Published in final edited form as:

Mol Carcinog. 2014 September ; 53(9): 711–721. doi:10.1002/mc.22023.

Putative Tumor Suppressor Gene *SEL1L* Was Downregulated by Aberrantly Upregulated hsa-mir-155 in Human Pancreatic Ductal Adenocarcinoma

Qian Liu¹, Jinyun Chen¹, Jin Wang², Christopher Amos¹, Ann M. Killary³, Subrata Sen², Chongjuan Wei¹, and Marsha L. Frazier^{1,*}

¹Department of Epidemiology, The University of Texas MD Anderson Cancer Center, Houston, Texas

²Department of Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas

³Departments of Genetics, The University of Texas MD Anderson Cancer Center, Houston, Texas

Abstract

Sel-1-like (SEL1L) is a putative tumor suppressor gene that is significantly downregulated in human pancreatic ductal adenocarcinoma (PDA). The mechanism of the downregulation is unclear. Here, we investigated whether aberrantly upregulated microRNAs (miRNAs) repressed the expression of *SEL1L*. From reported miRNA microarray studies on PDA and predicted miRNA targets, we identified seven aberrantly upregulated miRNAs that potentially target *SEL1L*. We assessed the expression levels of *SEL1L* mRNA and the seven miRNAs in human PDA tumors and normal adjacent tissues using real-time quantitative polymerase chain reaction. Then statistical methods were applied to evaluate the association between *SEL1L* mRNA and the miRNAs. Furthermore, the interaction was explored by functional analysis, including luciferase assay and transient miRNA overexpression. *SEL1L* mRNA expression levels were found to correlate inversely with the expression of hsa-mir-143, hsa-mir-155, and hsa-mir-223 ($P < 0.0001$, $P < 0.0001$, and $P = 0.002$, respectively). As the number of these overexpressed miRNAs increased, *SEL1L* mRNA expression progressively decreased ($P_{\text{trend}} = 0.001$). Functional analysis revealed that hsa-mir-155 acted as a suppressor of *SEL1L* in PDA cell lines. Our study combined statistical analysis with biological approaches to determine the relationships between several miRNAs and the *SEL1L* gene. The finding that the expression of the putative tumor suppressor *SEL1L* is repressed by upregulation of hsa-mir-155 helps to elucidate the mechanism for *SEL1L* downregulation in some human PDA cases. Our results suggest a role for specific miRNAs in the pathogenesis of PDA and indicate that miRNAs have potential as therapeutic targets for PDA.

Keywords

microRNA; gene expression regulation; reverse phase protein assay; Ingenuity Pathways Analysis

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States [1]. The most common form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDA), which accounts for more than 75% of all pancreatic tumors [2]. Unfortunately, the symptoms of pancreatic cancer usually do not manifest until local or distant metastasis has already occurred, and currently, there are no reliable biomarkers for early detection of this deadly disease.

Sel-1-like (SEL1L) is the human homolog of the *Caenorhabditis elegans sel-1* gene [3-5]. In *C. elegans*, this gene has been identified as an extragenic negative regulator of the lin-12/Notch family of receptor proteins [6,7]. The Notch signaling pathway, which is highly conserved, plays important roles in cellular differentiation, proliferation, and apoptotic events and has been associated with several cancers [8]. In addition to regulating Notch receptor proteins, *SEL1L* plays a role in modulating the expression of several other cancer-related proteins, including Smad4, activin A, and PTEN [9,10].

SEL1L messenger RNA (mRNA) is abundantly expressed in healthy pancreatic tissues in adult humans [3,4], and at the protein level, *SEL1L* is highly expressed in normal pancreatic acinar and islet cells [9]. *SEL1L* has been reported to be downregulated in a significant proportion of PDAs at both the mRNA and protein levels [3,9]. Overexpression of *SEL1L* causes a decrease in tumor growth both in vitro and in vivo; this suggests that *SEL1L* has a tumor-suppressive role [9,11].

To date, however, the mechanisms of *SEL1L* downregulation in PDA remain unclear. Determining these mechanisms could lead to the discovery of new biomarkers for early detection and/or new targets for treatment of PDA, but so far, neither functional mutations nor genomic changes in the *SEL1L* gene have been detected [3,12]. In this study, we sought to determine whether aberrantly upregulated microRNAs (miRNAs) are responsible for *SEL1L* downregulation.

MiRNAs are endogenous noncoding RNAs about 18–24 nucleotides long. Generally, miRNAs bind to the 3' untranslated region (UTR) of their target genes through imperfect complementation and repress gene expression either by increasing mRNA degradation or by inhibiting translation [13]. Whether the binding of miRNA drives mRNA degradation or translational inhibition of the target gene depends on the structure of the miRNA–mRNA duplex [14]. Some studies have shown that miRNAs may repress target gene translation even when they do not affect the mRNA level [15]. Emerging evidence shows that deregulated miRNAs may play an oncogenic or tumor-suppressive role in different kinds of cancers by repressing the expression of oncogenes or tumor suppressor genes [15,16].

The 3' UTR of the *SEL1L* gene is larger than 4000 bp and thus has great potential in terms of binding targets for miRNAs. Using miRNA microarrays, three studies have investigated genomewide alterations in miRNA expression in PDA [17-19]. Eighteen miRNAs displayed upregulation in PDA in at least two of these studies.

Here, we report that upregulation of certain miRNAs are associated with the downregulation of a putative tumor suppressor *SEL1L*. The findings suggest that the miRNAs play an important role in the pathogenesis of PDA through regulating the expression of target tumor suppressor genes and through the subsequent cellular networks. Our findings provide a foundation for studying miRNAs as biomarkers for therapeutic targets for this dismal disease, as well as possible markers for early detection.

MATERIALS AND METHODS

Materials

PDA tumors and matched normal-appearing adjacent pancreatic tissues were collected from a total of 42 patients who underwent surgical resection at The University of Texas MD Anderson Cancer Center before treatment with chemotherapy or radiotherapy. All tissues were snap frozen and stored at -80°C until RNA and protein were extracted. Cell lines used in this study were either developed in our laboratory or purchased from American Type Culture Collection (Manassas, VA). This study was approved by and conducted in accordance with the policies of the Institutional Review Board at MD Anderson.

Cell Culture

PDA cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 1% penicillin/streptomycin (Mediatech). hTERT-HPNE cells were cultured in 75% Dulbecco's modified Eagle's medium without glucose (Mediatech), 25% Medium M3 Base (Incell Corporation, San Antonio, TX) supplemented with 10 ng/mL human recombinant epidermal growth factor (Sigma-Aldrich, St. Louis, MO), 5.5 mM D-glucose (Mediatech), 5% fetal bovine serum, and 0.75% puromycin (Mediatech).

Extraction of RNA and Protein

Total RNA including miRNA was extracted using the miRNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). RNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). All the RNA samples used in this study passed a quality check with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Protein for Western blotting was isolated from the phenol-ethanol supernatant obtained after precipitation of the RNA. The isolation procedures for protein followed the TRIzol reagent protocol (Invitrogen, Carlsbad, CA). For reverse phase protein assay (RPPA), the cells were washed twice in ice-cold phosphate-buffered saline (PBS) and then lysed in Radio-Immunoprecipitation Assay buffer [150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris (pH 8.0)] (Sigma-Aldrich) for 1 h on ice, and the supernatant was collected after 15 min

of centrifugation at 14 000 rpm. Protein concentrations were determined with bicinchoninic acid protein assay reagents (Pierce, Rockford, IL).

MiRNAs Predicted to Target *SEL1L*

We started with the 18 miRNAs that had displayed upregulation in PDA in miRNA microarray studies [17-19]. We then searched the micro-RNA.org resource (miRanda) to identify which of those miRNAs were predicted to potentially target *SEL1L*. MiRanda is a comprehensive resource for miRNA target predictions that computes the optimal sequence complementarity between a mature miRNA and an mRNA using a weighted dynamic programming algorithm [20]. From the searches, we identified seven aberrantly upregulated miRNAs that potentially target *SEL1L*: hsa-mir-143, hsa-mir-155, hsa-mir-181a, hsa-mir-181c, hsa-mir-205, hsa-mir-210, and hsa-mir-223.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

The expression levels of *SEL1L* mRNA and of the seven mature miRNAs were examined in the DNA Analysis Facility at MD Anderson. The TaqMan gene expression assay kit and the TaqMan miRNA assay kit (Applied Biosystems, Foster City, CA) were used for the assays. The assays were run using a 7900HT fast real-time PCR system (Applied Biosystems). RNA from the matched normal-appearing adjacent tissue was used as a calibrator for the tumor tissue. *SEL1L* mRNA expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and expression of the miRNAs was normalized to that of U6B small nuclear RNA. All of the assays were run in duplicate. The relative quantities (RQs) of mRNA or miRNA expression in the tumors were calculated as described by Livak and Schmittgen [21]. A gene was considered to be upregulated if $RQ \geq 2$, which means that the gene expression in the tumor was at least twice that in the normal-appearing adjacent tissue. Similarly, a gene was considered to be downregulated if $RQ \leq 0.5$, which means that the gene expression in the tumor was half or less than half that in the normal-appearing adjacent tissue.

Western Blotting

Protein samples of 30 μ g each were resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. A goat polyclonal antibody to *SEL1L* (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 0.8 μ g/mL in tris-phosphate buffered saline with 5% Blotto (GE Healthcare, Piscataway, NJ). Bovine anti-goat immunoglobulin G-horseradish peroxidase secondary antibody (Santa Cruz Biotechnology) was used at a dilution of 1:5000. GAPDH antibody (Abeam, Cambridge, MA) at 0.4 μ g/mL was used as a loading control. Goat anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology) was used as a secondary antibody to GAPDH at a dilution of 1:5000. Amersham ECL Plus Western blotting detection reagents (GE Healthcare) were used to detect the signals. The intensity of the bands on the western blots was measured with Quantity One analysis software, version 4.6.1 (Bio-Rad Laboratories, Hercules, CA). Desired bands from two exposure times (20 s and 1 min) were quantified, and their average intensities were calculated. The RQ of *SEL1L* protein expression in the tumor was normalized to that of GAPDH and compared with the RQ of *SEL1L* protein expression in the matched normal-

appearing adjacent tissue. We again used the RQs of 2 and 0.5 as the cutoff values for considering a gene to be up- and downregulated.

Statistical Analysis

Fisher's exact test was used to evaluate the association between the expression level of *SEL1L* mRNA and the expression levels of the miRNAs. The Kruskal-Wallis test was used to evaluate expression differences between the groups with 0, 1, 2, or 3 upregulated miRNAs. Pearson's correlation coefficient was used to measure the correlation between *SEL1L* mRNA expression and *SEL1L* protein expression in PDA tumors. All tests of statistical significance were two-sided. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using Stata version 10.1 software (Stata, College Station, TX).

Luciferase Assay

The pMIR-REPORT miRNA expression reporter vector system (Applied Biosystems) was used to determine whether the miRNA bound to the predicted binding site and regulated reporter expression. The predicted hsa-mir-155 binding site in the *SEL1L* gene and more than 100 bp of flanking sequences on either side of the predicted binding site were inserted into the cloning region at the 3' UTR of the firefly luciferase sequence (construct vector). The 100-bp flanking sequences help ensure that the secondary structure of the binding site will not be interrupted, which makes the experimental condition better represent the in vivo condition. A second vector, pMIR-REPORT beta-galactosidase (β -gal) reporter vector, was used for normalization of transfection efficiency.

MDAPanc3 [22] and PL18 PDA cells were plated onto 6-well plates and reached a cell confluence of 50–60% at the time of transfection. In each well, a mixture of 1.5 μ g of luciferase reporter plasmid DNA (with or without the binding sites) and 0.15 μ g of β -gal reporter plasmid DNA was transfected into the cells with 5 μ L of Lipofectamine LTX (Invitrogen) and 1.65 μ L of PLUS reagent according to the manufacturer's instructions (Invitrogen). Cells were washed with ice-cold PBS twice and collected after 24 and 48 h, respectively. Firefly luciferase activity and β -gal activity were determined by using the Dual-Light luciferase and β -gal reporter gene assay system (Applied Biosystems) according to the manufacturer's instructions. A 20/20n single tube luminometer (Turner Biosystems, Sunnyvale, CA) was used for the measurement. All the conditions were performed in triplicate, and the average activities were calculated. Then the firefly luciferase activities were normalized to β -gal activity.

Overexpression of hsa-mir-143, hsa-mir-155, and hsa-mir-223

Precursors that mimic the function of hsa-mir-143, hsa-mir-155, or hsa-mir-223, as well as negative control for the precursors were used for the miRNA overexpression study. These molecules were purchased from Ambion, Inc. (Austin, TX). MDAPanc3 cells were plated in six-well plates and reached a cell confluence of 50–60% at the time of transfection. In each well, 30 pmol of miRNA precursor or negative control (final concentration 30 nM) was transfected into the cells using 2 μ L of Oligofectamine (Invitrogen). The cells were washed with ice-cold PBS twice and collected after 48 and 72 h of transfection. Then total RNA and

protein were extracted for qPCR and Western blotting using the same methods described previously.

Reverse Phase Protein Assay (RPPA)

Cell lysates from the hsa-mir-155 overexpression experiment were denatured with 1% sodium dodecyl sulfate (with beta-mercaptoethanol) and diluted in five twofold serial dilutions in lysis buffer containing 1% sodium dodecyl sulfate. Serially diluted lysates were arrayed on nitrocellulose-coated slides (Grace Bio-lab, Bend, OR) using an Aushon 2470 arrayer (Aushon BioSystems, Billerica, MA). Each slide was probed with validated primary antibodies plus biotin-conjugated secondary antibodies. A total of 111 proteins were included in the data set (SEL1L antibody was not available for this experiment). Besides the regular antibody, 35 phosphorylation state-specific antibodies were used to assess phosphorylation at specific sites of target proteins. All of the proteins assessed are related to cancer pathogenesis. Each array included spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. The signal obtained was amplified using a Dako Cytomation-catalyzed system (Dako, Carpinteria, CA) and visualized by diaminobenzidine colorimetric reaction. The slides were scanned, analyzed, and quantified using a customized software, MicroVigene (VigeneTech, Inc., Carlisle, MA), to generate spot intensity. Protein levels for each sample were determined by interpolation of each dilution curve into a logistic model, Supercurve Fitting, developed by the Department of Bioinformatics and Computational Biology at MD Anderson [23]. This model fits a single curve using all the dilution series on a slide with the signal intensity as the response variable and the dilution steps as independent variables. The protein concentrations of each set of slides were then normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample. A relative protein expression level was calculated by comparing the normalized linear value in the sample transfected with miRNA precursor to that in the sample transfected with negative control at the same time point. A protein was considered to be upregulated if $RQ \geq 1.500$ and to be downregulated if $RQ \leq 0.667$.

Ingenuity Pathways Analysis (IPA)

The “Core Analysis” function in IPA (Ingenuity Systems, Inc., Redwood City, CA) was used to interpret the RPPA data for pathways and networks involved [24]. Molecules from the data set displaying expression variation and contained in the Ingenuity Knowledge Base were analyzed. The significance of the association between the data set and the canonical pathway was measured by Fisher’s exact test to calculate a *P*-value. These molecules were also overlaid onto a global molecular network developed from information in the Ingenuity Knowledge Base, and networks were then algorithmically generated on the basis of their connectivity.

RESULTS

Expression Levels of hsa-mir-143, hsa-mir-155, and hsa-mir-223 Are Inversely Correlated With *SEL1L* mRNA Expression in PDA Tumors

The RQs of the expression of *SEL1L* mRNA and the 7 miRNAs were initially assessed in 20 pairs of PDA tumors and normal-appearing adjacent tissues by qPCR. The preliminary data showed that only levels of hsa-mir-143, hsa-mir-155, and hsa-mir-223 correlated significantly with *SEL1L* mRNA expression. Subsequent analyses of an additional 22 pairs of samples for these 3 miRNAs corroborated these findings (Table 1).

SEL1L mRNA was downregulated in 55% of the PDA tumors, whereas the seven miRNAs were upregulated in the following percentages of the PDA tumors: hsa-mir-155, 52%; hsa-mir-205, 45%; hsa-mir-210, 45%; hsa-mir-181c, 40%; hsa-mir-143, 38%; hsa-mir-181a, 35%; and hsa-mir-223, 33%.

The downregulation of *SEL1L* mRNA was significantly associated with the upregulation of hsa-mir-143, hsa-mir-155, and hsa-mir-223 as evaluated using Fisher's exact test ($P < 0.0001$, $P < 0.0001$, and $P = 0.002$, respectively). As shown in Table 1, 14 (88%) of the 16 samples in which hsa-mir-143 was upregulated showed *SEL1L* mRNA downregulation, as did 18 (82%) of the 22 samples in which hsa-mir-155 was upregulated and 12 (86%) of the 14 samples in which hsa-mir-223 was upregulated. Further, of the 23 tumor samples in which *SEL1L* mRNA was downregulated, 20 (87%) displayed upregulation of at least 1 of these 3 miRNAs. The other four miRNAs (hsa-mir-181a, hsa-mir-181c, hsa-mir-205, and hsa-mir-210) did not show a significant correlation with *SEL1L* mRNA expression (Table 1).

Increased Number of Upregulated miRNAs Correlates With Decreased *SEL1L* Expression

Because only hsa-mir-143, hsa-mir-155, and hsa-mir-223 showed correlations with *SEL1L* mRNA expression, we focused on the upregulation of these three miRNAs. We divided the 42 PDA samples into four groups on the basis of the number (0, 1, 2, or 3) of upregulated miRNAs in each sample. The RQs of *SEL1L* mRNA expression in the tumors were compared for the groups. We found that as the number of overexpressed miRNAs increased, the median RQ of *SEL1L* mRNA expression decreased (Figure 1). The RQs of *SEL1L* mRNA expression among the four groups were significantly different ($P = 0.004$), and the trend of an increased number of upregulated miRNAs corresponding to decreased expression of *SEL1L* mRNA was also significant ($P_{\text{trend}} = 0.001$).

SEL1L Protein Expression Correlates Significantly With *SEL1L* mRNA Expression

Because mRNA expression and protein expression are not always parallel that many mechanisms may affect protein levels at the transcriptional, translational, and posttranslational levels [25], to determine whether *SEL1L* protein expression is influenced by the expression of *SEL1L* mRNA, using Western blotting we assessed *SEL1L* protein expression level in the same set of PDA samples. Protein samples extracted from 40 of the 42 sample pairs were available, and these were examined. As shown in Table 2, *SEL1L* mRNA was downregulated in 21 (52.5%) of the 40 PDA tumors, and *SEL1L* protein was

downregulated in 16 (76.2%) of these 21 tumors. *SEL1L* mRNA and SEL1L protein displayed the same trends of expression (both up, both down, or both normal) in 32 (80%) of the 40 cases. Pearson's correlation test showed that the RQ of SEL1L protein expression varied directly and significantly with the RQ of *SEL1L* mRNA expression ($r^2 = 0.605$, $P < 0.0001$).

Expression of hsa-mir-143, hsa-mir-155, and hsa-mir-223 in PDA Cell Lines by qPCR

The hTERT-HPNE cell line was used as a "normal" control. This immortalized cell line was derived from normal ducts of human pancreas and presents the closest thing to normal human pancreas in terms of cell lines. hTERT-HPNE cells have been found to be able to differentiate into pancreatic ductal cells [26]. We assessed the expression levels of hsa-mir-143, hsa-mir-155, and hsa-mir-223 in a series of pancreatic cancer cell lines and in the hTERT-HPNE cell line by qPCR. The RQs of these three miRNAs in the PDA cell lines were then compared to the corresponding RQ in the hTERT-HPNE cell line.

In the examined PDA cell lines, hsa-mir-143 and hsa-mir-223 displayed relatively low levels of expression, whereas hsa-mir-155 showed differential expression levels. Functional analyses of these three miRNAs were conducted on the base of these results.

Luciferase Assay Revealed Decreased Reporter Expression in the Cell Line Expressing High Level of hsa-mir-155

We determined by qPCR that PL18 cells have relatively high expression levels of hsa-mir-155 and MDAPanc3 cells have very low expression levels of this miRNA. When the construct vector containing predicted binding site to hsa-mir-155 was transfected into PL18 cells, we found significantly lower firefly luciferase expression level in these cells comparing to the reporter expression level in the PL18 cells transfected with empty vector. For the MDA-Panc3 cell line, we did not find a significant difference in luciferase expression between the cells transfected with the construct vector and those transfected with the empty vector (Figure 2A).

Of the PDA cell lines we examined, none showed high expression level of hsa-mir-143 or hsa-mir-223. Luciferase assay was not performed for these two miRNAs due to this lack of ideal cell model.

SEL1L Protein Expression Was Downregulated After Overexpression of hsa-mir-155 in MDAPancB Cells

We transfected hsa-mir-155 precursor in MDA-Panc3 cells and assessed the expression level of hsa-mir-155 after transfection using qPCR. The expression level increased markedly after transfection of the precursor comparing to the negative control. qPCR revealed that the expression level of *SEL1L* mRNA did not show significant change when hsa-mir-155 was overexpressed. However, Western blotting revealed that SEL1L protein expression was downregulated when hsa-mir-155 was overexpressed after 48 and 72 h (Figure 2B).

Since MDAPanc3 cells also displayed low expression levels of hsa-mir-143 and hsa-mir-223, we overexpressed these two miRNAs in MDAPanc3 cells as well. However,

SEL1L gene expression level did not show significant changes at both mRNA level and proteins level when either of these two miRNAs was overexpressed (data not shown here).

Sixteen Proteins Showed Expression Level changes After Overexpression of hsa-mir-155 in MDAPanc3 Cells

Because an miRNA can target different genes simultaneously and many of these genes including *SEL1L* are involved in different networks, it would be interesting to know what kind of influence the overexpressed hsa-mir-155 may bring to PDA cells. Therefore, we performed RPPA to investigate the transient responses of some other cancer-related proteins to the overexpression of hsa-mir-155 in MDAPanc3 cells. We found that of all the proteins for which expression was assessed, 12 had downregulated expression (both total HER2 and phosphorylated HER2 were downregulated) and 4 had upregulated expression (both total p38MAPK and phosphorylated p38MAPK were upregulated) (Table 3). Through the microRNA.org resource, we found that of the genes that encode the proteins in Table 3, only *kinase insert domain receptor (KDR)*, *epidermal growth factor receptor (EGFR)*, and *v-rel reticuloendotheliosis viral oncogene homolog A (RELA)* had potential binding sites to hsa-mir-155; therefore, only these three genes were possibly downregulated by the overexpression of hsa-mir-155.

We then uploaded the RPPA data to the IPA program. Through the use of IPA, five canonical pathways were found to be significantly associated with the RPPA data set, including pancreatic adenocarcinoma signaling and PTEN signaling (Table 4A). Three top candidate networks were also generated through the use of IPA and are summarized in Table 4B. *SEL1L* was shown to be associated with a network with function in cell death, gene expression, and organism development. Another downregulated gene, *ERCC1*, was shown to be in the same network [24].

DISCUSSION

Although in as early as 1997, the pancreatic-specific putative tumor suppressor gene *SEL1L* was reported to be downregulated at both the mRNA and protein levels in PDA [3,9], the mechanisms of the downregulation is still an unsolved mystery. In this study, we aimed to determine whether seven aberrantly upregulated miRNAs were involved in the downregulation of *SEL1L* in PDA. Using the miRNA target prediction tools miRanda, we found that these seven miRNAs were predicted to bind to *SEL1L* gene. However, the pairing match between an miRNA and a gene is not the only factor that influences the ability of a miRNA to bind and repress a target gene. Other characteristics, such as the binding position in the 3' UTR, the flanking sequences, and mRNA secondary structures, are also important factors in predicting whether a miRNA is likely to repress the expression of a target gene [27]. Therefore, whether these seven miRNAs can really regulate *SEL1L* expression needs to be confirmed by other methods. We used statistical methods and found that expression of three of these seven miRNAs (hsa-mir-143, hsa-mir-155, and hsa-mir-223) was inversely associated with *SEL1L* mRNA expression in a high proportion of PDA tumors. Further functional analysis revealed that one of the miRNA, hsa-mir-155, acted as a suppressor of *SEL1L* in PDA cell lines.

Other investigators have shown that multiple coexpressed miRNAs can target a single mRNA and work together to repress the gene expression [28-30]. Velu et al. [29] have recently demonstrated that two miRNAs, miR-21 and miR-196b, work synergistically in Lin⁻ bone marrow cells to increase the blocking of granulopoiesis. Similarly, the statistical data presented here demonstrated that overexpression of an increased number of miRNAs in PDA was associated with increased repression of *SEL1L* mRNA expression. This suggests that these three miRNAs may work cooperatively to regulate *SEL1L* expression.

Although a study on a genomic scale showed poor correlations between the level of mRNA and the level of protein [25], another study recently published in *Nature* demonstrated that for miRNA regulatory interactions, more than 84% of the decrease in protein production was associated with decreased mRNA levels. The results of this study indicated that destabilization of target mRNAs is the predominant mechanism by which miRNAs influence gene expression [31]. In our study, the significant correlation between the expression of *SEL1L* mRNA and SEL1L protein in PDA tumors also suggests that mRNA abundance is a key modulator of SEL1L protein expression in PDA. However, protein expression can be controlled by multiple mechanisms. For the small proportion of samples in which SEL1L protein expression was not decreased when its mRNA expression decreased, other molecules or networks which were not investigated in this study, such as p53 network [32], may be involved. As we know, tumors display great genetic plasticity and therefore, genes in these regulatory pathways likely play a role in modulation of the level of SEL1L protein that are independent of miRNAs.

Correlation between two variables does not provide proof that one directly causes the other, as there may be other contributing factors, but it does warrant further investigation. In our study, the results of statistical analysis provided the rationale for conducting further in vivo functional analyses to explore the role of specific miRNAs in the regulation of SEL1L expression. Therefore, we further investigated the relationship between the three miRNAs and SEL1L using molecular biological approaches.

For the luciferase assay, it was hypothesized that in a cell line with higher expression level of the miRNA, the reporter expression of vector containing the miRNA-mRNA binding site would be repressed. In PL18 cells, which had relatively high expression levels of hsa-mir-155, we did observe a significant decrease in luciferase expression in cells transfected with the reporter vector compared to cells transfected with the empty vector. No such decrease was observed in MDAPanc3 cells, which had very low levels of expression of hsa-mir-155. These results indicated that hsa-mir-155 likely does bind to the predicted binding site in the *SEL1L* gene. RNA pull-down assay would be necessary to confirm this conclusion.

When hsa-mir-155 was overexpressed in MDA-Panc3 cells through transfection of the cells with the precursor of hsa-mir-155, we found decreased expression of SEL1L protein. Unlike the results in PDA tumors, in the cell lines, decrease in SEL1L protein expression was not accompanied by a corresponding decrease in *SEL1L* mRNA expression. Larsson et al. [33] found that transcripts with high turnover rates are less affected by miRNA overexpression after transfection. Their finding may explain why we did not observe a decreased expression

level of the mRNA in cells that *SEL1L* mRNA might have a high turnover rate. In the tumor tissue, since it is not a transient status, the consistent downregulation of *SEL1L* transcripts might have overwhelmed the high turnover rate.

Although hsa-mir-143 and hsa-mir-223 showed statistical correlation with the expression of *SEL1L*, we could not confirm the correlation by biological methods. From the statistics results in Table 1, we can see that although the correlations are significant, when an miRNA was overexpressed *SEL1L* was not always downregulated. Therefore, it was not quite a surprise that we did not detect the downregulation of *SEL1L* when mir-143 and mir-223 were overexpressed in MDAPanc3. However, using the current available cell models, we could not demonstrate and conclude that these two miRNAs regulate the expression of *SEL1L*.

The results of our statistical and functional analyses suggest that hsa-mir-155 acts as a suppressor of *SEL1L*. Hsa-miR-155 has been reported to play an oncogenic role in the pathogenesis of numerous cancers, including leukemia, breast cancer, colon cancer, lung cancer, and PDA [34,35]. In our study, RPPA and IPA data revealed that besides regulating target genes directly, overexpressed hsa-mir-155 can also influence a series of pathways and networks. Interestingly, we found that several very important signaling pathways in PDA were influenced by hsa-mir-155, which demonstrates the importance of hsa-mir-155 in the pathogenesis of PDA. Because of the limited number of antibodies in the RPPA experiment, we could not detect all the genes that were affected by upregulation of this miRNA. Another limitation of the RPPA study is that the variation in protein expression needs to be further confirmed by Western blotting. This indicated that most of the protein expression changes that we observed may have been induced through cellular networks instead of direct regulation by hsa-mir-155.

Because miRNAs are shed into the serum and can be isolated and studied, they may serve as biomarkers for early detection and diagnosis of cancers and for evaluating patients' prognoses [36]. A recent study by our colleagues found elevated expression levels of hsa-mir-155 in the plasma of PDA patients compared with the plasma of healthy controls, suggesting that this miRNA may serve as a blood-based biomarker for the detection of PDA [37]. In addition, the functional involvement of miRNAs in tumorigenesis makes them potential therapeutic targets for the treatment of cancers [38,39]. Therefore, our findings may lead to the development of new treatment strategy of PDA.

In conclusion, this study combined statistical analysis with biological approaches to determine the relationships between several miRNAs and the *SEL1L* gene. It is possible that we might have left some important *SEL1L* targeting miRNAs by following this approach, one instance is hsa-mir-125b which has been reported by Le et al. [32] of targeting *SEL1L*. However, ours is the first report demonstrating that the expression of the putative tumor suppressor *SEL1L* is regulated by hsa-mir-155. It helps to elucidate the mechanism for *SEL1L* downregulation in some PDA cases. These findings also provide evidence in support of using miRNAs as a new approach for the detection, prevention, and treatment of pancreatic adenocarcinoma.

Acknowledgments

The authors would like to thank Domitila Patenia, Hongli Tang, and Yuling Lu at MD Anderson for their technical support.

Contract grant sponsor: National Cancer Institute (U01 Grant to A.M.K., M.L.F., and S.S.); Contract grant number: CA111302; Contract grant sponsor: Cancer Center Support Grant (to J.M.); Contract grant number CA016672; Contract grant sponsor: National Institutes of Health R25 Educational Grant (to R.M.C. and S.C.); Contract grant number: CA56452.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]
2. Cubilla AL, Fitzgerald PJ. Classification of pancreatic cancer (nonendocrine). *Mayo Clin Proc.* 1979; 54:449–458. [PubMed: 221755]
3. Biunno I, Appierto V, Cattaneo M, et al. Isolation of a pancreas-specific gene located on human chromosome 14q31: Expression analysis in human pancreatic ductal carcinomas. *Genomics.* 1997; 46:284–286. [PubMed: 9417916]
4. Harada Y, Ozaki K, Suzuki M, et al. Complete cDNA sequence and genomic organization of a human pancreas-specific gene homologous to *Caenorhabditis elegans sel-1*. *J Hum Genet.* 1999; 44:330–336. [PubMed: 10496078]
5. Biunno I, Bernard L, Dear P, et al. SEL1L, the human homolog of *C. elegans sel-1*: Refined physical mapping, gene structure and identification of polymorphic markers. *Hum Genet.* 2000; 106:227–235. [PubMed: 10746565]
6. Grant B, Greenwald I. The *Caenorhabditis elegans sel-1* gene, a negative regulator of lin-12 and glp-1, encodes a predicted extracellular protein. *Genetics.* 1996; 143:237–247. [PubMed: 8722778]
7. Grant B, Greenwald I. Structure, function, and expression of SEL-1, a negative regulator of LIN-12 and GLP-1 in *C. elegans*. *Development.* 1997; 124:637–644. [PubMed: 9043078]
8. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: Cell fate control and signal integration in development. *Science.* 1999; 284:770–776. [PubMed: 10221902]
9. Cattaneo M, Orlandini S, Beghelli S, et al. SEL1L expression in pancreatic adenocarcinoma parallels SMAD4 expression and delays tumor growth in vitro and in vivo. *Oncogene.* 2003; 22:6359–6368. [PubMed: 14508516]
10. Cattaneo M, Fontanella E, Canton C, Delia D, Biunno I. SEL1L affects human pancreatic cancer cell cycle and invasiveness through modulation of PTEN and genes related to cell-matrix interactions. *Neoplasia.* 2005; 7:1030–1038. [PubMed: 16331889]
11. Orlandi R, Cattaneo M, Troglio F, et al. SEL1L expression decreases breast tumor cell aggressiveness in vivo and in vitro. *Cancer Res.* 2002; 62:567–574. [PubMed: 11809711]
12. Larsen ZM, Angelo AD, Cattaneo M, et al. Complete mutation scanning of the human SEL1L gene: A candidate gene for type 1 diabetes. *Acta Diabetol.* 2001; 38:191–192. [PubMed: 11855798]
13. Eulalio A, Huntzinger E, Izaurralde E. Getting to the root of miRNA-mediated gene silencing. *Cell.* 2008; 132:9–14. [PubMed: 18191211]
14. Aleman LM, Doench J, Sharp PA. Comparison of siRNA-induced off-target RNA and protein effects. *RNA.* 2007; 13:385–395. [PubMed: 17237357]
15. Schickel R, Boyerinas B, Park SM, Peter ME. MicroRNAs: Key players in the immune system, differentiation, tumorigenesis and cell death. *Oncogene.* 2008; 27:5959–5974. [PubMed: 18836476]
16. Medina PP, Slack FJ. MicroRNAs and cancer: An overview. *Cell Cycle.* 2008; 7:2485–2492. [PubMed: 18719380]
17. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA.* 2007; 297:1901–1908. [PubMed: 17473300]

18. Szafranska AE, Davison TS, John J, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene*. 2007; 26:4442–4452. [PubMed: 17237814]
19. Lee EJ, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer*. 2007; 120:1046–1054. [PubMed: 17149698]
20. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: Targets and expression. *Nucleic Acids Res*. 2008; 36:D149–D153. [PubMed: 18158296]
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
22. Frazier ML, Pathak S, Wang ZW, et al. Establishment of a new human pancreatic adenocarcinoma cell line, MDA-Panc-3. *Pancreas*. 1990; 5:8–16. [PubMed: 1688394]
23. Object-Oriented Microarray and Proteomic Analysis. Available from: <http://bioinformatics.mdanderson.org/Software/OOMPA/>
24. Ingenuity Pathways Analysis. Available from: <http://www.ingenuity.com/>
25. Greenbaum D, Colangelo C, Williams K, Gerstem M. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol*. 2003; 4:117. [PubMed: 12952525]
26. Lee KM, Nguyen C, Ulrich AB, Pour PM, Ouellette MM. immortalization with telomerase of the Nestin-positive cells of the human pancreas. *Biochem Biophys Res Commun*. 2003; 301:1038–1044. [PubMed: 12589817]
27. Grimson A, Farh KK, Johnston WK, Garrett-Engle P, Lim P, Bartel DP. MicroRNA targeting specificity in mammals: Determinants beyond seed pairing. *Mol Cell*. 2007; 27:91–105. [PubMed: 17612493]
28. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. *Nat Genet*. 2005; 37:495–500. [PubMed: 15806104]
29. Velu CS, Baktula AM, Grimes HL. Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. *Blood*. 2009; 113:4720–4728. [PubMed: 19278956]
30. Wu S, Huang S, Ding J, et al. Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3' untranslated region. *Oncogene*. 2010; 29:2302–2308. [PubMed: 20190813]
31. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*. 2010; 466:835–840. [PubMed: 20703300]
32. Le MT, Shyh-Chang N, Khaw SL, et al. Conserved regulation of p53 network dosage by microRNA-125b occurs through evolving miRNA-target gene pairs. *PLoS Genet*. 2011; 7:e1002242. [PubMed: 21935352]
33. Larsson E, Sander C, Marks D. mRNA turnover rate limits siRNA and microRNA efficacy. *Mol Syst Biol*. 2010; 6:433. [PubMed: 21081925]
34. Till E, Croce CM, Michaille JJ. miR-155: On the crosstalk between inflammation and cancer. *Int Rev Immunol*. 2009; 28:264–284. [PubMed: 19811312]
35. Faraoni I, Antonetti FR, Cardone J, Bonmassar E. miR-155 gene: A typical multifunctional microRNA. *Biochim Biophys Acta*. 2009; 1792:497–505. [PubMed: 19268705]
36. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008; 18:997–1006. [PubMed: 18766170]
37. Wang J, Chen J, Chang P, et al. MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood based biomarkers of disease. *Cancer Prev Res*. 2009; 2:807–813.
38. Weiler J, Hunziker J, Hall J. Anti-miRNA oligonucleotides (AMOs): Ammunition to target miRNAs implicated in human disease? *Gene Ther*. 2006; 13:496–502. [PubMed: 16195701]
39. Wurdinger T, Costa FF. Molecular therapy in the microRNA era. *Pharmacogenomics J*. 2007; 7:297–304. [PubMed: 17189960]

Abbreviations

PDA pancreatic ductal adenocarcinoma

<i>SEL1L</i>	<i>sel-1-like</i>
mRNA	messenger RNA
miRNA	microRNA
UTR	untranslated region
RPPA	reverse phase protein assay
PBS	phosphate-buffered saline
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
RQ	relative quantity
qPCR	quantitative polymerase chain reaction
β-gal	beta-galactosidase
IPA	Ingenuity Pathways Analysis

Figure 1. RQ of *SEL1L* mRNA expression in PDA tumors according to number of upregulated miRNAs. In each column, the horizontal line of pluses (+) and the number next to it indicate the median fold change in *SEL1L* mRNA expression.

Figure 2.

Results of functional assays of the interaction between *SEL1L* and hsa-mir-155. (A) Luciferase assay. Empty: cells transfected with empty pMIR-REPORT luciferase reporter vector. Construct: cells transfected with the vector containing the putative binding sites. *Significant difference between cells transfected with empty vector and those transfected with the construct (t -test, $P < 0.05$). Error bars, standard deviation ($n = 3$). (B) Expression of *SEL1L* mRNA and protein after transfection of hsa-mir-155 precursor in MDAPanc3 cells. RQ: relative quantities determined by qPCR. NC, transfection of negative control. Pre-155, transfection of hsa-mir-155 precursor.

Table 1Expression of *SEL1L* mRNA and Seven miRNAs in PDA Tumors

A: miRNA	Total (n = 42 ^a)			Fisher's exact test
	<i>SEL1L</i> down (n = 23)	<i>SEL1L</i> normal (n = 16)	<i>SEL1L</i> up (n = 3)	
hsa-mir-143				
Up ^b (n = 16)	14	1	1	<i>P</i> < 0.0001
Normal (n = 20)	8	12	0	
Down (n = 6)	1	3	2	
hsa-mir-155				
Up (n = 22)	18	4	0	<i>P</i> < 0.0001
Normal (n = 15)	4	10	1	
Down (n = 5)	1	2	2	
hsa-mir-223				
Up (n = 14)	12	2	0	<i>P</i> = 0.002
Normal (n = 19)	8	11	0	
Down (n = 9)	3	3	3	
B: miRNA	Total (n = 20)			Fisher's exact test
	<i>SEL1L</i> down (n = 11)	<i>SEL1L</i> normal (n = 7)	<i>SEL1L</i> up (n = 2)	
hsa-mir-181a				
Up (n = 7)	4	3	0	<i>P</i> = 0.749
Normal (n = 8)	5	2	1	
Down (n = 5)	2	2	1	
hsa-mir-181c				
Up (n = 8)	5	3	0	<i>P</i> = 0.763
Normal (n = 7)	4	2	1	
Down (n = 5)	2	2	1	
C: miRNA	Total (n = 20)			Fisher's exact test
	<i>SEL1L</i> down (n = 11)	<i>SEL1L</i> normal (n = 9)	<i>SEL1L</i> up (n = 0)	
hsa-mir-205				
Up (n = 9)	5	4	0	<i>P</i> = 0.654
Normal (n = 8)	3	5	0	
Down (n = 3)	3	0	0	
hsa-mir-210				
Up (n = 9)	6	3	0	<i>P</i> = 0.469
Normal (n = 9)	3	6	0	
Down (n = 2)	2	0	0	

^aData in panels A, B, and C resulted from different sets of samples, which were randomly grouped.^bUp: RQ ≥ 2; normal: 0.5 < RQ < 2; down: RQ ≤ 0.5.

Table 2
RQs of the Expression of *SEL1L* mRNA and *SEL1L* Protein in 40 PDA Tumors

Sample #	RQ of <i>SEL1L</i> mRNA	RQ of <i>SEL1L</i> protein	Sample #	RQ of <i>SEL1L</i> mRNA	RQ of <i>SEL1L</i> protein
1	0.04	0.17	21 ^a	0.49	0.81
2	0.04	0.36	22	0.52	0.67
3	0.04	0.09	23	0.54	1.25
4	0.04	0.13	24	0.58	0.57
5	0.06	0.38	25	0.76	1.42
6	0.07	0.09	26 ^a	0.84	2.41
7	0.09	0.27	27	0.84	1.71
8	0.09	0.01	28	1.02	0.94
9 ^a	0.11	1.09	29	1.02	0.68
10	0.12	0.28	30	1.05	1.7
11	0.13	0.33	31	1.14	1.89
12	0.15	0.16	32	1.14	1.2
13	0.16	0.14	33	1.25	1.27
14	0.24	0.35	34	1.38	0.57
15	0.29	0.39	35	1.4	1.39
16	0.35	0.2	36 ^a	1.65	3.36
17 ^a	0.38	1.18	37	1.85	1.44
18	0.39	0.47	38	2.74	4.1
19 ^a	0.39	0.8	39 ^a	2.81	1.66
20 ^a	0.49	1.85	40	4.42	3.25

^a*SEL1L* mRNA and *SEL1L* protein display different trends of expression.

Table 3

Protein Expression Alterations in Response to hsa-mir-155 Overexpression

Protein	Gene	48 h	72 h
Caveolin1	CAV1	0.609	0.511
Claudin7	CLDN7	0.489	1.014
HER2	ERBB2	0.643	0.913
HER2-pY1248	ERBB2	0.603	0.995
LKB1	STK11	0.634	0.911
Stat3-pS705	STAT3	0.619	0.962
KU80	XRCC5	0.664	1.018
ERCC1	ERCC1	0.614	1.043
VEGFR2	KDR	0.599	1.136
Tau	MAPT	0.59	1.258
Rad50	Rad50	0.625	1.066
EGFR	EGFR	0.863	0.658
NFkB-pS536	RELA	0.867	0.351
Beta catenin	CTNNB1	1.71	1.538
YAP-pS127	YAP1	1.819	1.155
p38 MAPK-pT180/Y182	MAPK	1.524	1.224
p38 MAPK	MAPK	1.593	1.046
Shc-pY317	SHC1	1.089	1.516

Table 4

Top Pathways and Networks Associated With the RPPA Data Sets Analyzed by IPA

A: Pathway	P-value	Genes involved
Pancreatic adenocarcinoma signaling	3.14E-06	EGFR, ERBB2, RELA, STAT3
PTEN signaling	3.26E-06	EGFR, KDR, RELA, SHC1
EGF signaling	1.22E-05	EGFR, SHC1, STAT3
IL-15 signaling	3.01E-05	RELA, SHC1, STAT3
PDGF signaling	4.52E-05	CAV1, SHC1, STAT3

B. Network Associated Functions	Molecules in Network ^a
Cellular Development, Cancer, Cellular Growth and Proliferation	26s Proteasome, atypical protein kinase C, BCR, Calcineurin protein(s), Caspase, CAV1 , CLDN7 , Collagen Alpha1, Collagen type I, ERBB2 , Estrogen Receptor, Focal adhesion kinase, growth factor receptor , Hsp27, Hsp70, Hsp90, Integrin, JAK, KDR , Laminin, MAPT , Mek, NFkB (complex) , p85 (pik3r), Pdgf (complex), PDGF BB, Pdgfr, PLC gamma, PP2A, RAD50 , SHC1 , Sos, STAT3 , Ubiquitin, XRCC5
Cancer, Gastrointestinal Disease, Organismal Injury and Abnormalities	14-3-3, Akt, Ap1, Calmodulin, Calpain, Cbp/p300, CTNNB1 , EGFR , Endothelin, ERK, ERK1/2, Gsk3, Histone h3, IFN Beta, IgG, IL1, Insulin, Interferon alpha, Jnk, LDL, Mapk, NLRX1, P38 MAPK , PI3K (complex), Pka, Pkc(s), Ras, Ras homolog, RELA , She, SRC, STAT5a/b, STK11 , Vegf, YAP1
Cell Death, Gene Expression and Organismal Development	CXCL12, dihydrotestosterone, EGF, ERCC1 , ERCC5 , FOS, HNF4A, HRAS, HSPA5, JUN, PTEN, RPA, SEL1L , SYVN1, TERF2, TFIIH, TNF, TNFRSF1A, TP53, tretinoin

^aMolecules in network in bold are genes included in Table 3 (plus SEL1L).