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Inactivation of Ink4a/Arf leads to deregulated expression of miRNAs in K-Ras transgenic mouse model of pancreatic cancer

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Abstract

Human Pancreatic Cancer (PC) is an aggressive disease, which has been recapitulated in transgenic animal model that provides unique opportunity for mechanistic understanding of disease progression and also for testing the efficacy of novel therapeutics. Emerging evidence suggests deregulated expression of microRNAs (miRNAs) in human PC, and thus we investigated the expression of miRNAs in pancreas tissues obtained from transgenic mouse models of K-Ras (K), Pdx1-Cre (C), K-Ras; Pdx1-Cre (KC) and K-Ras; Pdx1-Cre; INK4a/Arf (KCI), initially from pooled RNA samples using miRNA profiling, and further confirmed in individual specimens by quantitative RT-PCR. We found over-expression of *miR-21*, *miR-221*, *miR-27a*, *miR-27b* and *miR-155*, and down-regulation of *miR-216a*, *miR-216b*, *miR-217* and *miR-146a* expression in tumors derived from KC and KCI mouse model, which was consistent with data from KCI-derived RInk-1 cells. Mechanistic investigations revealed a significant induction of EGFR, K-Ras, and MT1-MMP protein expression in tissues from both KC and KCI mouse compared to tissues from K or C, and these results were consistent with similar findings in RInk-1 cells compared to human MIPaCa-2 cells. Furthermore, *miR-155* knock-down in RInk-1 cells resulted in the inhibition of cell growth and colony formation consistent with down-regulation of EGFR, MT1-MMP and K-Ras expression. In addition, *miR-216b* which target Ras, and forced re-expression of *miR-216b* in RInk-1 cells showed inhibition of cell proliferation and colony formation, which was correlated with reduced expression of Ras, EGFR and MT1-MMP. These findings suggest that these models would be useful for preclinical evaluation of novel miRNA-targeted agents for designing personalized therapy for PC.

Keywords

Ink4a; K-Ras; miRNAs; EGFR; RInk-1

Introduction

Pancreatic cancer (PC) is the fourth leading cause of cancer related deaths in the United States (Siegel et al, 2011) and it is virtually fatal to all patients diagnosed with PC. Diagnosis of PC at an early stage is a key factor in determining improved survival. The major difficulty in treating PC is also because of its overt and early metastatic potential, late diagnosis, and profound resistance to conventional therapeutics. In order to gain mechanistic

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insight and also for testing the efficacy of novel therapeutics, many researchers have adopted transgenic animal models especially K-Ras derived models among others.

Ras is a small GTPase that plays a central role in protein kinase signaling pathways, and regulates cell proliferation and apoptosis (Pylayeva-Gupta et al, 2011). K-Ras mutations are frequently found in PC, and occurs during early stages of tumor initiation (Kim et al, 2011; Krantz et al, 2011; Mohri et al, 2011; Ray et al, 2011) which is believed to arise through the progression of low grade to high-grade pancreatic intraepithelial neoplasia (PanIN) (Hruban et al, 2006). Identifying these PC precursors and associated biomarkers, has the potential for diagnostic accuracy which will aid in the early detection of these lesions in individuals at high-risk for PC (Cheung et al, 2008). Hence several mouse models have been created to mimic human PC that has been proven useful for studying tumor progression, prevention and therapy (Aguirre et al, 2003; Cook et al, 2008; Frese and Tuveson, 2007; Hingorani et al, 2003; Hingorani et al, 2005; Maniati et al, 2011; Olive et al, 2009; Tuveson and Hingorani, 2005; Wang et al, 2011). One such model with K-Ras mutation in combination with Ink4a/Arf deficiency resulted in the activation of Notch and NF- κ B signaling as reported in our recent publication (Wang et al, 2011) showing tumor progression, which could in part be also due to deregulated expression of microRNAs (miRNAs) and their target genes.

Experimental evidence supports an increasing role for miRNAs in the molecular biology of PC. These small molecules are known to play significant roles in animals and humans at the post-transcriptional level and act as either oncogenes or tumor suppressors (Ali et al, 2010b; Bao et al, 2011). The deregulated expression of miRNAs and their target genes in tumor cells or in the plasma as documented in our previous studies (Ali et al, 2010a; Ali et al, 2010b; Bao et al, 2011; Li et al, 2010; Wang et al, 2010) could become useful for early diagnosis and for assessing therapeutic outcome in PC. Previous studies have shown up-regulation of epidermal growth factor receptor (EGFR) in many malignancies including PC (Gadgeel et al, 2009; Li et al, 2010; Pryczynicz et al, 2008), and its expression was found to be associated with large tumor size, decrease survival, advanced clinical stage (Li et al, 2010), and was also associated with lower expression of *miR-146a* (Edmonds et al, 2009; Kogo et al, 2011; Li et al, 2010), which provided strong evidence in support of the deregulated expression of miRNA and EGFR in PC. The blockade of EGFR pathway by gefitinib as a chemo-preventive agent in K-Ras transgenic mouse model suppressed tumor cell proliferation, by inhibiting PanIN formation (Mohammed et al, 2010). Interestingly, membrane type 1-matrix metalloproteinase (MT1-MMP) has been found to be associated with metastasis and its over-expression in PC contributed to gemcitabine resistance both *in vitro* and *in vivo* (ngi-Garimella et al, 2011; Ottaviano et al, 2006). The over-expression of MT1-MMP was also found to be associated with decreased expression of *let-7* miRNA in human pancreatic tumor specimens (ngi-Garimella et al, 2011). Moreover, increased Snail expression, a known marker for epithelial-mesenchymal transition (EMT) driven by increased TGF- β 1 signaling, promotes invasion of PC cells, which was found to be associated with increased expression of MT1-MMP (Shields et al, 2011), suggesting that re-expression of *let-7* could inactivate MT1-MMP and thereby reduce the potential of invasive characteristics of PC cells.

Recent studies have confirmed that miRNAs are present in remarkably stable form in cells and in tumors, and thus it can serve as biomarkers to distinguish cancer from non-cancer (Nakata et al, 2011; Zhao et al, 2011). A recent report from our laboratory showed a significant difference in the expression levels of several miRNAs in the plasma of PC patients compared to normal subjects (Ali et al, 2010b), suggesting that the assessment for the levels of deregulated expression of miRNAs and their targets either in the tumor specimens or in the plasma could be linked with the biology of the development and

progression of PC. Our previous studies have shown that the activated K-ras and Ink4a/Arf deficiency cooperate during the development of PC in the transgenic animal models by activation of Notch and NF- κ B signaling pathways (Wang et al, 2011). Therefore, the goal of this study was to assess differential expression of miRNAs in pooled pancreatic tissue samples (n=4) from K-Ras^{G12D} (K) and Pdx1-Cre (C), compared to pancreatic tumor tissues of K-Ras^{G12D}; Pdx1-Cre (KC) and K-Ras^{G12D}; Pdx1-Cre; Ink4a/Arf (KCI) mouse models.

We found differential expression of miRNA that could distinguish the four different groups, and thus we focused further studies using selected miRNAs whose expression was either lost or up-regulated in the tumors. The expression of the top 9 up- and down-regulated individual miRNAs was independently quantitated in individual samples by qRT-PCR, and their expression was further investigated in the KCI-derived RInk-1 cells. We also investigated the protein expression of EGFR, K-Ras and MT1-MMP in the animal tissues and in the RInk-1 cells. We found that *miR-216b* and *miR-155* possess a match for base pairing with the 3'-UTR of K-Ras with context score percentile of 79 and 77, respectively as assessed by miRNA target prediction algorithms (TargetScanHuman 5.2). Recent studies have shown that oncogenic Ras up-regulates matrix metalloproteinase expression through Mitogen-activated protein kinase (MAPK) and Jun-kinase (JNK) (Kranenburg et al, 2004; Pylayeva-Gupta et al, 2011), which in turn could be responsible for deregulated expression of certain miRNAs. Therefore, we chose to conduct further mechanistic studies by investigating the consequence of transfecting *pre-miR-216b*, and anti-sense oligonucleotide (ASO) for *miR-155* in RInk-1 cells. Here we report, for the first time, that transfection of *pre-miR-216b* and *ASO-miR-155* suppressed cell proliferation and clonogenicity, which was correlated with reduced expression of not only K-Ras but also EGFR and MT1-MMP.

Materials and Methods

Ethics Statement

This study was conducted in strict accordance with the recommendations in the guidelines for the care and use of laboratory animals of the National Institutes of health. The protocol was approved by the Committee on the Ethics of animal experiments of Wayne State University.

Mouse Model

The LSL-K-Ras^{G12D} strain was bred to the following strains: LSL-K-Ras^{G12D}; Pdx1-Cre, and LSL-K-Ras^{G12D}; Pdx1-Cre; INK4a/Arf^{lox/lox} as previously described (Carriere et al, 2009; Seeley et al, 2009). Pancreata were collected from LSL-K-Ras^{G12D} (K), Pdx1-Cre (C), LSL-K-Ras^{G12D}; Pdx1-Cre (KC), LSL-K-Ras^{G12D} Pdx1-Cre; INK4a/Arf (KCI) mouse models and were harvested for both protein and RNA extraction and used for further studies.

Genotyping

Genomic DNA was extracted from tail cuttings using the REDEExtract-N-Amp Tissue PCR kit (Sigma St. Louis, MO). PCR reactions were repeated three times to confirm the presence of the oncogenic *K-Ras*, and *Pdx1-Cre* transgenes.

Cells Culture

Human and mouse pancreatic cancer cell lines MIAPaCa-2, and RInk-1 were chosen for this study. RInk-1 murine pancreatic tumor cell line was generated from the LSL-K-Ras^{G12D}; Pdx1-Cre; INK4a/Arf (KCI) mice as described previously (Seeley et al, 2009). MIAPaCa-2 cell line has been tested and authenticated in the core facility "Applied Genomics Technology Center" at Wayne State University on March 13, 2009. The method used for

testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). The cells were stored in liquid nitrogen in multiple vials.

RNA Isolation

The RNA was isolated using mirVana miRNA isolation kit (Ambion, Inc.) according to the manufacturer's protocol. Briefly, the tissue/cells were homogenized with a douncer in lysis solution and incubated with 1/10 volume of miRNA homogenate additive for 10 minutes. Equal volume of Acid-Phenol:Chloroform was added to the mixture and vortexed for a minute before centrifugation to separate aqueous phase. About 1.25 volume of 100% ethanol was added to the aqueous phase before being applied directly to the filter cartridge. The RNA was washed with the buffers provided with the kit to remove impurities and eluted in a final volume of 100 µl.

MicroRNA (miRNA) profiling

The pooled purified RNA samples of pancreatic tissues from K, C, KC and KCI mice were analyzed by LC Sciences for miRNA microarray profiling (LC Sciences Houston, TX). Network analysis was performed with the Ingenuity pathway Analysis (IPA) software (Ingenuity Systems). Ingenuity Pathway Analysis is a web-based bioinformatics tool to identify pathways and functional networks.

TaqMan miRNA Real-Time quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR is the standard method for validation of microarray profiling data which provides quantitative analysis of miRNA expression in real time for the miRNAs of interest from the samples analyzed by microarray profiling. Hence the expression of five oncogenes and 4 tumor suppressor miRNAs of all four groups K, C, KC and KCI were determined using TaqMan MicroRNA Assay kit (Applied Biosystems) following manufacturer's protocol. About 10 ng of RNA from each sample was reverse transcribed as described earlier (Ali et al, 2010b) and RT-PCR reactions were then carried out in a total volume of 10 µl reaction mixture as described earlier (Ali et al, 2010b). All reactions, including controls were performed in triplicate using StepOnePlus Real-Time PCR (Applied Biosystems). Relative expression of miRNAs was analyzed using C_t method and was normalized by RNU6B expression.

Protein extraction and Western blot analysis

The total protein was extracted from all four groups K, C, KC and KCI of transgenic mice, and also from MIAPaCa-2, and RInk-1 cell lines and subjected to western blot analysis as described previously (Ali et al, 2010b) to evaluate the expression status of EGFR, K-Ras and MT1-MMP.

Anti-sense miR-155 and Pre-miR-216b oligonucleotide Transfection

RInk-1 cells were plated in 6 well plates overnight. Cells were transfected with either anti-sense *miR-155* oligonucleotide (ASO), or *Pre-miR-216b*, or control miRNA (Ambion, Austin, TX) at a final concentration of 20nM using DharmaFECT transfection reagent (Dharmacon), following the manufacturer protocol. After 48–72h of transfection, the transfected cells were measured for cell growth by MTT and clonogenic assay, and also harvested for the extraction of total RNA and protein using standard methods.

Cell Viability Assay of transfected cells

Standard assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed after transfection for 72 h, and experiments were repeated three times. The

color intensity was measured by TECAN's microplate fluorometer (TECAN, Research Triangle Park, NC) at 595 nm.

Clonogenic assay of transfected cells

Transfected cells were trypsinized and 1000 viable cells were plated in 100 mm petri dishes. The cells were then incubated for about 10–12 days at 37°C in a 5% CO₂/5% O₂/90% N₂ incubator. Colonies were stained with 2% crystal violet and quantitated.

Statistical Methods

Comparisons of treatment outcome were tested for statistical difference by the paired t- test. Statistical significance was assumed at a p value of < 0.05.

Results

Expression profiling of miRNA from pancreatic tissue samples characterized 187 miRNAs that were differentially expressed between LSL-K-Ras^{G12D}; Pdx1-Cre; Ink4a (KCI) and LSL-K-Ras^{G12D}; Pdx1-Cre (KC)

For the current study, 5 µl of RNA from 4 animal's pancreatic tissue specimens of each group K, C, KC and KCI were pooled separately and subjected to miRNA profiling. The miRNA expression profiling revealed 187 miRNAs that were differentially expressed, of which 109 were up-regulated and 78 were down-regulated, when compared to either K or C. We observed up-and down-regulation of several miRNAs, including *miR-21*, *miR-221*, *miR-27a*, *miR-27b*, *miR-155*, *miR-216a*, *miR-216b*, *miR-217* and *miR-146a* in KCI compared to KC animals. In order to understand the pathway and target genes of the deregulated miRNAs from KCI and KC groups compared to either K or C, we performed Ingenuity modeling of the miRNA profiling as presented in the following section.

Pathway analysis of miRNA expression level changes between KCI and KC animals compared with K or C animals

The underlying mechanism on the differences in the expression levels between the KCI and KC compared to either K or C was studied by Ingenuity pathway analysis as presented in Figure 1A. The data set of differentially expressed miRNAs in KCI, KC, K or C was uploaded into the IPA software. Network of these miRNAs were then algorithmically generated based on their connectivity. This network also showed that many of the miRNAs are regulated by each other either directly or indirectly, and are also regulated by several target genes or *vice versa*. We found up-regulation (red) and down-regulation (green) of several miRNAs. We also observed 16 bio-functional network groups which included cancer, inflammatory disease, and gastrointestinal disease (Figure 1B) as demonstrated through the Ingenuity pathway analysis. Of the nine miRNAs chosen for further analysis based on the miRNA profiling data, five were oncogenic (*miR-155*, *miR-21*, *miR-221*, *miR-27a*, and *miR-27b*) and four functions as tumor suppressors (*miR-216a*, *miR-216b*, *miR-217*, and *miR-146a*). We further validated these nine miRNAs in KCI, KC, K and C individually with real-time PCR. The data is presented in the subsequent sections.

Up-regulation of five oncogenic miRNAs in LSL-K-Ras^{G12D}; Pdx1-Cre; INK4a/Arf (KCI) tumors as assessed by qRT-PCR

The miRNA expression analysis by qRT-PCR was blinded to the source of the samples. The samples from all four groups; K, C, KC and KCI were tested individually in parallel to avoid batch-effects differences. We confirmed the findings of five oncogenic miRNAs; *miR-21*, *miR-221*, *miR-27a*, *miR-27b* and *miR-155* with real-time qRT-PCR, showing a significant

increase in KCI group compared to the other three groups; K, C and KC as shown in Figure 2.

Down-regulation of four tumor suppressor miRNAs in LSL-K-Ras^{G12D}; Pdx1- Cre; INK4a/Arf (KCI) tumors as assessed by qRT-PCR

The miRNA expression analysis of the four functional tumor suppressor miRNAs such as *miR-216a*, *miR-216b*, *miR-217* and *miR-146a* (Figure 3), were performed individually in all samples by qRT-PCR which showed significant reduction in the expression levels in KC and KCI samples compared to either K or C samples. The tissue-based findings from these animal tissue specimens were further tested in human pancreas cancer cells and also the cells derived from the KCI mouse as presented below.

Differential expression of miRNAs in MIAPaCa-2 and Rlnk-1 cell lines

We compared the basal levels of expression of miRNAs (the same five oncogenic and four tumor suppressor miRNAs) in human pancreatic cancer cell line MIAPaCa-2 and mouse Rlnk-1 cells derived from the KCI mouse. We found that all five oncogenic miRNAs were significantly up-regulated in Rlnk-1 cells compared to MIAPaCa-2 cell line (Figure 4). On the contrary, none of the tumor suppressor miRNAs which showed expression level in all four tumor groups remained undetermined in both Rlnk-1 and MIAPaCa-2 cells (due to complete loss of expression), supporting highly aggressive nature of these cells. The C_T values above 36 were considered undetermined.

Elevated levels of expression of EGFR, K-Ras and MT1-MMP proteins in LSL-K-Ras^{G12D}; Pdx1-Cre; INK4a/Arf (KCI), and K-Ras; Pdx1-Cre (KC) tissues as assessed by western blot analysis

We examined the basal levels of expression of EGFR, K-Ras and MT1-MMP proteins in pooled samples of all four groups; K, C, KC and KCI transgenic animal tissues. We found that the protein expression of EGFR, K-Ras and MT1-MMP were all highly elevated in the pancreatic tumors of KC and KCI animals compared to K or C animals (Figure 5A). Similarly, we also compared the protein expression in the K-Ras mutant MIAPaCa-2 cells compared to murine Rlnk-1 cells derived from the KCI animals. We found up-regulated expression of EGFR in Rlnk-1 cells compared to MIAPaCa-2 cells whereas MT1-MMP expression was higher in MIAPaCa-2 cells compared to Rlnk-1 cells. The level of expression of K-Ras was found to be very high in both the cell lines (Figure 5B).

Transfection of anti-sense miR-155 in Rlnk-1 cells reduced cell growth, colony formation, and protein expression

Since *miR-155* was found to be significantly up-regulated in KCI tumors, and also in Rlnk-1 cells (derived from KCI animal), we tested whether the inactivation of *miR-155* using anti-sense oligonucleotide *ASO-miR-155* transfection could have any effects on cell proliferation and clonogenic growth. The cells were seeded in both 96 and 6 well plates and were transfected with ASO-miR-155 for 48 to 72h. Inactivation of *miR-155* significantly inhibited cell viability and clonogenicity in the *ASO-miR-155* transfected cells compared to control miRNA transfected cells (Figure 6A), which was correlated with decreased expression of *miR-155* as assessed by qRT-PCR (Figure 6A). Interestingly, inactivation of *miR-155* led to decreased expression of EGFR, K-Ras and MT1-MMP in cells transfected with *ASO-miR-155*.

Transfection of pre-miR-216b precursor in Rlnk-1 cells reduced cell growth, colony formation, and protein expression

Since the expression of *miR-216b* was found to be significantly down-regulated or undetermined in Rlnk-1 cells, we transfected these cells with *pre-miR-216b* precursor in both 96 and 6 well plated for 48 to 72h. Re-expression of *miR-216b* led to a significant decrease in cell viability and clonogenicity compared to control miRNA transfected cells (Figure 6B). Re-expression of *miR-216b* with *pre-miR-216b* was found to be consistent with increased *miR-216b* expression, which was further correlated with decreased expression of EGFR, K-Ras and MT1-MMP (Figure 6B).

Discussion

Pancreatic Cancer (PC) is a lethal disease for which novel therapeutics are urgently needed especially because conventional therapies have had limited success due to intrinsic and extrinsic resistance to cytotoxic agents (Sarkar et al, 2009; Wang et al, 2010; Xiong et al, 2006). Since proto-oncogene *K-Ras* mutations are the most common variation amongst many human cancers including PC (Smit et al, 1988), attempts has been made to target *K-Ras* with inhibitors like farnesyl transferase, which has shown to be completely ineffective (Macdonald et al, 2005). Several mouse models have been created to mimic human PC that has been proven useful for studying tumor progression, prevention and therapy (Aguirre et al, 2003; Cook et al, 2008; Frese and Tuveson, 2007; Hingorani et al, 2003; Hingorani et al, 2005; Maniati et al, 2011; Olive et al, 2009; Tuveson and Hingorani, 2005; Wang et al, 2011). One such model with K-Ras mutation in combination with Ink4a/Arf deficiency resulted in the activation of Notch and NF- κ B signaling as reported in our recent publication (Wang et al, 2011) showing tumor progression, which could in part be also due to deregulated expression of microRNAs (miRNAs) and their target genes.

In order to further understand the likely mechanism(s) involved in the progression of PC in the LSL-K-Ras^{G12D}; Pdx1-Cre; INK4a/Arf (KCI), and LSL-K-Ras^{G12D}; Pdx1-Cre (KC) mouse models, we analyzed a number of miRNAs by miRNA profiling compared to pancreata of the K-Ras^{G12D} (K), and Pdx1-Cre (C) mouse. Expression profiles of miRNAs could be highly informative in discriminating malignant from the normal pancreas because of the unique patterns of expression of miRNAs in tumors. We found 187 miRNAs that were differentially expressed in the KCI and KC tumors compared to K or C tissue specimens. A systems biology approach is a distinct approach for investigating networks of molecular measures that drive assorted signaling pathways resulting from the deregulations of miRNAs as demonstrated in figure 1. The knowledge gained from such pathway analysis provided the scientific rationale for choosing specific miRNAs for mechanistic studies as discussed later.

A recent report has shown that miR-21 is up-regulated by K-Ras and EGFR in PC cell lines (du Rieu et al, 2010), and here we report for the first time our results demonstrating higher expression of K-Ras, EGFR and MT1-MMP in the tumors of KCI mouse and these results were associated with up-regulated expression of several miRNAs including *miR-21*, *miR-221*, *miR-155*, miR-27a, b in KCI and KC mouse tumors. Moreover, we found down-regulated expression of *miR-216a*, *b*, and *miR-217* in KCI and KC mouse tumors compared to the pancreata of K or C mouse. The role of EGFR over-expression has been well established in many human cancers (Gadgeel et al, 2007; Gadgeel et al, 2009; Mohammed et al, 2010; Pryczynicz et al, 2008), and transgenic mouse model (Ohashi et al, 2009). EGFR promotes *miR-21* production, a key oncogenic miRNA in the multi-step progression of PC precursor lesions (du Rieu et al, 2010), and is also linked with the activation of the AP-1 transcription factor in response to Ras (Talotta et al, 2009). The increased levels of miR-21 in the plasma of PC patients has been found to correlate with worst survival as documented

in our recent publication (Ali et al, 2010b), suggesting that over-expression of *miR-21* found in the tumors of KCI and KC mouse is highly relevant to human PC and as such *miR-21* targeted agents could become a newer therapeutic approach for the treatment of human PC. To that end, we have recently reported the therapeutic effects of our novel synthetic analog of curcumin, CDF in preclinical *in vitro* and *in vivo* studies documenting that CDF could inactivate the expression of *miR-21* (Bao et al, 2011). In a recent study, *miR-217* was found to be down-regulated in PC both *in vitro* and *in vivo* and was further associated with *Ras* (Zhao et al, 2010). Our current findings clearly show significant down-regulation in the expression of *miR-217* in the tumors derived from KCI and KC mouse, which is consistent with the above published report.

We also found deregulated expression of *miR-155*, which is consistent with findings reported in chronic myeloid leukemia where the expression of *miR-155* was linked with the expression of K-Ras and EGFR (Machova et al, 2011). Other studies evaluated the role of *miR-155* in normal tissues, premalignant lesions and invasive carcinoma in the mouse model showing an increased expression of *miR-155* during the progression of normal tissues to invasive PC, which was further confirmed in tissues and blood samples of PC patients (LaConti et al, 2011). These results are consistent with our findings showing that over-expression of *miR-155* is associated with increased expression of K-Ras and EGFR, which could indeed be responsible for tumor aggressiveness. Our data clearly show that inactivation of *miR-155* in RInk-1 cells by *ASO-miR-155* transfection could down-regulate the expression of EGFR and K-Ras, which was correlated with inhibition of cell growth and clonogenicity. However, in addition to being expressed in cancer cells, *miR-155* is also expressed in a variety of immune-modulating cells, including macrophages, leukocytes and T cells, and its expression is known to be increased by pro-inflammatory cytokines (O'Connell et al, 2007; Rodriguez et al, 2007). These observations raise the possibility that in KCI mice the pancreatic tumors express high levels of *miR-155* due to the abundance of cancer cells and inflammatory cells within the tumor microenvironment in this model.

Another important key player in tumor progression is membrane type 1-matrix metalloproteinase (MT1-MMP) that stimulate cell migration and proliferation (D'Alessio et al, 2008). According to a recent study, down-regulation of MT1-MMP in orthotopic model of breast cancer significantly inhibited lung metastasis, but not tumor growth (Perentes et al, 2011). The expression of MT1-MMP is also considered as an independent predictive marker of overall survival in women with breast cancer (McGowan and Duffy, 2008). Here, we report for the first time, showing increased expression of MT1-MMP in both KC and KCI transgenic mouse models whose expression could be attenuated in cells transfected with *pre-miR-216a*, resulting in the inhibition of cell growth and clonogenicity. These results suggest the biological implication of targeted re-expression of *miR-216a* or inactivation of *miR-155* by novel approach could become newer therapy for the treatment of human PC in the future. In conclusion, we have provided experimental evidence, using the KC and KCI mouse models as well as by using the RInk-1 cells derived from the KCI mouse, supporting that targeting miRNAs could be a novel approach for therapy. Therefore, it is tempting to speculate that inactivation of Ras, EGFR and MT1-MMP by targeted re-expression of miRNAs or down-regulation of miRNAs would be useful for the treatment of PC.

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Figure 1. Ingenuity network analysis showing up (red) and down-regulation (green) of miRNAs as analyzed by miRNA profiling that are involved in pancreatic cancer (PC) and their target genes (A). The solid lines connecting genes represent a direct relation and dotted lines represent indirect relationship. We also observed 16 bio-functional network groups that included cancer, genetic disorder, and gastrointestinal disease (B).

Figure 2.

Comparative level of expression of *miR-21*, *miR-221*, *miR-27a*, *miR-27b*, and *miR-155* in the pancreata of K-Ras (K), Pdx1-Cre (C), K-Ras; Pdx1-Cre; (KC), and K-Ras; Pdx1-Cre; INK4a/Arf (KCI) mouse. There was a significant up-regulation in the expression of *miR-21*, *miR-221*, *miR-27a,b* and *miR-155* in KCI tumors compared to either K or C or KC pancreata. *P* values represent comparison between K and KCI as calculated by the paired *t* test.

Figure 3.

Comparative level of expression of *miR-216a*, *miR-216b*, *miR-217* and *miR-146* in the pancreata of K-Ras (K), Pdx1-Cre (C), K-Ras; Pdx1-Cre (KC), and K-Ras; Pdx1-Cre; INK4a/Arf (KCI) mouse. There was a significant down-regulation in the expression of *miR-216a*, *miR-216b*, *miR-217* and *miR-146a* in KCI tumors compared to either K or C or KC pancreata. *P* values represent comparison between K, and KCI as calculated by the paired *t* test.

Figure 4.

Comparative levels of expression of *miR-21*, *miR-221*, *miR-27a*, *miR-27b* and *miR-155* in MIAPaCa-2 and RInk-1 cells. There was a significant up-regulation in the expression of *miR-21*, *miR-221*, *miR-27a*, *miR-27b*, and *miR-155* in murine RInk-1 cells compared to human MIAPaCa-2 PC cells. *P* values represent comparison between MIAPaCa-2 and RInk-1 cells as calculated by the paired *t* test.

Figure 5.

Basal level of expression of EGFR, K-Ras and MT1-MMP in the pancreata of K, C, KC and KCI (A), and MIPaCa-2 and Rink-1 cells as assessed by western blot analysis. A significant over-expression of EGFR, K-Ras and MT1-MMP was found in KC and KCI tumors compared to either K or C pancreata. Similarly, in Rink-1 cells EGFR was over-expressed whereas the expression of MT1-MMP was lower in Rink-1 cells compared to MIPaCa-2 cells.

Figure 6. Inactivation of *miR-155* expression by *ASO-miR-155* transfection (A), and re-expression of *miR-216b* by *pre-miR-216b* precursor transfection (B) led to reduced cell viability as assessed by MTT assay, and clonogenicity as determined by colony formation assay, which was associated with decreased levels of EGFR, K-Ras and MT1-MMP expression as assessed by western blot, consistent with decreased and increased expression of *miR-155* and *miR-216b*, respectively as assessed by qRT-PCR.