

# Epigenetic Silencing of MicroRNA miR-107 Regulates Cyclin-Dependent Kinase 6 Expression in Pancreatic Cancer

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## Key Words

microRNA · Promoter methylation · miR-107

## Abstract

Aberrant expression of microRNAs (miRNAs) has emerged as an important hallmark of cancer. However, the putative mechanisms regulating miRNAs per se are only partially known. It is well established that many tumor suppressor genes in human cancers are silenced by chromatin alterations, including promoter methylation and histone deacetylation. We postulated that miRNAs undergo similar epigenetic inactivation in pancreatic cancer. Two human pancreatic cancer cell lines – MiaPACA-2 and PANC-1 – were treated with the demethylating agent, 5-aza-2'-deoxycytidine (5-Aza-dC) or the histone deacetylase inhibitor, trichostatin A, as well as the combination of the two. Expression of miRNAs in control and treated cell lines was assessed using a custom microarray platform. Fourteen miRNAs were upregulated two-fold or greater in each of the cell lines following exposure to both chromatin-modifying agents, including 5 that were in common (miR-107, miR-103, miR-29a, miR-29b, and miR-320) to both MiaPACA-2 and PANC-1. The differential overexpression of miR-107 in the treated cancer cell lines was confirmed by Northern blot assays. Methylation-specific

PCR assays for assessment of CpG island methylation status in the 5' promoter region of the miR-107 primary transcript demonstrated complete loss of methylation upon exposure to 5-Aza-dC. Enforced expression of miR-107 in MiaPACA-2 and PANC-1 cells downregulated *in vitro* growth, and this was associated with repression of the putative miR-107 target, cyclin-dependent kinase 6, thereby providing a functional basis for the epigenetic inactivation of this miRNA in pancreatic cancer.

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## Introduction

MicroRNAs (miRNAs) are a diverse class of 18–24 nucleotide RNA molecules that demonstrate remarkable evolutionary conservation [1]. The principal function of these noncoding RNAs is to regulate the stability and translation of miRNA transcripts. Physiologic regulation of expression by miRNAs plays a critical role in development and homeostasis. Aberrant expression of miRNA is widespread, if not ubiquitous, in human cancers, with the identification of both overexpressed and underexpressed miRNAs in neoplastic cells compared to their normal counterparts [2]. While such misexpression may be con-

strued as an epiphenomenon of the cancer phenotype, an increasing body of evidence suggests that these miRNA alterations are causal to tumorigenesis. Analogous to cancer-associated coding genes, both oncogenic miRNAs (onco-miRs) and tumor-suppressor miRNAs (TSG-miRs) have been identified in human cancers [3, 4]. As can be expected, candidate onco-miRs are typically overexpressed (for instance, miR-21, or the miR-17-92 polycistron) [5, 6], while putative TSG-miRs such as miR-15a, miR-16, and the *let-7* family are downregulated in cancer cells [7, 8]. Many of the coding gene targets altered by misexpressed miRNAs in human cancers have been identified. For example, the critical *RAS* oncogene is a target of the *let-7* miRNA family, and decreased expression of *let-7* members results in elevated *RAS* transcripts in adenocarcinomas of the lung [9]. Conversely, the miR-17-92 polycistron inhibits the expression of the TSG *phosphatase and tensin homolog deleted on chromosome ten* (PTEN), which likely contributes to the oncogenic phenotype associated with this miRNA cluster [6, 10, 11].

Even as miRNAs have emerged as master regulators of the cellular transcriptome, a critical question pertains to the mechanisms regulating these noncoding elements themselves. In some instances, misexpressed miRNAs are a reflection of genomic alterations in the neoplastic cells. Thus, reduced expression of miR-15 and miR-16 often correlates with deletions of chromosome 13q14 in B cell lymphomas, while conversely, recurrent amplifications of 13q31 result in overexpression of the miR-17-92 polycistron contained within this amplicon [12-14]. In other instances, bona fide transcription factors such as C-myc, p53, and Twist have been identified as direct transcriptional regulators of miRNA, as evidenced by binding to the promoter region of the corresponding miRNA primary transcript [15-17]. Since the activity of these transcription factors is often altered in neoplastic cells, there is, not surprisingly, an associated disruption in miRNA homeostasis as well.

Epigenetic mechanisms, including promoter methylation and histone modification, play a critical role in the regulation of mammalian gene expression [18]. Aberrations in the 'epigenome' are widespread in human cancers, with transcriptional silencing of tumor suppressor genes (TSGs) by promoter methylation observed in nearly all malignancies [19, 20]. In many of these instances, restitution of gene expression through chromatin modification leads to growth inhibition, underscoring the importance of epigenetic silencing in tumor suppression [21]. Not surprisingly, several recent studies have identified that subsets of miRNAs are also regulated via epigenetic mecha-

nisms in human cancers, typically through methylation of CpG islands within the miRNA primary transcript promoter region [22-34]. Thus, in addition to genomic alterations and transcription factor regulation, epigenetic mechanisms have emerged as another 'cog in the wheel' for the control of miRNA expression [35, 36]. In this study, we have utilized an unbiased approach to identify epigenetically regulated miRNAs in pancreatic cancer. We confirm that the miR-107 promoter undergoes methylation in pancreatic cancer cells, which can be reversed with chromatin-modifying agents. Further, we show that enforced expression miR-107 in pancreatic cancer cells inhibits *in vitro* growth, and represses cyclin-dependent kinase 6 (CDK6) levels, which links epigenetic inactivation of this miRNA in cancers with cell cycle progression.

## Materials and Methods

### *Treatment of Pancreatic Cancer Lines with Chromatin-Modifying Agents*

MiaPACA-2 and PANC-1 pancreatic cancer cell lines were maintained as previously described [37]. The cell lines were treated with the demethylating agent, 5-aza-2-deoxycytidine (5-Aza-dC; Sigma, St. Louis, Mo., USA) and histone deacetylase inhibitor, trichostatin A (TSA; Sigma), either alone or in combination, as previously described [38, 39]. Briefly, the cells were exposed to either 5-Aza-dC (1  $\mu$ M) for 4 days, or to TSA (1  $\mu$ M) for 24 h. Mock-treated cells were cultured with the equivalent volume of PBS. For the combination treatment, these cells were cultured in the presence of 5-Aza-dC (1  $\mu$ M) for 3 days and then treated for another 24 h with TSA (0.5  $\mu$ M), as described [38, 39].

### *Monitoring the Efficiency of Epigenetic Derepression*

In order to confirm the efficiency of drug treatment, we performed RT-PCR for two previously described epigenetically silenced genes in MiaPACA-2 and PANC-1 cells, NPTX2 and UCHL1, as described [38]. Total RNA was extracted from pancreatic cancer cell lines using TRIzol reagent (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions, and reverse transcribed using Superscript II (Invitrogen, Grand Island, N.Y., USA). Semi-quantitative RT-PCR was performed under the following conditions: (a) 95°C for 5 min; (b) 35 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s, and (c) a final extension of 4 min at 72°C. Primer sequences were 5'-CATCGAGCTGCTCATCAAC-3' (forward) and 5'-CTGCTCTTGCCAAG-GATC-3' (reverse) for NPTX2, 5'-CTTCATGAAGCAGACCATTG-3' (forward) and 5'-ATCATGGGCTGCCTGTATG-3' (reverse) for UCHL1. *GUSB* was utilized as a housekeeping gene.

### *MiRNA Analysis Using a Custom Microarray Platform*

Microarray analysis for differentially expressed miRNAs in 5-Aza-dC-, TSA-, and combination-treated, and control PBS-treated, MiaPACA-2 and PANC-1 cells was performed using a custom Combimatrix microarray platform as we have previously described [16, 40]. Briefly, these custom microarrays contain oli-

gonucleotide probes complementary to 474 human miRNAs. Probes containing 2 mismatches were included for all miRNAs. The hybridized arrays were scanned using a GenePix 4000B microarray scanner (Axon) and signal intensities were extracted using the Combimatrix Microarray Imager software. The background value was determined by calculating the median signal from the mismatch probes and this value was subtracted from all perfect-match probes. Signals that were less than 1.5 times background were removed and datasets were median-centered prior to calculating fold-change values. Fold-change was calculated relative to signals in the PBS-treated cells.

#### Northern Blot Analysis for Validation of miR-107 Expression

Northern blot assays were performed in control and treated MiaPACA-2 and PANC-1 cells as follows: 20 µg of total RNA was separated on 15% denaturing polyacrylamide gels (Invitrogen), transferred to GeneScreen Plus membranes (Invitrogen), and hybridized using UltraHyb-Oligo buffer (Ambion, Foster City, Calif., USA). The mature miR-107 sequence was obtained from Sanger institute miRBase web site (<http://microrna.sanger.ac.uk/sequences/index.shtml>), and complementary oligonucleotide end-labeled with T4 Kinase (Invitrogen) were used as probes.

#### Identification of Conserved CpG Islands in the miR-107 Regulatory Domain

The pri-miR-107 sequence is located at chromosome 10q23.31, within an intron of *PANK1*, a coding gene. The putative promoter region of pri-miR-107 was localized using our previously described in silico strategy for identifying miRNA promoters [16, 41, 42]. Briefly, the VISTA Browser (May 2004, <http://pipeline.lbl.gov/cgi-bin/gateway2>) is used to localize the predicted regulatory domain upstream of the pri-miRNA transcription site that is conserved across 6 nonhuman species – dog (May 2005), mouse (August 2005), rat (June 2003), cow (September 2004), opossum (October 2004) and chicken (February 2004). Arbitrarily, at least 50% or more conservation across 3 or more species is used as criterion to determine interspecies conserved sequences, as we have previously described [16, 41, 42]. The conserved regions are then overlaid with the CpG island track on the UCSC Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>), in order to delineate highly conserved CpG islands within the regulatory domain.

#### Methylation-Specific PCR for Reversible CpG Island Methylation

The methylation status of the CpG island upstream of miR-107 was determined by methylation-specific PCR (MSP). DNA samples were treated with sodium bisulfite (Sigma) for 3 h at 70°C, and purified with the Wizard DNA clean-up system (Promega, Madison, Wisc., USA). Thereafter, 1 µl of bisulfite-modified DNA was amplified using sequencing primers or primers specific for methylated or unmethylated DNA. MSP primers were designed to detect the sequence differences between methylated and unmethylated DNA as a result of bisulfite modification, and each primer pair contained at least 4 CpG sites to provide optimal specificity [38]. Primer sequences used in this study are shown in table 1. PCR conditions were as follows: (a) 94°C for 5 min; (b) 40 cycles of 95°C for 30 s, 57°C 62°C for 30 s, and 72°C for 30 s; and (c) a final extension of 5 min at 72°C. Finally, 10 µl of each PCR product were loaded onto 3% agarose gels and visualized by ethidium bromide staining.

**Table 1.** Methylation specific PCR primers for assessment of CpG island methylation in the miR-107 promoter

Methylated forward	TGTGTAGTAGTTTCGTTTATAGC
Methylated reverse	GACTCTACGACTACTAAATCG
Unmethylated forward	TGTGTAGTAGTTTGTTTATAGTG
Unmethylated reverse	CCAACTCTACAACACTACTAAATC

#### Retroviral Expression of miR-107 in Pancreatic Cancer Cells

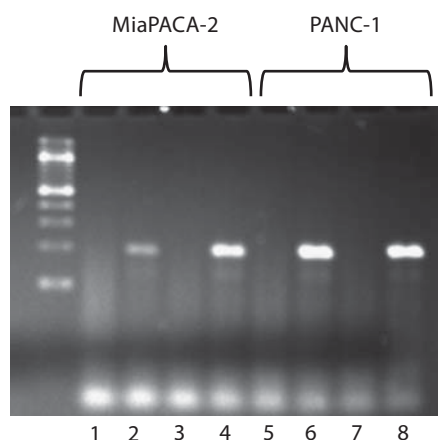
The miR-107 primary transcript was amplified with primers (forward 5'-ATACCGCTCGAGTGCCATGTGTCCACTGAAT and reverse 5'-ATACCGCTCGAGTTCCATGCCTCAACTCC-TCT) and cloned into the *XhoI* site of the retroviral vector pMSCV-PIG, as we have previously described [16, 42]. Phoenix packaging cells (obtained from G. Nolan, Stanford University, Stanford, Calif., USA) were transfected with 6 µg of DNA using Eugene 6 (Roche) according to the manufacturer's protocol. Following transfection, retroviral supernatants were collected, filtered, and added to MiaPACA-2 and PANC-1 cells for 8 h in the presence of 6 µg/ml polybrene. Two days after infection, puromycin was added to the media at 1 µg/ml and cell populations were selected for 48 h, subsequent to which, cells were trypsinized and counted. The infected cells were then plated and growth rates measured over 6 days using the Cell Counting Kit-8 (CCK-8, Dojindo, Rockville, Md., USA). Cells were infected with an empty MSCV-PIG vector as a control for these experiments.

#### Western Blot Analysis for CDK6

Western blot analysis for CDK6 expression was performed in PANC-1 cells with enforced miR-107 expression compared to cells with mock pMSCV-PIG infection, using anti-CDK6 (Santa Cruz).

## Results

We treated MiaPACA-2 and PANC-1 cells with the chromatin-modifying agents 5-Aza-dC, TSA or the combination, as described [38]. The efficiency of treatment was confirmed by the reexpression of two epigenetically silenced genes, *NPTX* and *UCHL1*, whose promoters are methylated in pancreatic cancer, as we have previously described [38]. Specifically, in both cell lines, reexpression was seen in the cells receiving 5-Aza-dC and combination therapy (fig. 1). We then performed microarray analysis on the Combimatrix platform, with 4 sets of microarrays (control, 5-Aza-dC, TSA, and combination) for each of the cell lines. In both cell lines, we identified independent panels of 14 miRNAs that were upregulated two-fold or greater upon combination therapy, compared to control cells (table 2). Of these, there were 5 miRNAs that were upregulated in both cell lines: miR-29a, miR-



**Fig. 1.** Assessing the efficiency of reexpression of epigenetically silenced genes in pancreatic cancer lines. RT-PCR for *NPTX* and *UCHL1* in MiaPACA-2 and PANC-1 cells confirms reexpression in cells treated with 5-Aza-dC and combination (5-Aza-dC and TSA) while no expression is seen in the PBS-treated cells and the TSA-only treated cells. Lanes 1 and 5: PBS-treated MiaPACA-2 and PANC-1, lanes 2 and 6: 5-Aza-dC treated; lanes 3 and 7: TSA-treated; lanes 4 and 8: combination treated.

29b, miR-103, miR-107, and miR-320. For further validation studies, we selected miR-107 due to several reasons, including its known association with human cancer [43, 44], a well-characterized primary transcript, and the existence of a conserved CpG island in the upstream sequence (see below) that would facilitate MSP analysis.

Northern blot analysis for miR-107 in treated MiaPACA-2 and PANC-1 cells confirmed the upregulation of the mature miRNA upon 5-Aza-dC and combination therapy (fig. 2); in MiaPACA-2 cells, miR-107 was also upregulated with TSA alone. In silico analysis of the miR-107 primary transcript showed the presence of an evolutionarily conserved CpG island upstream of the *PANK1* transcription start site (fig. 3), highly suggestive of a regulatory role for this region. We designed MSP primers for discrimination of the methylation status of this CpG island in control pancreatic cancer cells compared to those with 5-Aza-dC treatment. Treatment with 5-Aza-dC was accompanied by complete loss of CpG island methylation in both MiaPACA-2 and PANC-1 cells (fig. 4), consistent with epigenetic regulation of the miR-107 promoter. In the control cells, the presence of a product using MSP primers for the detection of unmethylated sequence suggested that methylation of the CpG island was partial.

Having established that the transcription of miR-107 is epigenetically regulated in pancreatic cancer cells, we next wanted to determine the phenotypic consequences

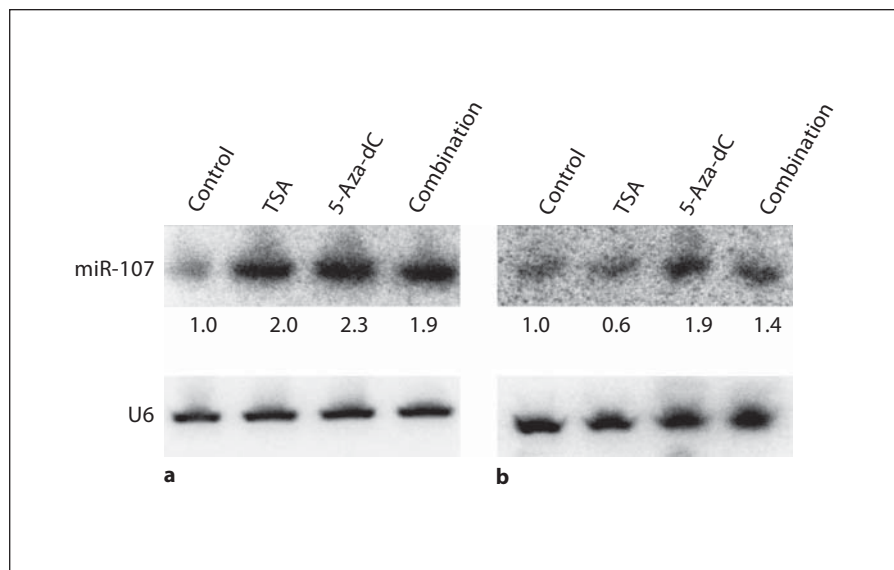
**Table 2.** Differentially upregulated miRNAs in MiaPACA-2 and PANC-1 cells treated with chromatin modifying agents

MiRNA	5-Aza-dC	TSA	5-Aza-dC + TSA
<b>MiaPACA-2</b>			
miR-193a	2.5	1.4	3.5
miR-214	2.0	3.0	2.9
miR-22	1.4	2.0	2.5
<b>miR-320</b>	1.5	2.3	2.5
miR-191	1.6	1.4	2.4
<b>miR-29b</b>	1.5	2.0	2.2
<b>miR-107</b>	1.4	1.6	2.2
miR-182	1.1	1.5	2.2
miR-27a	1.0	1.7	2.1
miR-31	1.3	1.8	2.1
<b>miR-29a</b>	1.3	2.6	2.0
miR-30b	1.1	1.5	2.0
<b>miR-103</b>	1.3	1.4	2.0
miR-19a	1.1	2.6	2.0
<b>PANC-1</b>			
<b>miR-29a</b>	2.4	3.1	5.2
<b>miR-29b</b>	1.5	1.8	2.9
miR-16	1.6	1.9	2.8
miR-23b	1.5	1.9	2.7
<b>miR-107</b>	2.0	1.1	2.5
<b>miR-103</b>	1.9	1.2	2.4
miR-20b	1.6	1.5	2.3
miR-93	1.7	1.7	2.2
<b>miR-320</b>	1.2	1.7	2.2
miR-25	1.6	1.5	2.1
miR-181d	1.5	1.3	2.0
miR-24	1.1	1.7	2.0
miR-494	1.7	1.9	2.0
miR-20a	1.5	1.2	2.0

Numbers represent fold upregulation compared to control MiaPACA cells, as measured on the Combinatrix miRNA microarray platform. Fold changes are rounded to 1st decimal place. miRNAs with at least 2-fold upregulation in combination treatment condition were selected. miRNAs in bold are present in both differentially upregulated lists.

of miR-107 reexpression in cells with low endogenous levels of the mature miRNA. Retrovirally induced enforced expression of miR-107 in PANC-1 and MiaPACA-2 lines led to in vitro growth inhibition at 6 days, compared to cells infected with an empty vector (fig. 5). The effect on growth was particularly pronounced in PANC-1, which is a highly treatment-resistant metastatic pancreatic cancer cell line [45]. In order to identify a putative coding gene target of miR-107 that might underlie the observed growth phenotype, we conducted an in silico search for genes with miR-107 'seed' sequences on TargetScan

**Fig. 2.** Northern blot assay for validation of miR-107 reexpression in pancreatic cancer cells. Relative quantification of the miRNA signals under each condition, normalized to U6 snRNA expression, is indicated below the Northern blot. Note the low endogenous expression of miR-107 in both cell lines, and its upregulation in the 5-Aza-dC and combination therapy conditions. **a** MiaPACA-2 cells. **b** PANC-1 cells.



(<http://www.targetscan.org>) [46]. CDK6, a cell cycle progression antigen that functions as a retinoblastoma kinase [47], contains miR-107 binding sites in its 3'UTR. Western blot analysis for CDK6 in PANC-1 cells with or without enforced miR-107 expression confirmed repression of CDK6 in the presence of exogenous miR-107 (fig. 6), providing a potential mechanistic link between cell growth and epigenetic silencing of this candidate 'TSG-miR' in pancreatic cancer.

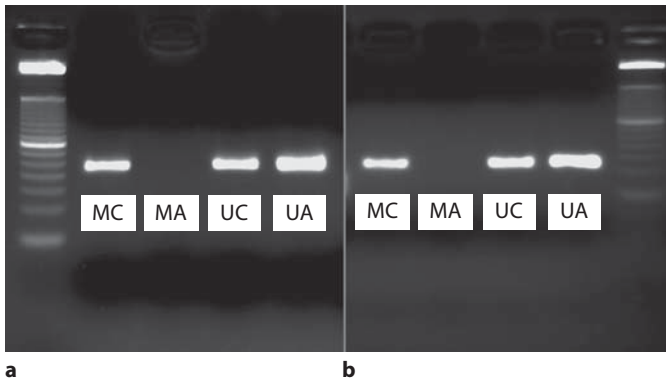
## Discussion

Recent reports have underscored the role of epigenetic regulation of noncoding miRNAs in human cancer, akin to what has been reported over the past decade in the context of coding genes [22–34]. One of the first examples for this phenomenon was reported by Jones et al. [22] in bladder cancer, where miR-127 was shown to be reversibly silenced by promoter methylation in the neoplastic cells. Further, these authors demonstrated that reexpression of miR-127 in bladder cancer causes translational repression of the antiapoptotic protein BCL-6, providing a functional basis to the observed phenotype of growth inhibition. More recently, epigenetic regulation of miRNAs has been documented in breast [23, 29, 33], colon [25, 27, 30], ovarian [24], brain [34], liver [32], and oral cancers [28], as well as in hematological malignancies [26], underscoring the rather ubiquitous nature of this phenomenon in human cancer. An miRNA methylation signature associated with cancer metastases has also been reported,

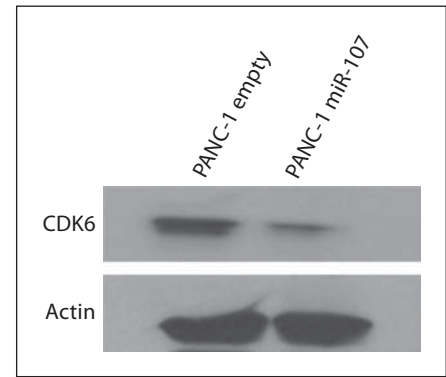
with epigenetic silencing of miR-148a, miR-34b/c and miR-9 in primary cultures obtained from lymph nodal metastases [31]. Of note, reexpression of these silenced miRNAs in highly metastatic head and neck cancer cells blocked systemic tumor metastases in vivo [31]. The majority of these aforementioned studies for identification of epigenetically inactivated miRNAs have relied upon an unbiased microarray-based approach using cancer cell lines treated with chromatin-modifying agents, validating the rationale for our current strategy with pancreatic cancer cells.

To the best of our knowledge, this is the first report on global epigenetic regulation of miRNAs in pancreatic cancer. We have identified miR-107 as a candidate miRNA that undergoes transcriptional silencing through methylation of a conserved CpG island in the promoter sequence. Recently, this miRNA was shown to be upregulated during retinoic-acid-induced differentiation in acute promyelocytic leukemia cells [43]. An empirical study examining the effects of a large panel of miRNA inhibitors on cell growth in A549 lung carcinoma found acceleration of growth upon miR-107 inhibition although the functional basis for this effect was not further examined [44]. Of note, miR-107 is *overexpressed* in nonductal tumors of the pancreas (pancreatic endocrine and acinar cell tumors) [48] while miR-155, a commonly upregulated miRNA in ductal adenocarcinomas [49–51], is essentially absent in nonductal tumors. This raises the rather intriguing possibility that ductal adenocarcinomas (also known as 'pancreatic cancers') are characterized by a '*miR-155 positive, miR-107 negative*' pattern, while non-



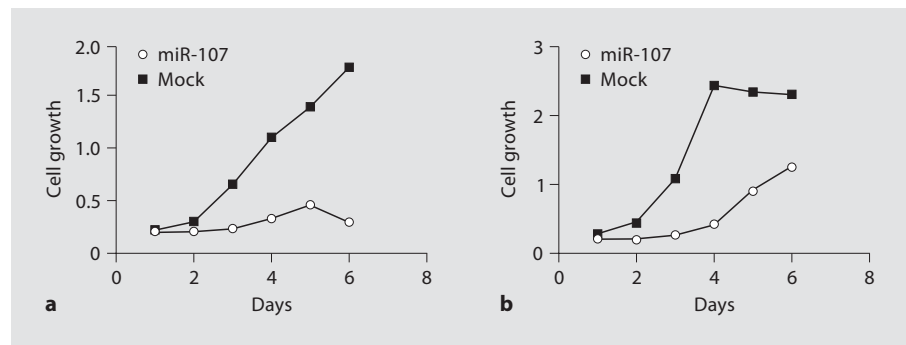


**Fig. 4.** MSP analysis of the conserved CpG island in the miR-107 promoter. MSP confirms complete loss of CpG island methylation upon 5-Aza-dC treatment in MiaPACA-2 (a) and PANC-1 (b) cells. The presence of an unmethylated band in the control cells confirms partial methylation of the CpG island in the miR-107 promoter. Note the qualitative increase in the unmethylated product upon 5-Aza-dC treatment, underscoring an alteration in the ratio of methylated:unmethylated alleles in treated cells. MC = Methylated primer, control; MA = methylated primer, 5-Aza-dC; UC = unmethylated primer, control; UA = unmethylated primer, 5-Aza-dC.



**Fig. 6.** Enforced miR-107 expression represses CDK6 in pancreatic cancer cells. Western blot analysis for CDK6 was performed in PANC-1 cells with enforced retroviral miR-107 expression or cells infected with the empty retrovirus. Actin is used as loading control.

**Fig. 5.** Enforced expression of miR-107 in pancreatic cancer cells inhibits in vitro growth. Retrovirally expressed miR-107 in PANC-1 (a) and MiaPACA-2 (b) cells inhibits in vitro growth at 6 days of culture, compared to cells infected with the empty virus. Growth was measured daily, beginning at day 1 after puromycin selection, using the Cell Counting Kit-8 (CCK-8, Dojindo).



the cell cycle, recent studies have also identified a novel function for CDK6 in blocking cellular differentiation, which is not shared by its functional homolog, CDK4 [reviewed in ref. 55]. Given the prior observation that miR-107 is induced during retinoic-acid-mediated differentiation [43], it is worth speculating that epigenetic silencing of miR-107 and the secondary elevation of CDK6 in pancreatic cancer have effects beyond growth promotion alone. Of note, miR-107 is not the only putative ‘TSG-miR’ that appears to target CDK6; a recent study on miR-34a has demonstrated that restitution of this epigenetically silenced miRNA also represses CDK6 in cancer cells and inhibits in vitro growth [56]. Our group and others have previously identified miR-34a as a p53-regulated miRNA that is downregulated in many cancers, includ-

ing pancreatic cancer [16, 57, 58]. Thus, it is likely that there are manifold mechanisms for the observed elevation of CDK6 in pancreatic cancer, both genetic (loss of function mutations of the CDK inhibitor, p16) [59, 60] and epigenetic (methylation of miR-107 and miR-34a promoters, respectively).

Finally, one needs to mention that, in addition to miR-107, our study also found microarray-based evidence for epigenetic silencing of other miRNAs in pancreatic cancer, including miR-29a, miR-29b, miR-103, and miR-320. A subset of these miRNAs has already been documented to have a putative TSG-like role in other malignancies, underscoring the rationale for their epigenetic silencing in pancreatic cancer. For example, miR-29b is highly expressed in normal cholangiocytes, but significantly

downregulated in cholangiocarcinoma cells [61]. Enforced miR-29b expression in cholangiocarcinoma represses the antiapoptotic protein and putative miR-29b target, Mcl-1, and sensitizes the cancer cells to tumor-necrosis-factor-related apoptosis-inducing ligand cytotoxicity. On the same lines, loss of miR-320 expression correlates with significantly lower recurrence-free survival in stage II microsatellite stable colorectal cancers, highlighting an association between miRNA loss of function and tumor progression [62]. From a therapeutic standpoint, one is encouraged by the premonition that restituting the expression of one or more of these epigenetically

silenced miRNAs will also emerge as a therapeutic strategy in pancreatic cancer, as recently documented with other disease models [63].

### Acknowledgements

A.M. is supported by NIH P50CA062924 SPORE in GI Cancers, the Sol Goldman Pancreatic Cancer Research Center, and the Michael Rolfe Foundation for Pancreatic Cancer Research. J.M. is supported by the Leukemia and Lymphoma Society, Rita Allen Foundation Society, the Sol Goldman Pancreatic Cancer Research Center, and the NIH (R01CA120185).

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