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CD133+ tumor initiating cells (TIC) in a syngenic murine model of pancreatic cancer respond to Minnelide

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Abstract

Purpose—Pancreatic adenocarcinoma is the fourth leading cause for cancer-related mortality with a survival rate of less than 5%. Late diagnosis and lack of effective chemotherapeutic regimen contribute to these grim survival statistics. Relapse of any tumor is largely attributed to the presence of tumor-initiating cells (TIC) or cancer stem cells (CSC). These cells are considered as hurdles to cancer therapy as no known chemotherapeutic compound is reported to target them. Thus, there is an urgent need to develop a TIC-targeted therapy for pancreatic cancer.

Experimental design—We isolated CD133+ cells from a spontaneous PDAC mouse model and studied both surface expression, molecular markers of pancreatic TICs. We also studied tumor initiation properties by implanting low numbers of CD133+ cells in immune competent mice. Effect of Minnelide, a drug currently under Phase I clinical trial, was studied on the tumors derived from the CD133+ cells.

Results—Our study showed for the first time that CD133⁺ population demonstrated all the molecular markers for pancreatic TIC. These cells initiated tumors in immunocompetent mouse models and showed increased expression of pro-survival and pro-invasive proteins compared to the CD133[−] non-TIC population. Our study further showed that Minnelide, was very efficient in downregulating both CD133[−] and CD133⁺ population in the tumors, resulting in a 60% decrease in tumor volume compared to the untreated ones.

Conclusion—As Minnelide is currently under Phase I clinical trial, its evaluation in reducing tumor burden by decreasing TIC as well as non-TIC population suggests its potential as an effective therapy.

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Author contribution: SB, AN, VS, VD and RC participated in and conducted experiments, SB and AN designed the experiments, performed experiments and analyzed the data. SB, SMV and AKS helped design the study and analyzed and interpreted the data. All authors participated in the writing and editing of the manuscript. AKS was the PI and supervised the project.

Keywords

Triptolide; Minnelide; pancreatic tumor initiating cells; CD133; NF-kB

Background and Aims

Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth most frequent cause of cancer related deaths (1). Aggressive biology, chemo-resistance and recurrence of the tumor make PDAC one of the most devastating cancers.

Since their discovery, tumor-initiating cells (TICs) have referred to a quiescent population of cells that is capable of causing recurrence, both local and distal to the site of tumor.

Pancreatic TICs were first isolated as a CD24+CD44+ESA+ population from human xenografts by Li et al in 2007 (2). Subsequently, CD133 was identified as a TIC for PDAC (3). Both groups demonstrated tumor-initiating properties in the cells enriched for these markers. Pancreatic TICs have been further characterized using other surface markers including CXCR4 (3, 4), Met (5), aldehyde dehydrogenase activity (6–8) or ABCG2 (9, 10).

CD133, a transmembrane pentaspan protein, was initially described as a surface antigen specific for human hematopoietic stem cells, (11, 12). Although the biological function of CD133 remains unknown, CD133 is well-recognized as a stem cell marker for normal and cancerous tissues. It has recently been demonstrated that CD133 is a marker of putative pancreatic progenitor cells in mice (13). In humans, CD133 is expressed on terminal ductal cells in the adult pancreas and carcinoma cells in pancreatic ductal adenocarcinoma (14, 15).

TICs are hypothesized to arise by either accumulation of mutations in stem cells or progenitor cells resulting in dysregulation of several self-renewal pathway genes. Consistent with this, Pancreatic TICs have been reported to show increased expression of developmental transcription factors like Sox2, Nanog, Oct4 (16) and several Hedgehog and Notch pathway genes (2, 17). Apart from the above features, resistance to apoptosis by upregulation of Bcl-2 and Survivin genes has been associated with pancreatic TICs (18). TICs are also associated with increased invasiveness. Pancreatic TIC population CD133+/CXCR4+ or Met+/CD44+ has been reported to be more invasive than either population alone.

Most studies on pancreatic TICs have involved isolation of cells from patient tumor tissue and propagation in immunocompromised mouse models such as SCID mice (Severe Combined Immuno Deficient) or athymic nude mice (2, 3, 19). However, xenografts in immunocompromised mice lack the syngenic interface between tumor and host microenvironment crucial for understanding tumor dynamics. One of the immunocompetent mouse models for pancreatic carcinogenesis is based on the pancreas specific expression of endogenous mutant *Kras* and *Trp53* alleles (LSL-KrasG12D; LSL-Trp53R172H; Pdx-1-Cre or KPC mice). These KPC mice, spontaneously develop primary pancreatic tumors that recapitulate the clinical and histopathologic features of the human disease (20, 21).

TICs often display resistance to cytotoxic cancer therapies, permitting the repopulation of tumors after radiation or chemotherapy. Several groups have demonstrated that TICs from multiple cancer types exhibit resistance to conventional cancer therapies (22). PDAC is known to be resistant to most chemotherapeutic drugs. However, triptolide, a diterpene triepoxide from the Chinese plant *Tripterygium wilfordii*, downregulates heat shock genes (23, 24) and induces apoptotic death in pancreatic cancer cells (25–27) has been an exception to this. Triptolide and its water soluble pro-drug Minnelide was recently reported to be very effective in tumor regression in a number of murine models (26). Other authors have demonstrated the efficacy of triptolide by inhibition of proliferation within a number of additional malignancies, including cholangiocarcinoma (28, 29) osteosarcoma (30) and neuroblastoma (31). However, efficacy of triptolide has not been tested on CSCs.

In this study, we have identified a population of CD133⁺ cells from the tumors developed from the KPC transgenic mouse model of PDAC. We have shown that this population expresses a number of CSC markers (surface markers, transcriptional markers and developmental markers); has a significantly higher expression of pro-survival genes like the heat shock proteins, Bcl-2 and Survivin; higher NF- κ B activity and has tumor initiating properties in a syngenic, immunocompetent system. We have further shown that these cells, and the tumors derived from these cells, respond to Minnelide, which effectively lowers the pro-proliferative pathways and induces cell death.

RESULTS

KPC tumors and cells exhibit a population of CD133⁺ tumor initiating cells

We analyzed 3 primary KPC tumors and two cell-lines derived from KPC mouse tumors for PDAC for the different stem cell markers. A flow-cytometric analysis showed these cells had 6–9% of CD133⁺ population (Fig. 1A, Supplementary Table 1). A population of CD24⁺/CD44⁺/ESA⁺ cells were also present but to a much lesser percent (3–4%) compared to the CD133⁺ population (Fig. 1B). A minor population of these cells (~2%) also showed higher Aldh1 activity (Fig. 1C). To put our study in perspective, we studied TIC markers from a classical TIC model: human tumors transplanted in mice as well as one that was freshly isolated tumor. These tumors showed 3–4% CD133⁺ cells while 1–2% CD24⁺CD44⁺ESA⁺ cells.

To see if the % of CD133⁺ cells differed in metastatic tumors or in accumulated ascitic fluid in KPC tumor bearing animals, we analyzed cells from accumulated ascitic fluid from these animals. Our analysis showed ~2% CD133⁺ cells were present in ascitic fluid from 3 animals tested. Similarly, cells from a liver metastasis nodule from a pancreatic tumor bearing KPC animal also showed ~2% CD133⁺ cells. The relative abundance of CD133⁺ cells from primary tumor, malignant ascites or metastatic nodule is summarized in Table 1.

Our results showed that CD133⁺ population was higher (6–9%) in the primary tumor while it was ~2–3% in malignant ascitic cells or metastasizing cells. We next looked for expression of genes involved in development of the pancreas and known to be upregulated in pancreatic TICs. KPC cells showed several hundred fold overexpression of CD133, Sox2, Nanog, Aldh1, CXCR4, SHH, Gli and Notch genes (Fig. 1D) compared to the normal

pancreatic ductal cells. These data indicated that KPC cells harbor a population of TICs characterized by the same markers as was previously reported from the human tumor xenografts and pancreatic cancer cell lines (2, 3).

CD133⁺ population in KPC cells includes ALDH1⁺ and CD24⁺/CD44⁺/ESA⁺ subpopulations

Since ~6–9% of cells derived from the KPC tumor were found to be CD133⁺, we used magnetic cell sorting (MACS) with anti-mouse CD133 (AC133) coupled microbeads to isolate this population. The enrichment for CD133 was verified by flow-cytometry using CD133 antibody (AC141) against a different epitope after purification. Approximately 97% purity was achieved consistently (Fig. 2A) by this cell separation technique. Sorted cells were labeled with CD24, CD44 and ESA antibody and assayed for Aldh1 activity. It was observed that CD133⁺ population from KPC001, KPC023 as well as cells isolated from KPC primary tumors, included the CD24⁺CD44⁺ESA⁺ population, whereas the CD133[−] did not (Fig. 2B Supplementary Fig 1A). It was also seen that CD133⁺ population harbored cells with maximum Aldh1 activity while the CD133[−] population lacked any Aldh1 activity (Fig. 2C Supplementary figure 1A). This indicated that both CD24⁺CD44⁺ESA⁺ population and Aldh1⁺ population were subpopulations of CD133⁺ cells. This was further confirmed with immunofluorescence, which showed that only CD133⁺ cells co-stained with Aldh1 (Fig. 2D).

Expression of CD133 in pancreatic cancer cell lines correlate with their invasiveness

CD133 expression has been correlated with poor prognosis in hepatocellular carcinoma (33). To evaluate if higher levels of CD133⁺ cells correlated with increased aggressiveness in pancreatic cancer cells, CD133 expression in normal pancreatic ductal cells was compared to cancer cell lines (MIA-PaCa2, BxPC3, Capan1, S2013 and S2VP10, AsPC1), human patient tumor xenografts and KPC tumor derived cells. KPC001, KPC023 derived from the tumor had increased (6–9%) expression of CD133 compared to 1%–4% in all the other cells (Supplementary Fig 1B). No significant expression of CD133 was seen in normal pancreatic ductal cells (0.01%) and 0.5–1% expression of CD133 was observed in the less aggressive pancreatic cancer cells like MIA-PaCa2. The KPC001 cells (with 8% CD133) showed increased rate of migration (assayed as a measure of wound healing ability) compared to the other pancreatic cancer cell lines (Supplementary Fig 1C). Migration was measured by ECIS and the data was represented as changing impedance over time. Since KPC cells showed increased invasiveness and since they had higher CD133 expressing cells, we studied the expression of genes involved in EMT in CD133⁺ and CD133[−] cells isolated from the KPC cell lines (both KPC001 and KPC023). Consistent with the functional assay, expression of EMT associated transcription factors like SNAIL, SLUG and Twist were 10–80 fold higher in CD133⁺ cells (Fig. 1D).

CD133⁺ cells show tumor initiating property in a syngenic model at low cell numbers

Tumorigenicity of CD133⁺ population was next tested by injecting 100, 50 and 10 MACS sorted cells subcutaneously in both flanks of C57BL/6 mice. Tumor volume was measured weekly to monitor tumor growth (Fig. 3A). Tumors formed from 10 CD133⁺ cells in these mice within 3 weeks of cell injection. Neither CD133[−] cells nor unsorted cell (Fig. 3B) population formed tumors up to 2 months post-inoculation at these cell numbers. Unsorted

KPC cells did not show tumor formation until 20,000 cells were injected (Supplementary Table 1). Tumorigenicity of CD133⁺ cells is summarized in Table 2. To put our study in perspective, we isolated CD133⁺ cells from 3 human tumor xenografts propagated in mice and implanted these at low numbers in immunocompromised SCID mice. CD133⁺ cells showed tumor initiation at as low as 100 cells while CD133⁻ cells did not even at 1000 cells (Supplementary figure 2A–D).

Since one of the hallmarks of cancer stem cells is their ability to generate serially transplantable tumors that recapitulate the cellular and histological structure of the parent tumor (3, 32), we injected 100 CD133⁺ cells subcutaneously into the flanks of the C57BL/6 mice. Tumors formed in an average of 21 days. 1mm³ tumor pieces were implanted into a new set of C57BL/6 mice and observed for a month once the parental tumor reached a volume of 700–800mm³. Similar transplantation was done with the second-generation tumors, once they reached a size of 700–800mm³ (Supplementary Fig 3A, B). The tumors from all the 3 generations were histologically identical as seen by H&E staining (Supplementary Fig. 3C), and showed identical proliferation pattern as seen by Ki67 staining (Supplementary Fig. 3D). This indicated that tumor initiating CD133⁺ cells had tumorigenic properties that were sustained with serial transplantation in animals.

CD133⁺ cell population promoted early tumorigenesis in an orthotopic mouse model

Five hundred CD133⁺ cells were implanted in the tail of the pancreas in 20 mice and tumorigenesis was compared to 5000 unsorted KPC cells. Groups of 5–7 mice were sacrificed at days 10, 20 and 40 after implantation and tumor development was assessed upon necropsy. Mice with CD133⁺ cells developed visible and palpable tumors by Day 20 while the mice with 5000 unsorted KPC cells showed a delayed tumor growth (Fig. 3C). By day 40 of implantation, tumors derived from CD133⁺ cells had an average volume of ~900 mm³, while tumors derived from unsorted KPC cells had an average volume of ~300 mm³. The experiment was terminated at day 40 owing to morbidity in the CD133⁺ group. CD133⁺ mice also showed increased ascites volume (0.978 +/-0.234ml) compared to unsorted KPC cells (0.098 +/- 0.05ml) indicating increased aggressiveness of the CD133⁺ derived tumor (Supplementary Fig. 3E). Histology of the pancreas in the two groups at day 20 and day 40 after tumor implantation showed early onset of tumor in CD133⁺ inoculated animals compared to the unsorted KPC cells (Fig. 3D). These data indicated that CD133⁺ cells are responsible for early tumor formation resulting in increased accumulation of ascitic fluid.

CD133⁺ cells have higher expression of HSPs and anti-apoptotic genes and show increased NF-κB activity

We next analyzed the expression of pro-invasion and survival genes in the CD133⁺ population of KPC cells. Anti-apoptotic and pro-survival genes Bcl-2 and Survivin were 4–5 fold higher in the CD133⁺ population compared to the CD133⁻ population (Fig. 4A). Similarly 3–4 fold higher expression of TIC specific genes like Nanog (4 fold) and Sox2 (2 fold) were also observed in CD133⁺ population versus the CD133⁻ population (Fig. 4B). CD133⁺ cells also showed ~4 fold increased expression of CXCR4 compared to CD133⁻ cells. Heat shock proteins are overexpressed in pancreatic cancer allowing increased survival. HSP70, GRP78, HSP27, HSF1 were found to be at least 3–4 fold higher in

CD133⁺ cells compared to CD133⁻ cells (Fig. 4C). This increase in mRNA expression corresponded to an increase in protein expression of GRP78, HSP70 and Bcl-2 in CD133⁺ cells versus CD133⁻, unsorted KPC or normal pancreatic ductal cells (Fig. 4E). Both GRP78 and HSP70 (Supplementary figure 4A–C) showed increased staining in immunohistochemistry CD133⁺ cell-derived tumor.

A gene expression profile of CD133⁺ cells as compared to CD133⁻ cells was performed using an Illumina microarray on RNA isolated from these cells. One of the genes overexpressed in CD133⁺ subset was IDH1, a metabolic enzyme that has been as a potential oncogene in a number of cancers (34, 35). Increased expression of IDH1 in CD133⁺ was confirmed by qRT-PCR (Supplementary Figure 5).

Since NF- κ B signaling pathway regulates survival, invasion and apoptosis in tumor cells, we analyzed DNA binding activity of CD133⁺ cells and compared it to the CD133⁻ cells. CD133⁺ cells had increased (~2.5 fold) NF- κ B DNA binding activity compared to CD133⁻ cells (Fig. 4D).

Minnelide induced apoptosis and decreased proliferation in CD133⁺ pancreatic cancer cells and caused CD133⁺ cell derived tumor regression

To study if CD133⁺ cells were resistant to other cytotoxic chemotherapeutic agents like paclitaxel, 5FU or current standard of care gemcitabine, KPC cells were sorted for CD133 and both positive and negative cells were treated with different concentration of 5FU, paclitaxel, triptolide and Gemcitabine. 5FU and paclitaxel reduced viability of CD133⁻ cells but did not have any effect on CD133⁺ cells. Gemcitabine did not affect either positive or negative population at a lower dose and reduced the viability of CD133⁻ cells at a higher concentration. Interestingly, triptolide was seen to reduce viability of CD133⁺ (0.4+/0.02) similar to CD133⁻ cells (0.32+/- 0.04) (Fig. 5A).

To test the effect of Minnelide on CD133⁺ derived tumors, CD133⁺ cells were implanted in flanks of C57BL/6 mice. Treatment with therapeutic dose of Minnelide (0.42mg/kg body wt) was started once implanted tumors reached a volume of 300mm³. Tumor progression as well as endpoint measurements of tumor volumes of treated tumors showed ~60% decrease in tumor volume in Minnelide treated tumors compared to untreated ones (Fig. 5B). Minnelide treated tumors also showed decreased number of Ki-67 positive (~30/field) (Supplementary figure 6 AC) indicating decreased proliferation and increased TUNEL staining (~60/field) (Supplementary figure 6D–F) indicating apoptosis.

To test the efficacy of Minnelide in human patient tumor derived xenograft, we sorted CD133⁺ cells from a human tumor and generated subcutaneous tumors on the flanks of NOD/SCID mice (n=12 tumors). Treatment with Minnelide (0.42mg/kg body weight) was started when the tumors reached ~1000mm³ in volume. Tumor regression (871+/-102 mm²) was apparent after 2 weeks of Minnelide treatment (Supplementary Figure 6G).

Minnelide reduced tumor-initiating population and inhibited pro-survival genes

Minnelide reduced the expression of the TIC markers in the CD133⁺ cell generated tumors as studied by flow-cytometry. Minnelide treated tumors were seen to express less CD133

(~4%) compared to the untreated tumors (~8%) (Fig. 5C). A similar decrease ($1.2\% \pm 0.32$ compared to 3.5 ± 1.2) was observed in the expression of CD24⁺/CD44⁺/ESA⁺ and in Aldh1 activity (0.3 ± 0.02 in Minnelide compared to 1.43 ± 0.78 in untreated) in tumors treated with Minnelide as compared to control (Data not shown).

To see if similar effects of this drug were obtained in primary KPC tumor bearing mice, we analyzed the primary tumors treated with Minnelide. Our analysis showed that Minnelide treated animals has 3–4% CD133⁺ cells compared to the untreated animals that had 6–9% CD133⁺ cells (Fig. 5C).

Minnelide also decreased the expression of genes involved in Hedgehog signaling (3%), Notch signaling, stemness like Nanog (19% of untreated), ALDH1 (2%), CXCR4 (23%) pro-survival genes like HSP70 (26%) and GRP78 (16%) and anti-apoptotic genes like Bcl2 (8%) and Survivin (21%) (Supplementary figure 6 H, I) compared to saline treated animals. Additionally, NF- κ B activity was decreased in Minnelide treated tumors (~ 500 RLU/mg protein) compared to untreated (2670 RLU/mg protein) (Fig. 5D). Thus, Minnelide appeared to downregulate pro-proliferative pathways and decrease the CD133⁺ tumor initiating population in pancreatic cancer cells.

Similarly, Minnelide also decreased expression of the genes involved in the Hedgehog signaling (9% of untreated), Notch signaling (18% of untreated) and stemness genes (21–24% of untreated) in KPC animals bearing primary tumors. Consistent with our results obtained from implanted cells in C57BL6 mice, Minnelide also decreased expression of HSP70 (40% of untreated), GRP78 (32% of untreated) and other anti-apoptotic genes like Survivin (41% of untreated) and Bcl-2 (26% of untreated). Along with this, Minnelide also downregulated NF- κ B activity in the primary tumors (876RLU/mg protein) compared to untreated tumor (3712RLU/mg protein) (Fig. 5D).

DISCUSSION

Tumor initiating cells are a population of quiescent cells within a tumor that evade apoptosis and cell death induced by standard chemotherapy, often resulting in tumor recurrence. As a result, there is a need to develop therapy that can reduce this population and contribute to tumor regression. In pancreatic cancer, TICs have been isolated using a number of markers including surface expression of CD133 (2, 3, 5, 10, 36).

However, all of these studies have been performed in either immunocompromised, athymic nude mice or in SCID mice. Though transplantation into these mice is the best way of assessing tumorigenicity, such studies often cannot address the extent to which these cells might be positively or negatively regulated by the actual physiology of animal. As development of solid tumors is a complex phenomenon involving intricate interaction with the host system, use of a syngenic model is a closer representation to actual tumor development of PDAC from tumor initiating cells. In the current study we report isolation and characterization of CD133 expressing TIC from a cell line derived from the KPC genetic mouse model for pancreatic cancer in a syngenic host.

As reported by other groups, genetically engineered KPC mice are the closest clinical representation of pancreatic adenocarcinoma (20). Our study revealed that 6–9% of the cells in primary pancreatic tumors from KPC mice as well as in the cell lines isolated from the KPC tumors are CD133⁺. The CD133⁺ population was also responsible for initiation of a fresh tumor in both subcutaneous and orthotopic models (Fig. 3). The CD133⁺ cells showed early tumorigenesis and increased invasiveness in both these models. Particularly, in the orthotopic mouse model, CD133 derived tumors showed increased accumulation of abdominal ascites compared to unsorted KPC cell derived tumor bearing animals (Supplementary Fig 3E). The CD133⁺ cells derived from the KPC model formed tumors from as low as 10 cells which reflected their increased tumorigenicity when compared with that of CD133⁺ cells isolated from human tumors (that formed tumors from a minimum of 100 cells). In all these studies, the CD133⁻ population did not form tumors in almost twice the amount of time.

The CD133⁺ population in KPC cells also included a smaller subset of Aldh⁺ population, previously described as a TIC marker in SCID mice (Fig 2C, D) (8). Further, the CD133⁺ population also contained an independent subset of CD24⁺/CD44⁺/ESA⁺ cells, shown previously in human pancreatic cancer xenografts as a population of TICs (Fig. 2B) (2).

Though pancreatic TICs have been well-characterized, there has been limited study on the dysregulation of molecular pathways in these cell populations. Biological function of CD133 is not till date. Our study revealed that in addition to an expected increase in expression of Bcl2 and Survivin genes, the CD133⁺ population had increased expression of heat shock proteins; HSP27, HSP70, and GRP78. Previous studies from our group have shown that HSP70 is overexpressed in pancreatic cancer cells and downregulation of HSP70 results in loss of viability in PDAC cells (24, 25).

HSP70 thus functions as an inhibitor of apoptosis in pancreatic cancer. Similarly, HSP27 has been shown to have an increased expression in breast cancer stem cells (37). GRP78, a heat shock protein family that is a gatekeeper of ER stress in eukaryotic cells, has been found to be overexpressed in PDAC and involved in its cell survival. GRP78 was found to be crucial for embryonic stem cell precursor survival and also highly expressed in hematopoietic stem cells (38).

Additionally, GRP78 has been shown to be upregulated in TICs in other cancers (39, 40). Our studies show an increased expression of HSP27, HSP70 and GRP78 in CD133⁺ cells isolated from the tumor, as well as in immunohistochemical studies on tumors derived from these cells (Supplementary figure 4A–C). This may be of significant clinical relevance as therapy targeted towards these proteins can be used for complete eradication of pancreatic TICs.

Along with overexpression of heat shock proteins, the pro-survival NF-κB pathway was also overexpressed in CD133⁺ KPC cells (Fig. 4D). The NF-κB pathway has been reported to also regulate multiple phenomena in TICs from other cancers (41). In the KPC tumor derived pancreatic cancer cell line, increased expression of NF-κB in the CD133⁺ TICs indicate the role of this pathway in promoting tumorigenesis. Minnelide also reduced the

expression of genes that define tumor initiating cells like Hedgehog and Notch signaling pathway genes in CD133⁺ derived tumors (Supplementary Fig 6H, I).

As expression of CD133 in different cell lines correlated with increased invasion, we studied the expression level of EMT genes like SNAI1, SLUG, Twist and Vimentin. CD133⁺ cells showed ~5–10 fold increased expression of SLUG and Twist genes whereas almost 50–80 fold increased expression of SNAI1 and Vimentin genes compared to CD133[–] cells (Supplementary Fig 1D).

Overexpression of the IDH1 gene was seen in the gene expression microarray for CD133⁺ cells, as compared to CD133[–] (Supplementary Fig 5). However, there is no report of IDH1 overexpression in this disease. IDH1 is a NADP⁺ dependent isocitrate dehydrogenase, which catalyzes the oxidative decarboxylation of isocitrate to alpha ketoglutarate. Overexpression of IDH1 in CD133⁺ cells of pancreatic cancer may indicate increased metabolic activities in TICs.

It is now well established standard chemotherapy targets rapidly proliferating cells as a result of which the quiescent TICs evade the cytotoxic effect of the drug. Our studies showed that CD133⁺ cells were indeed very slow proliferating compared to the CD133[–] cells (Supplementary Fig. 1E). Our lab has previously demonstrated that Minnelide treatment prevents tumor recurrence and causes tumor regression in a number of mouse models (26). In this study, Minnelide was effective in decreasing CD133⁺ population as well. Our in vitro study showed that the CD133⁺ population responded equally to triptolide (the active compound) as the CD133[–] population, while both populations remained relatively resistant to gemcitabine, the current first line of therapy for pancreatic cancer. Our study also showed that cytotoxic agents 5FU and Paclitaxel decreased the viability of CD133[–] cells where as CD133⁺ cells were unaffected (Fig. 5A).

Consistent with our in vitro studies, a 50–60% decrease tumor volume within the Minnelide treated group was observed in our animal studies along with almost 50% decrease in the expression of stem cell marker CD133⁺ (Fig. 5C). Similar regression was also observed on tumors derived from CD133⁺ population of the human tumors (Supplementary Fig 6G). Analysis of the CD133⁺ derived tumors showed a decrease in tumor volume and less proliferation in Minnelide treated animals and increased cell death by the drug (Fig 5A, Supplementary Fig 6). Treatment with Minnelide also showed a similar decrease in expression of pro-survival genes (HSP70, GRP78, Bcl-2, Survivin) in tumors (Supplementary Fig. 6H, I). NF-kB activity, which was upregulated in CD133⁺ population, was decreased in Minnelide treated tumors (Fig. 5D). Effect of other compounds targeting TICs have been studied in pancreatic cancer (Supplementary Table 2). However, all of these previous studies have been done in immunocompromised models. The current study is the first report showing the effectiveness of Minnelide in an immunocompetent syngenic system.

CONCLUSION

Our study on TIC in a syngenic animal system show that cells derived from KPC tumors have a high percentage of CD133+ cells forming tumors from very low cell numbers. This study shows that Minnelide regresses CD133+ derived tumor volume and reduces the number of TICs in tumor. It also downregulates all the pro-survival proteins overexpressed in these cells. The observation that Minnelide can reduce the TIC population strengthens our earlier observation that it can prevent recurrence of pancreatic tumor. The current study is the first report of pancreatic TIC characterization in an immunocompetent syngenic system as Minnelide is being evaluated in a Phase I Clinical trial.

MATERIALS AND METHODS

Mouse model, cell isolation and establishment of primary tumor cell line

Moribund animals of the KRasG12D; Trp53R172H; Pdx-1-Cre genetic background was sacrificed and single cell suspension was isolated by digestion with collagenase B and dispase II. Cells were plated in medium containing growth factors and 2% serum. Medium was replaced with serum free medium after 48h and cells were maintained in it for 2–3 weeks in the absence of serum until all fibroblasts were removed. Cells were then grown in DMEM with 10% serum for all experiments. The two cell lines derived were named KPC001 and KPC023. All experiments were carried out in 2 derived cell lines and 2–3 primary tumors from KPC animals.

Isolation of CD133+ population

The CD133+ population was separated from the mouse progenitor cells and other CD133– cells using MACS separation (Miltenyi Biotech) using manufacturers protocol. Single cell suspension was generated from tumors in KPC mice according to the Li et al (36). Non-epithelial progenitor cells were removed using anti-CD31-Biotin (BD Biosciences) and anti-CD45 Biotin (BD Bioscience) using MACS technique. The flowthrough free from the mouse progenitor cells was bound to anti-mouse CD133–Microbeads for 10 min on ice and positively purified for CD133+ cells by MACS. The purity of separation was tested for each batch by performing a FACS analysis using Anti-CD133-PE antibody AC141 (Miltenyi Biotech). The separated populations were used for RNA, Protein and FACS analysis. Cells growing in culture were scraped gently into centrifuge tube and washed once in Wash Buffer (PBS, 0.5% BSA, 2mM EDTA) before binding to Anti-mouse CD133 microbeads and proceeded as described above.

Flow cytometric analysis of isolated samples

For flow cytometric analysis of tumor tissues, single cell suspension was prepared according to the protocol of Li et al (36). Cells isolated from tumor, as well as those growing in culture, were stained with directly conjugated monoclonal antibodies in the presence of FcR blocking reagent (Miltenyi Biotech) with anti- CD133-PE (Miltenyi Biotech), anti- CD44-FITC (BD Biosciences), ESA-APC (BD Bioscience) and CD24-PE (BD Biosciences). The Aldefluor assay was performed per manufacturer's instructions (Stem Cell Technologies) complete with DEAB controls. IgG isotype controls corresponding to each directly

conjugated fluorophore were utilized to identify, quantify, and positively select desired cell populations. All FACS analyses were performed on a BD FACSCanto II (BD Biosciences) using FACSDiva (BD Biosciences) and FlowJo (Tree Star) software. Debris and cell clusters were excluded during side-scatter and forward-scatter analyses.

Migration Assay

Migration assay for different cell lines was conducted by Electric Cell-substrate Impedance Sensing (ECIS) (Applied Biophysics). In ECIS, the cells are grown on the surface of small and planar gold-film electrodes and the AC impedance of the cell-covered electrode is measured continuously at a frequency of 64 kHz. 4×10⁴ pancreatic cancer cells (MiaPACA-2, S2VP10, S2013, AsPC-1 and KPC001) were seeded into an 8E1W ECIS array (Applied Biophysics containing a 250μM electrode. Upon confluence a high field current with frequency 48 kHz and amplitude 5 was applied for 10 seconds killing cells overgrowing the electrode, creating “wounds” in the wells devoid of cells.

The migration was compared between each cell line at the end of each experiment, which was presented as a measure of changing impedance over time after normalizing to the time for wounding.

Immunohistochemistry

For immunohistochemistry, paraffin tissue sections were deparaffinized in xylene and hydrated through graded ethanol. Slides were steamed with a Reveal Decloaker (Biocare Medical) to minimize background staining. Sniper Universal Blocking Sera (Biocare Medical) were used throughout the protocol. The slides were stained using anti- Ki67, anti-HSP 70 antibody (Abcam) and anti-GRP78 antibody (Cell signaling). Staining was detected using anti-rabbit secondary antibody conjugated to HRP followed by diaminobenzidine peroxidase (DAB) substrate kit (Vector Laboratories). The tissue sections were counterstained with Gill's hematoxylin (Vector Laboratories). The primary antibody was omitted for the negative controls. TUNEL was performed using the In situ cell death determination kit (Roche Diagnostics) according to manufacturer's protocol. For immunofluorescence, fluorescent antibody conjugates were used after primary antibody staining. Slides were counterstained with DAPI and visualized in a Nikon fluorescent microscope. Tissue samples were incubated with mouse IgG1 isotype controls (BD Biosciences) and did not demonstrate any specific staining.

NF-κB Assay

NF-κB activity assay was performed on CD133+ sorted cells and Minnelide treated CD133+ generated tumors. DNA binding and downstream activation of NF-κB pathway was assessed using a transcription factor binding ELISA (Thermo Scientific) according to manufacturer's instruction.

Illumina microarray for gene expression study of CD133+ and CD133-

Microarray studies were performed at the University of Minnesota Biomedical Genomics Center. RNA samples from 3 independent cell sorts were submitted to the facility for

Illumina microarray analysis. Results were analyzed using GeneData Expressionist software at the Minnesota Supercomputing Institute.

Animal experiment

All animal experiments were performed according to the University of Minnesota Animal Care Committee guidelines.

Subcutaneous animal model—Sorted cells were washed with serum-free HBSS and suspended in serum free-DMEM/Matrigel mixture (1:1 volume). 10, 50, 100, 500 cells (CD133+/CD133-/Unsorted) were injected subcutaneously into the right and left flank of age and gender matched C57BL/6 mice (Jackson Laboratories). 8–10 mice were used in each group. Tumors were measured weekly and tumor volume was documented. Tumors were allowed to grow until they reached a volume of 1cm³, at which the mice were sacrificed and the tumor tissue was harvested and processed for other experiments.

For serial transplantation, tumor measuring ~700–800mm³ was chopped up into approximately 1–2mm. Tumor pieces were implanted subcutaneously into right and left flank of age matched 10 C57BL/6 mice. Tumors were measured weekly to monitor their progress.

Treatment with Minnelide—Minnelide treatment was started 5 days after tumor implantation following randomization of animals. 0.42mg Minnelide/kg body weight was administered intraperitoneally every day for 25 days. Tumors were measured as stated above. At the end of study, animals were sacrificed according to the University of Minnesota Animal Care guidelines.

Orthotopic animal model—For orthotopic model, 500 CD133+ cells or 5000 unsorted KPC cells were injected into the pancreas of 24 C57BL/6 mice. 5–6 mice were sacrificed every 10 days to monitor development of tumor in each group.

Statistical analysis

Values are expressed as the mean $\pm$ SEM. All in vitro experiments were performed at least three times. Statistical significance of results was calculated using the Student's t-test. Columns represent Mean; bars represent Standard Error (n=4; *p<0.05)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation

TIC Tumor initiating cells

CSC	Cancer stem cells
PDAC	Pancreatic Adenocarcinoma

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA: a cancer journal for clinicians*. 2009; 59:225–49. [PubMed: 19474385]
2. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. *Cancer research*. 2007; 67:1030–7. [PubMed: 17283135]
3. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell stem cell*. 2007; 1:313–23. [PubMed: 18371365]
4. Balic A, Dorado J, Alonso-Gomez M, Heeschen C. Stem cells as the root of pancreatic ductal adenocarcinoma. *Experimental cell research*. 2011
5. Li C, Wu JJ, Hynes M, Dosch J, Sarkar B, Welling TH, et al. c-Met is a marker of pancreatic cancer stem cells and therapeutic target. *Gastroenterology*. 2011; 141:2218–27. e5. [PubMed: 21864475]
6. Kahlert C, Bergmann F, Beck J, Welsch T, Mogler C, Herpel E, et al. Low expression of aldehyde dehydrogenase 1A1 (ALDH1A1) is a prognostic marker for poor survival in pancreatic cancer. *BMC cancer*. 2011; 11:275. [PubMed: 21708005]
7. Kim MP, Fleming JB, Wang H, Abbruzzese JL, Choi W, Kopetz S, et al. ALDH activity selectively defines an enhanced tumor-initiating cell population relative to CD133 expression in human pancreatic adenocarcinoma. *PloS one*. 2011; 6:e20636. [PubMed: 21695188]
8. Rasheed ZA, Yang J, Wang Q, Kowalski J, Freed I, Murter C, et al. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. *Journal of the National Cancer Institute*. 2010; 102:340–51. [PubMed: 20164446]
9. Wang F, Xue X, Wei J, An Y, Yao J, Cai H, et al. hsa-miR-520h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations. *British journal of cancer*. 2010; 103:567–74. [PubMed: 20628378]
10. Olempska M, Eisenach PA, Ammerpohl O, Ungefroren H, Fandrich F, Kalthoff H. Detection of tumor stem cell markers in pancreatic carcinoma cell lines. *Hepatobiliary & pancreatic diseases international : HBPD INT*. 2007; 6:92–7. [PubMed: 17287174]
11. Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, et al. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood*. 1997; 90:5013–21. [PubMed: 9389721]
12. Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*. 1997; 90:5002–12. [PubMed: 9389720]
13. Hori Y, Fukumoto M, Kuroda Y. Enrichment of putative pancreatic progenitor cells from mice by sorting for prominin1 (CD133) and platelet-derived growth factor receptor beta. *Stem cells (Dayton, Ohio)*. 2008; 26:2912–20.
14. Oshima Y, Suzuki A, Kawashimo K, Ishikawa M, Ohkohchi N, Taniguchi H. Isolation of mouse pancreatic ductal progenitor cells expressing CD133 and c-Met by flow cytometric cell sorting. *Gastroenterology*. 2007; 132:720–32. [PubMed: 17258722]
15. Immervoll H, Hoem D, Sakariassen PO, Steffensen OJ, Molven A. Expression of the “stem cell marker” CD133 in pancreas and pancreatic ductal adenocarcinomas. *BMC cancer*. 2008; 8:48. [PubMed: 18261235]
16. Wen J, Park JY, Park KH, Chung HW, Bang S, Park SW, et al. Oct4 and Nanog expression is associated with early stages of pancreatic carcinogenesis. *Pancreas*. 2010; 39:622–6. [PubMed: 20173672]
17. Tang SN, Fu J, Nall D, Rodova M, Shankar S, Srivastava RK. Inhibition of sonic hedgehog pathway and pluripotency maintaining factors regulate human pancreatic cancer stem cell characteristics. *International journal of cancerJournal international du cancer*. 2011

18. Du Z, Qin R, Wei C, Wang M, Shi C, Tian R, et al. Pancreatic cancer cells resistant to chemoradiotherapy rich in “stem-cell-like” tumor cells. *Digestive diseases and sciences*. 2011; 56:741–50. [PubMed: 20683663]
19. Lonardo E, Hermann PC, Mueller MT, Huber S, Balic A, Miranda-Lorenzo I, et al. Nodal/Activin signaling drives self-renewal and tumorigenicity of pancreatic cancer stem cells and provides a target for combined drug therapy. *Cell stem cell*. 2011; 9:433–46. [PubMed: 22056140]
20. Hingorani SR, Wang L, Multani AS, Combs C, Deramautd TB, Hruban RH, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer cell*. 2005; 7:469–83. [PubMed: 15894267]
21. Hruban RH, Adsay NV, Albores-Saavedra J, Anver MR, Biankin AV, Boivin GP, et al. Pathology of genetically engineered mouse models of pancreatic exocrine cancer: consensus report and recommendations. *Cancer research*. 2006; 66:95–106. [PubMed: 16397221]
22. Rich JN, Bao S. Chemotherapy and cancer stem cells. *Cell stem cell*. 2007; 1:353–5. [PubMed: 18371369]
23. Westerheide SD, Kawahara TL, Orton K, Morimoto RI. Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death. *The Journal of biological chemistry*. 2006; 281:9616–22. [PubMed: 16469748]
24. Dudeja V, Mujumdar N, Phillips P, Chugh R, Borja-Cacho D, Dawra RK, et al. Heat shock protein 70 inhibits apoptosis in cancer cells through simultaneous and independent mechanisms. *Gastroenterology*. 2009; 136:1772–82. [PubMed: 19208367]
25. Phillips PA, Dudeja V, McCarroll JA, Borja-Cacho D, Dawra RK, Grizzle WE, et al. Triptolide induces pancreatic cancer cell death via inhibition of heat shock protein 70. *Cancer research*. 2007; 67:9407–16. [PubMed: 17909050]
26. Chugh R, Sangwan V, Patil SP, Dudeja V, Dawra RK, Banerjee S, et al. A preclinical evaluation of minnelide as a therapeutic agent against pancreatic cancer. *Science translational medicine*. 2012; 4:156ra39.
27. Dudeja V, Chugh RK, Sangwan V, Skube SJ, Mujumdar NR, Antonoff MB, et al. Prosurvival role of heat shock factor 1 in the pathogenesis of pancreatobiliary tumors. *American journal of physiology Gastrointestinal and liver physiology*. 2011; 300:G948–55.
28. Clawson KA, Borja-Cacho D, Antonoff MB, Saluja AK, Vickers SM. Triptolide and TRAIL combination enhances apoptosis in cholangiocarcinoma. *The Journal of surgical research*. 2010; 163:244–9. [PubMed: 20691980]
29. Yang S, Chen J, Guo Z, Xu XM, Wang L, Pei XF, et al. Triptolide inhibits the growth and metastasis of solid tumors. *Molecular cancer therapeutics*. 2003; 2:65–72. [PubMed: 12533674]
30. Banerjee S, Thayanithy V, Sangwan V, Mackenzie TN, Saluja AK, Subramanian S. Minnelide reduces tumor burden in preclinical models of osteosarcoma. *Cancer letters*. 2013
31. Krosch TC, Sangwan V, Banerjee S, Mujumdar N, Dudeja V, Saluja AK, et al. Triptolide-mediated cell death in neuroblastoma occurs by both apoptosis and autophagy pathways and results in inhibition of nuclear factor-kappa B activity. *American Journal of Surgery*. 2013; 205:387–96. [PubMed: 23428154]
32. Shackleton M, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell*. 2009; 138:822–9. [PubMed: 19737509]
33. Sasaki A, Kamiyama T, Yokoo H, Nakanishi K, Kubota K, Haga H, et al. Cytoplasmic expression of CD133 is an important risk factor for overall survival in hepatocellular carcinoma. *Oncology reports*. 2010; 24:537–46. [PubMed: 20596644]
34. Borger DR, Tanabe KK, Fan KC, Lopez HU, Fantin VR, Straley KS, et al. Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. *The oncologist*. 2012; 17:72–9. [PubMed: 22180306]
35. Borger DR, Zhu AX. IDH mutations: new genetic signatures in cholangiocarcinoma and therapeutic implications. *Expert review of anticancer therapy*. 2012; 12:543–6. [PubMed: 22594888]
36. Li C, Lee CJ, Simeone DM. Identification of human pancreatic cancer stem cells. *Methods in molecular biology* (Clifton, NJ). 2009; 568:161–73.

37. Prinsloo E, Setati MM, Longshaw VM, Blatch GL. Chaperoning stem cells: a role for heat shock proteins in the modulation of stem cell self-renewal and differentiation? *BioEssays : news and reviews in molecular, cellular and developmental biology*. 2009; 31:370–7.
38. Luo S, Mao C, Lee B, Lee AS. GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. *Molecular and cellular biology*. 2006; 26:5688–97. [PubMed: 16847323]
39. Bartkowiak K, Effenberger KE, Harder S, Andreas A, Buck F, Peter-Katalinic J, et al. Discovery of a novel unfolded protein response phenotype of cancer stem/progenitor cells from the bone marrow of breast cancer patients. *Journal of proteome research*. 2010; 9:3158–68. [PubMed: 20423148]
40. Wu MJ, Jan CI, Tsay YG, Yu YH, Huang CY, Lin SC, et al. Elimination of head and neck cancer initiating cells through targeting glucose regulated protein78 signaling. *Molecular cancer*. 2010; 9:283. [PubMed: 20979610]
41. Jiang R, Li Y, Xu Y, Zhou Y, Pang Y, Shen L, et al. EMT and CSC-like properties mediated by the IKKbeta/IkappaBalpha/RelA signal pathway via the transcriptional regulator, Snail, are involved in the arsenite-induced neoplastic transformation of human keratinocytes. *Archives of Toxicology*. 2012

Translational relevance

Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth most frequent cause of cancer-related death in the United States owing to its propensity to invade and metastasize, and resist chemotherapy almost completely. Relapse of any tumor is largely attributed to the presence of tumor-initiating cells (TIC) or cancer stem cells (CSC). These CSCs or TICs are quiescent cells within a tumor that often evade conventional chemotherapy and result in tumor recurrence either locally or in a distant organ.

TICs from most cancers have been identified and isolated from patient tumors implanted in an immune-compromised mouse model. In this current study we isolate CD133+ pancreatic TIC from both cell lines and tumors derived from the spontaneous genetic mouse model (KPC) for pancreatic cancer. We further show that these CD133+ tumor cells can form tumors in C57/Bl6 immune competent mice from as low as 10 cells. Our study also shows that Minnelide (a water soluble pro-drug of triptolide, currently under Phase I clinical trial) decreases the volume of the tumors derived from the CD133+ cells. Minnelide further reduces the total CD133+ population in KPC primary tumors.

The translational significance of this study lies in the fact that Minnelide is able to decrease the CD133+ TICs in an extremely aggressive model for pancreatic cancer. Further, since this model is based on an immune-competent animal model, it is more clinically relevant compared to the immune-compromised TIC models reported earlier.

Fig. 1. KPC cells show expression pancreatic TIC markers

(A) Representative CD133 expression, (B) CD24⁺/CD44⁺/ESA⁺ expression and (C) Aldh1 activity in KPC cells. (D) Fold change in mRNA expression of a number of TIC markers in KPC cells compared to normal ductal cells.

Fig. 2. CD133⁺ subset in KPC cells expressed TIC markers associated with other cell types

(A) Enrichment of KPC CD133⁺ population by MACS; (B) CD44⁺CD24⁺ESA⁺ population within CD133⁻ cells and CD133⁺ population. (C) Aldh1 activity within CD133⁻ cells and CD133⁺ population. (D) Co-expression of CD133⁺/Aldh1⁺ by immunofluorescence.

Fig. 3. CD133⁺ cells formed serially transplantable tumors in immune-competent mice

(A) Tumor progression with different numbers of CD133⁺ cells. (B) Representative pictures of C57BL6 mice showing tumor from 10 CD133⁺ cells, while CD133⁻ and unsorted KPC did not form tumors with this number. CD133⁺ cells showed early tumorigenesis in an orthotopic model: Tumor progression in an orthotopic model of pancreatic cancer showed tumor formation in CD133⁺ cells as early as 20 days as compared to unsorted cells that did not form tumors (C). H&E staining showing histology of tumors in an orthotopic model (D).

Fig. 4. CD133 cells have higher expression of pro-survival proteins

Fold change in mRNA expression of (A) Bcl-2 and Survivin, (B) developmental markers, and (C) heat shock genes of CD133⁺ compared to CD133⁻ KPC cells. NF- κ B activity in CD133⁺ and CD133⁻ cells (D). Wild type and Mutant oligos were used as specificity controls for NF- κ B binding assay.

Fig. 5. KPC cells respond to triptolide

(A) Viability of CD133+ and CD133- cells with triptolide, paclitaxel, 5FU and gemcitabine. (B) Tumor progression after Minnelide treatment compared to untreated tumor. Minnelide reduces TIC population in vivo: (C) Expression of CD133 in primary KPC tumors and CD133+ implanted tumors after treatment with Minnelide, (D) NF-kB activity in Minnelide treated and untreated tumors from KPC primary tumors and CD133+ cell implanted tumors. 4-5 tumors were analyzed for each experiment.

Table 1

TIC markers cells in different pancreatic tumors and cell lines

	Nature of sample	CD133 (% positive)	CD44/24/ESA (% positive)	Aldh activity (net) (% positive)
KPC001	Cell line	8% (+/- 1.8%)	4.3% (+/- 1.6%)	2.2% (+/- 1.1%)
KPC023	Cell line	7.1% (+/- 1.2%)	3.8% (+/- 1.2%)	1.8% (+/- 0.8%)
KPC883	Tumor	6.9%	4.1%	0.9%
KPC820	Tumor	8.2%	3.2%	1.1%
KPC793	Tumor	7.8%	2.6%	0.8%
AsKPC883	Ascitis	2.2%	0	0
AsKPC820	Ascitis	2.8%	0	0
AsKPC793	Ascitis	2.1%	0	0
Liver001	Cell line	2.4% (+/- 0.7%)	0.5% (+/-0.2%)	0.8 (+/-0.4%)
hPDX1	Tumor xenograft	4.2% (+/- 1.6%)	1.2% (+/-0.3%)	2.8% (+/- 1.2%)
hPDX2	Tumor xenograft	3.5% (+/- 1.4%)	0.8% (+/-0.3%)	1.1% (+/- 0.9%)
hPDX3	Tumor xenograft	2.8% (+/- 0.9%)	0.3% (+/- 0.2%)	0.9% (+/- 0.4%)
hPDX4	Tumor (fresh)	4.5%	0.2%	0.8%