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The differentiation of pancreatic tumor-initiating cells by vitronectin can be blocked by Cilengitide

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Abstract

Objective—Pancreatic cancer is a leading cancer type and its molecular pathology is poorly understood. The only potentially curative therapeutic option available is complete surgical resection; however, this is inadequate as a majority of patients are diagnosed at an advanced or metastatic stage. Tumor-initiating cells constitute a subpopulation of cells within a solid tumor that sustain tumor growth, metastasis and chemo-/radio-resistance. Within pancreatic cancer, tumor-initiating cells have been identified based on the expression of specific cell surface markers.

Methods—We utilize a sphere formation assay to enrich for putative TICs and use human serum as a driver of differentiation. We demonstrate using specific blocking reagents that we can inhibit the differentiation process and maintain tumor-initiating cell associated markers and genes.

Results—We can induce differentiation of pancreatospheres with the addition of human serum and identified vitronectin as an inducer of differentiation. We inhibit differentiation by human serum using an arginine-glycine-aspartate specific peptide, Cilengitide; hence, demonstrating this differentiation is mediated via specific integrin receptors.

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Conclusions—Overall, our studies further the definition of pancreatic tumor-initiating cells and provide further insight into both the maintenance and differentiation of this lethal population.

Keywords

Pancreatic cancer; TICs; vitronectin; differentiation; Cilengitide

Introduction

Pancreatic cancer, the fourth leading cause of cancer related deaths ¹, is one of the most challenging solid tumors to diagnose and treat as it presents such a clinically challenging disease due to its ability to aggressively metastasize and its high resistance against both chemotherapy and radiation ². One of the most effective treatments to date for pancreatic cancer is complete surgical resection via a procedure known as the Whipple procedure. Unfortunately, the ability to perform the Whipple procedure is limited to roughly 20% of patients with local disease ². Currently, gemcitabine is the first-line treatment for pancreatic cancer patients presenting with locally advanced or metastatic adenocarcinoma and recently, Erlotinib, an EGFR tyrosine kinase inhibitor, has been used in pancreatic cancer therapy ³. An additional factor contributing to the poor survival rate and diagnosis of pancreatic cancer is the lack of efficient detectable markers for early prognosis.

The hypothesis that a small population of cells termed cancer stem cells (CSCs) or tumor-initiating cells (TICs) can give rise to the bulk tumor is currently under extensive investigation. The properties of TICs include the ability to undergo self-renewal, differentiation and initiate tumor formation ⁴. TICs have been identified in various solid tumors including breast ⁵, colon ⁶, brain ⁷, cervix ⁸ and prostate ^{9, 10} cancers. Recently, TICs have been identified in pancreatic cancer as well ^{11, 12}. Previous reports suggests that there are distinct populations of pancreatic cells that overlap displaying putative cancer stem cell properties which include populations characterized by either CD44+CD24+ESA+, CD133+CXCR4+ or c-Met+ cell surface markers ^{11–13}. Additionally, Jimeno et al ¹⁴ identified a TIC population, CD24+CD44+, which became enriched post gemcitabine treatment and prompted the repopulation of proliferating cells. Additionally, our laboratory has recently shown that an invasive pancreatic cell population representative of the TIC population has an increased ability to undergo DNA repair once challenged with gemcitabine ¹⁵. The putative TICs previously identified have been shown to be highly tumorigenic and possess TIC characteristics such as self-renewal and the ability to differentiate which are representative of a heterogeneous tumor. However, the biology which governs pancreatic TIC maintenance is complex and under investigation.

Our laboratory has recently demonstrated in a prostate cancer model that vitronectin (VN), a major component of the extracellular matrix (ECM) and a component of human serum can drive the differentiation of both breast and prostate TICs ¹⁶. Furthermore, we were capable of blocking VN induced differentiation by inhibiting the integrin receptor $\alpha V\beta 3$ and were able to attenuate TIC-driven tumorigenesis in mice by blocking $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins via a cyclic arginine-glycine-aspartate (RGD)-peptide ¹⁶. We demonstrated that TICs are responsible for tumor initiation formation and there is a requirement for extrinsic cues in order to drive these cells into a differentiated state to initiate tumor formation.

As previously stated, pancreatic cancer is characterized by its ability to metastasize aggressively. TICs are hypothesized to be responsible for both the aggressiveness and chemo-resistance often associated with pancreatic cancer. Additionally, the ability of TICs to differentiate is hypothesized to be the cause of tumor initiation; hence, we sought to investigate if we could drive differentiation of pancreatic TICs by human serum, specifically

by the ECM component vitronectin. Using a sphere formation assay to enrich for a pancreatic TIC population, we enriched for a putative TIC population in both immortalized and primary pancreatic cell lines. These pancreatospheres were cultured in a highly specialized stem cell media and were able to maintain previously identified TIC markers associated with pancreatic TICs. Additionally, we analyzed the global molecular signature of pancreatospheres and identified various pathways which may contribute to the maintenance of this TIC population. We further demonstrated that upon the addition of both human serum and vitronectin, pancreatospheres differentiate into cells with an epithelial-like morphology and lose expression of TIC related genes, specifically Hedgehog signaling pathway associated genes (SHH, Gli1 and Gli2). Furthermore, we demonstrate an ability to inhibit the differentiation process driven by human serum by specifically blocking the integrin α V β 3 and α V β 5 receptors using the cyclic RGD pentapeptide Cilengitide (Merck KGaA), a drug currently in clinical trial. Our data demonstrates a novel mechanism by which pancreatic TICs (pancreatospheres) can differentiate upon the addition of extrinsic factors such as human serum or vitronectin and that the ability to block this process by using an integrin antagonist provides further insight into the underlying molecular biology governing pancreatospheres. Furthermore, we believe these observations present potential grounds for further investigation into the clinical applications of this drug in targeting pancreatic TICs for the prevention and treatment of pancreatic cancer and metastasis.

Materials and Methods

Isolation and culture of TICs

Pancreatospheres (pancreatic TICs) were obtained from an immortalized cell line, PANC1 (ATCC, Manassas, VA, USA) and a primary pancreatic cell line PL3 (Panc 4.14), a generous gift from Dr. Elizabeth Jaffee (Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD). The primary pancreatic cancer cell line is a low-passage pancreatic carcinoma cell lines. Pancreatospheres were maintained in highly specialized stem cell media (SCM) (F12:Dulbecco's modified Eagle's medium supplemented 10 ng/ml bFGF, 20 ng/ml EGF, 5 μ g/ml insulin, 0.04% bovine serum albumin) as previously described⁹. SCM was supplemented with 1% knockout serum (Invitrogen, Carlsbad, CA, USA) and insulin-transferrin-selenium (ITS) (Invitrogen, Carlsbad, CA, USA). Pancreatic cell lines were enriched for pancreatic TICs by plating in conditions that favor nonadherent sphere formation (SCMITSKO media) as demonstrated for various cancer cell lines and primary samples^{9, 17–20}.

Soft Agar Colony Assay

Pancreatospheres were grown in SCMITSKO for 1–2 weeks and resuspended at 3000 cells in SCMITSKO containing 0.4% agarose and overlaid onto a 60-mm dish containing a solidified bottom layer of 0.4% agarose in SCMITSKO. Total pancreatic cells were resuspended at 3000 cells in DMEM 10% FBS media. Once the top layer solidified, 1 mL of medium was placed on top to keep the plates moist. Plates were incubated for 3 weeks until colonies were visible. The plates were stained with 1:75 dilution of 0.33% neutral red solution at 37C for 1 hour, and then counted using Gel Count Software (Oxford Optronics, Oxford, United Kingdom).

Quantitative Real Time RT-PCR

Gene expression was measured by quantitative real time polymerase chain reaction (qRT-PCR) using cDNA generated from cell lysates. For mRNA detection, cDNA was prepared using the SuperScript[®]III First-Strand Synthesis System (Invitrogen Corporation, Carlsbad, CA, USA) and qRT-PCR analysis was performed using a StepOne Real-time PCR machine (Applied Biosystems, Foster City, CA, USA) with TaqMan Gene Expression Assay reagents

and probes (Applied Biosystems, Foster City, CA, USA). Log2 ratios are shown and calculated using the relative quantification ($\Delta\Delta CT$) method and 18S rRNA was used as a normalization control.

Differentiation and Blocking Analysis

For differentiation studies, pancreatospheres were maintained in SCMITSKO. Human serum (Gemini Bio-Products, West Sacramento, CA, USA) was added as indicated and fractionated as previously described¹⁶. Antibodies and peptides were added at the same time as human serum when indicated. Blocking antibodies for integrins were obtained from Millipore (Millipore, Billerica, MA, USA), anti-integrin $\alpha V\beta 3$ (MAB1976) and anti-integrin $\alpha V\beta 5$ (MAB1961). The RGD peptide, cyclo (Arg-Gly-Asp-d-Phe-Lys), and RAD peptide, cyclo (Arg-Ala-Asp-d-Phe-Lys) were purchased from Peptides International (Louisville, KY, USA) and were dissolved in DMSO. Vitronectin-coated plates were purchased from R&D systems (R&D, Minneapolis, MN, USA). The integrin antagonist Cilengitide was directly obtained from Merck KGaA (Merck KGaA, Germany). Pancreatospheres were plated at a density of 1000 cells/mL, allowed to form spheres for 1–2 weeks then dissociated and replated for differentiation and blocking studies.

Microarray analysis

RNA was isolated from samples and labeled as previously described⁹, with the following modifications. Reverse transcriptase was heat inactivated at 65°C for 10 minutes followed by RNaseA RNA degradation at room temperature for 30 min. Universal Reference RNA (Stratagene, La Jolla, CA, USA) was labeled with Cy3-dUTP and experimental samples were labeled with Cy5-dUTP. Samples were hybridized to an Agilent whole genome gene expression array (Agilent, Santa Clara, CA, USA) following manufacturer's directions. Arrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) and analyzed using NCI mAdb (<https://madb.nci.nih.gov/index.shtml>), a microarray and data analysis system for NCI/CCR scientists/collaborators.

Results

Pancreatosphere isolation in immortalized and primary pancreatic cell lines

We first sought to determine if we could enrich for a TIC population in adherent pancreatic cell lines. Using sphere formation assay conditions, as described, we plated pancreatic cell lines 1000 cells/mL in highly specialized stem cell media (SCMITSKO) in low-attachment conditions. We examined 2 cell lines that included an immortalized and primary patient cell line: PANC1 cells (immortalized) and Panc 4.14 (primary patient derived), which has previously been used in both transcription profile and single-nucleotide polymorphism analysis^{10, 21}. Figure 1A compares the total adherent population to the spheres formed from these cell lines in the described culture conditions. The ability of both immortalized and primary patient cell lines to form pancreatospheres is in agreement with previously published data using the sphere model as an accepted model for the isolation of putative pancreatic TICs^{17, 22}.

To determine if the pancreatospheres formed displayed an increase in both previously identified TIC markers and stem associated genes, we performed further qRT-PCR on the immortalized cell line PANC1 and the primary patient cell line Panc 4.14. As previously shown, pancreatic TICs are believed to express the TIC related markers, CD44, CD133, CD24, EpCam (ESA) and CXCR4^{11, 12, 14}. To confirm expression of TIC markers in our spheres, specifically CD44, CD24 and EpCam, using qRT-PCR analysis, Figure 1B demonstrates that the pancreatospheres formed from both PANC1 and Panc 4.14 cells display an increase in these markers in comparison to the total adherent population. All data

was normalized to 18s rRNA expression. Interestingly, the most significant increases in expression were seen for CD24 and EpCam (Holm-Sidak analysis, $\alpha=5\%$) in PANC1 spheres and for EpCam expression in Panc 4.14 spheres (Holm-Sidak analysis, $\alpha=5\%$). However, in line with previous data, PANC1 total cells did not express CD133 and the CD133 expression in pancreatospheres derived from both PANC1 and Panc 4.1.4 cells was barely detectable (data not shown)²³. Interestingly, we have previously shown that CD133 expression in pancreatospheres is not significant unless challenged with gemcitabine¹⁵, which is in agreement with previous data demonstrating an enrichment for pancreatic TICs post-gemcitabine treatment as well¹⁴. Hence, we speculate that in agreement with previous data demonstrating there are distinct subpopulations of pancreatic TICs, we believe CD133, in these specific cell lines, does not serve as a useful TIC marker^{11, 12}. This data correlates with additional studies in other solid tumors, specifically in brain and colon cancers, which indicate that CD133 expression may not serve as a universal marker for the TIC population in tumor initiation but its expression may function in tumor progression^{24, 25}. To further confirm isolation of a putative TIC population, we performed qRT-PCR analysis and assayed for gene expression of Hedgehog pathway associated genes, Gli1, Gli2 and the receptor SHH, which have previously been shown to be increased in pancreatospheres¹⁷. Figure 1B demonstrates that in the immortalized PANC1 derived pancreatospheres, there is a significant increase in the Hedgehog target gene Gli1 and in the primary patient derived pancreatospheres from Panc 4.14, we see an increase in both Gli2 and SHH. In the case of the PANC1 pancreatospheres, we do not see a significant increase in SHH gene expression, which may be a result of the lack of extracellular signals from the microenvironment that is typically responsible for SHH ligand activation. However, as we see an increase in Gli1 expression, this supplies evidence for the hyperactivation of this pathway in pancreatospheres compared to the total adherent population.

A key property of TICs is the ability to grow in anchorage-independent growth conditions as this is representative of the TICs ability to function in tumorigenesis. Hence, we tested the ability of pancreatospheres to form colonies in soft agar in comparison to the total adherent population. We first utilized our sphere formation assay to enrich for the TIC population then proceeded to use this enriched population to determine if it had the ability form colonies. As seen in Figure 1C, the cells isolated from already formed pancreatospheres were able to form more colonies in comparison to the total adherent population. Specifically, in the case of the primary patient cell line Panc 4.14, the number of colonies formed was almost doubled and in addition, the colonies formed were generally larger in size compared to the colonies formed from the total cell population. These results indicate that the pancreatospheres represent a more aggressive population with respect to colony-initiating ability, a key property of TICs. Taken together with the previous observation that pancreatospheres have the ability to initiate tumor formation in mice^{17, 26}, the ability of both PANC-1 and Panc 4.14 cells to form spheres, an increase in TIC related markers, an increase in stem related genes and the ability to form colonies in anchorage-independent conditions indicates that the pancreatospheres are representative of a TIC population according to the accepted model used for TIC characterization.

To gain further insight into the molecular mechanism(s) which may govern the development of pancreatic TICs we performed whole genome microarray analysis on pancreatospheres derived from PANC1 and Panc 4.14 cells. Figure 1D is representative of the top 10 pathways upregulated in pancreatospheres based on Ingenuity Pathway Analysis (IPA) software. In the PANC1 derived pancreatospheres we see an upregulation of key regulatory pathways previously identified in other TIC models such as: PI3K/Akt and PTEN signaling (leukemia, prostate, hepatocellular, medulloblastoma, lung and breast TICs (reviewed in²⁷)); IGF-1 signaling (glial and colon TICs²⁸⁻³⁰); ERK5 signaling (promotion of EMT in breast cancer cells²⁵); PTEN and Wnt/ β -catenin signaling (extensively reviewed in^{27, 31}).

In the primary patient Panc 4.1.4 derived pancreatospheres we see an upregulation of pathways such as: prolactin signaling (prostate, breast, leukemia TICs^{32–34}) and BMP signaling (ovarian TICs³⁵). We believe this overview of additional pathways upregulated in this TIC population can give way to additional avenues of investigation in identifying TIC targets, specifically in the primary patient cell line. Lastly, we believe that the variation in gene profiles as demonstrated by the microarray analysis emphasizes the need to further investigate and increase use and establishment of primary patient cell lines. The use of immortalized cell lines has been invaluable to the scientific community; however, the genetic manipulation needed to ensure immortalization may possibly alter genotype. Hence, the differences in gene profiles should be further investigated and additional primary cell lines should be analyzed at a whole genome level to determine if there is significant difference between primary and immortalized cell lines.

The loss of stemness markers in pancreatospheres upon addition of human serum

Our laboratory has previously demonstrated that TICs derived from both LNCap cells and prostate cancer patients (PCSCs) have the capability of differentiating in the presence of human serum¹⁶. We sought to determine if this effect could be reproduced for pancreatic TICs as well. As demonstrated in Figure 2A, when human serum is added to the culture of pancreatospheres derived from PANC1, the cells become an adherent monolayer that is reminiscent of the total cell population (Figure 2A). However, in the case of Panc 4.14 spheres, after 48 hours exposure to human serum, a majority of the cells have formed an adherent monolayer but there is still a portion of the population that remain as spheres. To determine if this change in morphological phenotype was a result of serum induced downregulation of stems associated genes, we performed qRT-PCR analysis and compared gene expression levels in pancreatospheres to the spheres differentiated with human serum. Figure 2B demonstrates that in PANC1 derived pancreatospheres treated with human serum, there is a decrease in expression of both TIC associated markers and stem associated genes upon addition of human serum. Specifically, EpCam expression in PANC1 cells treated with human serum is significantly decreased (Holm-Sidak, $\alpha=5\%$). In line with the observation that a mixed population remains after exposure to human serum for 48 hours, pancreatospheres derived from Panc 4.14 cells did not demonstrate a decrease in stem associated markers and maintained a significant increase in expression for CD24 (Holm-Sidak, $\alpha=5\%$) (Figure 2C). Interestingly, the TIC associated marker Gli2 was no longer detected by qRT-PCR upon addition of human serum in comparison to spheres in Panc 4.14 cells, hence, we can conclude that although there is not an overall effect on TIC associated markers, there is a downregulation of a TIC associated pathway. We believe that the inability of the human serum to drive a decrease in the TIC associated markers at the mRNA level may be a result of the time exposure to human serum as 48 hours may not be sufficient to observe a decrease in mRNA levels of TIC associated markers. Additionally, it is possible that the concentration of human serum tested, 5%, may not be sufficient to drive complete differentiation. These possibilities are currently under further investigation in our laboratory. Furthermore, the Panc 4.14 cell line is a primary patient cell line and to the best of our knowledge, this is the first report using primary cells directly established from patients that have not been established by xenografts for pancreatic cancer to analyze the mechanism(s) that drive differentiation. Recently, Li et al identified a new population of highly tumorigenic pancreatic TICs expressing c-met^{high}CD44⁺³⁶ as well. It is clear that the expressions of specific cell surface markers currently used to isolate pancreatic TICs are not universal. This growing list of potential TIC markers, specifically in the pancreatic TIC model, further stresses the importance of the need to further characterize and investigate additional regulatory pathways that are responsible for TIC maintenance/regulation and the process of differentiation at a molecular level which are unique in comparison to a non-TIC population.

The loss of stemness markers in pancreatospheres upon addition of the extracellular matrix protein vitronectin

It is hypothesized that TICs reside within a highly specialized microenvironment/niche that is responsible for TIC regulation which include inflammatory molecules, factors involved in hypoxia and angiogenesis (reviewed in ¹⁵). Major components of this niche may also include extracellular matrix proteins as well that function in cellular structure and participate in the regulation of molecular pathways deemed necessary for survival, as seen in mesenchymal stem cells ³⁷. Previously, our laboratory used fractionated human serum to identify vitronectin as an inducer of differentiation in prostate TICs ¹⁶. Vitronectin, a major component of the extracellular matrix and a protein found in human serum, is known for its role in differentiation of the endoderm in mouse development ³⁸, role in neuronal differentiation ³⁹ and osteogenic differentiation of mesenchymal stem cells ⁴⁰. Furthermore, it has previously been shown that FG human pancreatic carcinoma cells have the ability to attach to vitronectin using the integrin receptor $\alpha V\beta 5$ and migration on a vitronectin substrate is stimulated in an EGFR-dependent manner and activation of Rap1, a downstream target of EGFR, is required ^{41, 42}. Specifically, in PANC1 cells, the integrin receptor $\alpha V\beta 5$ is the main receptor for VN and its expression correlates with the ability to migrate and metastasize ^{43, 44}. Furthermore, VN expression has been noted to function in the development of the human pancreas and modulate events necessary for islet neogenesis, the ability to develop β -cells from progenitors ⁴⁵. Cirulli et al demonstrated that both ductal cells and clusters of undifferentiated cells emerging from the ductal epithelium during human pancreatic development are characterized by high expression levels of both $\alpha V\beta 3$ and $\alpha V\beta 5$ integrin receptors ⁴⁵ that function in migration of endocrine progenitors.

Based on these observations, we chose to investigate if vitronectin was capable of inducing differentiation of PANC1 pancreatospheres. Figure 2D demonstrates that culturing of previously formed pancreatospheres on VN-coated plates resulted in an adherent monolayer PANC1 cells, similar to the effect of human serum addition. However, unlike the observation of complete differentiation as a result of human serum addition, we never observed complete adherence on VN-coated plates for PANC1 spheres and observed a mixed population. To determine if VN affected expression of TIC associated markers we performed qRT-PCR analysis on pancreatospheres cultured on BSA-coated plates (control) and VN-coated plates, Figure 2E. PANC1 pancreatospheres cultured on VN-coated plates did not display a significant decrease in TIC associated markers nor in SHH expression in comparison to the decrease seen upon differentiation by human serum. In addition, Gli1 was no longer detectable in pancreatospheres cultured on VN-coated plates. Hence, we suspect that VN is not a sole inducer of PANC1 pancreatosphere differentiation but there are additional differentiation drivers present within the human serum to still be investigated. Cirulli et al have previously shown that the ECM proteins fibronectin (FN) and collagen-IV are involved in islet development ⁴⁵ and serve in the differentiation process. Interestingly, for the primary patient cell line Panc 4.14, VN alone is capable of driving a decrease in TIC associated genes more efficiently in comparison to human serum (Figure 2F) and similarly to PANC1 cells, the Gli2 family member is no longer detected. Specifically, SHH expression is significantly decreased (Holm-Sidak, $\alpha=5\%$). We suspect that for Panc 4.14 cells VN is a major driver of differentiation but for PANC1 cells, the TICs require a network of ECM related proteins to undergo differentiation. Additionally, the inability to detect Gli1 or Gli2 at the mRNA level upon addition of VN may represent the inactivation of the SHH pathway, resulting in the downregulation of Gli-mediated transcription that contributes to loss of TIC maintenance and regaining of a differentiated state. A role between SHH and VN has previously been established as Pons et al described a synergistic relationship that was necessary for motor neuron differentiation ⁴⁶. Hence, this data demonstrates a unique mechanism by which pancreatic TICs are driven into a differentiated

state by an ECM protein which regulates a crucial TIC regulatory pathway. Furthermore, the differences in the ability to differentiate between PANC1 and Panc 4.14 cells provide further evidence for the differences in regulatory mechanism(s) which may exist between varying TIC populations in pancreatic cancers.

Blocking human-serum induced differentiation via the integrin-signaling axis

The observation that human serum can drive differentiation of pancreatospheres and that specifically, VN could partially drive this differentiation, led us to test if by blocking the integrin $\alpha V\beta 3$ and $\alpha V\beta 5$ receptors, we could inhibit the differentiation process in PANC1 cells. Using a cyclic RGD peptide (cyclicRGDfk), specific for the $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins, we were able to partially block these changes morphologically, however, the control RAD peptide, was unable to (Figure 3A). Interestingly, qRT-PCR analysis of TIC associated markers in PANC1 cells revealed that there was maintenance of TIC associated markers when blocking with RGD in comparison to the control peptide RAD and human serum alone, Figure 3B. Hence, we speculate that although morphologically we are able to partially block the shift from a pancreatosphere to an adherent phenotype by the RGD peptide, the partial block by the RGD peptide is not sufficient to maintain the total population of pancreatospheres. Interestingly, qRT-PCR analysis of TIC associated genes, specific for the HH pathway, reveal that partially blocking serum induced differentiation by RGD is not effective in maintaining a high expression level of SHH in comparison to serum alone and the control RAD peptide, Figure 4B. In addition, Gli1 expression is decreased at a level which is statistically significant (Holm-Sidak, $\alpha=5\%$) in comparison to serum alone indicating genes necessary for TIC maintenance are not maintained. The culturing of pancreatospheres yield a highly clustered, spherical form that is very 'sticky' and difficult to dissociate. It is possible that the ability of pancreatospheres to strongly bind and form these cell-cell contacts within this spherical cluster make it difficult for the RGD peptide to bind to the $\alpha V\beta 3$ and $\alpha V\beta 5$ integrin receptors within the cluster. Hence, this inability to bind results in a maintenance of pancreatospheres which cannot bind to RGD and undergo differentiation.

Subsequently, we wanted to determine if blocking the integrin receptors, $\alpha V\beta 3$ and $\alpha V\beta 5$, with receptor specific antibodies would be more efficient in blocking differentiation. Using antibodies specific to both $\alpha V\beta 3$ and $\alpha V\beta 5$, we observed a more efficient means of inhibiting the differentiation process. As seen in Figure 3C, both anti- $\alpha V\beta 3$ and - $\alpha V\beta 5$ are capable of blocking morphological changes driven by human serum, with anti- $\alpha V\beta 5$ having a greater effect. Furthermore, using qRT-PCR analysis, we were able to demonstrate that specifically, the blocking antibody to integrin $\alpha V\beta 5$ was able to block a decrease in expression of TIC associated markers and genes with greater significance compared to the antibody to integrin $\alpha V\beta 3$ (Figure 3D), and in comparison with the RGD peptide. This data is in agreement with previous data suggesting that the integrin $\alpha V\beta 5$ receptor, the main receptor for VN in pancreatic cells, is largely responsible for mediating the differentiation process in pancreatic cells^{45, 47}. The ability to block differentiation of pancreatospheres by human serum and ECM components via the inhibition of the integrin signaling axis provides evidence that this pathway is crucial in the regulation of TICs and possibly, their ability to initiate tumor formation upon differentiation by extrinsic cues.

Promoting and blocking differentiation of pancreatospheres with the integrin antagonist Cilengitide

As the data demonstrates that the differentiation of pancreatospheres by human serum is mediated by the integrin receptors, $\alpha V\beta 3$ and $\alpha V\beta 5$, we sought to determine if we could block this process by a compound currently in clinical trial, Cilengitide (EMD 121974, Merck KGaA). Cilengitide is a cyclic RGD pentapeptide that functions as a selective

integrin antagonist specific for $\alpha V\beta 3$ and $\alpha V\beta 5$ ⁴⁸. Cilengitide has exhibited the ability to inhibit various biological functions critical to cancer development such as angiogenesis and tumor growth⁴⁹. Cilengitide is under investigation in the clinic for treatment of cancers such as glioblastoma, lung, prostate⁴¹ and pancreatic⁵⁰. Hence, based on our data, we tested the effect of Cilengitide on serum induced differentiation of pancreatospheres. Figure 4A demonstrates that we are able to morphologically inhibit the differentiation of PANC1 spheres with the addition of Cilengitide. We then performed qRT-PCR analysis and demonstrate that Cilengitide was able to block a decrease in expression of TIC associated markers and genes upon addition of serum, Figure 4B. Specifically, EpCam expression was significantly decreased using 2.5 μ M Cilengitide (Holm-Sidak, $\alpha=5\%$). The success of Cilengitide in the clinic has come in the treatment of patients with glioblastoma using Cilengitide in addition to traditional cytotoxic therapies⁴⁹. Cilengitide has been tested in patients with pancreatic cancer as these tumors rely on sufficient blood supply via angiogenesis for tumor persistence using the integrin-axis. This initial study determined no significant difference between patients treated with gemcitabine alone (the current standard for pancreatic cancer patients) or gemcitabine plus Cilengitide⁵⁰.

To apply clinical relevance to the expression of the VN receptor, ITGAV (antigen CD51), and its role in aggressiveness and metastasis, we queried the Oncomine database to determine expression of ITGAV. Figure 4C represents a heat map of raw data from a number of pancreatic cancer studies comparing the gene expression level of ITGAV over 3 analyses comparing normal pancreas versus pancreatic carcinoma and pancreatitis including the Badea Pancreas⁵¹, Logsdon Pancreas⁵² and Segara Pancreas⁵³ datasets. As seen, there is a high expression level of ITGAV with a statistically significant p-value, $p=5.37E-12$. We further utilized the Oncomine database to determine the relationship between ITGAV and high grade pancreatic carcinoma. As demonstrated in Figure 4D, in the Buchholz Pancreas⁵⁴, studying pancreatic intraepithelial neoplasia, high PanIN grade, and in the Collisson Pancreas⁵⁵, studying high grade pancreatic ductal adenocarcinoma, we see a statistically significant expression ($p\text{-value}=0.015$) of ITGAV as well. Using Oncomine, we are able to conclude that ITGAV is indeed highly expressed in clinical samples and correlates with a high grade form of pancreatic cancer often defined by aggressiveness and metastasis. Hence, the use of blocking agents in the clinic to inhibit receptor binding to ECM proteins present within the niche may possibly function to block activation of signal transduction cascades which would promote differentiation.

Conclusions

In this report, we demonstrate that we are able to partially block serum induced differentiation of pancreatospheres using an RGD peptide, antibodies specific for integrin $\alpha V\beta 5$ and $\alpha V\beta 3$ and the drug Cilengitide which is currently in clinical trial. We have previously shown in a prostate cancer model system that the differentiation of prostate TICs appears to be induced by the ECM component vitronectin and largely regulated by its receptor integrin $\alpha V\beta 3$ ¹⁶. We propose that in our pancreatic cancer model, the mechanism(s) by which both human serum and vitronectin are able to drive partial differentiation of pancreatospheres (TICs) appears to be regulated and coordinated via the integrin $\alpha V\beta 5$ and $\alpha V\beta 3$ receptors and VN is a major driver of the differentiation process. However, our evidence also demonstrates that there may be additional components present within human serum aside from VN that may co-regulate the differentiation process suggesting the integrin signaling axis may not be the sole signalling pathway involved.

We propose that pancreatic TICs use various mechanism(s) to undergo human serum and vitronectin induced differentiation as our blocking studies using antibodies specific for $\alpha V\beta 5$ and $\alpha V\beta 3$, peptides specific for both $\alpha V\beta 5$ and $\alpha V\beta 3$ (RGD) and the integrin

antagonist Cilengitide only partially inhibits differentiation. If indeed pancreatic TICs have dual mechanism(s) to undergo differentiation in response to extracellular stimuli then the ability to initiate tumor formation is enhanced, cellular growth is increased, and the interaction of TICs with extracellular components may promote motility, invasion and metastasis. The connections which exist between TICs, motility and metastasis are under intense investigation as it serves as an appealing axis for targeted therapy. Our data suggests that by targeting the ability of pancreatic TICs to differentiate and possibly block processes regulating cell motility, this deadly population may be restrained. This inhibition would result in the obstruction of tumor formation and perhaps, metastasis, a trait deadly to those diagnosed with pancreatic cancer.

Hence, we believe this gives further evidence to the concept of TICs in pancreatic cancer and that there is indeed a unique population present within pancreatic cancer cells that have properties reminiscent of an undifferentiated stem cell, such as the ability to self-renew and differentiate. The ability to block this differentiation process can be utilized as a means in the clinic to prevent further tumor growth and serve as a new target for treatment.

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References

1. Jemal A, Siegel R, Xu J, et al. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60(5):277–300. [PubMed: 20610543]
2. Lonardo E, Hermann PC, Heeschen C. Pancreatic cancer stem cells - update and future perspectives. *Mol Oncol.* 2010; 4(5):431–442. [PubMed: 20580623]
3. Maitra A, Hruban RH. Pancreatic cancer. *Annu Rev Pathol.* 2008; 3:157–188. [PubMed: 18039136]
4. Lobo NA, Shimono Y, Qian D, et al. The biology of cancer stem cells. *Annu Rev Cell Dev Biol.* 2007; 23:675–699. [PubMed: 17645413]
5. Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A.* 2003; 100(7):3983–3988. [PubMed: 12629218]
6. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature.* 2007; 445(7123):111–115. [PubMed: 17122771]
7. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res.* 2003; 63(18):5821–5828. [PubMed: 14522905]
8. Feng D, Peng C, Li C, et al. Identification and characterization of cancer stem-like cells from primary carcinoma of the cervix uteri. *Oncol Rep.* 2009; 22(5):1129–1134. [PubMed: 19787230]
9. Hurt EM, Kawasaki BT, Klarmann GJ, et al. CD44+ CD24(-) prostate cells are early cancer progenitor/stem cells that provide a model for patients with poor prognosis. *Br J Cancer.* 2008; 98(4):756–765. [PubMed: 18268494]
10. Patrawala L, Calhoun T, Schneider-Broussard R, et al. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene.* 2006; 25(12):1696–1708. [PubMed: 16449977]
11. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res.* 2007; 67(3):1030–1037. [PubMed: 17283135]
12. Hermann PC, Huber SL, Herrler T, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell.* 2007; 1(3):313–323. [PubMed: 18371365]

13. Li C, Wu JJ, Hynes M, et al. c-Met Is a Marker of Pancreatic Cancer Stem Cells and Therapeutic Target. *Gastroenterology*. 2011; 141(6):2218–2227. e2215. [PubMed: 21864475]
14. Jimeno A, Feldmann G, Suarez-Gauthier A, et al. A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. *Mol Cancer Ther*. 2009; 8(2):310–314. [PubMed: 19174553]
15. Cabarcas SM, Mathews LA, Farrar WL. The cancer stem cell niche--there goes the neighborhood? *Int J Cancer*. 2011; 129(10):2315–2327. [PubMed: 21792897]
16. Hurt EM, Chan K, Serrat MA, et al. Identification of vitronectin as an extrinsic inducer of cancer stem cell differentiation and tumor formation. *Stem Cells*. 2010; 28(3):390–398. [PubMed: 19998373]
17. Gaviraghi M, Tunici P, Valensin S, et al. Pancreatic cancer spheres are more than just aggregates of stem marker-positive cells. *Biosci Rep*. 2010; 31(1):45–55. [PubMed: 20426768]
18. Klarmann GJ, Hurt EM, Mathews LA, et al. Invasive prostate cancer cells are tumor initiating cells that have a stem cell-like genomic signature. *Clin Exp Metastasis*. 2009; 26(5):433–446. [PubMed: 19221883]
19. Dimri G, Band H, Band V. Mammary epithelial cell transformation: insights from cell culture and mouse models. *Breast Cancer Res*. 2005; 7(4):171–179. [PubMed: 15987472]
20. Yuan X, Curtin J, Xiong Y, et al. Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene*. 2004; 23(58):9392–9400. [PubMed: 15558011]
21. Iacobuzio-Donahue CA, Ashfaq R, Maitra A, et al. Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. *Cancer Res*. 2003; 63(24):8614–8622. [PubMed: 14695172]
22. Gou S, Liu T, Wang C, et al. Establishment of clonal colony-forming assay for propagation of pancreatic cancer cells with stem cell properties. *Pancreas*. 2007; 34(4):429–435. [PubMed: 17446842]
23. Olempska M, Eisenach PA, Ammerpohl O, et al. Detection of tumor stem cell markers in pancreatic carcinoma cell lines. *Hepatobiliary Pancreat Dis Int*. 2007; 6(1):92–97. [PubMed: 17287174]
24. Shmelkov SV, Butler JM, Hooper AT, et al. CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. *J Clin Invest*. 2008; 118(6):2111–2120. [PubMed: 18497886]
25. Zhou C, Nitschke AM, Xiong W, et al. Proteomic analysis of tumor necrosis factor- α resistant human breast cancer cells reveals a MEK5/Erk5-mediated epithelial-mesenchymal transition phenotype. *Breast Cancer Res*. 2008; 10(6):R105. [PubMed: 19087274]
26. Bao B, Ali S, Kong D, et al. Anti-tumor activity of a novel compound-CDF is mediated by regulating miR-21, miR-200, and PTEN in pancreatic cancer. *PLoS One*. 2011; 6(3):e17850. [PubMed: 21408027]
27. Martelli AM, Evangelisti C, Follo MY, et al. Targeting the Phosphatidylinositol 3-Kinase/Akt/Mammalian Target of Rapamycin Signaling Network in Cancer Stem Cells. *Curr Med Chem*. 2011
28. Hsieh A, Ellsworth R, Hsieh D. Hedgehog/GLI1 regulates IGF dependent malignant behaviors in glioma stem cells. *Journal of Cellular Physiology*. 2011; 226(4):1118–1127. [PubMed: 20857406]
29. Hägerstrand D, He X, Bradic Lindh M, et al. Identification of a SOX2-dependent subset of tumor- and sphere-forming glioblastoma cells with a distinct tyrosine kinase inhibitor sensitivity profile. *Neuro-Oncology*. 2011; 13(11):1178–1191. [PubMed: 21940738]
30. Hart LS, Dolloff NG, Dicker DT, et al. Human colon cancer stem cells are enriched by insulin-like growth factor-1 and are sensitive to figitumumab. *Cell Cycle*. 2011; 10(14):2331–2338. [PubMed: 21720213]
31. Katoh M, Katoh M. WNT Signaling Pathway and Stem Cell Signaling Network. *Clinical Cancer Research*. 2007; 13(14):4042–4045. [PubMed: 17634527]
32. Kato Y, Iwama A, Tadokoro Y, et al. Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis. *J Exp Med*. 2005; 202(1):169–179. [PubMed: 15998795]

33. Rouet V, Bogorad RL, Kayser C, et al. Local prolactin is a target to prevent expansion of basal/stem cells in prostate tumors. *Proc Natl Acad Sci U S A*. 2010; 107(34):15199–15204. [PubMed: 20699217]
34. Hernandez-Vargas H, Ouzounova M, Calvez-Kelm FL, et al. Methylome analysis reveals Jak-STAT pathway deregulation in putative breast cancer stem cells. *Epigenetics*. 2011; 6(4)
35. McLean K, Gong Y, Choi Y, et al. Human ovarian carcinoma-associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production. *The Journal of Clinical Investigation*. 2011; 121(8):3206–3219. [PubMed: 21737876]
36. Li C, Wu JJ, Hynes M, et al. c-Met Is a Marker of Pancreatic Cancer Stem Cells and Therapeutic Target. *Gastroenterology*. 2011
37. Chen XD. Extracellular matrix provides an optimal niche for the maintenance and propagation of mesenchymal stem cells. *Birth Defects Res C Embryo Today*. 2010; 90(1):45–54. [PubMed: 20301219]
38. Wang YH, Li F, Luo B, et al. A side population of cells from a human pancreatic carcinoma cell line harbors cancer stem cell characteristics. *Neoplasia*. 2009; 56(5):371–378. [PubMed: 19580337]
39. Wechsler-Reya RJ. Caught in the matrix: how vitronectin controls neuronal differentiation. *Trends Neurosci*. 2001; 24(12):680–682. [PubMed: 11718852]
40. Salaszyk RM, Williams WA, Boskey A, et al. Adhesion to Vitronectin and Collagen I Promotes Osteogenic Differentiation of Human Mesenchymal Stem Cells. *J Biomed Biotechnol*. 2004; 2004(1):24–34. [PubMed: 15123885]
41. Huang M, Anand S, Murphy EA, et al. EGFR-dependent pancreatic carcinoma cell metastasis through Rap1 activation. *Oncogene*. 2011
42. Klemke RL, Yebra M, Bayna EM, et al. Receptor tyrosine kinase signaling required for integrin alpha v beta 5-directed cell motility but not adhesion on vitronectin. *J Cell Biol*. 1994; 127(3): 859–866. [PubMed: 7525598]
43. Ryschich E, Khamidjanov A, Kerkadze V, et al. Promotion of tumor cell migration by extracellular matrix proteins in human pancreatic cancer. *Pancreas*. 2009; 38(7):804–810. [PubMed: 19893454]
44. Ricono JM, Huang M, Barnes LA, et al. Specific cross-talk between epidermal growth factor receptor and integrin alphavbeta5 promotes carcinoma cell invasion and metastasis. *Cancer Res*. 2009; 69(4):1383–1391. [PubMed: 19208836]
45. Cirulli V, Beattie GM, Klier G, et al. Expression and function of alpha(v)beta(3) and alpha(v)beta(5) integrins in the developing pancreas: roles in the adhesion and migration of putative endocrine progenitor cells. *J Cell Biol*. 2000; 150(6):1445–1460. [PubMed: 10995448]
46. Pons S, Marti E. Sonic hedgehog synergizes with the extracellular matrix protein vitronectin to induce spinal motor neuron differentiation. *Development*. 2000; 127(2):333–342. [PubMed: 10603350]
47. Kaido T, Perez B, Yebra M, et al. Alphav-integrin utilization in human beta-cell adhesion, spreading, and motility. *J Biol Chem*. 2004; 279(17):17731–17737. [PubMed: 14766759]
48. Dechantsreiter MA, Planker E, Matha B, et al. N-Methylated cyclic RGD peptides as highly active and selective alpha(V)beta(3) integrin antagonists. *J Med Chem*. 1999; 42(16):3033–3040. [PubMed: 10447947]
49. Paolillo M, Russo MA, Serra M, et al. Small molecule integrin antagonists in cancer therapy. *Mini Rev Med Chem*. 2009; 9(12):1439–1446. [PubMed: 19929817]
50. Friess H, Langrehr JM, Oettle H, et al. A randomized multi-center phase II trial of the angiogenesis inhibitor Cilengitide (EMD 121974) and gemcitabine compared with gemcitabine alone in advanced unresectable pancreatic cancer. *BMC Cancer*. 2006; 6:285. [PubMed: 17156477]
51. Badea L, Herlea V, Dima SO, et al. Combined gene expression analysis of whole-tissue and microdissected pancreatic ductal adenocarcinoma identifies genes specifically overexpressed in tumor epithelia. *Hepatogastroenterology*. 2008; 55(88):2016–2027. [PubMed: 19260470]
52. Logsdon CD, Simeone DM, Binkley C, et al. Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. *Cancer Res*. 2003; 63(10):2649–2657. [PubMed: 12750293]

53. Segara D, Biankin AV, Kench JG, et al. Expression of HOXB2, a retinoic acid signaling target in pancreatic cancer and pancreatic intraepithelial neoplasia. *Clin Cancer Res.* 2005; 11(9):3587–3596. [PubMed: 15867264]
54. Buchholz M, Braun M, Heidenblut A, et al. Transcriptome analysis of microdissected pancreatic intraepithelial neoplastic lesions. *Oncogene.* 2005; 24(44):6626–6636. [PubMed: 16103885]
55. Collisson EA, Sadanandam A, Olson P, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med.* 2011; 17(4):500–503. [PubMed: 21460848]

Figure 1. Pancreatic cells are enriched for TICs in serum-free conditions

(A) The human pancreatic cell lines Panc1 (immortalized) and primary patient line, Panc 4.14, were cultured in sphere forming conditions utilizing highly specialized SCM-ITSKO (stem cell media) which enriches for a TIC population and compared to cells grown in adherent conditions. (B) qRT-PCR was performed on both Panc1 and Panc 4.14 cells (total and pancreatospheres) and TIC-associated cell surface markers and genes were measured. The assay was performed in triplicate, and all data was normalized to 18s rRNA expression. Log2 ratios of TIC (spheres) versus non-TIC (total) are shown, with standard error indicated. Bars in black represent Panc1 pancreatospheres and bars in grey represent Panc 4.14 pancreatospheres. qRT-PCR data was analyzed with an unpaired t-test using Holm-Sidak method with the criteria of statistical significance of $\alpha=5\%$, significance is indicated by “*”. (C) Both Panc1 and Panc 4.14 (total and pancreatospheres) were plated to determine if they were capable of anchorage-independent growth using the soft-agar assay. Colonies were stained with neutral red and counted using Gelcount Software. (D) Panc1 and Panc 4.14 cells (total and pancreatospheres) were subjected to whole genome microarray analysis using Agilent 4x44k arrays. To elucidate the pathways associated with upregulated genes in pancreatospheres, the data from the Agilent whole genome array analysis was

analyzed using Ingenuity Pathway Analysis (IPA). We analyzed the top 10 pathways associated with upregulated genes.

Figure 2. Pancreatospheres differentiate upon addition of human serum

(A) Morphology of pancreatospheres isolated from Panc1 and Panc 4.14 grown in SCM (left panels) and after exposure to 5% human serum (right panels). (B & C) Log2 values for fold-change of TIC associated markers and genes of Panc1 and Panc 4.14 pancreatospheres treated with human serum compared with SCM. qRT-PCR was done in triplicate, normalized to 18S with standard error shown. (D) Morphology of pancreatospheres grown on either bovine serum albumin-coated (BSA) (left panels) or VN-coated (right panels). (E & F): Log2 values for TIC associated markers and genes of Panc1 and Panc 4.14 cells grown on VN-coated plates compared with BSA-coated plates. qRT-PCR was performed in triplicate, normalized to 18s rRNA, and shown relative to the respective pancreatospheres grown on BSA-coated plates. qRT-PCR data was analyzed with an unpaired t-test using Holm-Sidak method with the criteria of statistical significance of $\alpha=5\%$, significance is indicated by “*”.

Figure 3. Promoting and blocking differentiation of PANC1 pancreatospheres

(A) Growth of pancreatospheres in the presence of human serum with the following additions, as indicated: RAD or RGD peptides. (B) Log2 values are shown for qRT-PCR performed in triplicate, normalized to 18s rRNA and shown relative to the respective pancreatospheres treated with human serum. Bars in white represent PANC1 cells treated with serum + RAD, bars in gray represent PANC1 cells treated with serum + RGD. (C) Growth of pancreatospheres in the presence of human serum with the following additions, as indicated, preimmune IgG, anti- $\alpha V\beta 3$ and anti- $\alpha V\beta 5$. (D) Log2 values are shown for the qRT-PCR performed in triplicate, normalized to 18s rRNA and shown relative to the respective pancreatospheres treated with human serum and preimmune IgG. Bars in gray represent PANC1 cells treated with serum + anti- $\alpha V\beta 3$, bars in gray represent PANC1 cells treated with serum + anti- $\alpha V\beta 5$. qRT-PCR data was analyzed with an unpaired t-test using Holm-Sidak method with the criteria of statistical significance of $\alpha=5\%$, significance is indicated by “*”.

Figure 4. Promoting and blocking differentiation of PANC1 pancreatospheres with Cilengitide
 (A) Growth of pancreatospheres in the presence of human serum with the addition of Cilengitide. (B) Log2 values are shown for qRT-PCR performed in triplicate, normalized to 18s rRNA and shown relative to the respective pancreatospheres treated with human serum. Bars in white represent PANC1 cells treated with serum + 2.5 μ M Cilengitide and bars in gray represent PANC1 cells treated with serum + 5 μ M Cilengitide, respectively. qRT-PCR data was analyzed with an unpaired t-test using Holm-Sidak method with the criteria of statistical significance of $\alpha=5\%$, significance is indicated by “*”. (C) Oncomine analysis in pancreatic cancer studies comparing the gene expression level of ITGAV in normal versus pancreatic carcinoma and pancreatitis. The expression level of ITGAV (VN receptor) is statistically significant (p-value=5.37E-12). (D) Oncomine analysis in pancreatic studies focusing on pancreatic intraepithelial neoplasia, high PanIN grade and high grade pancreatic ductal adenocarcinoma. The expression level of ITGAV is statistically significant (p-value=0.015). (www.oncomine.org)