Published in final edited form as:

Cancer Biol Ther. 2009 November ; 8(21): 2013–2024.

A resource for analysis of microRNA expression and function in pancreatic ductal adenocarcinoma cells

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Abstract

MicroRNAs (miRNAs) are 21-24 nucleotide RNA molecules that regulate the translation and stability of target messenger RNAs. Abnormal miRNA expression is a common feature of diverse cancers. Several previous studies have classified miRNA expression in pancreatic ductal adenocarcinoma (PDAC), although no uniform pattern of miRNA dysregulation has emerged. To clarify these previous findings as well as to set the stage for detailed functional analyses, we performed global miRNA expression profiling of 21 human PDAC cell lines, the most extensive panel studied to date. Overall, 39 miRNAs were found to be dysregulated and have at least two-fold or greater differential expression in PDAC cell lines compared to control non-transformed pancreatic ductal cell lines. Several of these miRNAs show comparable dysregulation in first-passage patient-derived xenografts. Initial functional analyses demonstrate that enforced expression of miRNAs derived from the miR-200 family and the miR-17-92 cluster, both of which are overexpressed in PDAC cell lines, enhances proliferation. In contrast, inhibition of the miR-200 family, the miR-17-92 cluster, or miR-191 diminishes anchorage independent growth. Consistent with a known role for the miR-200 family in negatively regulating an epithelial-to-mesenchymal transition (EMT), the abundance of these miRNAs correlated positively with E-cadherin expression and negatively with the EMT-associated transcription factor and established miR-200 target ZEB1. Finally, reconstituted expression of miR-34a, a miRNA whose expression is frequently lost in PDAC cell lines, abrogates growth, demonstrating that the anti-proliferative activity of this miRNA is operative in PDAC. These results, and the widespread availability of PDAC cell lines wherein the aforementioned data were

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The Authors have no conflict of interest or financial disclosure statements.

generated, provide a valuable resource for the pancreatic cancer research community and will greatly facilitate functional studies essential for elucidating the consequences of miRNA dysregulation in pancreatic cancer.

Keywords

microRNA; miR-200; pancreatic ductal adenocarcinoma; microarray; oncogene; gene expression

Introduction

MicroRNAs (miRNAs) are 21-24 nucleotide RNA molecules that regulate the translational efficiency and stability of target messenger RNAs (mRNAs). First discovered as regulators of developmental timing in *C. elegans*¹⁻³, miRNAs have since been found in diverse plant and animal species where they participate in a wide range of functions including the regulation of cellular proliferation, differentiation, and apoptosis.^{4,5} In humans, over 500 miRNAs have been identified and are predicted to regulate at least one third of all mRNA transcripts. Despite this significant progress in miRNA identification, the biological roles of most miRNAs remain to be characterized.

Numerous studies have documented that dysregulated miRNA expression is a very frequent, if not ubiquitous, feature of human cancers. Furthermore, miRNA expression signatures are not only highly characteristic of specific cancer subtypes and therefore useful for tumor classification, but also have been associated with prognosis, staging, and response to therapy. Moreover, specific miRNAs exhibit oncogenic and tumor suppressor activity, supporting a causative role for altered miRNA expression in cancer pathogenesis.⁶ Examples of known oncogenic miRNAs include the miR-17-92 cluster, which accelerates tumorigenesis in models of both solid and hematopoietic malignancies,^{7,8} miR-21, which has pro-proliferative and anti-apoptotic activity in several tumor types,⁹ and miR-155, which promotes lymphomagenesis.^{10,11} On the contrary, miRNAs with tumor suppressing activity include the miR-16-1/15a cluster, which is frequently deleted in chronic lymphocytic leukemia¹² and the let-7 family, which downregulates Ras and c-Myc.^{13,14} Recently, miR-34a has been shown to be induced by p53 and exhibit potent anti-proliferative and pro-apoptotic activity.¹⁵⁻¹⁹ Given the diagnostic utility of miRNA expression patterns as well as the potential for identifying novel lesions that functionally contribute to tumorigenesis, continued classification of global miRNA expression in cancers remains a priority.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human malignancies, with a five year survival rate of less than 5%.²⁰ Recently, several independent studies have assessed miRNA expression profiles in PDAC tumor tissue, chronic pancreatitis, and normal pancreas.²¹⁻²³ Surprisingly, these studies reported disparate sets of miRNAs that exhibit differential expression between the sources of pancreatic material. The minimal overlap between these studies may be due to the highly heterogeneous nature of pancreatic tissue, which contains not only pancreatic ducts from which adenocarcinoma arises, but also stroma, acini, and infiltrating inflammatory cells. Thus, differences in tissue sampling may confound this type of expression profiling analysis. To extend and clarify these previous findings and overcome problems with tissue heterogeneity, we have analyzed global miRNA expression patterns in a panel of 21 PDAC cell lines and two non-transformed ductal epithelial cell lines. Of note, nearly all of these pancreatic lines, including the two non-neoplastic lines, are widely available to the scientific community (for example, refs. 24-28, etc.). Our studies have identified 39 miRNAs that exhibit a two-fold or greater up- or down regulation in the PDAC cell lines. We further demonstrate that the miR-200 family and the miR-17-92 cluster, both of which are over expressed in PDAC cell lines and patient-derived xenografts, confer a growth advantage when

expressed in non-transformed pancreatic epithelial cells. Inhibition of these miRNAs in multiple PDAC cell lines results in a significant negative effect on anchorage independent growth in soft agar. Since the miR-200 family is known to inhibit an epithelial-to-mesenchymal transition (EMT),²⁹⁻³² we used a linear regression analysis to confirm that miR-200 family expression is positively associated with E-cadherin expression in PDAC cell lines and negatively correlated with expression of the EMT-promoting transcription factor ZEB1. We have also demonstrated that enforced expression of miR-34a, which is lost in PDAC cell lines and in xenografts, causes a significant growth arrest in several PDAC cell lines. These results provide the most extensive catalogue to date of miRNA expression in a panel of well described PDAC cell lines, establishing a resource for future functional analyses of the consequences of miRNA dysregulation in pancreatic cancer. Moreover, these findings suggest that the miR-200 family and the miR-17-92 cluster may act as oncogenes and that miR-34a may act as a tumor suppressor in the development of PDAC.

Results

Identification of dysregulated miRNAs in PDAC cell lines

We used a custom microarray to monitor the expression of 321 human miRNAs in a panel of 21 PDAC cell lines. As controls, we examined miRNA expression in two non-transformed human pancreatic ductal epithelial cell lines, HPDE and HPNE. These non-transformed lines were derived from cultures of primary pancreatic epithelial cells immortalized with the HPV-E6E7 genes or with human telomerase reverse transcriptase (hTERT), respectively. The HPDE and HPNE cell lines are non-tumorigenic in immunocompromised mice and have wild-type function of Kras and p16^{INK4A}, proteins which are mutated or abnormally expressed in nearly all PDAC cells.³³⁻³⁵ Henceforth, HPDE and HPNE cells will be referred to as “controls” for brevity.

Expression data was filtered such that only miRNAs that fulfilled one of the following criteria were included in subsequent analyses: 1) expression of the miRNA was present in one or both controls regardless of its expression in PDAC cell lines, or 2) expression of the miRNA was present in at least three PDAC cell lines regardless of its expression in the controls. 143 miRNAs fulfilled these criteria and are shown in a heat map organized by hierarchical clustering analysis of miRNA expression (Figure 1A). Hierarchical clustering of the cell lines based on the expression of these 143 miRNAs using a Euclidean distance similarity metric revealed two distinct groups: one group containing the two control cell lines and a second group containing all the PDAC cell lines (Figure 1B). Therefore, miRNA expression patterns are sufficient to distinguish non-transformed pancreatic epithelial cells from PDAC cells.

We computed the signal-to-noise ratio of miRNA expression in PDAC cell lines to determine which miRNAs were significantly dysregulated. The signal-to-noise value reflects the difference in miRNA expression between cancer and controls relative to the standard deviation of miRNA expression within cancer and controls.³⁶ The absolute value of signal-to-noise ratio indicates the amplitude of distinction between PDAC and control cells, while the nature of differential expression (up or down in PDAC *versus* controls) is indicated by the mathematical sign preceding the ratio. The median signal-to-noise value was 0.31 for the 143 miRNAs which fulfilled our inclusion criteria. We estimated that a conservative cut off would be exhibited by miRNAs that had a signal-to-noise value double the median value and greater than two fold expression change. Of the 143 miRNAs included in this analysis, 39 miRNAs demonstrated signal-to-noise scores greater than the ± 0.6 cutoff and greater than two fold expression change (Figures 1C and 1D, Table 1).

Confirmation of dysregulated miRNAs in pancreatic cancer cell lines and xenografts

We next experimentally validated the array analysis for a subset of the differentially expressed miRNAs by northern blot analysis on total RNA isolated from 15 PDAC cell lines and the two control lines (Figure 2A and Supplemental Figure 1). The northern results correlated very well with the expression data obtained by microarray, demonstrating the reliability of this large dataset.

In order to confirm that the miRNA expression changes identified in PDAC cell lines are not an artifact of *in vitro* culture, we performed northern blot analysis of total RNA isolated from first-passage xenografts established directly from patients with pancreatic cancer. Tissue from surgically resected pancreatic carcinomas was implanted in nude mice, and the resulting xenografts were maintained and propagated as previously described.³⁷ Following establishment of adequately sized carcinomas, tumors were removed and total RNA was extracted and analyzed by northern blot (Figure 2B). We analyzed miRNAs isolated from 5 different patient derived xenografts and 4 normal bulk pancreas tissues. Although significant variability in miRNA expression was observed in these samples, miRNA expression in xenografts generally correlated well with expression in PDAC cell lines for several key miRNAs tested (Figure 2B).

Pro-proliferative effects of overexpressed miRNAs in pancreatic ductal epithelial cells

In order to begin to assess the functional consequences of miRNA overexpression in PDAC cells, we examined the effects of enforced expression of 3 groups of miRNAs: 1) the miR-200 family, the most highly upregulated miRNA family in PDAC cell lines; 2) miR-191, another highly upregulated miRNA; and 3) the miR-17-92 cluster, known to have oncogenic properties in other settings. Because mutational activation of the *KRAS2* oncogene is a central event in pancreatic cancer pathogenesis,³⁸ miRNAs were co-expressed in HPNE cells with constitutively active mutant Kras (HPNE-Kras^{G12D}). Notably, expression of Kras^{G12D} in HPNE cells alone is not sufficient to transform this cell line.³⁹ Retroviral vectors were used to stably express the complete miR-200b cluster (consisting of miR-200b, miR-200a, and miR-429), miR-200a alone, the complete miR-17-92 cluster (consisting of miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92), or miR-191 alone (Figure 3A). Enforced expression of the miR-200b cluster, miR-200a, and the miR-17-92 cluster resulted in significantly accelerated proliferation (Figure 3B). In contrast, miR-191 expression did not affect proliferation rate in this cell line. Overexpression of the miR-200b cluster or miR-200a was not sufficient to endow HPNE-Kras^{G12D} cells with anchorage-independent growth, as assayed by growth in soft agar (data not shown).

miRNA inhibition abrogates anchorage-independent growth of PDAC cell lines

We next performed miRNA inhibition experiments to assess the necessity of miRNA activity for maintaining the transformed phenotype of PDAC cells. We first interrogated the effects of inhibition of the miR-200 family in Panc-2.03, PK9, and Su86.86 cell lines, each of which overexpresses several miR-200 family members. Cells were transfected with a pool of miRNA inhibitors (Dharmacon) that target all five miRNAs derived from the two distinct miR-200 clusters (miR-200a, miR-200b, miR-200c, miR-141, and miR-429). miRNA inhibition was confirmed by northern blotting (Figure 3C). In Panc2.03 cells, at least two-fold knockdown of each mature miRNA was observed in inhibitor treated cells, with the exception of miR-429 which was not detectable by northern blotting (data not shown). Similar knockdown was observed for representative miRNAs in PK9 and Su86.86 cells treated with inhibitors (Supplemental Figure 2A). Although inhibition of the miR-200 family had no effect on the rate of cell proliferation (Supplemental Figure 2B and data not shown), a significant reduction in colony formation in soft agar was observed for all three tested cell lines (Figure 3D). Thus, the miR-200 family is essential for anchorage-independent growth of multiple PDAC cell lines.

We also tested the effects of inhibiting the miR-17-92 cluster and miR-191 cluster in Panc-2.03, PK9, and Su86.86 cells. Cells were transfected with a pool of inhibitors that target all six miRNAs derived from the miR-17-92 cluster or inhibitors that target both miRNAs derived from the miR-191 cluster (miR-191 and miR-425). Inhibition of a representative miRNA from each cluster was confirmed by northern blotting (Supplemental Figure 2A). Although inhibition of the miR-17-92 cluster or the miR-191 cluster did not effect colony formation of Panc-2.03 cells (data not shown), a dramatic reduction in colony formation was observed when either cluster was inhibited in PK-9 and Su86.86 cell lines (Figure 3D). Inhibition of either cluster in these cell lines had no effect on the rate of proliferation in culture (data not shown). These results establish that miRNA activity is essential to maintain the transformed phenotype in multiple PDAC cell lines.

miR-200 expression correlates with an epithelial phenotype in PDAC cells

In other tumor types, expression of the miR-200 family has been shown to inhibit EMT by negatively regulating the ZEB1 and ZEB2 transcription factors.²⁹⁻³² To assess whether miR-200 performs a similar function in PDAC cells, we characterized expression of miR-200 family members, E-cadherin (an epithelial marker), ZEB1, and ZEB2. Using a linear regression analysis, we documented a significant strong positive association between the sum of expression levels of all five miR-200 family members (as determined by microarray) and E-cadherin expression in 13 randomly selected PDAC cell lines ($p=7.5e-05$, Figure 4A). Furthermore, a significant positive correlation was also observed between the mean of miR-200a and miR-200b expression and E-cadherin levels, indicating that expression of miRNAs from the miR-200b cluster alone can predict E-cadherin positivity ($p=1.3e-04$, Figure 4B). We used a similar linear regression analysis to assess the relationship between the sum of expression of all five miR-200 family members and ZEB1 and ZEB2 expression. As predicted, we detected a significant negative association between miR-200 expression and ZEB1 levels in PDAC cell lines ($p=0.034$, Figure 4C). ZEB2 expression showed greater variance and was not significantly correlated with miR-200 expression; however, the data followed a negative trend (Figure 4D). In summary, these data are consistent with the existing literature documenting that ZEB1 and ZEB2 are targets of miR-200 family but also emphasize that other factors are clearly important in establishing the expression levels of ZEB1, ZEB2, and E-cadherin.

We next directly assessed the ability of miR-200 family members to regulate an epithelial phenotype in pancreatic ductal cells through gain- and loss-of-function experiments. HPNE-Kras^{G12D} cells with enforced expression of miR-200a or the miR-200b cluster exhibited reduced levels of ZEB1 and ZEB2 mRNAs (Figure 4E). In this cell line E-cadherin is not expressed at levels detectable by qRT-PCR and expression of these miRNAs did not result in induction of E-cadherin expression (data not shown). When miR-200 expression was inhibited in Panc-2.03 cells, ZEB1 and ZEB2 expression were dramatically enhanced and E-cadherin was strongly repressed (Figure 4F), consistent with a loss of epithelial identity.

Anti-proliferative effects of miR-34a in PDAC cell lines

miR-34a, which is frequently downregulated in PDAC cell lines, has recently been demonstrated to be an integral component of the p53 network with potent anti-proliferative and pro-apoptotic properties.¹⁵⁻¹⁹ We therefore investigated the effects of reconstituted miR-34a expression in pancreatic cancer cells. A retroviral construct was used to express miR-34a in Panc-1 or MiaPaCa2 cells, two cell lines with low endogenous miR-34a expression (Figure 5A). Expression of this miRNA dramatically inhibited cellular proliferation (Figure 5B), documenting that the anti-tumorigenic properties of miR-34a are operative in PDAC cells. However, inhibition of miR-34a in HPNE-Kras^{G12D} cells did not result in a measurable change in cellular proliferation at 3 days or 6 days post transfection (Supplemental Figure 2C).

Discussion

In this study, we used a custom microarray to monitor miRNA expression in an extensive panel of PDAC cell lines and the two non-transformed immortalized pancreatic cell lines HPDE and HPNE. Clustering analysis revealed that miRNA expression is sufficient to distinguish HPDE and HPNE from the PDAC cell lines. Moreover, these results allowed us to identify 39 miRNAs that exhibit at least a two-fold increase or decrease in expression in PDAC cells (Table 1). Several of these miRNAs also exhibited dysregulation in first-passage patient derived xenografts, demonstrating that their altered expression is not simply due to growth in culture. In multiple cases, our analyses documented coordinated dysregulation of more than one member of the same miRNA transcription unit providing an internal measure of the reliability of these data (Table 1). For example, we have shown that multiple members of the miR-200b/200a/429, miR-15a/16-1, miR-183/96/182, miR-30d/30b, and miR-99b/let-7e/125a clusters are similarly dysregulated in many cell lines. Collectively, these data provide a valuable resource for the research community, extensively documenting miRNA expression patterns in numerous commonly utilized PDAC cell lines and setting the stage for detailed functional studies of the pro- and anti-tumorigenic properties of these miRNAs.

Recently, four independent studies have examined miRNA expression profiles in biopsy material from pancreatic adenocarcinoma, chronic pancreatitis, and normal pancreas, as well as in a limited set of pancreatic cancer cell lines.^{21-23,40} Interestingly, all four studies reported very dissimilar sets of dysregulated miRNAs. These disparate results may be in part due to the difficulties involved in sample collection. Pancreatic tumor tissue is highly heterogeneous in nature, containing stroma, acini, and infiltrating inflammatory cells in addition to the adenocarcinoma cells. Thus, it is difficult to definitively conclude that each aberrantly expressed miRNA reflects a difference between non-neoplastic epithelium and cancer, rather than differences among cell types within a bulk tumor.²² Our data generated using a large panel of cell lines not complicated by contaminating cell types may begin to provide some clarity to our understanding of miRNA expression in pancreatic cancer. Indeed, several of the miRNAs we found to be abnormally expressed in PDAC cell lines were found to be similarly dysregulated in at least one of the earlier pancreatic cancer profiling studies (Table 1). One potential pitfall of our approach, however, is that miRNA profiles in cell lines may be highly influenced by *in vitro* passaging. Notably, in the study by Szafraska et al., the authors confirmed that miRNA expression profiles in PDAC cell lines more closely resemble that of primary tumors than normal pancreas tissue. Additionally, we have confirmed that several of the top dysregulated miRNAs in PDAC cell lines are similarly dysregulated in patient-derived low-passage xenografts that have never been propagated *in vitro*. Therefore, despite important caveats, many dysregulated miRNAs in PDAC cell lines are likely to exhibit comparably altered expression *in vivo*.

Two miRNAs in particular, miR-155 and miR-222, deserve special consideration as both the present study as well as 3 out of 4 of the previous studies found them to be overexpressed in PDAC cells and tumors. Overexpression of miR-155 is a frequent event in a variety of tumor types including B-cell lymphoma, and breast, lung, colon and thyroid cancers.^{10,41,42} Further, miR-155 is known to have potent pro-tumorigenic activity in several cancer models. For example, miR-155 accelerates *MYC*-mediated lymphomagenesis in a chicken model⁴³ and transgenic mice with enforced B-cell-specific miR-155 expression develop a polyclonal B-cell malignancy.¹¹ We have documented that miR-155 is upregulated by approximately 3-fold in 11 of 21 PDAC cell lines. Recently, we also demonstrated that miR-155 is overexpressed in intraductal papillary mucinous neoplasms (IPMNs), which are precursor lesions of PDAC.⁴⁴ Furthermore, the p53 regulated stress-induced gene TP53INP1, whose downregulation in PDAC correlates with tumor progression, has been shown to be a target of miR-155.⁴⁵ Together, the available data provide compelling evidence that hyperactivity of miR-155 is a

common event in pancreatic cancer which very likely contributes to tumorigenesis in this setting.

We have also shown that miR-222, which is co-transcribed in a cluster with miR-221, is upregulated by almost 4-fold in 9 of 21 cell lines. The miR-221/222 cluster has been shown to be upregulated in prostate carcinoma and human thyroid papillary carcinomas.⁴⁶⁻⁴⁷ Enforced expression of miR-221/222 in multiple cancer cell lines represses expression of the cell cycle inhibitor p27 (Kip1), promotes cell-cycle progression, and enhances anchorage-independent growth.⁴⁶⁻⁴⁸ The miR-221/222 cluster likely functions similarly to promote proliferation in PDAC.

The most highly overexpressed miRNA in PDAC cell lines is miR-200a. miR-200a is a member of the miR-200b cluster consisting of miR-200b, miR-200a, and miR-429, all of which exhibited coordinated upregulation in these cell lines. Prior studies documented overexpression of miR-200 family members in colorectal and papillary thyroid carcinoma.^{49,50} In contrast, expression of the miR-200 family was shown to be lost in invasive breast cancer cell lines with mesenchymal phenotype.³⁰ The miR-200 family has been shown to repress the ZEB1 and ZEB2 transcription factors, thereby inhibiting EMT, a key step in the metastasis of epithelial-derived tumors.²⁹⁻³² Specifically, expression of the miR-200 family, individually or as clusters, reduced EMT in metastatic breast cancer cells and primary ovarian cancer cells.^{31,32} Furthermore, it has been shown that miR-200 expression correlates highly with E-cadherin positivity in cancer cells.³² We have similarly demonstrated that expression of the miR-200 family in PDAC cell lines is positively correlated with E-cadherin expression and negatively correlated with ZEB1 expression. Enforced expression of the miR-200a cluster reduces ZEB1 and ZEB2 transcripts in HPNE-Kras^{G12D} cells while inhibition of endogenous miR-200 family members in Panc-2.03 cells results in increased expression of these transcription factors and reduction in E-cadherin levels. Based on these results, it is clear that at least in some PDAC cells, sustained expression of the miR-200 family is necessary to maintain a fully-established epithelial phenotype. However, it remains possible that in other cell lines, the miR-200 family and E-cadherin are independently regulated and their expression levels reflect the epithelial differentiation status of the cells rather than a direct interdependent regulatory relationship. Although the overexpression of miR-200 in a highly metastatic tumor like PDAC might seem counter-intuitive, emerging studies have suggested that this miRNA family, and its regulatory network, might be required for metastatic colonization *in vivo*.⁵¹

We have found that miR-34a is strongly downregulated or absent in the vast majority of PDAC cell lines examined in this study. Although it has been found that transcription of the miR-34a primary transcript is directly activated by p53 in response to DNA damage and oncogenic stress,¹⁵⁻¹⁹ it is unlikely that p53 loss directly accounts for the reduced miR-34a expression observed in PDAC. Previously, we have reported that there is not a direct correlation between biallelic p53 loss and miR-34a down regulation in PDAC cell lines.¹⁵ It is likely other mechanisms contribute to miR-34a loss such as deletion of the genomic region encompassing miR-34a (1p36) which is known to occur frequently in diverse cancer types.^{52,53} Additionally, miR-34a expression is silenced in several types of cancer due to aberrant methylation of the miR-34a promoter.⁵⁴ Previous studies have demonstrated that miR-34a exhibits potent anti-proliferative and pro-apoptotic activity. Based on the results reported here, these anti-tumorigenic properties of miR-34a are clearly operative in pancreatic cancer cells.¹⁵⁻¹⁹

In summary, the present study provides the most detailed analysis to date of miRNA expression in PDAC cell lines. These results provide a comprehensive resource that will facilitate miRNA gain- and loss-of-function experiments as well as target identification, studies that will be essential to fully elucidate the consequences of miRNA dysregulation in pancreatic cancer.

Materials and Methods

Cell lines

The HPNE cell line (obtained as a generous gift from Dr. Ouellette at the University of Nebraska Medical Center) was cultured in DMEM (low glucose) with 25% M3F Base Media (IncCell) supplemented with 5% (v/v) fetal bovine serum (FBS), 10ng/ml EGF, and 25µg/ml gentamicin. The HPDE cell line (obtained from as a generous gift from Dr. Tsao at the University of Toronto) was cultured in serum free keratinocyte media supplemented with supplied growth factors according to the manufacturer's instructions. Twenty-one pancreatic cancer cell lines, AsPc1, BxPc3, CAPAN1, CAPAN2, COLO357, E3LZ10.7, L3.6PL, MiaPaCa2, Panc-1, Panc-2.03, Panc-327, PK8, PK9, PL3, PL5, PL11, Su86.86, XPA1, XPA2, XPA3, and XPA4 were utilized in this study.²⁵ All cell lines were cultured in DMEM (4.5 mg/ml glucose) supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) with the exception of E3LZ10.7, L3.6PL, Panc-203, PL3, PL5, and PL11 which were cultured in MEM supplemented with 10% (v/v) FBS, 5% (v/v) MEM vitamin solution, 2mM L-glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate, and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The HPNE-Kras^{G12D} cell line was generated and characterized as described.³⁹ HPNE-Kras^{G12D} cells were cultured in HPNE media supplemented with 200µg/ml hygromycin.

miRNA expression profiling

Total RNA was isolated from cells with TRIZOL (Invitrogen) according to the manufacturer's protocol. RNA labeling, microarray hybridization, and data extraction were performed as described.¹⁵ Background subtracted, normalized expression profiling data is provided in Supplemental Table 1 for the miRNAs that passed our inclusion criteria (expressed in either HPDE or HPNE or at least 3 PDAC cell lines).

Analysis of microarray expression data

Signal-to-noise ratios were calculate by first adding the baseline signal (the smallest non-zero value on each array) to each miRNA expression value from that cell line (based on Watts et al. 55). Next, all resulting miRNA values for a given cell line were normalized to all other cell lines by setting the baseline value to 1. After this normalization, signal-to-noise computations were generated for each miRNA as follows: $[M1, \sigma1]$ and $[M2, \sigma2]$ represent the means (M) and standard deviations (σ) of the background normalized miRNA expression levels in PDAC (class 1) and HPDE and HPNE (class 2), respectively and the signal-to-noise ratio is defined as $[M1 - M2]/[\sigma1 + \sigma2]$ (Supplemental Table 2).

Hierarchical clustering

The expression levels of 143 miRNAs in HPDE and HPNE cell lines and 21 PDAC cell lines were converted to a rank-based matrix and standardized. The hierarchical clustering analysis was carried out using the Cluster 3.0 software⁵⁶ and the Java Treeview⁵⁷ visualization tool and was performed based on Euclidean distance similarity metric using the complete linkage clustering algorithm.

Northern blot analysis

For northern blotting, 20µg of total RNA was fractionated on 15% denaturing polyacrylamide gels, electro-transferred to GeneScreen Plus membranes, and hybridized using UltraHyb-Oligo buffer (Ambion). DNA oligonucleotides complementary to mature miRNA sequences were end labeled with T4 kinase (Invitrogen). Blots were exposed to a phosphor screen overnight, scanned with a Personal Molecular Imager FX (BioRad), and quantified using Quantity One

(BioRad) software. Blots were successively stripped and reprobed a maximum of five times. U6 snRNA served as a loading control.

RNA isolation from xenografts

Excess tissues from resected pancreatic carcinomas were implanted in nude mice to generate xenografts as described.³⁷ After harvesting, RNA was extracted from xenografts using the miR-VANA kit (Ambion) according to the manufacturer's protocol and subjected to northern blot as described above.

PCR amplification of miRNA precursors

The miR-17-92 cluster, the miR-200b cluster, miR-200a, miR-191, and miR-34a were amplified from genomic DNA using Pfu polymerase and cloned into the XhoI site in the MSCV-Neo or MSCV-Puro vectors (Clontech). Primers for the miR-17-92 cluster were described previously.⁵⁸ Primers for miR-34a were described previously.¹⁵ The primers used for the miR-200b cluster, miR-200a and miR-191 are provided in Supplemental Table 3. The sequences of the amplified products were confirmed by sequencing.

Retroviral infection and growth rates

Retroviruses were generated by transfecting Phoenix cells with miRNA-MSCV vectors using FUGENE6 (Roche) according to the manufacturer's protocol. After 24 hours, retroviral supernatants were collected, filtered, and added to target cells in the presence of 4µg/mL polybrene (Sigma). After 48 hours, transfected cells were selected by growing in 300µg/ml Neomycin or 2 µg/ml Puromycin. Cells were selected for 7-14 days for creation of stable cell lines (Figure 3) or transiently selected for 48 hours (Figure 5). Growth rates were measured using the CKK-8 kit (Dojindo).

miRNA inhibitors

miRIDIAN miRNA Hairpin Inhibitors (obtained from Dharmacon) were resuspended in 1× universal buffer (60mM KCl, 6mM HEPES-KOH pH 7.5, 0.2 mM MgCl₂), heated to 90°C for 3 minutes and cooled on ice. Hairpin Inhibitors (0.05 µM each) were complexed with DharmaFECT2 (Panc-2.03 cells) or DharmaFECT4 (PK9 and Su86.86 cells) (Dharmacon) in OPTIMEM serum free media at room temperature for 20 minutes. Complexes were then added to PDAC cells plated in antibiotic free media at a final concentration of 10nM. 48 hours later, RNA isolation, colony formation analysis, and growth rate measurements were performed. mMiRIDIAN microRNA Hairpin Inhibitor Negative Control #1 (IN-001000-01-05, Dharmacon) was used as a negative control inhibitor for experiments shown in figures 3D and 4F.

Colony formation

Cells transfected with miRNA Hairpin Inhibitors were resuspended at a concentration of 5000 cells/mL in warm 4% agarose (Invitrogen) in DMEM (4.5 mg/ml glucose) supplemented with 10% FBS in the absence of antibiotics and layered on a 4% agarose/DMEM base layer. Cells were grown for 14 days and then counted using BioRad Quantity One software.

RT-PCR and qPCR

Total RNA was isolated using Trizol (Invitrogen) and subjected to DNase I digestion. Reverse-transcription was performed using the SuperScript First-strand Synthesis System (Invitrogen) with random hexamers. Quantitative PCR was performed using an ABI 7900 Sequence Detection System with the SYBR Green PCR core reagent kit (Applied Biosystems).

Eukaryotic beta-actin was used as an internal standard. Primer sequences are provided in Supplementary Table 3.

Linear regression analysis

We analyzed the following 13 randomly selected cell lines for regression analysis: PK9, ASPC-1, E3LZ-10.7, MIAPACA, CAPAN-1, PL1, BXPC3, PANC1, PL8, SU8686, Panc203, PL3, and L3.6PL. The relationship between miR-200 family expression and E-cadherin, ZEB1 and ZEB2 mRNA levels in PDAC cell lines was assessed using a linear regression analysis using the open source statistical program R.⁵⁹ The model equation fitted for each transcript was $y_i = \beta_0 + \beta_1 x_i + e_i$, where y_i is the response variable (mean of 2 independent real time PCR measurements in log scale), x_i is the explanatory variable (sum of the miR-200a, miR-200b, miR-200c, miR-141, and miR-429 expression levels or mean of miR-200a and miR-200b expression levels as determined by microarray) and e_i is the independent and normally distributed error terms (with mean = 0 and constant variance).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Michel Ouellette at the University of Nebraska for HPNE cells and Ming-Sound Tsao at the University of Toronto for HPDE cells. We also thank Alex Amiet and Devin Leake at Thermo Fisher Scientific (Dharmacon) for microRNA inhibitors, transfection reagents and consultation. This work was supported by grants from The Lustgarten Foundation for Pancreatic Cancer Research (to J.T.M.), The Sol Goldman Center for Pancreatic Cancer Research (to J.T.M.), The Michael Rolf Foundation for Pancreatic Cancer Research (to A.M.), and the NIH (R01CA120185 to J.T.M., R01CA113669 and P50CA062924 to A.M., P01CA134292 to J.T.M. and A.M., and P30CA006973 to M.F.O.). J.T.M. is a Rita Allen Foundation Scholar and a Leukemia and Lymphoma Society Scholar. O.A.K. is a Life Sciences Research Foundation Fellow supported by Pfizer.

Abbreviations

EMT	epithelial-to-mesenchymal transition
miRNA	microRNA
PDAC	pancreatic ductal adenocarcinoma

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Figure 1. miRNA expression in pancreatic ductal adenocarcinoma cell lines. (A) Heat map of expression of 143 miRNAs in 21 PDAC cell lines and HPDE and HPNE control cell lines. (B) Enlarged dendrogram from (A) demonstrating the hierarchical clustering of cell lines based on miRNA expression. (C) Plot of miRNA signal-to-noise ratio versus fold change. miRNAs in the grey boxes represent the top 39 dysregulated miRNAs in PDAC. (D) Summary of miRNA expression changes in PDAC.

Figure 2.

Validation of dysregulated miRNA expression in PDAC cell lines and xenografts. (A) Northern blot analysis of miRNA expression in PDAC cell lines. Fold expression relative to HPDE is indicated below each lane. For this and subsequent figures, U6 served as a loading control. (B) Northern blot analysis of miRNAs isolated from normal bulk pancreas and human pancreatic tumor xenografts.

Figure 3.

Functional analyses of the miR-200 family, the miR-17-92 cluster, and miR-191 in pancreatic cell lines. (A) Northern blot demonstrating miRNA expression in parental HPNE-Kras^{G12D} cells or cells transduced with empty retrovirus or virus expressing the indicated miRNAs. (B) Growth rates of HPNE-Kras^{G12D} cell lines transduced with empty vector or the indicated miRNA-expressing vector. All growth measurements were performed simultaneously and the identical empty vector growth curve is depicted in all graphs. The effect of each miRNA is shown in a separate graph for clarity. Error bars represent the standard deviation for each data point from 6 independent wells. (C) Northern blot demonstrating knockdown of the miR-200 family in Hairpin Inhibitor-treated Panc-2.03 cells. Transfection with a non-targeting Hairpin Inhibitor (Negative) served as negative control. (D) Anchorage-independent growth of indicated miRNA inhibitor- or control (Mock and/or Negative)-treated Panc-2.03, PK9 and Su86.86 cells.

Figure 4. miR-200 expression correlates with an epithelial phenotype in PDAC cell lines. (A) Linear regression analysis of the sum of expression of miR-200 family members versus log-transformed E-cadherin levels. (B) Linear regression analysis of the mean of miR-200a and miR-200b expression versus log-transformed E-cadherin levels. (C-D) Linear regression analysis of the sum of expression of miR-200 family members versus log-transformed ZEB1 (C) or ZEB2 (D) levels. (E) Quantitative real time PCR (qPCR) analysis of ZEB1 and ZEB2 expression in HPNE-Kras^{G12D} cells transduced with empty retrovirus or retrovirus expressing miR-200a or the entire miR-200b cluster. (F) qPCR analysis of ZEB1, ZEB2, and E-cadherin expression in Panc-2.03 cells that were mock transfected or transfected with negative control

inhibitor or a pool of anti-miR-200 family inhibitors. Transcript expression was normalized to β -actin. Error bars represent standard deviations.

Figure 5. Restitution of miR-34a expression in PDAC cell lines inhibits cell growth. (A) Northern blot analysis demonstrating expression of miR-34a in Panc-1 and MiaPaCa2 cells infected with empty retroviral vector or MSCV-miR-34a (B) Photomicrographs and growth rates of retrovirally-infected cell lines.

Table 1

Top dysregulated miRNAs in Pancreatic Ductal Adenocarcinoma.

miRNA	Cluster	Signal to noise	Fold change	Cell lines (1)	Noted in prior studies (Ref)
miR-200a	200b/200a/429	1.15	10.8	8	
miR-29b	29b-1/29a, 29b-2/29c	0.74	5.9	6	
miR-182	183/96/182	1.08	4.4	8	
miR-141	141/200c	0.62	4.3	6	23
miR-222	222/221	0.76	3.8	9	21,23,34
miR-191	191/425	1.04	3.6	6	
miR-15a	15a/16-1	0.85	3.4	8	
miR-361		1.68	3.2	12	
miR-17-3p	17/18a/19a/20a/19b-1/92	1.15	3.2	11	
miR-200b	200b/200a/429	0.66	3.1	10	34
miR-26b		1.09	3	10	
miR-155		0.75	2.9	11	21-23
miR-16	15a/16-1	0.75	2.7	9	22
miR-96	183/96/182	0.67	2.7	8	
miR-30a-5p	30a-5p/30c-2	0.72	2.6	9	
miR-7		0.72	2.6	10	
miR-429	200b/200a/429	0.7	2.5	9	
miR-30c	30c/30c-1,30a-5p/30c-2	0.7	2.5	10	
miR-30b	30d/30b	0.77	2.5	8	
miR-345		1.02	2.4	12	
miR-31		0.6	2.4	9	23
miR-193b	193b/365-1	1.06	2.3	13	
miR-30d	30d/30b	0.87	2.3	8	
miR-26a		0.98	2.2	7	
miR-134	487a/328/134/668/485/453	0.81	2.1	9	
miR-151		0.84	2.1	10	
let-7c	99a/let7c	-0.99	-2.1	12	
let-7b	let7a-3/let7b	-2.01	-2.1	13	

miRNA	Cluster	Signal to noise	Fold change	Cell lines (1)	Noted in prior studies (Ref)
miR-99b	99b/let7e/125a	-0.69	-2.2	11	
miR-204		-0.71	-2.2	21	
miR-125a	99b/let7e/125a	-0.67	-2.3	12	21
miR-143	143/145	-0.71	-3	21	21,23
let-7e	99b/let7e/125a	-2.07	-3.2	12	
miR-34a		-0.79	-3.7	15	
miR-145	143/145	-0.71	-5.4	21	23
miR-199a		-0.67	-7.6	20	21
miR-424	424/503	-1.24	-10.1	18	22
miR-199a*		-0.67	-14.6	20	

¹ The number of PDAC cell lines (of a possible 21) exhibiting expression greater (for upregulated miRNAs) or less (for downregulated miRNAs) than the mean expression for that miRNA in the control cell lines.