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Original Article

Contribution of microRNAs in understanding the pancreatic tumor microenvironment involving cancer associated stellate and fibroblast cells

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Abstract: Understanding of molecular events associated with tumor microenvironment in pancreatic cancer (PC) is an active area of research especially because of the rich desmoplasia seen in human PC. Desmoplasia is contributed by several cell types including cancer-associated fibroblast (CAF) and stellate cells (PSCs), which are believed to play critical roles in conferring aggressiveness to PC. The aberrant expression of microRNAs (miRNAs) in PSCs and CAF cells appears to play a pivotal role in the development and progression of PC. In this study, expression analysis of *miR-21/miR-221* in conditioned media derived from PSCs/CAF cells, and from PSCs/CAF cells showed up-regulation of both miRNAs compared to MIAPaCa-2 PC cells. In addition, *miR-21* expression in stellate cells derived from normal pancreas was substantially lower when compared to PSCs or CAF cells. COLO-357 PC cells cultured in the presence of conditioned media derived from PSC/CAF cells led to a significant increase in clonogenicity and pancreatosphere formation. Furthermore, inhibition of *miR-21* with antisense oligonucleotide (ASO) transfection resulted in decreased migration/invasive capacity of PSCs. Similarly, the effect of ASO-*miR-221* transfection in CAF cells reduced the expression of NF-κB and K-Ras (target of *miR-221*) along with inhibition of migration/invasion. Moreover, miRNA expression profiling of PSCs, MIAPaCa-2, and COLO-357 cells, and further validation by real-time PCR, showed several differentially expressed miRNAs, among which four was significantly up-regulated. Collectively, these results suggest a crosstalk between PSCs/CAF cells and PC cells, resulting in the up-regulation of *miR-21/miR-221* expression which in part may confer aggressiveness to PC. We conclude that targeting these miRNAs could be useful for developing precision medicine for the prevention of tumor progression and/or for the treatment of PC.

Keywords: Stellate cells, CAF cells, exosomes, miRNA, pancreatic cancer

Introduction

Although substantial progress has been made over the past decades in understanding the biology of human pancreatic cancer (PC) and therapeutics, it still remains the fourth leading cause of cancer related deaths in the United States [1]. PC patients continue to exhibit incurable and/or chemo-resistant tumors with a median survival period of less than 6 months. Many targeted therapies and novel combinations of drugs have prolonged life, but only by a few weeks [2]. Recent studies in PC have led to the acceptance of the role of cancer stem cells (CSCs) and epithelial-to-mesenchymal transition (EMT) cells in defining aggressiveness of PC [3, 4]. Other studies have reported a facilitatory role for stromal components such as cancer-associated stellate cells (PSCs), and cancer-associated fibroblasts (CAF) in tumor progression, metastasis and chemoresistance in PC [5, 6]. In addition, the staining of PC tissue sections with activation marker α-smooth muscle actin (α-SMA) has revealed the presence of PSCs, now established as the main source of collagen in the desmoplastic reaction surrounding cancer cells. Notably, PSCs have been shown to stimulate pancreatic cancer cell proliferation and migration, resulting in increased local tumor growth as well as distant metastasis [7]. The CAF is derived from heterogeneous population of cells type that contribute to many of the equivalent functions of activated stellate cells having activation of α-SMA and myofibroblast-like phenotype and are equally responsible for desmoplastic reaction [8]. Moreover,

Scarlett *et al* demonstrated that circulating bone marrow derived stem cells (BMDC) can merge into pancreas and contribute significantly to the activated PSCs both in pancreatitis and PC suggesting that BMDC may play an important role in promoting carcinogenesis [9]. Although many *in vitro* studies demonstrated significant sensitivities to chemotherapy to several solid tumors, chemo-sensitivity to PC is dismal which suggest the presence of an exceptional tumor microenvironment [10]. Emerging evidence suggests that hypoxia, a cancer driver which confers aggressiveness to PC [11], also causes activation of PSCs to their myofibroblast-like phenotype with consequent increase in the synthesis and deposition of extracellular matrix (ECM) proteins in the stroma [5].

Mounting evidence suggests that the deregulated expression of microRNAs (miRNAs) plays a pivotal role in the progression of various types of diseases, including diabetes, cardiovascular disease and cancer [3, 12, 13]. MicroRNAs are stable, small non-coding RNAs with thousands of predicted mRNA targets, and they play a diverse role in numerous cellular processes including tumor cell proliferation and invasion, and thus can serve as early diagnostic markers in many cancers including PC [14-18]. A recent study in a murine model of chronic pancreatitis suggested that the interaction between cellular and exosomal expression of *miR-21* and connective tissue growth factor (CCN2) in activated PSCs leads to the up-regulation of *miR-21* and CCN2 expression via a positive feedback loop [19]. Another study revealed that isolated PSCs from pancreas tissue of male rats showed deregulation of several known miRNAs including *miR-221*, *miR-143* and *miR-146a* between day 1 quiescent PSCs and day 14 activated PSCs, targeting pathways such as p38 mitogen-activated and extracellular-signal-regulated kinase [20]. The down-regulation of *miR-15b* and *miR-16* was inversely correlated with their target gene Bcl-2, while reinstating their expression decreased Bcl-2 expression and induced apoptosis of activated rat PSCs [21]. The activation of PSCs was not only observed in animal model but also in human PSCs co-cultured with PC cells, which showed altered expression of several miRNAs including *miR-210*. Inhibition of *miR-210 in PSCs* resulted in decreased cell migration of PC cells accompanied by increased expression of EMT markers such as vimentin and snail, suggesting an interaction between PSCs and PC cells [22], all of which could be important in the biology of tumor microenvironment.

In the present study, we compared the expression level of *miR-21/miR-221* in the human PC cell line MIAPaCa-2 and normal stellate cells (nhPSCs) with that in cancer associated stellate cells (PSCs) and fibroblast (CAF) cells, as well as in conditioned media. Furthermore, PC cells cultured with and without conditioned media from PSCs and CAF were assessed for colony formation and the formation of pancreatospheres. In addition, the putative roles of *miR-21* and *miR-221* expression were studied by inhibiting the expression of *miR-21* in PSCs and *miR-221* in CAFs using antisense oligonucleotide transfection. We found that inhibition of *miR-21* and *miR-221* expression led to decreased invasion and migration of PSCs and CAF cells respectively. Moreover, miRNA microarray profiling of PC cells and PSCs/CAF cells revealed several dysregulated miRNAs, of which four were significantly up-regulated in PSCs/CAF cells compared to the pancreatic cancer cell lines MIAPaCa-2 and COLO-357 cells, which was chosen based on our experience working with these cells. These results suggest that the activation of PSCs is likely to be regulated by miRNAs, which in turn may play a significant role in PC progression through deregulation of tumor microenvironment.

Materials and methods

Cells culture

Human normal and cancer associated stellate cells were generated by Professor Minoti Apte and her collaborators, University of New South Wales, Sydney, Australia and were grown in IMDM containing 20% FBS and antibiotics. Human cancer associated fibroblast cells CAF-19 were a gift from Dr. Anirban Maitra from Johns Hopkins University, Baltimore, MD and were grown in DMEM containing 10% FBS as described previously by the same group [23, 24]. Human PC cell lines COLO-357 and MIAPaCa-2 were maintained and grown as described earlier and were chosen for this study. Cell lines were tested and authenticated as described earlier [17].

RNA isolation from cells

RNA from human PSCs, CAF, and PC cell lines was isolated using the Trizol (Invitrogen, Carlbad, CA) method according to the manufacturer's protocol and as described earlier [17].

RNA isolation from cell culture media

RNA from culture media obtained from human PSCs, CAF, and PC cell lines were isolated using ExoMir Kit (BI00 Scientific Corporation, Austin, TX) following manufacturer's protocol. Briefly about 10 ml of conditioned media was centrifuged @ 2000 x g for 10 min and was passed slowly through the syringe fitted with the filter provided in the kit. The captured particles on the filter were then attached to 1 ml syringe containing BI00Pure-MP (provided in the kit) and were slowly depressed to recover lysate into the micro centrifuge tube. 200 µl of chloroform was mixed with lysate and centrifuged to separate layers. 3 µl of co-precipitant provided with the kit was added to the aqueous layer before the addition of isopropanol, vortexed and stored at -20°C to precipitate RNA. After centrifugation at maximum speed 17,000 x g, the pellet was washed with 75% ethanol and resuspended in 50 µl of resuspension solution provided with the kit. RNA concentration was measured and its quality was evaluated by the absorption ratio at 260/280 nm using NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA). The RNA was then used for quantitative real-time PCR as described below.

Quantitative real-time RT-PCR of miRNA

To examine basal expression of *miR-21* and *miR-221* in human PSCs, CAF, PC cells and conditioned media, TaqMan microRNA Assay kit was used (Applied Biosystems) per manufacturer's protocol. 10 ng of RNA from each sample were reverse transcribed as described earlier [17]. All reactions were performed in triplicate using StepOnePlus Real-Time PCR (Applied Biosystems). Relative expressions of miRNAs were analyzed using C_t method and were normalized by RNU48 expression.

Immunostaining and confocal imaging microscopy

The expression of alpha-smooth muscle actin (α-SMA) in MIAPaCa-2, CAF, and PSCs was assessed by immunostaining. 1000 cells were plated in a 4-chamber culture glass slide and cultured till 50% confluent. The cells were then washed, fixed with 4% paraformaldehyde solution and permeabilized with 0.05% Tween 20 solution. After blocking the cells with 1% BSA for 30 min at room temperature, they were incubated with α-SMA (Abcam) primary antibody for 1 h followed by 30 min with secondary antibody. Nuclear staining was performed with DAPI solution (Invitrogen). The antibody-labeled cells were photographed using confocal Imaging Microscope with 40X magnification (EVOS Imaging System, Life Technologies).

In vitro co-culture experiments

Interactions between PC cells (COLO-357 cells) and cancer associated PSCs and CAFs were studied by exposing cancer cells for 72 h to conditioned media derived from both PSCs and CAF-19 cells, and this co-culturing was continued for additional six days for assessing optimal effects. PC cells cultured without conditioned media served as controls. Clonogenic and pancreatosphere formation assays were performed as detailed below.

Clonogenic assay of co-cultured cells

After incubation with and without conditioned medium for 72 hours as described above, PC cells were trypsinized and 1000 viable cells were plated in 100 mm petri dishes. Cells were then incubated in a 5% CO_2/5% O_2/90% N_2 incubator for about 10-12 days at 37°C. The colonies formed were stained with 2% crystal violet and photographed.

Pancreatosphere formation assay

After incubation with and without conditioned medium for 72 hours as described above, PC cells were trypsinized and 1000 viable single cells were plated in the ultralow attached 6 well plate with 2 ml of sphere formation media and were incubated for about a week. The spheres formed termed as "pancreatospheres" were harvested by centrifugation (300 X g) for 5 minutes and were counted under a converted microscope and were also photographed.

Anti-sense miR-21 oligonucleotide transfection

All PSCs (200,000/well) isolated from three different patients were plated in 6 well plates and incubated for about a week. The cells were then transfected twice with ASO-*miR-21* or control ASO-*miRNA* (Life Technology) using DharmaFECT transfection reagent (Thermo Scientific, Pittsburgh, PA) following the manufacturer's protocol, and as described previously [17]. After 72 h of transfection, inhibition of *miR-21* expression was assessed by qRT-PCR (as described above). In addition, migration and invasion of PSCs was measured by chamber cell invasion assay as discussed below.

Invasion assay

Chamber Cell invasion assay was conducted using the 24-well transwell permeable support system (Corning, Lowell, MA) according to the manufacturer's protocol. 50,000 cells transfected with control ASO-*miRNA* and ASO-*miR-21* were plated in a serum-free medium in the upper chamber coated with matrigel. The lower chamber was filled with complete media and cells were incubated for 24 h. The cells in the upper chamber were then removed and the matrigel-invading cells were stained with Calcein AM (Invitrogen) for one hour. The fluorescently labeled cells were photographed using a fluorescence microscope. Fluorescence of the invading cells was measured at excitation/emission wavelengths of 405/535 nm using a Microplate Reader (Tecan, Durham, NC).

Migration assay

Similar to the invasion assay, migration assay was conducted using the 24-well transwell permeable support system (Corning, Lowell, MA) according to the manufacturer's protocol. 50,000 transfected cells with control ASO-*miRNA* and ASO-*miR-21* were plated in serum-free medium in the upper chamber (no matrigel coating), and the lower chamber was filled with complete media and was incubated for 24 h. The cells in the upper chamber were removed and the migrated cells were stained with Calcein AM (Invitrogen) for one hour. Fluorescently labeled cells were photographed and fluorescence measured as described above.

Anti-sense miR-221 oligonucleotide transfection

Cancer associated fibroblasts (CAF-19) were plated at a seeding density of 200,000 cells per well in a 6 well plate and incubated for 72 h. The cells were transfected ASO-*miR-221* or control ASO-*miRNA* (Life Technology) using DharmaFECT transfection reagent (Thermo Scientific, Pittsburgh, PA) following the manufacturer's protocol, and as described previously [17]. After 72 h of transfection, these cells were transfected again for two more rounds and RNA was extracted for assessing of the expression of *miR-221* qRT-PCR, and cell migration and invasion was measured as described above. Transfected cells were also harvested for mRNA expression by qRT-PCR and protein expression by western blot analysis.

Quantitative real-time RT-PCR of mRNA

To examine basal expression of K-Ras and NF-κB in human CAF-19 cells transfected with control ASO-*miRNA* and ASO-*miR-221,* RNA was isolated as described above. 1 μg of RNA from each sample was reverse transcribed using a High Capacity RNA-to-cDNA assay kit (Applied BioSystems) per manufacturer's protocol. All PCR reactions were performed in triplicate with SYBR Green PCR master mix using StepOnePlus Real-Time PCR (Applied BioSystems). Relative expressions of mRNAs were analyzed using C_t method and were normalized by GAPDH expression.

Protein extraction and western blot analysis

Total protein was extracted from CAF-19 cells, transfected with control ASO-*miRNA* and ASO-*miR-221* and subjected to western blot analysis as described previously to assess K-Ras and NF-κB expression [17]. The data was normalized against β-actin expression.

MicroRNA profiling

Total RNA was isolated from human PC cell lines and PSCs using a miRNeasy Kit (QIAGEN) according to the manufacturer's protocol. Purified RNA samples from all cell lines were analyzed by LC Sciences for miRNA microarray profiling, utilizing miRBase version 20 (LC Sciences Houston, Tx). Data were normalized using selected housekeeping genes.

Statistical methods

Data are expressed as mean ± SD. Paired Student's t test was used for statistical analysis. Statistical significance was assumed at a p value of < 0.05.

Results

Expression of miR-21 was up-regulated in cancer associated stellate cells compared to PC cells

Since *miR-21* has proven to be an oncogenic miRNA in many solid tumors and cancer cells [15, 25-28], we extracted exosomal RNA from conditioned media and analyzed the expression of *miR-21* in the conditioned media derived from MIAPaCa-2 PC cells and compared it with four separate samples of cancer associated stellate cells (PSCs) and one cancer-associated fibroblast cell (CAF-19). Total RNA was efficient-

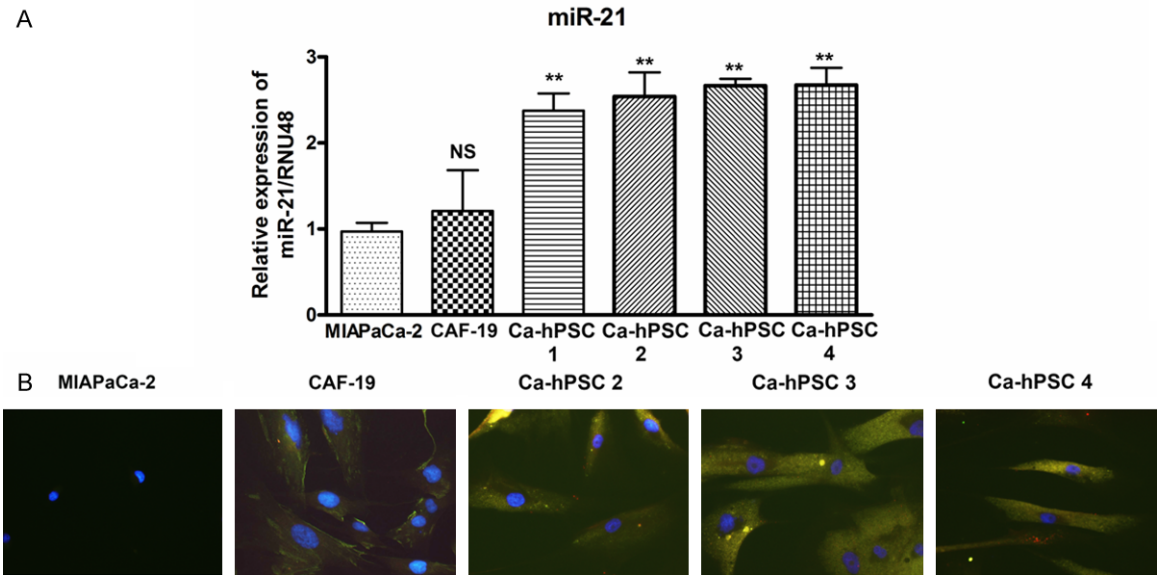

Figure 1. Comparative expression of *miR-21* (A) and α-smooth muscle actin (α-SMA) (B) in human PC cell line MIAPaCa-2, cancer associated fibroblast (CAF-19) and four cancer-associated stellate cells (PSCs). There was a significant up-regulation in the expression of *miR-21* in all four PSCs tested followed by CAF-19 compared to MIAPaCa-2 cells. RNU48 was used as control miRNA. As expected, activation marker α-smooth muscle actin (α-SMA) was expressed in CAF-19 and in all four PSCs tested, but not in MIAPaCa-2 cells. *p values* represent comparison between MIAPaCa-2 with CAF-19 and PSCs. **≤ 0.005 and NS = non-significant.

ly extracted from conditioned media, and was compared across multiple cell lines. There was a slight increase in *miR-21* expression in CAF-19 cells compared to MIAPaCa-2 cells. Interestingly, *miR-21* expression showed a significant increase in all four PSCs compared to MIAPaCa-2 cells, suggesting that *miR-21* may be functioning as an oncogenic miRNA in PSCs (**Figure 1A**), which is consistent with the oncogenic role of *miR-21* in PC as reported earlier [15, 29].

Cancer associated stellate and fibroblast cells exhibited alpha smooth muscle actin expression

MIAPaCa-2 cells, CAF-19, and PSCs isolated from four different patients were assessed by immunostaining for the expression of alpha-smooth muscle actin (α-SMA). As expected α-SMA (a mesenchymal marker) expression was observed in all four PSCs and in CAF-19 cells, but not in MIAPaCa-2 cells as shown in **Figure 1B**.

Co-culture of PSCs and CAF-19 with PC cells exhibited increased clonogenicity and pancreatosphere formation

The effect of co-culture of PC cells with PSC (**Figure 2A**) and CAF-19 (**Figure 2B**) conditioned media was assessed by a clonogenic assay which showed a significant increase in colony formation by PC cells when compared to cells without conditioned media. These findings indicate the high clonogenic potential of conditioned media derived from PSCs and CAF-19 compared to PC cells. Similarly, there was an increase in the cancer-stem cell (CSC) self-renewal capacity with increased number of pancreatospheres formed by PC cells after 7 days of incubation with PSC but not with CAF-19 conditioned media, as compared to the number of pancreatospheres formed by PC cells unexposed to conditioned media (**Figure 2**), suggesting that secreted factors from PSCs have a remarkably higher potential to induce self-renewal of PC cells.

Expression of miR-21 was up-regulated in cancer associated stellate cells compared to stellate cells isolated from normal human pancreas

Since we observed a significant increase in *miR-21* expression in all PSCs tested, we assessed its expression in normal stellate cells (nhPSCs). Both normal (non-cancerous pancreatic tissue derived stellate cells) and cancer associated stellate cells were isolated by the laboratory of Dr. Apte as described earlier [30]. We observed significantly lower expression of

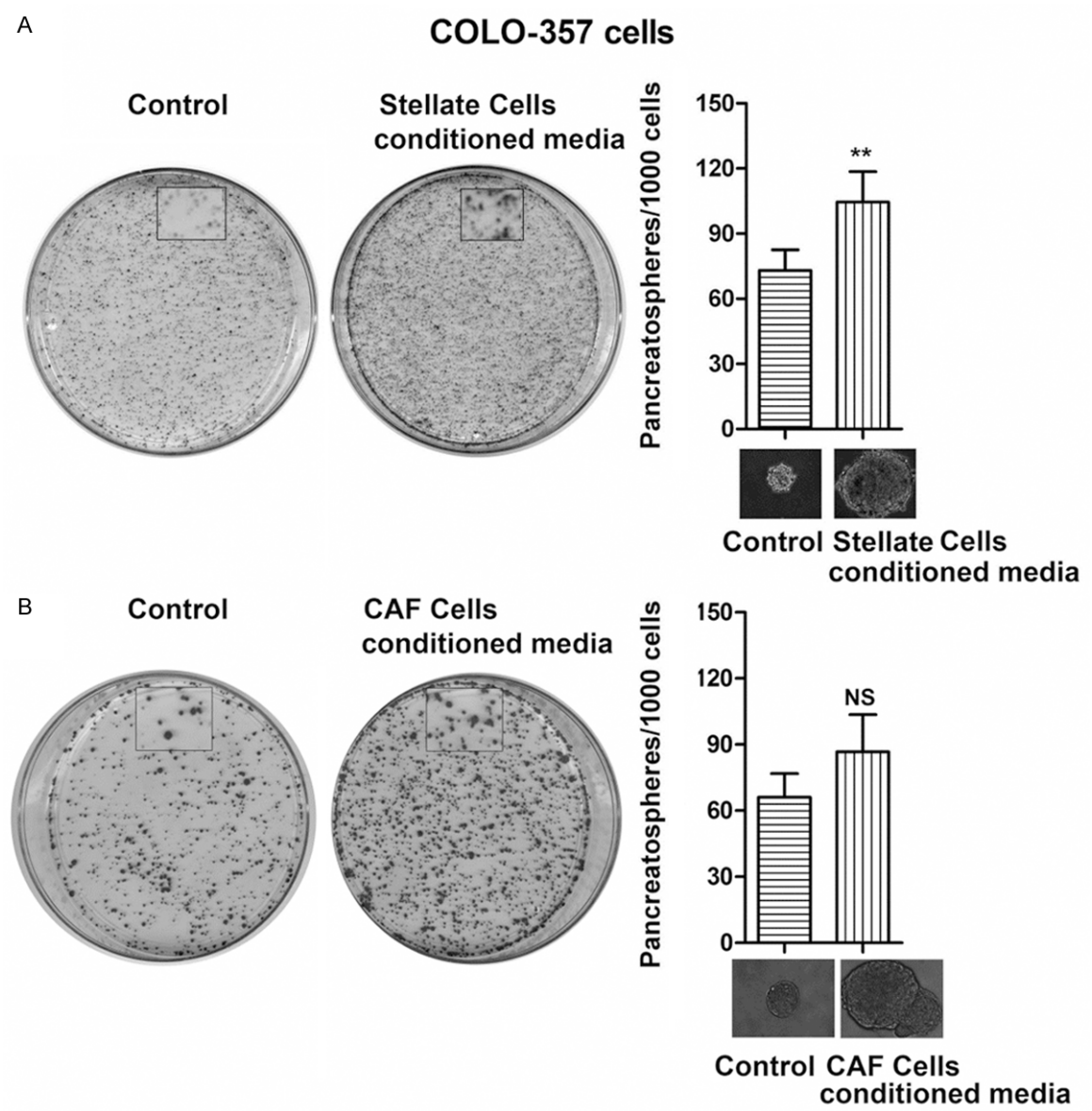

Figure 2. Effect of co-culture of PC cell line COLO-357 with conditioned media derived from PSCs (A) and CAF-19 cells (B). There was a significant increase in colony formation of PC cells cultured with conditioned media derived from both PSCs and CAF-19 cells compared to untreated COLO-357 cells (A and B). Similarly there was an increase in pancreatosphere formation of PC cells cultured with conditioned media derived from both PSCs and CAF-19 cells compared to untreated COLO-357 cells (A and B). *p* values represent comparison between control cells and cells treated with conditioned media. **≤ 0.01 and NS = non-significant.

miR-21 in all three nhPSCs compared to cancer associated PSCs and to conditioned media from two of the PSCs tested as detailed under figure legend. Due to sample limitation, we were only able to compare *miR-21* expression with only one cancer associated PSCs, and conditioned media derived from two preparations of PSC cells. Similarly, *miR-21* expression in MIAPaCa-2 cells was higher than nhPSCs as presented in **Figure 3**. This increase in the expression of *miR-21* in PSCs may play important roles in the tumor microenvironment associated with tumor aggressiveness of PC.

Transfection of anti-sense miR-21 in cancer associated stellate cells decreased cell migration and invasion

Significantly higher expression of *miR-21* could be responsible for many biological processes

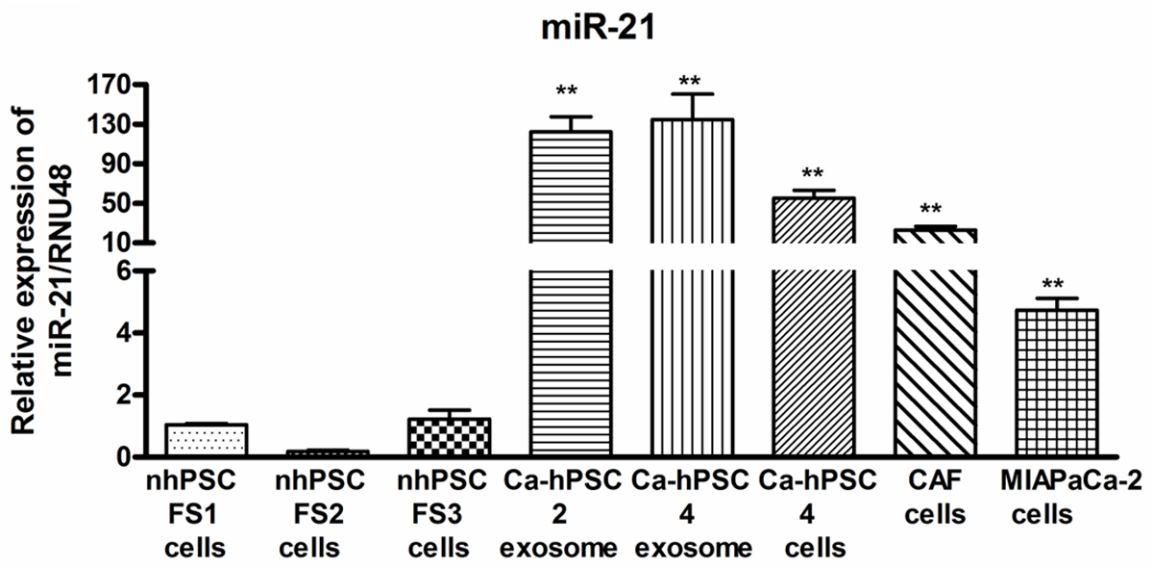

Figure 3. Comparative expression of *miR-21* in the conditioned media derived from nhPSC and PSCs/CAF-19, and the cells (PSCs, CAF-19 cells and PC cell line MIAPaCa-2). There was a significant up-regulation in the expression of *miR-21* in both RNA preparation from intact cells and conditioned media derived from PSCs, followed by CAF-19 cells and MIAPaCa-2 cells compared to all three nhPSCs. RNU48 was used as control miRNA. *p* values represent data from two different experiments in triplicate and was compared against nhPSCs. **≤ 0.0001.

as documented by numerous publications including chemo-resistance in colon cancer cells due to enriched undifferentiated cancer stem like cells [28]. The significant increase in migration and invasion in non-small cell lung cancer cell lines YTMLC-90 and NCI-H157 was observed, which was suppressed by knock-down of *miR-21* expression [31]. Hence, we studied the effect of *miR-21* ASO transfection on the migration and invasion of PSCs. The cells were transfected twice with ASO-*miR-21* for 72 h. The transfected cells were used for cell migration and invasion assays. The transfection of ASO-*miR-21* was confirmed by qRT-PCR, which revealed reduced expression of *miR-21* as shown in **Figure 4A**. We also found that lowering the expression of *miR-21* resulted in decreased cell migration compared to control ASO-*miRNA* transfected cells, as shown in **Figure 4B**. Likewise, the suppression of *miR-21* resulted in decreased invasive capacity of these cells (**Figure 4C**). These results suggest that *miR-21* is an oncogenic miRNA in PSCs and that lowering the expression of *miR-21* leads to decreased cell migration and invasion. All PSCs isolated from three different patients showed similar results. **Figure 4** data represents results from one of the three PSCs treated with ASO-*miR-21*.

Transfection of anti-sense miR-221 in CAF-19 cells decreased cell migration, invasion, and the expression of K-Ras and NF-κB

Relative levels of *miR-221* expression were measured both in the exosomal RNA and RNA derived from PC cell line MIAPaCa-2, as well as from cancer-associated fibroblast and stellate cells as presented in **Figure 5A**. As can be seen from the figure, the expression level of *miR-221* was significantly higher in CAF-19 compared to PSCs and MIAPaCa-2 cells and in conditioned media. Thus, we wanted to assess whether lowering the expression of *miR-221* by ASO transfection on CAF-19 cells would have any effect on migration, invasion, and the expression of K-Ras and NF-κB which are *miR-221* targets [32, 33]. The cells were transfected with anti-sense *miR-221* (inhibitor) twice for 72 h. Transfection of ASO-*miR-221* was confirmed by qRT-PCR showing reduced expression of *miR-221* as depicted in **Figure 5B**. We also found that lowering the expression of *miR-221* resulted in decreased cell migration and invasion compared to control inhibitor (control ASO-*miRNA*) transfected cells as demonstrated in **Figure 5D**. Furthermore, the suppression of *miR-221* resulted in decreased expression of K-Ras, and NF-κB both at the mRNA level

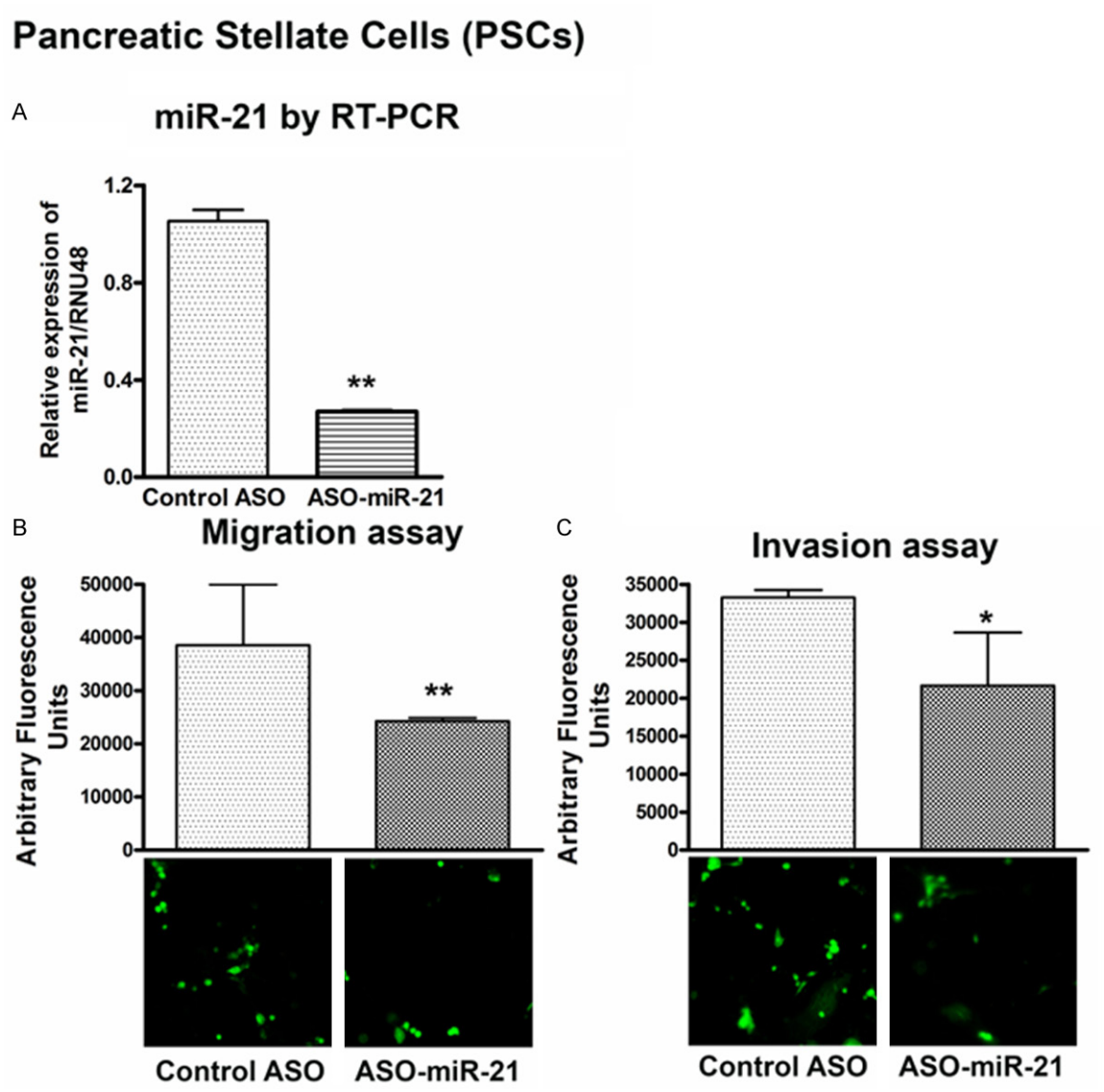

Figure 4. Treatment of PSCs with ASO *miR21*, led to a significant reduction in the expression of *miR-21* as assessed by qRT-PCR (A), decreased cell migration (B), and decreased cell invasion (C) as assessed by chamber cell invasion assays. *p* values represent comparison between control ASO and ASO-*miR-21.* **≤ 0.007 and *≤ 0.05.

(**Figure 5C**) and at the protein level (**Figure 5E**). These results suggest that inhibiting the expression of *miR-221* in CAF-19 cells led to the inhibition of cell migration, invasion, and the expression of its targets K-Ras and NF-κB. Hence targeting *miR-221* in patients may serve as a novel treatment option for PC.

Differential expression of miRNAs was observed by profiling of RNA derived from PSCs, CAF-19 and PC cells

RNA extracted from cancer-associated stellate, fibroblast and PC cell line MIAPaCa-2 and COLO-357 were profiled for miRNA analysis by LC Sciences, Houston, TX. Expression profiling revealed several differentially expressed miRNAs in PSCs, CAF-19, MIAPaCa-2 and COLO-357 cells. Four of the significantly up-regulated miRNAs in CAF-19 cells includes *miR-99a*, *miR-100*, *miR-125b* and *miR-4488*, whose expression was further validated by qRT-PCR using RNU48 as a control miRNA as presented in **Figure 6**. As evident from the Figure, these four miRNAs were found to be significantly increased in PSCs and CAFs compared to the cancer cell lines.

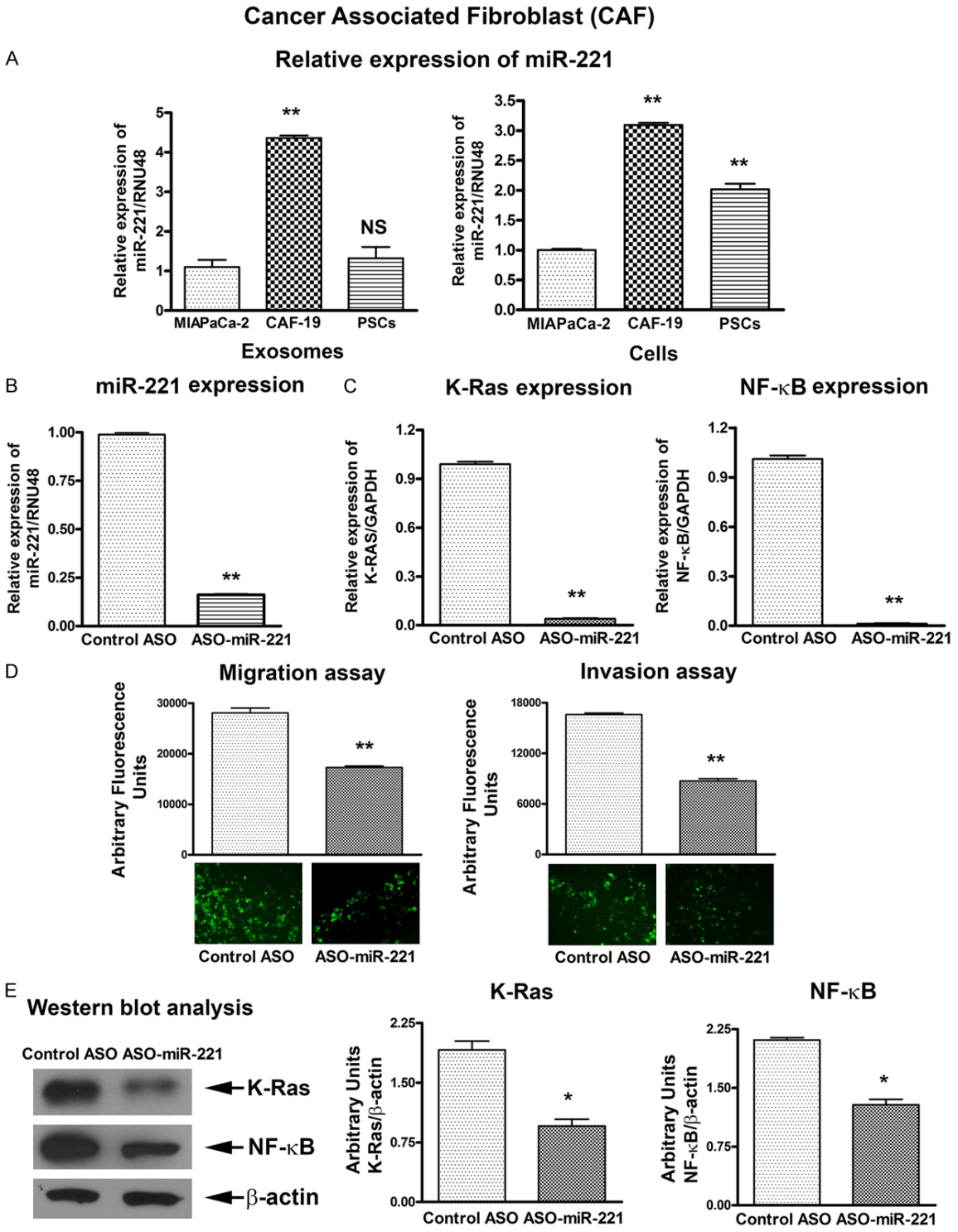

Figure 5. Relative expression of *miR-221* was compared in MIAPaCa-2, CAF-19 and PSCs both from cells and conditioned media (A), inactivation of *miR-221* expression by ASO in CAF-19 cells led to reduced expression of *miR-221* as assessed by qRT-PCR (B), decreased K-Ras and NF-κB mRNA expression assessed by qRT-PCR (C), decreased cell migration and invasion of cells by chamber cell invasion assays (D), and decreased K-Ras and NF-κB protein expression as assessed by western blot analysis and were quantified against β-actin (E) Controls used are: for miRNA (RNU48), mRNA (GAPDH), and protein (β-actin). *p* values represent comparison against MIAPaCa-2 in 5A, comparison against control ASO in 5B-E. **≤ 0.001, *≤ 0.01 and NS = non-significant.

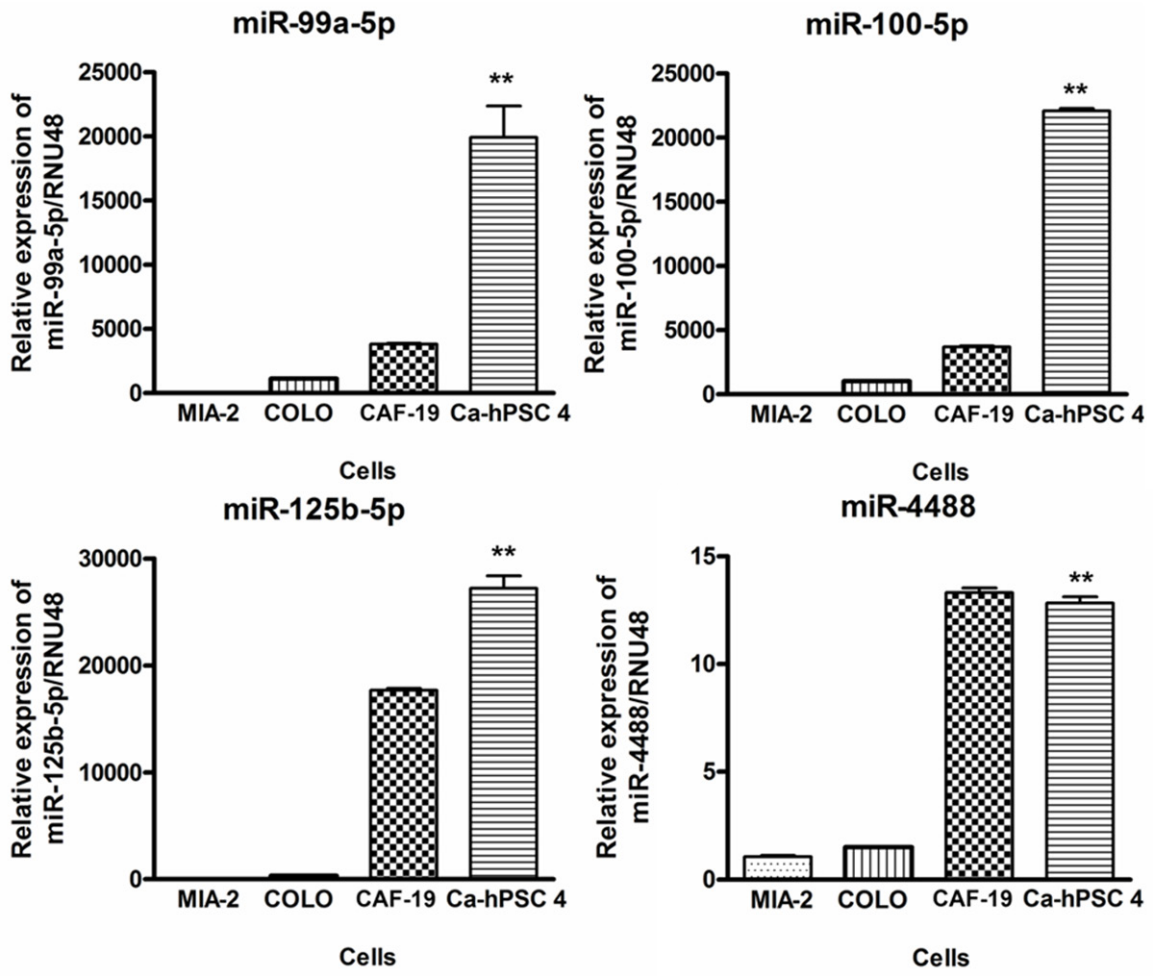

Figure 6. Comparative expression of *miR-99a-5p, miR-100-5p*, *miR-125b-5p*, and *miR-4488* in MIAPaCa-2, COLO-357, CAF-19, and Ca-hPSC 4 by qRT-PCR. There was a significant up-regulation in the expression of *miR-99* and *miR-100* in Ca-hPSC-4 compared to CAF-19 and both PC cell lines. The other two miRNAs *miR-125* and *miR-4488* showed significant up-regulation in both CAF-19 and Ca-hPSC4 compared to both PC cell lines. *p* values represent comparison between PC cells and Ca-hPSC 4. **≤ 0.0001.

Discussion

Emerging evidence suggests the importance of the interaction of tumor-stroma in the initiation and progression of pancreatic ductal adenocarcinoma (PDAC) or PC [34, 35]. This signifies an inter-dependent relationship between PC and PSCs, resulting in an overall increase in tumor growth [34, 35]. A schematic representation of the involvement of activated PSCs, CAF cells with PC cells in EMT, miRNAs and hypoxia in the tumor microenvironment that leads to tumor progression, survival and metastases is presented in **Figure 7**. The interaction of PSCs with neighboring cells in the tumor microenvironment such as cancer cells and cancer stem cells can increase ECM production, leading to increased fibrosis, proliferation and migration, which have been documented as typical desmoplasia seen in human PC. Cancer cells can corrupt the tumor microenvironment for its benefit and this process can be accomplished through partners in crime such as stellate cells and fibroblast and other cells, also involving exosomes and many soluble factors as reviewed recently [36].

Upon exposure to conditioned media derived from PSCs and CAF-19 cells, COLO-357 PC cells, showed increased colony formation and formation of pancreatospheres compared to PC cells not exposed to conditioned media. This suggests that soluble factors are produced and secreted by PSCs and CAF cells that can confer tumor aggressiveness. Similarly, *in vivo* studies showed increased tumor growth and

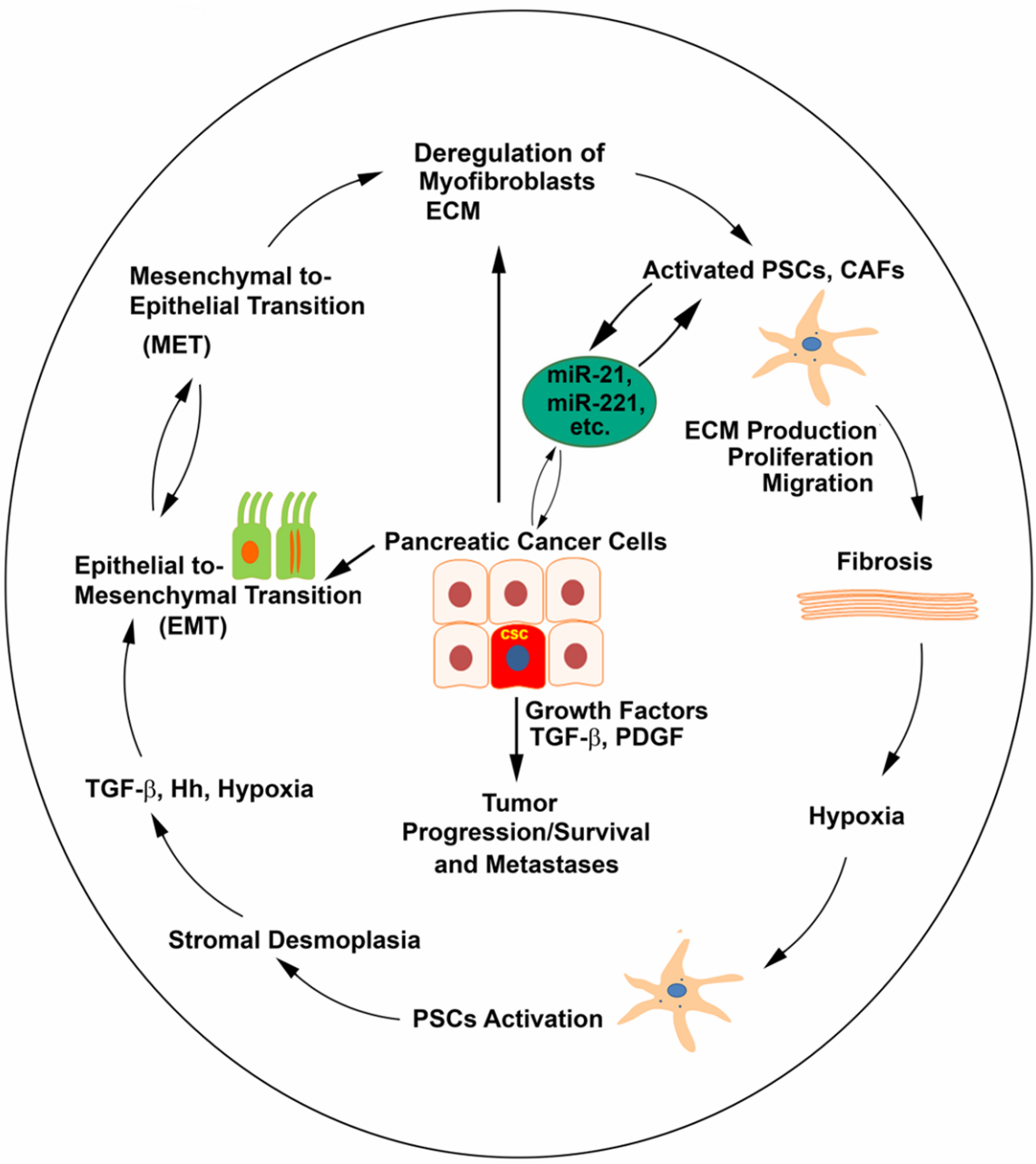

Figure 7. A schematic representation of the tumor microenvironment involving activated PSCs, CAF cells and their interactions with PC cells, EMT, miRNAs and hypoxia-all of which may lead to tumor progression, survival and metastases (tumor aggressiveness).

metastases in mouse models of PC co-injected with PC cells and PSCs when compared to models injected with PC cells alone, indicating the interaction of stromal fibroblasts and PC cells in promoting tumor progression in PC model [34, 37].

Although there are few reports documenting up-regulation of *miR-21* resulting in the activation of hepatic stellate cells causing liver fibrosis [38-41]; however, similar studies on human PSCs have not yet been reported. Human pancreatic cancer associated stellate cells (PSCs) are a subset of pancreatic cancer-associated myofibroblast-like cells that express the activation marker α-SMA and when activated these cells secrete ECM components and play a role in the pathogenesis of pancreatitis and PC. To the best of our knowledge, our current study is the first to demonstrate over-expression of

miR-21 in cancer associated human PSCs compared to normal human PSCs and PC cells. The inhibition of *miR-21* by ASO significantly decreased both cell migration and invasion of PSCs, suggesting that therapeutic targeting of this miRNA may interfere with the recruitment of PSCs to the vicinity of cancer cells, thereby inhibiting the tumor promoting cross-talk between the two cell types. The over-expression of *miR-21* was also shown in lung fibroblasts in response to TGF-β, and inhibition with ASO-*miR-21* diminished the harshness of lung fibrosis in mice, providing yet another example of miRNA targeted therapeutic approach for treating pulmonary fibrosis [42].

In spite of the discovery of exosomes three decades ago, the interest in these vesicles increased significantly only after finding the presence of mRNA and miRNA [43]. Exosomes are small membrane vesicles of endocytic origin that are released extracellularly in their secretions (conditioned media) of cultured cells [43]. A recent report by Charrier *et al*. in murine model of alcoholic pancreatitis revealed the presence of *miR-21* and CCN2 expression in PSC-derived exosomes that stimulated delivery to other PSC [19]. Recent development suggests that cancer-associated stellate and fibroblast cells are present in the stroma of pancreatic tumors causing desmoplasia, and may lead to tumor invasion, metastasis and also resistance to therapy, suggesting that targeting these molecules associated with desmoplasia could serve as precision medicine.

Another study demonstrated the over-expression of *miR-221* in paired samples of normal fibroblast when compared to cancer-associated fibroblast in six resected human breast cancer tissue samples. Their relationship with *miR-221* was established with the activation of TGF-β and IL-6 signaling pathways [44]. In this study over-expression of *miR-221* was correlated directly with over-expression of K-Ras and NF-κB in CAF which is another tissue contributing to desmoplastic reaction. The over-expression of K-Ras and NF-κB at the mRNA and protein level was reduced at both levels upon treatment with ASO-*miR-221*, which significantly decreased the migration and invasiveness of CAF-19 cells, suggesting that miRNA treatment might be a novel therapeutic approach to inhibit K-Ras signaling pathway. Hence, understanding the biological functions of miRNAs especially in the context of PC microenvironment will significantly help in the development of new and novel targeted therapies.

We also compared the expression profiling of PC cells MIAPaCa-2 and COLO-357 cells with PSCs and CAF cells, and found several miRNAs that were differentially expressed in the PSCs/CAF cells compared to both PC cells. Of the several deregulated miRNAs, we chose four up-regulated miRNAs for further validation using qRT-PCR and found that the expression of *miR-125b* and *miR-4488* was significantly higher in both PSCs and CAF-19 cells compared to PC cells. In contrast, the expression of *miR-99a* and *miR-100* was significantly up-regulated in PSCs compared to CAF-19 and PC cells, suggesting that although CAF and PSCs share many of the similar functions such as myofibroblast-like phenotype and α-SMA expression, these cells appears to be distinct from each other and there is a vast difference in the expression of *miR-99a* and *miR-100*.

In conclusion, this pre-clinical study provides a clear rationale for targeting PSCs and CAF cells within the tumor microenvironment using miRNA targeted therapeutics such as antisense oligonucleotides to inhibit migration and invasion, which will in turn inhibit tumor progression. Hence, effective methods should be developed to interrupt the interaction of PSCs/CAF cells with PC cells by developing novel approaches in order to prevent cancer progression which would likely improve the therapeutic outcome in the treatment of PC patients.

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Disclosure of conflict of interest

All the authors declare no competing conflict of interest.

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