50 ng of pRL-TK (Promega) was used to correct for the transfection efficiency. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega). Promoter activities were expressed as the ratio of *Firefly* luciferase to *Renilla* luciferase activity. Chemically synthesized RNAs, including negative control (NC), miR-132 mimics and miR-132 inhibitor, were obtained from GenePharma (GenePharma, Shanghai, China). For transfection, the cells were transfected with 1 μg of the chemically synthesized RNA.

*Quantitative chromatin immunoprecipitation*

Cells and pancreatic tissues were used for the chromatin immunoprecipitation (ChIP) assays. We used the EZ ChIP Kit (Upstate, Biotechnology, Lake Placid, NY) and followed the manufacturer's instructions. Briefly, $5 \times 10^6$ cells or 30 mg of the tissues were cross-linked by 1% formaldehyde, lysed in 400 μl lysis buffer and sonicated to ~500 bp fragments. ChIP was conducted with antibodies against RNA polymerase (RNA Pol) II, RNA PolIII, Sp1, CREB and IgG. Input control DNA or immunoprecipitated DNA was amplified in a 15 μl reaction volume containing 2 μl of the eluted DNA template. We designed eight sets of primers targeting different regions in the pri-miR-212/132 promoter (supplementary Table 2 is available at *Carcinogenesis* Online). Both the immunoprecipitated fragments and the inputs were amplified by real-time PCR. The results for the immunoprecipitated fragments were calculated and compared with the $C_t$ values obtained for the input samples in each case. The results are expressed as a percentage of the input.

*Cell proliferation assays*

Cells were seeded in a 96-well plate at a density of $1 \times 10^4$ cells per well and incubated for 96 h. *In vitro* growth was measured using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). The optical density (OD) at 450 nm was measured using a Microplate Reader (Bio-Rad, Hercules, CA), and the proliferation index was calculated as experimental OD value/control OD value. Three independent experiments were performed in quadruplicate.

*Colony formation*

PANC1 and SW1990 cells transfected with the indicated RNAs were plated at low density (1000 cells/6 cm plate), incubated for 10 days and fixed and stained with crystal violet. Foci and colonies containing >50 cells were counted using a microscope.

*Western blot*

Treated cells in a six-well culture cluster were washed twice with ice-old phosphate-buffered saline and then directly lysed in 200 μl of 2× sodium dodecyl sulfate cell lysis buffer in each well. The lysates were boiled, centrifuged at 10 000 r.p.m. and then loaded onto a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The samples were electrophoresed for 4 h and then transferred to a Millipore Immobilon transfer membrane in Bio-Rad blot apparatus. After blocking with 5% non-fat milk in phosphate-buffered saline-Tween-20 for 1 h at room temperature, the membranes were blotted with the appropriate Akt, pAkt, P70, external signal-regulated kinase (ERK), pERK, cyclin D1 or pJNK primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000–1:2000 dilution. Membranes were then incubated with the appropriate secondary antibody linked to horseradish peroxidase at a 1:2000 dilution for 1 h at room temperature. After TBST washes, the blot was incubated in detection reagent (ECL Advance Western Blotting Detection Kit, Amersham Bioscience, Freiburg, Germany) and exposed to a Hyperfilm ECL film (Pierce, Rockford, IL). β-Actin served as the loading control and was detected with a mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology).

*Statistical analysis*

Data from at least three independent experiments are expressed as the mean ± standard error of the mean. Standard error bars are included for all data points. The differences between groups were analyzed using Student's *t*-test when only two groups were present or by one-way analysis of variance when more than two groups were compared. Correlation analysis was performed using the Pearson test. All tests performed were two sided. Data were considered significant if $P < 0.05$ (indicated by '*') and $P < 0.001$ (indicated by '**').

## Results

*miRNA-132 is downregulated in pancreatic cancer*

To search for miRNAs that may be involved in the development of pancreatic cancer, especially among Chinese patients, we collected three different human clinical specimens and used miRNA array analysis to screen for differentially expressed miRNAs between pancreatic cancer tissues and respective adjacent normal tissues. The raw array measures are accessible through Gene Expression Omnibus (GEO) Series accession number GSE28862 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28862). We found that many

miRNAs were deregulated in these cancer samples, including miR-21, miR-365, miR-103 and miR-141 (Table I). In addition, the expression of miR-132, which had not been suggested to be involved in pancreatic cancer in previous studies, was significantly downregulated in pancreatic malignant tissues. To further confirm the expression of miR-132, we extended our sample to twenty different clinical specimens. Taqman-based real-time PCR analysis showed that miR-132 was significantly decreased in 16 of 20 carcinoma tissues compared with the matched adjacent tissues ($P < 0.0001$; Figure 1A), suggesting that miR-132 may be a novel factor associated with the development of pancreatic cancer. Because miR-132 and miR-212 are derived from the same precursor pri-miR-212/132, we also detected the expression of pri-miR-212/132 and mature miR-212. The expression of pri-miR-212/132 was significantly reduced in cancerous tissues ($P = 0.006$; Figure 1B), and its expression correlated with mature miR-132 ($r = 0.467$, $P = 0.024$). However, mature miR-212 did not show a significant alternation between tumors and the non-tumor counterparts ($P = 0.065$, Figure 1C). Interestingly, the expression of mature miR-212 correlated with that of its precursor ($r = 0.452$, $P = 0.045$) but not with that of mature miR-132 ($r = 0.024$, $P = 0.919$).

**Table I.** Summary of significantly differentially expressed miRNAs in pancreatic cancer tissues compared with matched normal adjacent pancreatic tissues

| Upregulated miRNAs | | | Downregulated miRNAs | | |
|---|---|---|---|---|---|
| miRNA ID | Fold change[a] | *P*-value | miRNA ID | Fold change[a] | *P*-value |
| hsa-miR-21 | 1.650198 | 0.032628 | hsa-miR-103 | 0.578887 | 0.02360 |
| hsa-miR-138 | 1.645756 | 0.043586 | hsa-miR-132 | 0.328297 | 0.02459 |
| hsa-miR-365 | 1.746684 | 0.047170 | hsa-miR-141 | 0.335244 | 0.03184 |
| hsa-miR-545 | 1.655292 | 0.038122 | hsa-miR-379 | 0.521367 | 0.03609 |
| hsa-miR-603 | 1.893133 | 0.031299 | hsa-miR-299-3p | 0.409409 | 0.00293 |
| hsa-miR-631 | 1.606680 | 0.035403 | hsa-miR-24-2* | 0.614162 | 0.03496 |

[a]Cancer tissues versus adjacent normal tissues.

*The miR-132 promoter is hypermethylated in pancreatic carcinomas*

Transcriptional regulation plays a pivotal role in miRNA expression. The human *miR-212/132* cluster is far from the annotated genes in chromosome 17 and thus is postulated to be located intergenically and transcribed via its own promoter. We first predicted the presence of CpG islands in the pri-miR-212/132 promoter. The CpG Island Searcher program (http://www.uscnorris.com/cpgislands2/cpg.aspx) helped us to identify a dense CpG island near the transcription start site of pri-miR-212/132, which indicates that the mature miR-132 and its precursor may potentially be regulated through DNA methylation. To clarify the possible roles of this epigenetic mechanism of miR-132 silencing in cell lines, we treated two pancreatic cancer cell lines (PANC1 and SW1990) with 5-Aza, a methyltransferase inhibitor. We found that the treatment of cells with 5-Aza significantly elevated

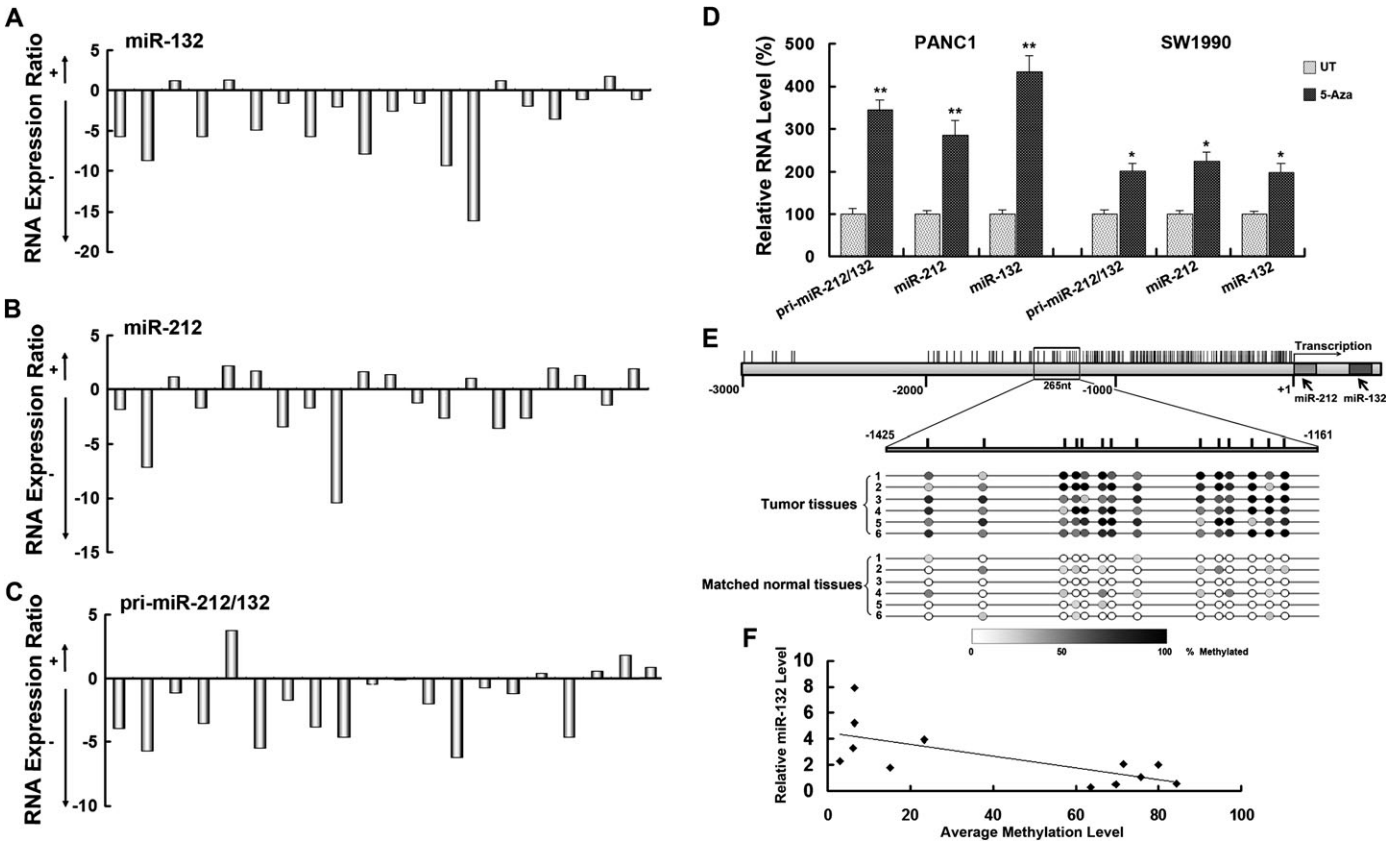


**Fig. 1.** Relative expression ratios of miR-132, miR-212 and pri-miR-212/132 in 20 pancreatic tumor biopsies and the methylation status of the pri-miR-212/132 promoter in the tissue samples. The 20 coupled biopsies were obtained from fresh surgical resection. The expression level of miR-132 (**A**), miR-212 (**B**) and pri-miR-212/132 (**C**) was measured in triplicate by Taqman real-time PCR analysis. T (tumor)/N (non-tumor) relative miRNA expression ratios between normalized values were calculated. T/N values $<1$ were transformed to the inverse value N/T. $+$ and $-$ indicate that the expression is higher or lower, respectively, in the tumor sample than in the respective adjacent control tissue. (**D**) DNA methylation inhibitor 5-aza stimulated the expression of pri-miR-212/132, miR-212 and miR-132 compared with untreated (UT) samples in PANC1 and SW1990 cell lines. (**E**) The promoter of miR-132 was hypermethylated in pancreatic tumors. Schematic map of the miR-132 promoter indicates CpG doublets (vertical tick marks, CpG sites). After bisulfite treatment of DNA, 10 clones of each PCR product were sequenced. The color of circles for each CpG site represents the percentage of methylation. (**F**) Correlation analysis between relative miR-132 expression level and average methylation level of the 14 CpG sites.

the expression of pri-miR-212/132 and its two mature products (miR-132 and miR-212) in both cell lines (Figure 1D), indicating the existence of epigenetic regulation in their promoter.

To further illustrate the methylation status of the pri-miR-212/132 promoter, sodium bisulfite sequencing assay was used. Genomic DNA from six pancreatic cancer tissues showed hypermethylation in the miR-132 promoter, whereas only a few methylated cytosines in CpG dinucleotides were detected in the respective non-cancerous tissues (Figure 1E). Statistical analysis further revealed that the mean methylation level negatively correlated with miR-132 expression ($r = -0.668$, $P = 0.013$; Figure 1F) but not with miR-212 expression. These data indicated that methylation was involved in the reduced expression of miR-132 during pancreatic cancer development.

*The miR-132 promoter contains cis*-modulating elements and *trans*-factors

To find the *cis*-regulatory elements of the miR-132 promoter, fragments from positions −2668, −1205, −754 and −445 to position −74 in the miR-132 promoter were cloned into a luciferase reporter plasmid and their transcriptional activity was assessed in pancreatic cancer cells (Figure 2B). We found that in both PANC1 and SW1990 cells, deletion of the region from −2668 to −1205 did not obviously affect the reporter activity, whereas further deletion to position −754 significantly reduced the activity (Figure 3B), suggesting that the region between −1205 and −754 in the promoter is a *cis*-activating element for miR-132 expression.

To further explore the *trans*-factors of miR-132 transcription, bioinformatic tool Alibaba2.1 (available at http://www.gene-regulation.com/pub/programs/alibaba2/index.html; Min mat. Conservation 80% and minimum number of sites 5) was used to predict potential transcription factors in the pri-miR-212/132 promoter. The results revealed that abundant putative binding sites for the transcription factor Sp1 were present (Figure 2A), indicating that this *cis*-element is a major transcription factor in pri-miR-212/132 transcription. Other *trans*-acting factors such as activator protein 2 and CREB were also predicted.

To examine whether Sp1 and CREB could regulate the expression of miR-212 and miR-132, the pGL3-2668 vector was cotransfected with different transcription factors in PANC1 and SW1990 cells. The expression of the reporter was found to be remarkably upregulated to 8.1-fold upon Sp1 cotransfection in PANC1 and to 6.8-fold in SW1990 cells (Figure 2C), suggesting that the interplay between Sp1 and the pri-miR-212/132 proximal promoter is involved in the

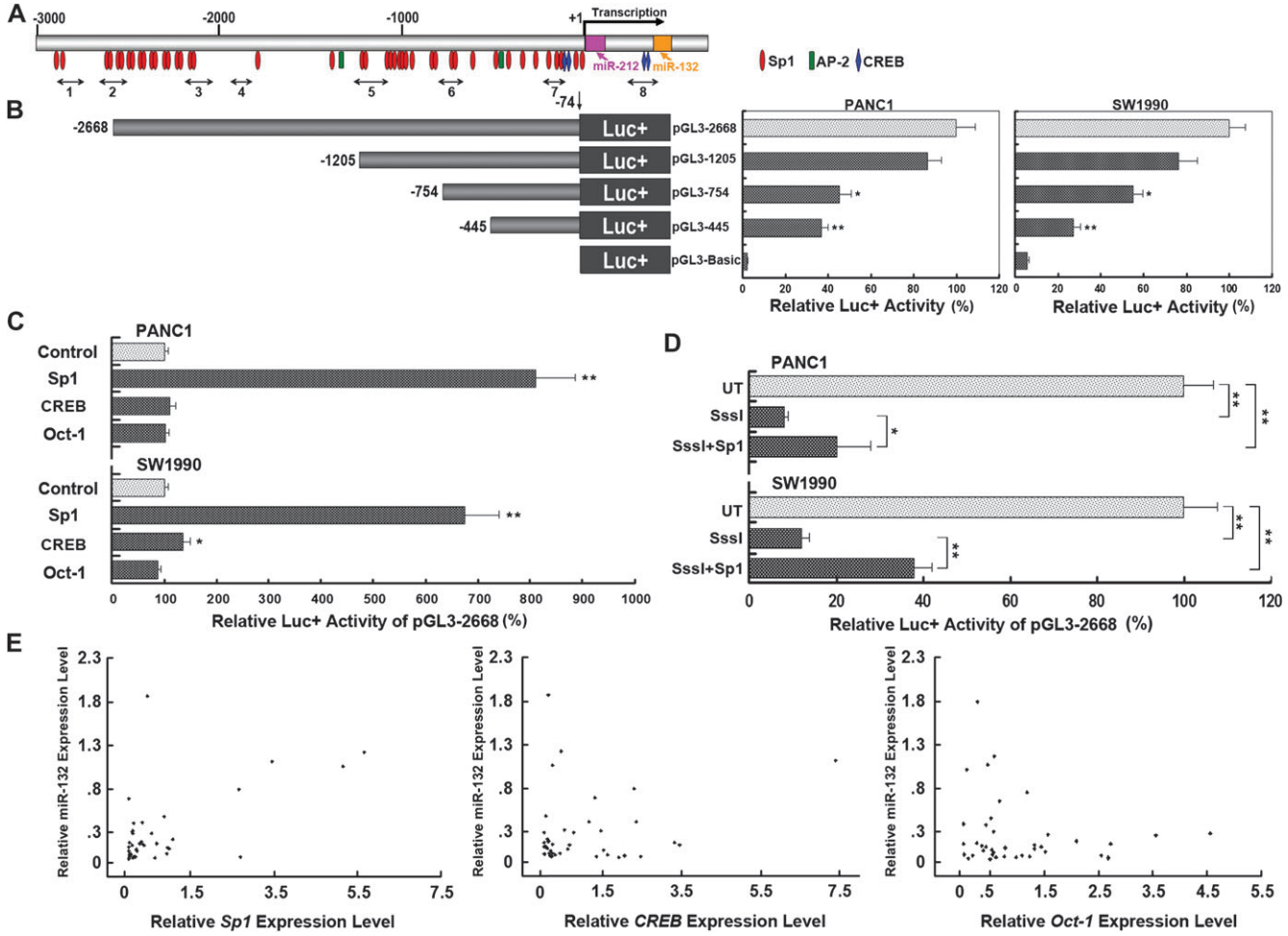


**Fig. 2.** The expression of miR-132 promoters and correlation between the transcription factors and miR-132 expression levels in 20 carcinoma tissues and matched adjacent tissues. (**A**) A diagram indicates the relative positions of transcription factors (Sp1, red; AP-2, green and CERB blue) and the location of the miR-212/132 cluster. (**B**) Truncated promoters were cloned upstream of the firefly luciferase reporter gene. We named each recombinant vector pGL3-X, where X is the first base of each truncated promoter. (**C**) Transcription factors were involved in the transactivation of the pri-miR-212/132 promoter. The pGL3-2668 vector coupled with the pri-miR-212/132 was cotransfected with the transcription factor overexpression vector or control vector in PANC1 and SW1990 cells. Each value represents the average of at least three independent experiments. (**D**) Sp1 and methylation status affected the pri-miR-212/132 promoter activity. The reporter constructs examined were either untreated (UT) or treated with SssI to methylate the DNA. (**E**) mRNA levels of endogenous miR-132 (relative to U6) showed a significant positive correlation with Sp1 expression (relative to glyceraldehyde 3-phosphate dehydrogenase; $P < 0.0001$, $r = 0.589$) but not with that of CREB ($P = 0.205$, $r = 0.204$) or Oct-1 ($P = -0.191$, $r = 0.237$).

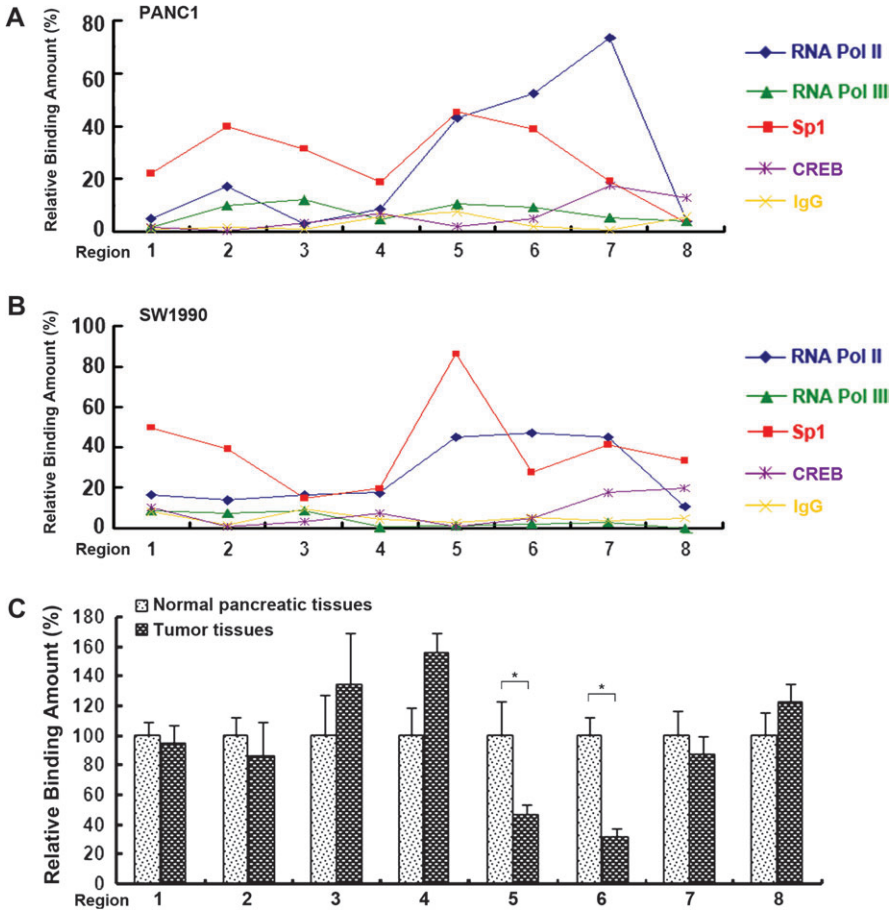


**Fig. 3.** ChIP analysis of binding affinity for RNA polymerases and transcription factors in cells and tissues. ChIP assays were performed in PANC1 (**A**) and SW1990 cells (**B**). The eight areas (depicted in Figure 2A) were examined by real-time PCR, and the results are shown as ratios of immunoprecipitated DNA (IP) to input DNA. Each value represents the average of three independent experiments. (**C**) The relative presence of Sp1 in 10 pancreatic tissue samples compared with the matched non-tumor counterpart.

regulation of miR-132 in pancreatic cancer. In addition, CREB significantly increased the reporter expression to 1.4-fold in SW1990 cells.

We were intrigued by whether the expression levels of miR-132 correlated with that of the transcription factors in the tissue samples. Using quantitative real-time PCR, we found that endogenous miR-132 levels significantly correlated with that of *Sp1* ($P < 0.0001$, $r = 0.589$; Figure 2E) but not with *CREB* ($P = 0.205$, $r = 0.204$) or *Oct-1* ($P = -0.191$, $r = 0.237$).

### *Hypermethylation eliminates Sp1 binding in the miR-132 proximal promoter*

Hypermethylation may destroy the accessibility of transcription factors to promoters. Therefore, we treated the pGL3-2668 construct with the methylase SssI to mimic the hypermethylation status of the pri-miR-212/132 promoter *in vivo* and then examined its activity in PANC1 and SW1990 cells. Methylated pGL3-2668 showed remarkably reduced luciferase activities relative to the unmethylated reporter, indicating that hypermethylation could substantially cripple its expression (Figure 2D). Moreover, Sp1 stimulation of the methylated pGL-2668 reporter (<3-fold) was much lower than that of the unmethylated construct, suggesting that hypermethylation of the pri-miR-212/132 promoter might abrogate Sp1 binding and transactivation.

Next, using ChIP assays, we explored the active region for the binding of RNA polymerases and transcription factors within ~3 kb upstream of the pri-miR-212/132 promoter. In both PANC1 and SW1990 cells, we observed significant enrichment of PolII binding from −1269 bp, whereas PolIII was unlikely to show binding affinity to the pri-miR-212/132 promoter (Figure 3A and B). Moreover, the transcription factor Sp1 displayed the highest binding affinity at Region 5 (region labeled '5' in Figure 2A; −1269 to −1133 relative to the transcription start site). The occupancy for CREB was relatively weak at Region 7 and Region 8. Additionally, we used quantitative ChIP to detect the relative binding amount of Sp1 in both normal and cancerous pancreatic tissues at the eight regions in 10 matched tissues. We found that the *in vivo* Sp1 binding was reduced to ~52% in Region 5 and 38% in Region 6 for pancreatic cancer tissues relative to normal tissues (Figure 3C); this result may be a consequence of hypermethylation in the pri-miR-212/132 proximal promoter.

### *Inhibition of miRNA-132 in pancreatic cancer cell promotes cell proliferation and colony formation*

Because our above results indicated that miR-132 expression was reduced in pancreatic cancers and that miR-132 might act as a tumor suppressor, we next tried to explore the function of miR-132 in pancreatic cancer development. The CCK-8 proliferation assay showed that miR-132 mimics significantly repressed the proliferation of PANC1 cells (Figure 4A). Colony formation analysis consistently demonstrated that miR-132 mimics inhibited SW1990 and PANC1 cells and causes them to grow less and in smaller colonies (Figure 4B). Conversely, anti-miR-132 transfection in PANC1 and SW1990 cells significantly stimulated cell proliferation (Figure 4A) and resulted in increased PANC1 or 1990 PANC1 colony foci (Figure 4B). These results suggest that miR-132 attenuates the proliferation and anchorage-independent growth of pancreatic cancer cells *in vitro*.

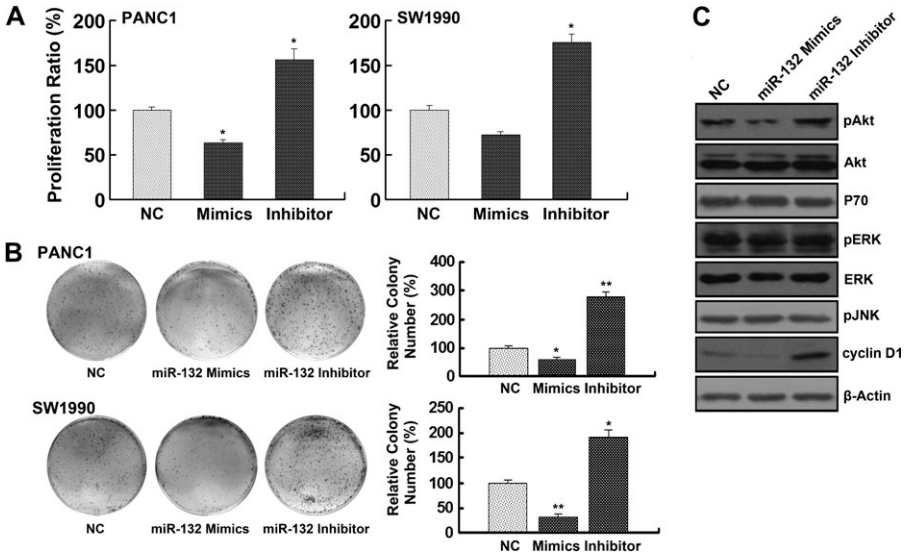


**Fig. 4.** The effect of miR-132 on cell proliferation and colony formation. (**A**) SW1990 and PANC1 cell lines were seeded in a 96-well plate. After transfection, the cells were incubated for 96 h. Cell proliferation was measured by CCK-8. (**B**) About 1000 cells were seeded on each plate and transfected with indicated RNA. After 10 days, cells were stained with crystal violet. Colonies consisting of >50 cells were counted. The data are presented as the means ± SEM normalized to the miRNA-negative control (NC) transfected cells. (**C**) Western blot analysis of total and phosphorylated levels of Akt, ERK, JNK, P70 and cyclin D1 in PANC1 cells.

*The Akt-signaling pathway is involved in miR-132-induced cancer cell suppression*

To investigate the pathways implicated in the miR-132-induced suppression of cancer cells, we analyzed the expression of signaling of pathways using western blot analysis. In agreement with the growth disadvantage, the phosphorylation of Akt and expression of cyclin D1 were strongly downregulated after miR-132 transfection, whereas the protein level of Akt remained unchanged (Figure 4C). In contrast, the expression and/or phosphorylation of ERK, JNK and P70 were not changed. These results demonstrated that the Akt pathway is involved in miR-132-induced suppression of cancer cells.

## Discussion

In this study, using miRNA array analysis of human clinical specimens, we found that six miRNAs were overexpressed and six were underexpressed in pancreatic cancer tissues compared with respective adjacent benign pancreatic tissues. Our finding shared some differentially expressed miRNAs in common with previous studies. Notably, similar to earlier reports, miR-21 is uniquely overexpressed in pancreatic cancers; this miRNA plays a role in increasing cell proliferation, invasion and chemoresistance to gemcitabine (14–16). miR-365 is upregulated in primary breast cancer compared with normal adjacent tumor tissues (17). miR-141, a member of the miR-200 family, is downregulated in gastric, pancreatic, prostate, hepatocellular and renal cell carcinoma (18–20). Our data add to the list of miRNAs with aberrant expression in pancreatic cancer. The decreased expression of miR-132 was consistent upon further validation in 20 pairs of tissues. miR-132 and miR-212 are derived from the same pri-miRNA transcript. In the microarray analysis and Taqman real-time PCR analysis, we did not observe a significant correlation between the expression of the two miRNAs and miR-212 did not show a significant alternation in expression between pancreatic tissues and matched non-tumor tissues. It was found that the *miR-212/132* cluster was found to be able to produce four miRNAs in mice: miR-132, miR-132*, miR-212 and miR-212*. Among the four miRNAs, miR-132 is found to be the predominantly active product in hippocampal neurons (21). We postulate that the processing of miR-132 and miR-212 in human pancreatic tissues was imbalanced and that posttranscriptional regulation of miRNA plays a role in this imbalance.

Rat miR-132 is located in an intron but is transcribed independently (22). Our results show that the upstream region of miR-132 harbors RNA PolII-binding sites and can mediate transcription of a luciferase reporter, suggesting that it is transcribed via its own promoter. RNA PolIII was initially believed to mediate miRNA transcription because it transcribes most small RNAs such as tRNAs and U6 snRNA (23,24). However, evidences have shown that pri-miRNAs are structurally analogous to mRNAs (25). Pri-miRNAs are mainly transcribed by RNA PolII, yet some miRNAs are transcribed by RNA PolIII (26,27). Here, using ChIP analysis, we found that the miR-212/132 promoter could recruit RNA PolII but not RNA PolIII, suggesting that pri-miR-212/132 is transcribed via RNA PolII. Besides RNA Pol, transcription factors are required for the activation of miR-132. CREB protein has been identified in the regulation of miR-132 in rat neurons and human primary lymphatic endothelial cells by a genome-wide screen (22,28). One of the CREB-binding sites lies in a region between miR-212 and miR-132. This CREB-binding region is conserved between human and rat. Consistent with this finding, we detected CREB binding in Regions 7 and 8. Importantly, transcription of miR-132 is influenced more by Sp1 than by CREB in human pancreatic cancer cells. Furthermore, our study explored the reason for the miR-132 expression difference. CpG methylation is one of the epigenetic mechanisms for miRNA deregulation in cancer. We found that promoter hypermethylation and reduced Sp1-binding affinity resulted in the underexpression of miR-132 in tumor tissues. Because Sp1 can recruit histone deacetylase and DNA methyltransferase to repress transcription (29,30), it is not clear whether methylation of the miR-132 promoter is induced by Sp1 or whether hypermethylation of the promoter abrogates Sp1 binding.

The functions and target genes of miR-132 have gradually been illustrated. miR-132 regulates neuronal morphogenesis by decreasing levels of the GTPase-activating protein, p250GAP[21] (22) and regulates antiviral innate immunity through suppression of the p300 transcriptional coactivator (28). In addition, miR-132 acts as an angiogenic switch by suppressing endothelial p120RasGAP expression, leading to Ras activation and the induction of neovascularization (31). Since each miRNA can regulate numerous target protein-coding genes, its function can be interpreted as the sum of the functions of the genes it regulates (32). In this study, we did not investigate the

direct targets of miR-132. Instead, we analyzed the consequence of miR-132 deregulation in pancreatic cancer progression. Ectopic expression of miR-132 inhibited cell proliferation colony formation, suggesting a tumor suppressor role. Several signaling pathways regulate cell proliferation and cell-cycle progression such as the mitogen-activated protein kinase–ERK and the phosphatidylinositol 3 kinase–Akt pathways (33,34). Overexpression of miR-132 reduced Akt phosphorylation and cyclin D1 expression but not expression or phosphorylation of ERK and JNK, indicating that miR-132 exerts its tumor repressing function through inactivation of the Akt-signaling pathway. Activation of Akt occurs frequently in pancreatic cancer and is of significant prognostic value; this protein has been suggested as a target for pancreatic cancer therapy (35,36). Cyclin D1, a downstream effector of the Akt pathway, promotes pancreatic cancer cell proliferation (37). Because genes involved in the Akt pathway are not predicted to be direct targets of miR-132 by the bioinformatic tools TragetScan 5.1 and PicTar, inactivation of the Akt-signaling pathway may be a consequence of the miR-132 regulatory network.

Taken together, our study illustrates the mechanism driving miR-132 transcription and the role of miR-132 in pancreatic cancer. miR-132 acts as a tumor suppressor via inactivation of the Akt pathway. This report contributes to the understanding of the role of miR-132 in pancreatic cancer progression and the diagnostic and therapeutic potential for pancreatic cancer.

## Supplementary material

Supplementary Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

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