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Original Research Article

miRNA profiling in pancreatic cancer and restoration of chemosensitivity

Saurabh Singh [a], Deepak Chitkara [a,c], Virender Kumar [a], Stephen W. Behrman [b], Ram I. Mahato [a,*]

[a] Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, TN 38163, United States
[b] Department of Surgery, University of Tennessee Health Science Center, Memphis, TN 38163, United States
[c] Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research, SAS Nagar, Mohali, Punjab 160 062, India

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ABSTRACT

Pancreatic cancers relapse due to small but distinct population of cancer stem cells (CSCs) which are in turn regulated by miRNAs. The present study identifies a series of miRNAs which were either upregulated (e.g. miR-146) or downregulated (e.g. miRNA-205, miRNA-7) in gemcitabine resistant MIA PaCa-2 cancer cells and clinical metastatic pancreatic cancer tissues. Gemcitabine resistant MIA PaCa-2 cells possessed distinct ALDH-positive CSC fraction expressing stem cell markers OCT3/4 and CD44 and chemoresistance marker class III β-tubulin (TUBB3) which decreases on transfection with miR-205 resulting in the restoration of chemosensitivity to gemcitabine.

1. Introduction

Pancreatic cancer is the fifth leading cause of cancer related mortalities in the United States [1]. Gemcitabine (Gemzar®) is the first line of chemotherapy in pancreatic cancer but is significantly metabolized in plasma and therefore requires high doses leading to toxicity [2–4]. While the drug is initially effective in increasing survival in pancreatic cancer patients, the possibility of reemergence of secondary tumors that are resistant to initial chemotherapeutic drug is very high. In fact, most patients will eventually progress to advanced form of the disease marked by chemoresistance, metastasis and poor prognosis [5]. For these reasons, the overall prognosis for pancreatic cancer remains rather bleak based on the current therapy regimens.

Most solid tumors, including pancreatic carcinomas are composed of bulk and cancer stem cells (CSCs) with the latter forming a small but distinct subpopulation. Evidence suggests that CSCs may be involved not only in tumorigenesis but also survive the initial chemotherapy and utilize their self-renewal potential to regenerate chemoresistant secondary tumors [6,7]. Chemoresistance to anticancer drugs including gemcitabine by these cells may be controlled and contributed by microRNAs (miRNAs) which are noncoding RNA molecules and have recently emerged as molecular switches for a number of life processes including development, and carcinogenesis. The development of chemoresistance through an increase in the number of CSCs has been attributed to alterations at the level of miRNAs in pancreatic and other solid tumors [8–12]. Several lines of evidence now link miRNA dysregulation to carcinogenesis, progression, chemoresistance and recurrence in several cancers [13–15], making them targets for therapy as well as biomarkers for disease progression. For instance, we have recently identified miR-200c and 34a as mediators of chemoresistance in a model of advanced prostate cancer [16].

Alcohol dehydrogenase (ALDH) is a family of enzymes required for detoxification of xenobiotics such as alkylating agents [17–19]. Cells with higher expression of ALDH have been shown to be insensitive to various chemotherapeutic drugs and will survive thereby allowing disease recurrence when therapy is withdrawn [19–21]. These CSCs are thus considered a viable target for improved therapeutic intervention and preventing chemoresistance and cancer relapse.

In our present study, we hypothesized that chemoresistance to gemcitabine in highly invasive pancreatic adenocarcinoma cell line could be due to the altered miRNA expression and that replenishment of anticancer miRNAs may effectively restore chemosensitivity. Thus, while chemotherapy with gemcitabine eventually fails due to the emergence of chemoresistance mediated by CSCs, our proposed approach would be to first restore miRNA to prechemoresistant levels and then treat with initial chemotherapeutic drug. This will allow the antimetabolite drugs to attack bulk tumor cells after suppressing chemoresistant CSCs that are maintaining the tumors. Towards this end, we have identified altered miRNAs in advanced pancreatic cancer clinical samples as well as in pure populations of CSCs isolated from a gemcitabine resistant pancreatic cancer cell line using a fluorescent substrate of ALDH1 [22]. One of these was miR-205 that was consistently downregulated

* Corresponding author. Address: Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, 19 South Manassas, CRB RM224, Memphis, TN 38103-3303, United States. Tel.: +1 901 448 6929; fax: +1 901 448 2099.
URL: http://www.uthsc.edu/pharmacy/rmahato/ (R.I. Mahato).

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in clinical pancreatic cancer samples as well as in isolated CSC populations. Following its replacement, treatment with gemcitabine was effective in killing MIA PaCa-2 cancer cells hitherto resistant to its effects. Our approach not only allows the identification of novel targets that modulate chemoresistance in pancreatic cancer, but also demonstrates the validity of miRNA replacement regimen. Compared to chemotherapy drugs which are non-specific and target all cells, miRNA replacement treatment is likely to yield better outcomes since this strategy is immune to the development of chemoresistance and by targeting mRNAs can provide therapy outcomes at low doses than normally used for chemotherapy.

2. Materials and methods

2.1. Materials

Gemcitabine was purchased from LC Labs (Woburn, MA). SYBR Green real-time PCR master mix and reverse transcription reagents were purchased from Applied Biosystems (Foster city, CA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO) and used as received, unless stated otherwise.

2.2. Cell lines

The human pancreatic adenocarcinoma cell line MIA PaCa-2 was purchased from ATCC. Gemcitabine-resistant version of these cells (MIA PaCa-2^R) was a kind gift of Dr. Fazlul H. Sarkar (Wayne State University). Both cell lines were passaged in DMEM culture media supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Gibco) in a humidified incubator containing 5% CO_2 at 37 °C. Gemcitabine was added to the culture medium used for maintaining drug resistant cancer cells.

2.3. Human pancreatic tissue

Human pancreatic tissues (cancerous and benign) were obtained from surgical resections following established protocols and in accordance with the informed consent waiver provided by the Institutional Review Board (IRB) at the Baptist Hospital. Pancreatic tissue was rinsed and flash frozen in liquid nitrogen and then stored at −80 °C. Tissues were classified as malignant, localized adenocarcinoma or benign based on the diagnosis made by a pathologist.

2.4. Aldeflour analysis and cell sorting by FACS

Identification and analysis of cell lines was performed using Aldeflour reagent based (Stem Cell Technologies, Vancouver, Canada) flow cytometry method according to the manufacturer's instructions. This method relies on the increased alcohol dehydrogenase (ALDH) activity of CSCs. Briefly, adherent cells were trypsinized and washed with phosphate buffered saline (PBS). Cells (1×10^6 cell/ml) were then suspended in cell suspension media provided in the kit. Aldeflour reagent was then added to the cell suspension followed by the incubation for 40 min at 37 °C. For each cell type tested, a negative control comprising cells treated with ALDH-inhibitor diethylamino-benzaldehyde (DEAB) was also included. Cells were recovered by centrifugation and washed twice with PBS to remove unbound reagent and finally suspended in ice cold cell suspension media.

Cell sorting/analysis were carried out using a FACSCalibur flow cytometer (Becton Dickinson). Cells treated with DEAB (negative control) were used to set up a gated region which was then populated by ALDH+ cells. These were quantified by calculating the percentage of total cells that displayed greater fluorescence compared with the negative control. Post-sorting, 10 μl of each isolated fraction was immediately analyzed to reconfirm the purity of isolated populations.

2.5. Real time RT-PCR

Total RNA was extracted using RNeasy RNA isolation kit (Qiagen, MD) followed by reverse transcription into cDNA template [16]. Real-time PCR was then performed to amplify cDNA templates using SYBR Green dye universal master mix on a Light Cycler 480® (Roche, Indianapolis) using the primers for genes of interest for forty cycles. S19 was kept as housekeeping gene and relative amount of mRNA was calculated using Crossing point (Cp) values and scaled relative to control samples set at a value of 1.

2.6. miRNA profiling of pancreatic cancer cells and human pancreatic tissues

miRNA profiling was done using standardized protocols as described earlier [16]. Briefly, total RNA including small non-coding miRNA was isolated from MIA PaCa-2, MIA PaCa-2^R, CSCs and human pancreatic tissues using miRNEasy RNA isolation kit (Qiagen, MD). RNA quality was then determined using a Nanodrop 2000 UV–Vis spectrophotometer (Thermo Scientific) and converted to cDNA template using the miScript II RT Kit. A SYBR green based pathway-focused miScript miRNA PCR Array (catalog number 102ZF, Qiagen, MD) was performed as per manufacturer's protocol. This array allows detection of 84 miRNAs previously identified in various human cancers. RNA quality controls and housekeeping genes were also included in the array. The plates were run on a Roche Light Cycler 480® and the expression of individual miRNAs was analyzed using the Cp values. Non-CSCs, as well as benign pancreatic samples were considered as 'controls' to calculate the fold change in CSCs and cancerous pancreatic tissue, respectively. Endogenous controls, controls for reverse transcription reaction, and DNA contamination controls were also tested to ensure high quality of data.

2.7. Transfection studies for miRNA mimic replenishment

Gemcitabine resistant MIA PaCa-2^R cells were used in the miRNA transfection study. Cells were transfected with synthetic miR-205 mimic or miR7 and scrambled miRNA using Lipofectamine® 2000 reagent according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates (1.25×10^5 cells/well) in DMEM media containing 10% FBS. After 12 h of incubation at 37 °C/5%CO_2 the media was replaced with Opti-MEM® (without serum and antibiotics) and cells again incubated 24 h. At 50% cells confluency, transfection was done with final miRNA concentration of 30 nM/well. Media was replaced with DMEM containing FBS 10% and antibiotics after 6 h post transfection. After 48 h cells were trypsinised, lysed and gene expression levels of OCT3/4, CD44 and TUBB3 were determined using real time RT-PCR as described above.

2.8. Drug sensitivity and apoptosis assays in pancreatic cancer cells

To determine the reversal of gemcitabine sensitivity following miRNA replenishment therapy, MIA PaCa-2^R cells were seeded in 96 well cell culture plates and incubated for 24 h to allow cell attachment. Cells were then transfected with miR-205 mimic as described in Section 2.7. Following transfection, the media was replaced with the fresh media containing gemcitabine (20–500 nM) and incubated at 37 °C/5% CO_2. Cell viability was assessed after 72 h by MTT assay. Briefly, cells were washed with PBS followed by incubation with fresh media containing 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (0.5 mg/ml; 200 μl) at 37 °C/5% CO_2 for 4 h. After 4 h, plates were centrifuged at 1000 rpm for 5 min and media was removed. Formed formazan crystals were then dissolved in DMSO and absorbance was measured at 560 nm after subtraction for cell debris at 655 nm. Cell viability was calculated using the following formula:

$$\text{Cell viability} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

2.9. Statistical analysis

The Student's unpaired *t*-test was used to compare individual group means. A p value of <0.05 was considered as statistically significant. All values in the figures and text were expressed as the mean ± S.D.

3. Results

3.1. Aldeflour staining and cell sorting

Following staining with Aldeflour reagent which contains a substrate for ALDH enzyme, MIA PaCa-2 cells, treated with DEAB or not, did not show significant fluorescence (Fig. 1A and B, respectively). In case of gemcitabine-resistant MIA PaCa-2 population, however, almost 20 ± 2.2% of total viable cells present in the gated area (P2) of the dot-plot were fluorescent (ALDH+) (Fig. 1C). Nearly 2–3% of total fluorescent cells at the leading edge were brighter than the rest of the cells in P2 and designated as ALDH++.

Isolation of three distinct populations with respect to their ALDH activity was carried out by flow sorting. Fig. 2A demonstrates three peaks corresponding to three levels of ALDH-activity in gemcitabine-resistant MIA PaCa-2 cells, namely ALDH-ve, ALDH+ and ALDH++. Each of these populations was confinable to a specific region with minimal bleed over confirming a high degree of purity. This was confirmed by reanalyzing pure populations (Fig. 2B) which matched the pattern seen in 2A. In contrast to these results, non-resistant MIA PaCa-2 cells demonstrated a single peak which consists of mostly non-fluorescent cells (Fig. 2C).

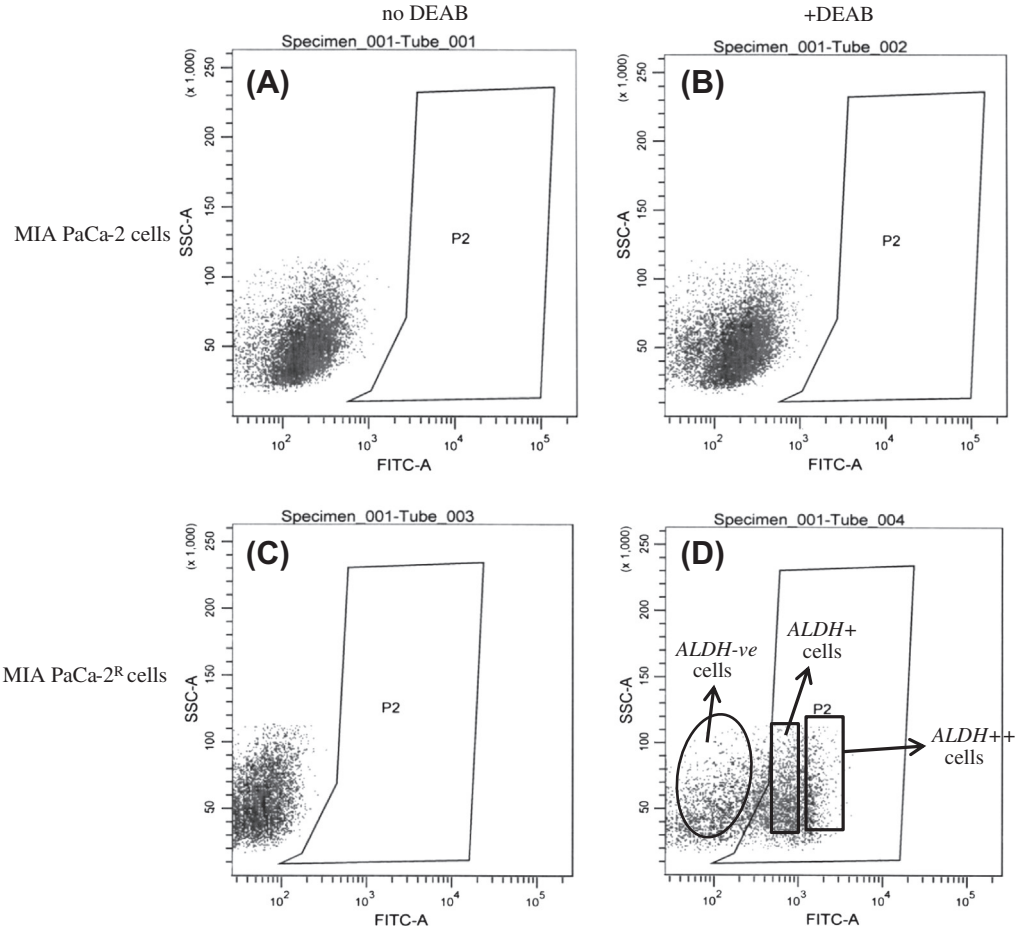

Fig. 1. Aldeflour staining of gemcitabine sensitive and resistant MIA PaCa-2 pancreatic cancer cells. Following aldeflour staining of suspended cells, flow cytometry was carried out to identify ALDH positive cells. ALDH++ cells are at the leading edge of all ALDH-positive cells in the dot plot and represent the fraction believed to be the pure cancer stem cells. DEAB treatment was used as negative control to inhibit ALDH activity and set up the identification grid P2.

3.2. Culture and characterization of purified cancer cells

Following their separation by flow sorting, each fraction was transferred to a 6-well plate and allowed to grow for 30 h after which the cells were photographed. As can be seen in Fig. 3, ALDH-ve (non-stem cells or NSCs) cells possess the typical flattened epithelial phenotype. ALDH++ (CSCs) cells possess a significant number of spindle shaped, elongated cells (shown by arrows) presenting a mesenchymal phenotype. Intermediate cells (ALDH+) area shows mixture of these two groups.

Molecular characterization of NSCs and CSCs was carried out by real time RT-PCR to assess gene expression of ALDH1 (chemoresistance/CSC marker) [17], CD44 (CSC marker) [6], ABCG2 (chemoresistance marker) [23–25], Oct3/4 and Nanog (stem cell markers) [26] and MCM7 (cell cycle/proliferation marker) [27]. A 3-fold increase in the expression of ALDH1 transcript was seen in CSCs (Fig. 4). For all other transcripts, a 2-fold increase was observed in CSCs. MCM7 expression was 4-fold higher in NSCs as compared to that in CSCs.

3.3. Profiling of cells and clinical pancreatic tissues for identifying chemoresistance miRNAs

We isolated total RNA from NSCs and CSCs and used it for preparing cDNA which was used as a template for identifying miRNAs that are specifically altered between the two populations. Fig. 5 shows the 14 differentially altered transcripts of which five (miR-15, 134, 205, 7 and 32) were downregulated in CSCs with miR-205-a tumor suppressor molecule – the most reduced transcript (6-fold). The nine upregulated transcripts were miR-143, 214, 10a, 10b, 146a, 146b-5p, 34a and 100.

To increase the clinical relevance of CSC profiling data, we carried out miRNA profiling of clinical pancreatic cancer tissue and compared it to non-cancerous pancreatic tissue. Fig. 6 shows upregulated and downregulated transcripts compared to the expression in non-cancerous sample. There are 38 up- and 14 downregulated miRNAs and miR-205 was highly downregulated (~85-fold) signifying its role as a potential marker in advanced pancreatic cancer. Since metastases are a major complication in pancreatic cancer recurrence, we assessed metastatic pancreatic cancer tissue and identified altered miRNA transcripts which may have a role in metastasis. In this case, localized pancreatic cancer sample was used as a normalizer control. Fig. 7A depicts the distribution of significantly altered transcripts in a scatter plot arranged about a central axis. Increasing distance from the axis indicates greater alteration in the transcript level. As can be seen in Fig. 7B and C, a number of miRNAs are altered in metastatic pancreatic tissue; including miR-205 which was downregulated at least two folds. miR-146a is again upregulated in metastatic sample, signifying its consistent role in pancreatic cancer in general.

3.4. miRNA replenishment therapy and restoration of chemosensitivity

To establish the biological significance of our profiling studies, we selected miR-205 and miR-7, both of which were downregulated in CSCs. miR-205 is also consistently downregulated across

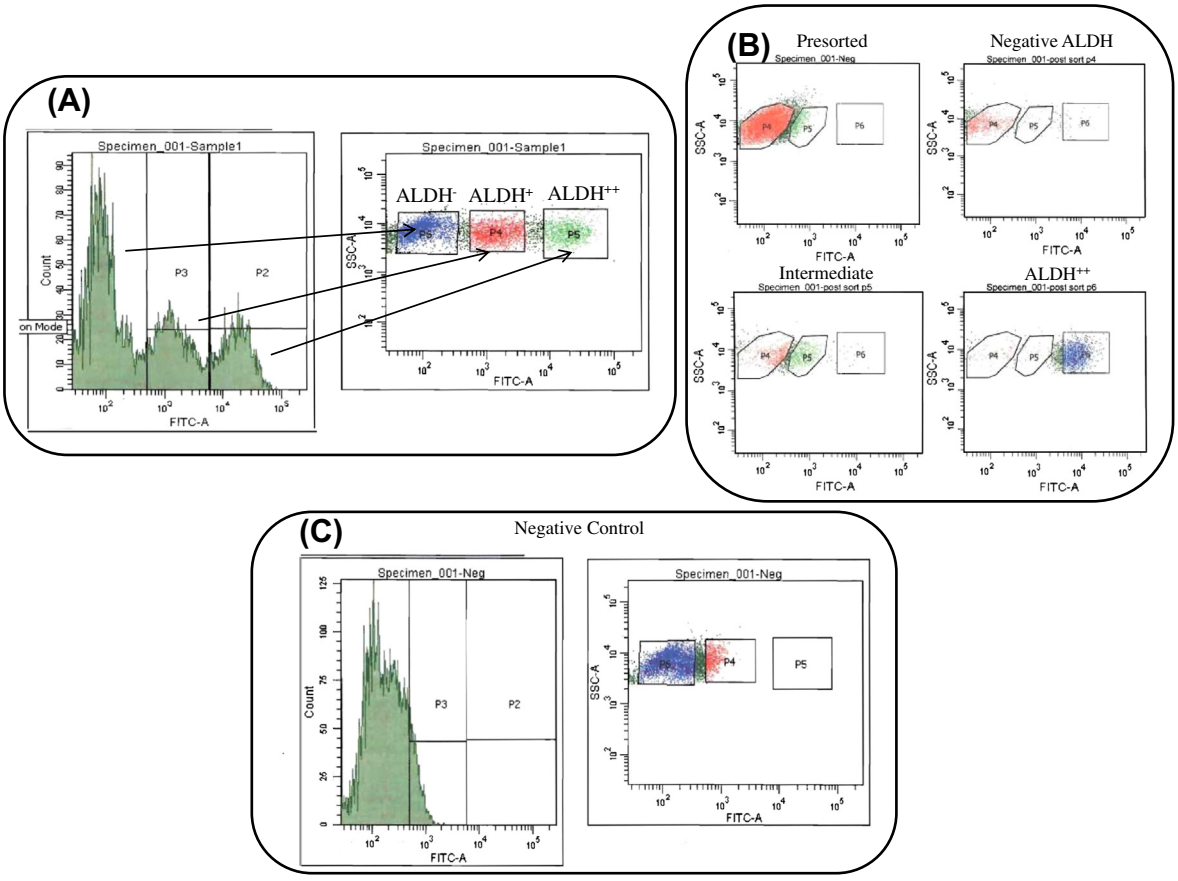

Fig. 2. Isolation of ALDH-positive cells by flow cytometry. (A) Plot of aldeflour-stained, gemcitabine resistant MIA PaCa-2 cells demonstrating three individual peaks corresponding to ALDH negative, ALDH+ and ALDH++ cells (arrows), respectively. (B) Post-isolation samples were immediately tested for extent of ALDH-staining to determine purity. (C) DEAB-treated gemcitabine-resistant MIA PaCa-2 cells show a single peak and small number of ALDH + cells. No ALDH++ cells were observed.

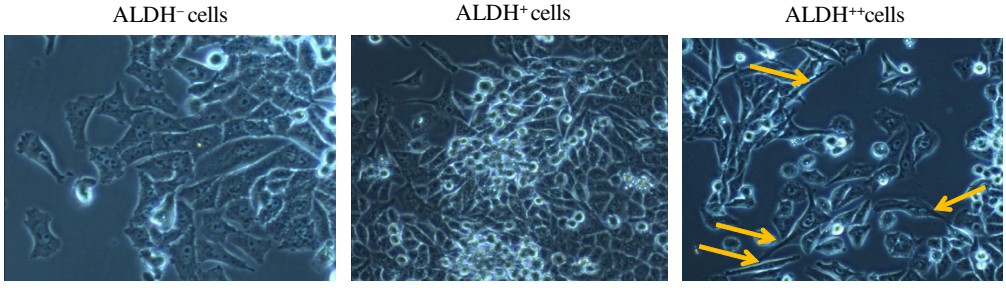

Fig. 3. Morphology of isolated CSC and non-cancer stem cells (NSCs) populations. Post-sorting, cells were incubated in culture media and allowed to grow for 30 h. NSCs demonstrate typical flattened epithelial morphology while intermediate (ALDH+) cells exhibit a mixture of flattened cells and spindle shaped cells with projections. ALDH++ cells are mostly elongated, spindle shaped (arrows) structures with projections.

pancreatic CSCs and clinical cancer samples, making it a suitable therapeutic target. Gemcitabine-resistant MIA PaCa-2 cells were treated with synthetic mimic constructs delivered using lipofectamine 2000 following which gene expression of stem cells and chemoresistance markers was tested. Replenishment of miR-205 in drug-resistant cells resulted in a significant reduction in the expression of pluripotency/stem cell marker OCT 3/4 and CSC marker CD44 compared to non-specific ('scrambled') mimic construct ($p < 0.05$ (Fig. 8A). Additionally, the expression of TUBB3 was significantly ($p < 0.05$) reduced which is known to modulate chemoresistance to anticancer drugs such as taxol and is a target of miR200 cluster [28]. Fig. 8B shows the effect of replacement of miR-7 on gene expression of various markers. Both OCT3/4 and CD44 were significantly reduced ($p < 0.05$) compared to scrambled sequence. Pak-1, a known target of miR-7 [29] and a marker of proliferation had its transcript levels significantly reduced ($p < 0.05$) following restoration of its regulator. This data not only validates our overall approach, but also identifies potential therapy targets for reversing chemoresistance.

To further assess whether this decrease in gene expression markers results in chemosensitization, we treated the miRNA-205 transfected gemcitabine resistant MIA PaCa-2^R cells with gemcitabine and cell viability was assessed and compared to non-transfected and scramble transfected controls (Fig. 9). Cell viability was reduced to less than 50% upon treatment with gemcitabine following transfection with miRNA-205 while no cell death

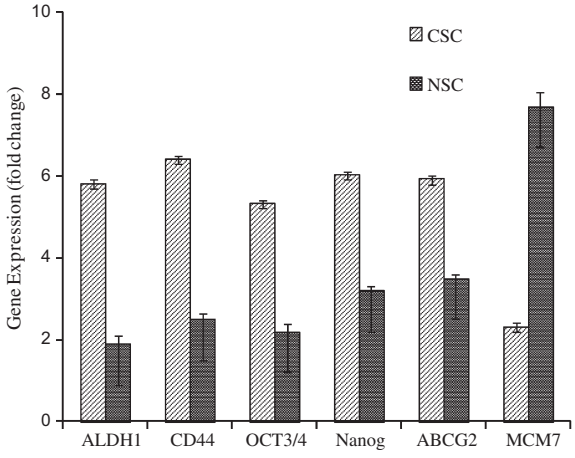

Fig. 4. Characterization of purified non-cancer stem cells (NSCs) and cancer stem cells (CSCs) by real time RT-PCR. Isolated populations of NSCs and CSCs were used for RNA extraction which was then converted to cDNA as described in 'Methods'. Expression of stemness marker OCT3/4 and Nanog, cancer stem cell markers ALDH1 and CD44, chemoresistance marker ABCG2 and cell cycle marker MCM7 were assessed. CSCs had higher expression of all markers except MCM7, indicating their increased chemoresistance and self-renewal potential. Since CSCs are slow-cycling, significantly reduced levels of MCM7 were observed in this fraction.

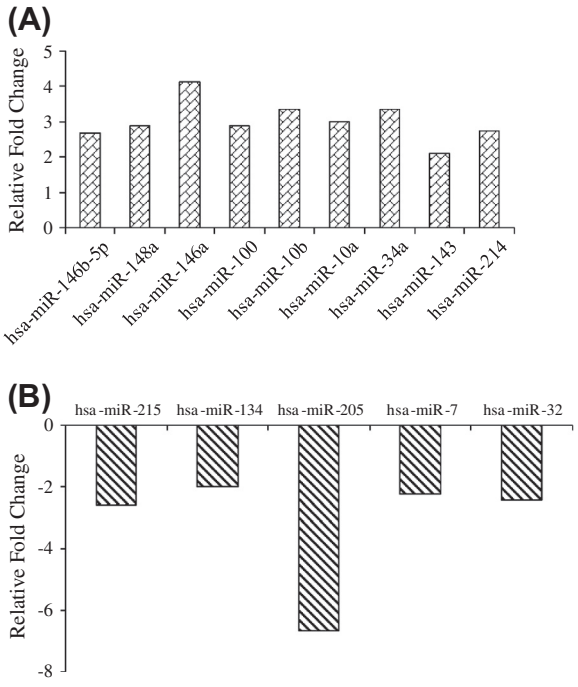

Fig. 5. miRNA profiling of cancer stem cells (CSCs). Profiling and data analysis was carried out as described in 'Methods'. (A) Upregulated miRNA scripts, and (B) downregulated scripts. In both cases, relative fold change with respect to NSCs was used to calculate the final fold change.

was observed in non-transfected MIA PaCa-2^R indicating that gemcitabine sensitivity was indeed induced on miRNA replenishment.

4. Discussion

Pancreatic adenocarcinoma is a highly aggressive micrometastatic disease usually diagnosed in an advanced state and has extremely poor outcomes [14,30–34]. This is due to the fact that small molecule drugs that form the heart of current therapy can kill cancer cells that are differentiated ('bulk' cells) but fail in killing CSC populations which are highly chemoresistant [23,31]. CSCs are only a small subpopulation (<5%) of the tumor cells that make up solid tumors but are believed to trigger aggressive profile, cellular metastasis, drug resistance and subsequent relapse [11,35,36]. Essentially, CSCs are able to protect themselves from the highest concentrations of chemotherapy drugs used clinically by activating their multidrug resistance transporters. Most of the identification efforts rely on the presence of specific cell surface proteins such as CD44, CD24, epithelial specific antigen (ESA) and EpCam using antibody coupled-FACS method [20]. However, since trypsinization is a vital step in this process, there is an increased possibility of loss of these markers, underestimating the final number of CSCs. Recently, the use of ALDH1 has been used to isolate cancer cells that demonstrate higher chemoresistance and possess stem cell nature. High ALDH expression has been correlated to poor prognosis in breast cancer patients with metastasis in lymph nodes and correlates with chemoresistance and poor outcome in ovarian cancer [37,38]. It has also been demonstrated that as few as 100 ALDH positive cells from clinical pancreatic cancer samples could initiate tumor formation in nude mice [39]. We utilized this strategy to identify and isolate pancreatic CSCs from a gemcitabine-resistant pancreatic adenocarcinoma cell line (MIA PaCa-2^R). By using DEAB, an inhibitor of ALDH, a gated region was set up which would be populated by ALDH expressing cells (Fig. 1). Once the method was standardized, MIA PaCa-2 cells were treated with high concentration of gemcitabine, resulting in an increase in the percentage of ALDH-positive cells. This is not surprising since chemotherapy drugs such as cyclophosphamide and gemcitabine can enrich for ALDH-positive cells [40,41]. In Fig. 2, MIA PaCa-2 cells were distributed in three distinct peaks and were flow sorted on the basis of fluorescence corresponding to the activity of ALDH. Among ALDH bright populations, approximately 2% of the cells on the leading edge were brighter than the rest of ALDH-positive cells and were designated as ALDH++ while the rest of ALDH-bright cells were designated as ALDH+ or intermediate populations. After three distinct populations were isolated, immediate reanalysis confirmed that most of the isolated cells, particularly ALDH++ were highly pure. This observation confirmed that this approach is valid and subsequent experiments, including miRNA profiling can actually identify unique targets.

Purified subpopulations were cultured for 30 h to ensure cell adherence and remove non-viable cells without allowing significant reduction in the number of slow-cycling CSCs. CSCs being chemoresistant and slow-cycling are believed to have undergone epithelial to mesenchymal transition (EMT) and thus have a distinct morphology. Fig. 3 demonstrates the presence of elongated structures in ALDH++ cells, most of which were spindle shaped, a characteristic of cells that have undergone EMT [42]. It is known that pancreatic cell lines exhibiting EMT features are resistant to gemcitabine, cisplatin and 5 fluorouracil [43]. In contrast, pancreatic cell lines with high epithelial morphology were sensitive to these anticancer agents. More importantly, reports in the literature suggest EMT characteristics are acquired as pancreatic cancer cells increasingly become resistant to gemcitabine [14,37,44].

To establish the purity of isolated cells at molecular level gene expression analysis was carried out. Compared to the non-CSCs, CSCs from gemcitabine-resistant versions of MIA PaCa-2 cells had higher gene expression of pluripotency markers OCT4 and NANOG [45] and increased expressions of CSC markers ALDH1 and CD44 (Fig. 4). Clinical pancreatic cancer samples have revealed that pancreatic CSC populations with higher expression of CD44+ are more resistant to gemcitabine and correlate with poor prognosis [25,46]. This result is expected as CSCs possess self-renewal potential which is retained in cultured cells even after flow-sorting. Comparison of expression of ABCG2 and MCM7 transcripts also differed significantly between CSCs and NSCs. While expression of efflux

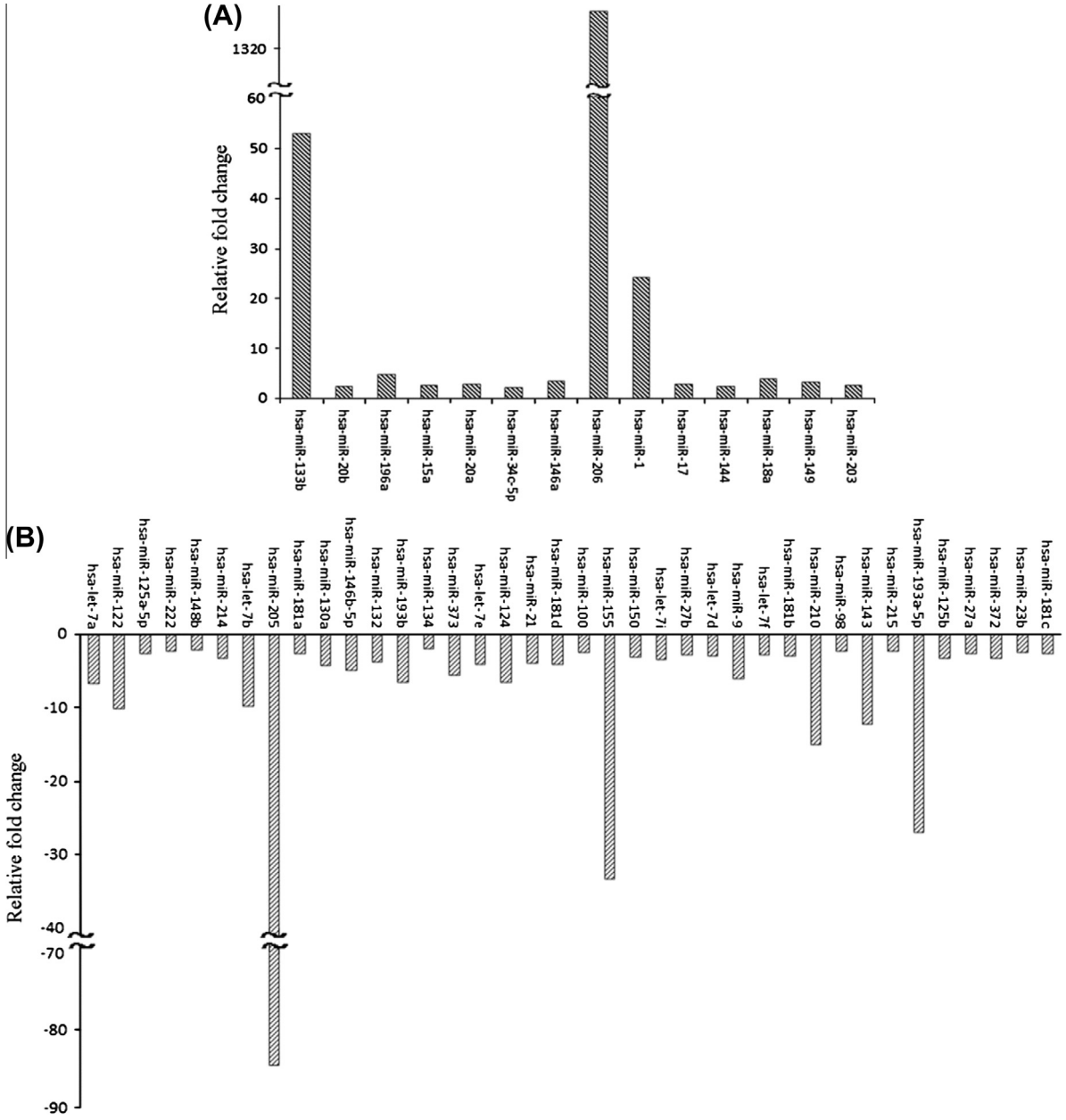

Fig. 6. miRNA expression in serially implanted pancreatic cancer tissue isolated from xenografts. (A) Several miRNAs such as miR-133b and miR-146a were increased compared to noncancerous pancreatic tissue. Relative fold change with respect to normal pancreatic tissue from a second patient was used to calculate the final fold change. (B) Several miRNAs such as miR-205 and 150 were decreased compared to noncancerous pancreatic tissue. Relative fold change with respect to normal pancreatic tissue was used to calculate the final fold change.

pump and chemoresistance marker ABCG2 was higher in the former, proliferation marker MCM7 [47] was highly expressed in NSCs. Resistance of CSCs to chemotherapy could be partly attributed to the overexpression of efflux pumps [48,49]. Previous reports also suggested the role of efflux pumps transporting dFdU in gemcitabine resistance [50]. Higher MCM7 expression is in accordance with our previous study [16] and studies from other groups who have demonstrated that the inherent stem cell properties of CSCs include a propensity for quiescent phase [44,51]. This property allows CSCs to bypass the G1/S checkpoint and maintain their self-renewal property. In addition to this, CSCs are pluripotent cells that have the potential to differentiate into bulk cells and stem cells. In our previous study [16], we demonstrated that isolated CSCs gave rise to a mixed cell population after prolonged culture. CSCs have a role in relapse following initial therapy; therefore it stands to reason that the initial population will give rise to a mixed population composed of bulk tumor cells and CSCs. This data suggests that while there may be technical limitations to the extent to which pure populations can be obtained, the approach for separating and utilizing only ALDH++ cells for further miRNA analysis is valid and will yield specific molecular targets. While more work needs to be done to fully understand the mechanisms that regulate drug resistance in CSCs, therapeutic strategies targeting CSCs are a promising approach to treat advanced pancreatic cancer.

Recent studies in our lab and by other groups [14,52,53] have pointed to the important role played by miRNAs in regulating CSCs in etiology and progression of various cancers, including that of the pancreas. Also, in pancreatic cancer cells specific miRNA signatures may correlate with chemoresistance. For example, alterations in

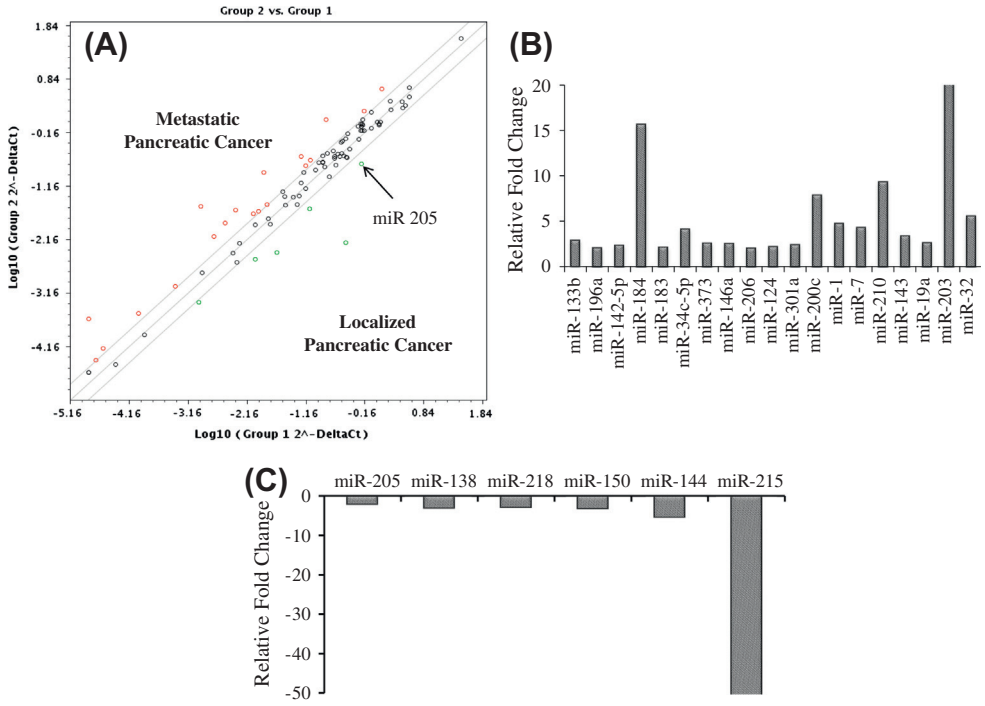

Fig. 7. miRNA profiling of metastatic clinical pancreatic cancer. (A) Scatter plot of miRNAs significantly overexpressed in metastatic (*Y*-axis) and localized pancreatic tumors (*X*-axis). A fold change of two or more was considered significant. miRNA profiling was carried out using the Qiagen miscript miRNA PCR array system. miR-205 is indicated by a black arrow. (B) Upregulated miRNA scripts, and (C) downregulated scripts. In both cases, relative fold change with respect to localized adenocarcinoma from a different patient was used to calculate the final fold change.

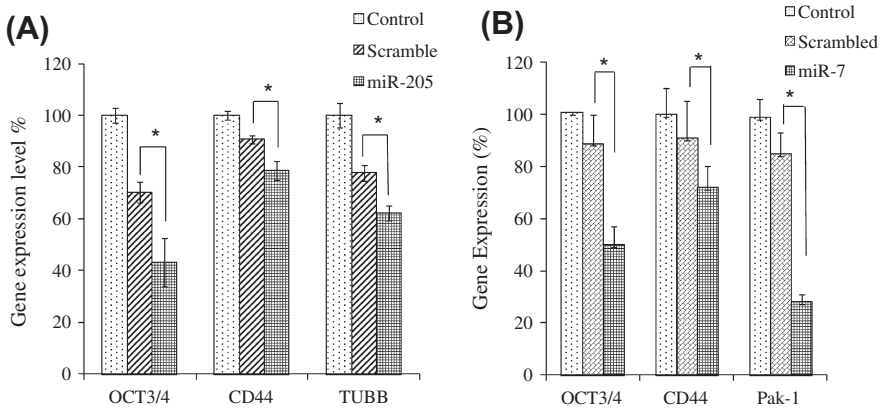

Fig. 8. Effect of miRNA replenishment on expression of stem cells, cancer stem cells (CSCs) and chemoresistance. Gemcitabine-resistant MIA PaCa-2^{R}cells were transfected with (A) miR-205 or (B) miR-7 mimic constructs as described in 'Methods'. Subsequently, total RNA was extracted, converted to cDNA which was used as template for assessing expression of self-renewal (stemness) marker OCT3/4 and CSC marker CD44. Expression of chemoresistance marker TUBB3 for miR-205 transfected cells and of proliferation marker Pak-1 for miR-7 transfected cells was also carried out. Compared to scrambled mimic control, a significant decrease in stemness, self-renewal capacity, chemoresistance and proliferation markers was observed. $^{*}p < 0.05$.

the level of miR-200a, miR-200b and miR-200c are observed in gemcitabine-resistant pancreatic cancer cells [54,55]. Gemcitabine treatment resulted in an increase in expression of miR-155 levels in pancreatic carcinoma and pancreatic cancer patients with reduced expression of miR-21 were responsive to pancreatic cancer treatment. However, as miRNAs express tissue, and disease specificity, it is entirely possible for the same miRNA to act differently in solid tumors present in different organs. Fig. 5 is a documentation of significantly altered cancer-associated miRNAs in CSCs. To obtain this list, the expression of the transcript in NSCs as a normalizing control was used. It was observed that in CSCs expression levels of miR-205 and miR-7 were highly reduced.

Since our future goals include implantation of clinical cancer samples to generate tumors in nude mice and test miRNA based treatments. This would require reassurance that implantation will not significantly alter the microenvironment of the xenografts resulting in alterations of molecular targets making it impossible to co-relate the *in vitro* results with *in vivo*. Towards this end, one clinical cancer sample was serially implanted in NSG mice and isolated after two generations of serial implantations. Subsequent profiling of the isolated tumor sample (Fig. 6) again revealed miR-205 to be the most downregulated transcript. Interestingly, in this sample a reduced expression of nearly all members of the let7 anticancer family, namely miR let7-a, b, c, d, f and i was observed.

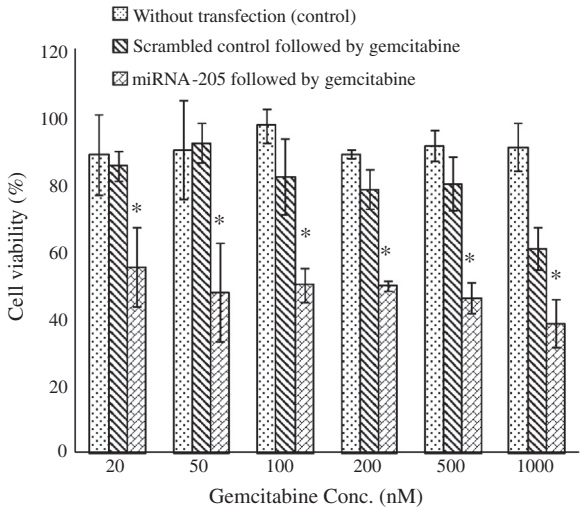

Fig. 9. Cytotoxicity assay of gemcitabine on miR-205 transfected MIA PaCa-2^R cells. Gemcitabine-resistant MIA PaCa-2^R cells were transfected with miR-205 followed by treatment with gemcitabine as described in the 'Methods' section. Cell viability was assessed after 72 h using MTT assay. $^*p < 0.05$ versus control.

In head and neck cancers, combined low levels of miR-205 and let-7d expression are associated with poor survival [56]. Studies have also shown that EMT type pancreatic cancer cells resistant to gemcitabine have downregulated let7 members [57]. miRNA profiling of a clinical pancreatic tissue resected from a stage IV pancreatic tumor with metastasis was then carried out. Expression of miR-205 was found to be reduced in advanced stage pancreatic cancer tissue identifying it as a potential therapy target (Fig. 7). On co-relating the miRNA profiling data of CSCs and pancreatic cancer tissues, it was observed that miRNA-205 is downregulated in pancreatic cancer tissues by ~2-fold as against ~85-fold in CSCs. Also, miRNA-215 was ~50-fold downregulated in cancer tissue in contrast to ~2-fold in CSCs. Since cell lines are by their very nature extremely different from actual clinical samples, the discovery of a miRNA that downregulated in both cell lines and tissues is significant. Further, it is not the extent of downregulation but rather the nature of expression that is critical for identifying new therapeutic targets. In the present studies we have focused on miRNA-205 replenishment since TUBB3 is its downstream target which is known to involve in chemo-resistance.

Once the target was identified, restoration of chemosensitivity in gemcitabine-resistant MIA PaCa-2^R cells was studied. A lipofectamine-based transfection protocol was used for replenishing levels of miR-205 (Fig. 8A) and miR-7 (Fig. 8B) to demonstrate that this strategy is independent of miRNA mimic sequence, and can deliver any functional or control (scrambled) miRNA mimic. miR-205 replenishment resulted in a decreased expression of OCT3/4 and CD44 which is in accordance with the literature where OCT3/4 deficiency causes cell dedifferentiation in primary lung tumors [58]. It is also known that CD44-positive cells are responsible for gemcitabine resistance and poor prognosis in pancreatic cancer, suggesting that reduction of CD44 by miR-205 replenishment is a potential strategy for treating pancreatic cancer by targeting CSCs. In addition, studies have demonstrated that TUBB3 regulates chemoresistance to chemotherapy drugs such as paclitaxel and vinorelbine in several types of cancer and is associated with poor quality of life and prognosis [59–61]. TUBB3 is a known target of miR-200c [28] and present study establishes miR-205 also as its regulator at least in gemcitabine-resistant MIA PaCa-2^R cells. This is a significant finding and may help in identifying the underlying mechanisms that modulate chemoresistance in pancreatic cancer. miR-205 is a known anticancer miRNA [62] and thus a valid potential target based on present data and several lines of evidence in the literature. It has recently been shown that miR-205 inhibition established chemoresistance and its overexpression in metastatic melanoma cells increases cellular apoptosis by decreasing pro-survival gene BCL2 and reduces tumor volume [23]. In prostate cancer, downregulation of miR-205 was associated with poor clinical outcome [63,64] and its replacement hampered cancer progression [65]. Fig. 9 shows the reversal of chemoresistance in MIA PaCa-2^R cells after transfection with miRNA-205. It was observed that there is very less difference in cell viability in miRNA-205 transfected cells treated with various concentration of gemcitabine. Gemcitabine has a very low IC50 value in MIA PaCa-2 cells (>10 nM). Since, we have transfected the cells with a constant concentration of miRNA-205 which resulted in sensitization of particular percentage of resistant cancer stem cells in MIA PaCa-2^R cell line. These were then subsequently killed by even 20nM concentration of gemcitabine hence very less difference was observed on further increasing gemcitabine concentration.

In view of the important role played by miRNAs in CSC proliferation, tumor formation and chemoresistance, there is a pressing need to identify specific miRNAs responsible for cancer relapse and chemoresistance. Targeting these miRNAs for their inhibition/re-expression can potentially reverse chemoresistance and kill chemoresistant CSCs responsible for relapse and aggressive disease profile. The reversal of chemoresistance is an important strategy to ensure better clinical outcomes for pancreatic cancer patients. While various anticancer agents capable of targeting CSCs have been investigated [66], our present approach which has identified miR-205 as a specific miRNAs responsible for drug resistance, and experimentally altering their expression to suppress CSCs will be useful for treating patients diagnosed with pancreatic cancer. Apart from its therapeutic potential, profiling also provides a diagnostic and prognostic tool for monitoring cancer progression and efficacy of the treatment. However, unlike small molecule drugs, delivery of nucleic acids, such as miRNAs to tumor cells *in vivo* remains a challenge and efforts are needed to be focused on this aspect of therapy. The effect of miRNA mimic in modulating cellular functions is transient in nature and thus may require multiple dosing to achieve the therapeutic outcome [67]. However the present strategy of reversing the chemo-resistance using a miRNA based approach, although being transient, will be beneficial since co-administration of chemotherapeutic drug will act on these sensitized cells. Further work in this direction is needed before the presented strategy can transition to the clinic.

Pancreatic cancer presents a challenge of dense desmoplasmic microenvironment resulted from increased fibrosis within the stroma thereby limiting the chance for complete excision of these tumors [68]. Most of the current approaches of nano-therapeutics are essentially based on designing the carrier to deliver the chemotherapeutic drug to target bulk tumor cells, which often failed to kill tumor initiating CSCs resulting in relapse. In the present manuscript we moved to first establish the relationship between CSCs, miRNAs and chemo-resistance. Identification of potential miRNA involved in chemo-resistance could be further targeted using miRNA mimics and/or small molecules using the tailor-made nanocarriers. These nanomedicines hold a great potential since they can overcome barriers to cancer therapy owing to their small size and can target the specific cell population which is resistant to chemotherapy.

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