Cancer Stem Cell Marker Phenotypes Are Reversible and Functionally Homogeneous in a Preclinical Model of Pancreatic Cancer

Joseph S. Dosch¹,², Elizabeth K. Ziemke¹,², Amrith Shettigar¹,², Alnawaz Rehemtulla²,³, and Judith S. Sebolt-Leopold¹,²,⁴

Abstract

Survival rates associated with pancreatic cancer remain dismal despite advancements in detection and experimental treatment strategies. Genetically engineered mouse models of pancreatic tumorigenesis have gained considerable attention based on their ability to recapitulate key clinical features of human disease including chemotherapeutic resistance and fibrosis. However, it is unclear if transgenic systems exemplified by the KrasG12D/Tp53R172H/Pdx-1-Cre (KPC) mouse model recapitulate the functional heterogeneity of human pancreatic tumors harboring distinct cells with tumorigenic properties. To facilitate tracking of heterogeneous tumor cell populations, we incorporated a luciferase-based tag into the genetic background of the KPC model. We isolated pancreatic cancer cells from multiple independent tumor lines and found that roughly 1 out of 87 cells exhibited tumorigenic capability. Notably, this frequency is significantly higher than reported for human pancreatic adenocarcinomas. Cancer stem cell (CSC) markers, including CD133, CD24, Sca-1, and functional Aldefluor activity, were unable to discriminate tumorigenic from nontumorigenic cells in syngeneic transplants. Furthermore, three-dimensional spheroid cultures originating from KPC tumors did not enrich for cells with stem-like characteristics and were not significantly more tumorigenic than cells cultured as monolayers. Additionally, we did not observe significant differences in response to gemcitabine or salinomycin in several isolated subpopulations. Taken together, these studies show that the hierarchical organization of CSCs in human disease is not recapitulated in a commonly used mouse model of pancreatic cancer and therefore provide a new view of the phenotypic and functional heterogeneity of tumor cells. Cancer Res; 75(21); 4582-92. ©2015 AACR.

Introduction

It is commonly accepted that cancers are comprised of heterogeneous cell populations that are often defined by differences in the expression of cell surface markers, activity of key enzymes and transcription factors, or quiescent versus active states. One model of tumor heterogeneity suggests that tumor cells follow a hierarchical organization in which tumorigenic cells, termed cancer stem cells (CSC), differentiate and give rise to nontumorigenic cells within the tumor. The use of FACS has made it possible to separate distinct populations of tumor cells on the basis of cell surface markers or specific cellular activity in order to test for subpopulations that have increased tumorigenic potential. This approach has been used to identify CSC populations in leukemia (2) and solid tumors of the breast (3), colon (4), brain (5), and pancreas (6, 7). Thousands of bulk tumor cells were needed to re-establish these tumors as opposed to only 100 cells or fewer for specific CSC subsets, suggesting that the cells responsible for tumor formation are rare. It has also been reported that CSCs are resistant to chemotherapy and radiation and are directly involved in metastasis (5, 8, 9).

An alternative view of cancer heterogeneity is a stochastic model in which there exists significant plasticity in the CSC phenotype that is driven by intrinsic factors or dynamic changes in the tumor microenvironment. Investigations of breast cancer models have led to the identification of conditions where normal and non-neoplastic breast epithelial cells can convert to cells with a cancer stem–like phenotype either spontaneously or by induction of epithelial-to-mesenchymal transition (10). For some human cancers, including melanoma and acute myeloid leukemia, tumorigenic potential was found to be common (1:4 and 1:10 cells, respectively) and not driven by rare subpopulations of cells (11, 12). High tumor-initiating frequencies have also been observed in various genetically engineered mouse models (GEMM) of cancers, including melanoma (13) and peripheral neural sheath tumors (14). Irrespective of whether tumorigenic cells are rare or common, a better understanding of tumor heterogeneity is critical to the development of therapies to eradicate all tumor cells.

¹Translational Oncology Program, University of Michigan Medical School, Ann Arbor, Michigan. ²Department of Radiology, University of Michigan Medical School, Ann Arbor, Michigan. ³Department of Radiation Oncology, University of Michigan Medical School, Ann Arbor, Michigan. ⁴Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Judith S. Sebolt-Leopold, University of Michigan, 1600 Huron Parkway NCRC 520-1365, Ann Arbor, MI 48109. Phone 734-615-7326; Fax: 734-764-9017; E-mail: jssl@med.umich.edu

doi: 10.1158/0008-5472.CAN-14-2793
©2015 American Association for Cancer Research.
Mouse models of pancreatic ductal adenocarcinoma (PDAC) have been critical in advancing our understanding of the genetic pathways responsible for initiating and driving progression of the disease. Multiple lines of evidence suggest that a GEMM of pancreatic cancer, LSL-Kras\(^{G12D}\)/\(^{+}\), LSL-Trp53\(^{R172H}\)/\(^{-}\); Pdx-1-Cre (KPC), might be an ideal model system for the disease as these animals recapitulate many of the clinical features, histopathology, therapy resistance, and invasive nature of the human disease that are not consistently observed in patient-derived xenograft models (15–17). Although KPC-derived tumors appear to mimic the phenotype of human PDAC, it is unknown if this model may have lost the hierarchal structure owing to expression of mutant Kras\(^{G12D}\) and Trp53\(^{R172H}\) in pancreatic epithelial cells at early embryonic stages unlike the spontaneous tumors that arise in adult human disease. The tumorigenic potential of human PDAC cells transplanted into immune-compromised mice has been reported to range from 1/2,500 to 1/7,700 bulk cells (6, 18). In addition, human pancreatic CSCs were enriched with a mixture and injected with a single i.p. dose of 150 mg/kg \(\alpha\)-luciferin (Promega). At 10 minutes after luciferin injection, images were acquired for 1 to 60 seconds using an IVIS imaging system (Perkin Elmer). Images and region of interest values were obtained using Living Image software (Perkin Elmer). Changes in KPC animal bioluminescence were plotted as fold change from day 1 of imaging (Initial BL) to images collected just prior to euthanasia for end-stage disease symptoms. Bioluminescence images were also collected from adult Pdx1/Luc control animals from 12 to 24 weeks for comparison.

**Flow cytometry analysis**

Single-cell suspensions were prepared from excised KPC tumors using Mouse Tumor Dissociation Kits (Miltenyi Biotec). Tumor digests were passed over 40 µm nylon cell strainers (BD Biosciences) and washed 2× with Hank’s Balanced Salt Solution containing 2% FBS, followed by lysis of red blood cells in ACK lysis buffer (Invitrogen). Experimental details on the antibodies and reagents used for cell sorting analysis can be found in Supplementary Materials. Sorting was performed at the University of Michigan Flow Cytometry Core using an iCyt sy3200 instrument (Sony Biotechnology). Images and FACS data were analyzed using the WinList software (Verity Software House).

**Limiting dilution assays**

Viable and lineage-depleted KPCL cells were isolated by FACS and resuspended 1:1 in DMEM/F12 and Matrigel (BD Biosciences). Cells were injected subcutaneously in syngeneic littermates at 1,000, 100, and 10 cells per injection. Animals were monitored by palpation twice weekly and in some cases by bioluminescence imaging for at least 10 weeks following injection to detect tumor formation. Tumors were counted as detectable at 50 mm\(^3\) in calculated size using the formula: tumor volume = \((\text{length} \times \text{width}^2)/2\). For orthotopic injections, an incision was made in the left abdominal side, the tail region of the pancreas was exteriorized, and the appropriate cell suspension was injected via 29G syringe. Successful injection was verified by the appearance of a wheal at the injection site with no leakage through the pancreatic capsule. The abdominal wall was sutured with absorbable 4.0 Vicryl suture (Ethicon), and the skin was closed with wound clips. Mice were euthanized at the indicated time points. The frequency of tumorigenic cells and the 95% confidence interval were calculated using ELDA (Extreme Limiting Dilution Analysis; ref. 27).

**Materials and Methods**

**KPCL mouse model establishment**

Characteristics of the genetically engineered mouse strain, "KPC" that carries the Pdx-1-Cre, Kras\(^{G12D}\), and Trp53\(^{R172H}\) alleles, have been described previously (22). B6.FVB-Tg(p1f1-cre)1Tuv (Pdx-1-Cre; ref. 23), B6.129-Kras\(^{G12D}\)/\(^{-}\) (LSL-Kras\(^{G12D}\); ref. 24), and 129S4-Trip53\(^{R172H}\) (Trip53\(^{R172H}\); ref. 25) mice were interbred to obtain LSL-Kras\(^{G12D}\)/\(^{-}\); LSL-Trp53\(^{R172H}\)/\(^{-}\); Pdx-1-Cre triple-mutant animals on a mixed 129/ FVB/C57BL/6 background. To noninvasively track tumor growth, the LSL-ROSA2\(^{Mm8}\)/\(^{-}\) luciferase allele (26) was introduced to construct KPCL quadruple transgenic mice. At the onset of distress and/or abdominal distension, animals were sacrificed and tumors were excised from normal pancreatic tissue. Passage 0 (P0) tumors were passaged subcutaneously in 6- to 8-week-old Pdx-1-Cre-negative littermates. KPCL tumors were passaged no more than 3 times, and tumor material was collected and banked at each passage. All studies were conducted in compliance with the University of Michigan’s Committee guidelines on the Use and Care of Animals.

**In vivo imaging of luciferase activity**

Mice were anesthetized using a 2% to 2.5% isoflurane/air mixture and injected with a single i.p. dose of 150 mg/kg \(\alpha\)-luciferin (Promega). At 10 minutes after luciferin injection, images were acquired for 1 to 60 seconds using an IVIS imaging system (Perkin Elmer).
Histology
Pancreatic tissues from KPC and KPCL mice were fixed over-night in 10% neutral-buffered formalin, embedded in paraffin, and sectioned. Embedding and sectioning were performed by the University of Michigan Cancer Center Histopathology Core. Hematoxylin and eosin (H&E), Periodic Acid Staining (PAS), and Masson’s trichrome staining were done with assistance from the lab of Marina Pasca di Magliano and were performed as previously described (28). Images were taken with a Nikon E-800 microscope, Olympus DP71 digital camera, and DP Controller software.

Clonogenic assays
KPCL cells were dissociated and stained at 1:50 with allopbycicocyanin (APC)–anti-mouse CD133 (eBioscience; clone: 13A4) for 20 minutes on ice. Only viable CD133+ or CD133− cells were sorted into 96-well, white-walled, clear-bottom plates (Corning). Cells were deposited using the automatic cell deposition unit at 1 cell/well using an iCyt sy3200 (Sony Biotechnology Inc.). Assessment of positive colony formation was performed by microscopic evaluation and by bioluminescence imaging on an Envision plate reader (Perkin Elmer).

Chemosensitivity assays
Isolated KPCL subpopulations or bulk-sorted cells (2.5 x 10^5) were plated in 96-well, white-walled clear-bottom plates and allowed to adhere for 24 hours. Cells were then treated with increasing doses of gemcitabine (Selleck) or salinomycin (Selleck). After 72 hours, viability was assessed by addition of Cell Titer Glo (Promega), and luminescence data were collected using an Envision plate reader (Perkin Elmer). Dose response curves were generated using Prism software (GraphPad).

Statistical analysis
Survival analysis of KPCL versus KPC animals using log-rank test and t test of clonogenic assays was performed using Prism Software (GraphPad). For limiting dilution analysis and calculation of tumor-initiating frequency rates, ELDA program was used (27).

Results
Establishment of a bioluminescence mouse model of PDAC
To facilitate noninvasive assessment of pancreatic disease progression, we engineered a well-characterized mouse model of PDAC to express a bioluminescence transgene. The KPC mouse model of PDAC utilizes a Pdx1-Cre transgenic strain to activate expression of mutant Kras^{LSL-G12D} and Trp53^{R172H} alleles in early pancreatic and gut endoderm progenitor cells (22). These animals recapitulate the stages of pancreatic cancer development, including the formation of precursor pancreatic intraepithelial neoplasms (PanIN), progression to adenocarcinoma, and metastatic spread to the liver and lungs (22). We developed a quadruple cross using Pdx1-Cre (23), LSL-Kras^{G12D/+} (24), LSL-Trp53^{R172H/+} (25), and LSL-RosA2Luc^{LSL-Luc/+} (26) to obtain "KPCL" bioluminescent reporter animals (Fig. 1A). Coexpression of firefly luciferase in the pancreatic epithelium along with mutant Kras and Trp53 enabled us to noninvasively track progression of the disease over time for individual animals using a Xenogen IVIS imaging system.

Consistent with previous reports, KPCL animals began to develop varying grades of PanIN lesions between 8 and 12 weeks of age (Fig 1B and C). Advanced disease symptoms were apparent at 18 weeks on average, and included cachexia, abdominal distension with ascites from as early as 7 weeks to as late as 36 weeks. Median survival was not statistically different between KPC and KPCL mice (Supplementary Fig. S1A and S1B). Histologic analysis of KPCL tumors revealed a mixture of differentiated ductal lesions and undifferentiated cells surrounded by dense collagen-rich fibrosis, consistent with the strong desmoplastic reaction observed in human PDAC (Fig. 1D). Most ductal lesions exhibited luminal budding and were highly positive for the accumulation of mucins, confirmed by PAS staining (Fig. 1E).

Assessment of pancreatic bioluminescence in KPCL animals following intraperitoneal injection of α-luciferin was compared at initial time points (4–12 weeks old) and measured weekly until the time at which individual KPCL animals exhibited end-stage disease symptoms. Bioluminescence from animals with only Pdx1-Cre/ROSA2Luc^{LSL-Luc/+} (Pdx1/Luc) transgenes was also measured over time as a control. As expected, the signal intensity from the pancreas of KPCL animals significantly increased [48.0 ± 9.0 fold (n = 6)] at the terminal disease stage, whereas pancreatic bioluminescence in Pdx1/Luc control animals did not change significantly with up to 12 weeks of imaging (Fig. 1F and G). However, consistent bioluminescence measurements were difficult to obtain as we often observed plateaus or decreases in bioluminescence signal during tumor progression (Supplementary Fig. S1C). Upon necropsy of KPCL animals with end-stage disease, we observed cystic lesions in 4 of 7 KPCL tumors that were not consistently observed in KPC animals (Fig. 1H). These cystic structures, along with tumor necrosis and poor perfusion of the tumor as the disease progressed, typical for KPC tumors (15), affected our ability to obtain consistent bioluminescence measurements for tracking tumor progression in KPCL animals. Overall, background bioluminescence emitting from normal pancreatic epithelium compounded by the physical characteristics of this tumor model limits the utility of bioluminescence imaging to track changes in nascent KPCL tumor development as a reliable system for disease staging.

Tumorigenic potential of KPCL cells is high
Despite imaging limitations, the KPCL model could prove informative to investigate whether tumors arising from this disease model system mimic the hierarchal phenotype of cancer stem-like cells found in human PDAC (6). As tumorigenic potential and progression could be altered by defects in the host immune system, the tumor-initiating frequency of KPCL cells was assessed in an immune-competent background by using syngeneic littermates as hosts. To ensure that normal mouse pancreatic cells were excluded in our analysis, primary tumor fragments from separate KPCL tumors were initially passaged in syngeneic littermates. These tumors were histologically identical to the parent tumor and were devoid of any remnant normal pancreatic endocrine or exocrine cells (data not shown). Five individual KPCL allografts and one KPC allograft were dissociated and depleted of cells expressing hematopoietic (CD5, CD45R (B220), CD11b, anti-Gr-1, Ly-6G/C, 7–4, and Ter-119), vascular markers (CD31), and nonviable cells (DAPI) using flow cytometry (Fig. 2A). Cells were then injected subcutaneously in limiting dilutions into syngeneic littermates that were negative for mutant Kras^{G12D/+} and Trp53^{R172H/+} alleles. Palpable tumors from the injection of
Tumor-Initiating Cells are Common in KPC Mouse Model

1,000 cells were detected as early as 15 ± 4 days after injection. Monitoring of animals continued for at least 10 weeks after transplantation of KPC tumor cells; however, all arising tumors appeared <5 weeks after implantation. Every KPC allograft tested exhibited a high frequency of tumorigenic cells ranging from 1:27 to 1:199, with an average frequency of 1:87 cells (Table 1). KPC cells lacking the LSL-ROSA26<sup>Luc</sup> transgene were also highly tumorigenic (1:59 cells), indicating that luciferase expression does not significantly affect tumorigenicity.

To explore whether the high rate of tumor-initiating frequency was a function of the site of implantation, an orthotopic limiting dilution assay was carried out using cells derived from a KPC allograft. In line with our previous experiments, viable, lineage, and vascular-depleted cells were isolated from a KPC allograft and injected in limiting dilutions into the pancreas of syngeneic littersmates. Bioluminescence imaging enabled tracking of tumor growth in these animals (Fig. 2B). We observed 5 of 5 animals at 1,000 cells, 4 of 5 animals at 100 cells, and 3 of 5 animals with tumors at 10 cells injected at the terminal endpoints. The rate of tumor-initiating frequency for subcutaneous implantation of the same KPC tumor line (1:29) versus orthotopic implantation (1:37) was not significantly different (P value 0.76). These data demonstrate that the tumorigenic potential of KPC cells is high and not dependent on the site of implantation for determining read-out of tumor-initiating frequency.

Tumor-initiating cells in KPC tumors are phenotypically heterogeneous

We next sought to identify candidate CSC populations in KPC tumors based on cell surface and functional markers previously identified in pancreatic cancer CSC studies, including CD44, CD24, c-Met, CD133, Sca-1, and Aldefluor activity (6, 7, 19, 29, 30). Four of these markers, CD133, CD24, Sca-1, and Aldefluor activity, were heterogeneously expressed in our KPC allografts (Supplementary Fig. S2). However, CD44 expression was nearly uniform (>90%), in agreement with a previous study (29).

In contrast, c-Met expression was nearly undetectable (<1%) in most KPC allografts. Based on these uniformly high or low expression patterns, CD44 and c-Met were excluded from additional functional studies.
To evaluate the tumor-initiating frequency of each marker population, KPCL allograft tumors were dissociated, depleted of dead/lineage/CD31− cells, and separated by flow cytometry based on varying levels of CD133, CD24, Sca-1, and Aldehyfluor activity. Isolated subpopulations were then injected in limiting dilutions in syngeneic littermates to determine the tumor-initiating frequency of each marker group (Table 2). Recent reports have indicated that CD133 (Prominin-1), a marker of normal pancreatic ductal cells and linked to cells with a CSC phenotype in several human cancers, may also mark cells with CSC properties in KPC-derived tumors (31, 32). Interestingly, KP-4053 cells elicited a statistically significant increase rather than the anticipated decrease in tumorigenicity in the CD133− population (1:196) compared with CD133+ cells (1:879). Although not statistically significant, a similar trend was observed in the KPCL-3804 line upon implantation of CD133+ or CD133− cells (1.263 vs. 1.93, P value 0.13). Aldehyde dehydrogenase (ALDH) activity was also analyzed using the ALDEFLUOR reagent, whereupon we were able to determine the distribution of KPCL tumor cells with high or low Aldehyfluor activity. Specific activity was confirmed by treatment of an identical tumor cell sample with diethylamino-benzaldehyde (DEAB), a specific ALDH inhibitor. For both KPC-4053 and KPCL-3887 allografts, ALDHhigh and ALDHlow subpopulations were equally tumorigenic. We also carried out in vivo studies for CD24 as well as Sca-1, a marker associated with identifying tumor cells with increased metastatic capability in KPC-derived tumors (33). Again, for each of these markers, CD24+/− and Sca-1+/− groups were equally tumorigenic in each allograft that we tested.

The ability of distinct tumorogenic cell populations to give rise to the heterogeneity of the original tumor is another distinguishing feature of CSCs. To test if different marker subpopulations gave rise to phenotypically different tumors, we compared the marker expression and histologic phenotype of the secondary tumors to the original parent tumor for CD133+/− and ALDHhigh/low-derived tumors. Both CD133+ and CD133− KPC-4053 tumor cells resulted in tumors with a mixed ductal and undifferentiated histologic phenotype that was consistent with the parent tumor (Fig. 3A). Likewise, ALDHhigh and ALDHlow cells from the KPCL-3887 allograft displayed an undifferentiated tumor phenotype that was consistent with the parent allograft.

Table 1. Tumor-initiating cell frequency of mouse pancreatic tumor cells isolated from individual KPC and KPCL allografts.

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Number of tumors/number of injected animals</th>
<th>Tumor-initiating cell frequency (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPC-4053</td>
<td>5/5</td>
<td>157 (1/22/1/522)</td>
</tr>
<tr>
<td>KPL-4277</td>
<td>4/5</td>
<td>199 (1/69/1/576)</td>
</tr>
<tr>
<td>KPC-3887</td>
<td>5/5</td>
<td>127 (1/42/1/385)</td>
</tr>
<tr>
<td>KPC-3900</td>
<td>4/4</td>
<td>127 (1/8/1/89)</td>
</tr>
<tr>
<td>KPC-3804</td>
<td>5/5</td>
<td>129 (1/70/1/84)</td>
</tr>
<tr>
<td>Total</td>
<td>27/28</td>
<td>187 (1/57/1/33)</td>
</tr>
</tbody>
</table>
Tumor-Initiating Cells are Common in KPC Mouse Model

In addition, analysis of CD133 expression or Aldefluor activity from secondary tumors revealed that each of these tumors contained both CD133+/− or ALDHhigh/low cells regardless of which subpopulation was used to initiate the tumor (Fig 3B and D). These results indicate that these phenotypes are reversible within KPC tumor subpopulations. Overall, we were unable to find a marker that differentiated between tumorigenic and non-tumorigenic KPC and KPCL tumor cells. Furthermore, for the markers that we analyzed, each subpopulation had the ability to recapitulate the heterogeneity of the parent allograft.

**Spheroid-forming ability does not reflect increased tumor-initiating properties of KPC cells**

The ability of cells to initiate spheroid growth in suspension culture is often used as an in vitro surrogate assay to define stem-like characteristics of CSC populations and enrich for cells with high tumorigenic capacity (34). As we were unable to identify a specific marker to enrich for tumorigenic cells, we aimed to utilize functional properties of CSCs to observe changes in tumorigenic capacity. Primary cell lines were first established from several KPCL tumors followed by serial passaging in spheroid culture to explore whether three-dimensional (3D) culture increased the ability of these cells to self-renew. KPCL cells grown as monolayers in 10% FBS supplemented media from two cell lines were dissociated and plated in 3D culture in defined serum-free media, including methylcellulose to promote clonal spheroid formation (Fig 4A). Cells were then serially cultured for several passages to assess changes in spheroid self-renewal. Ability to form spheroids did not change significantly for KPCL-3297 cells but varied highly across several passages of the KPC-4053 line (Fig 4B).

The expression of CSC markers was then compared in cells grown either as monolayers or as tumorspheres. CD133 expression was upregulated in KPC and KPCL spheres compared with the matching adherent cell line and was homogeneous for the expression of CD44 (Fig. 4C). Aldefluor activity was not significantly changed in monolayer versus spheroid culture (data not shown). Although CD133 expression did not discriminate KPCL cells with enriched tumor-initiating frequency, the upregulation of CD133 in 3D spheroid culture could indicate that CD133 expression identifies cells with CSC properties and increased spheroid formation in vitro. Single-cell clonogenic assays were performed, but in both KPCL cell lines we tested, there was not a clonogenic advantage between CD133+ or CD133− cells (Fig. 4D). As we were unable to identify a cell surface or functional marker that defined tumorigenic versus nontumorigenic cells, we investigated whether spheroid cultures of KPCL tumor cells would enrich for cells with increased tumorigenic capacity compared with adherent culture. KPCL cells were cultured concurrently as either monolayers or tumorspheres. Cells harvested from both growth conditions were then injected subcutaneously in limiting dilutions in syngeneic mice. Tumor growth was monitored for at least 10 weeks. No statistically significant difference in the rate of tumor formation was seen when comparing the incidence of tumors arising from cells grown as monolayers versus tumorspheres (Fig. 4E). These results indicate that spheroid formation alone is not a reliable assay for enriching for tumor cells with cancer stem-like properties in KPCL tumors.

**Sensitivity of KPCL subpopulations to chemotherapy**

One of the key features of CSCs is the ability to resist chemotherapy (35, 36). Regardless of tumorigenic frequency, the possibility remained that certain subpopulations of KPCL tumors could prove to be more resistant than others to chemotherapy treatment. To explore this possibility, the response of KPCL subpopulations to gemcitabine, a chemotherapeutic drug commonly used in the clinic to treat pancreatic cancer, and salinomycin, an antimicrobial agent shown to have specific anti-CSC effects in breast and pancreatic cancer, was evaluated (37, 38). Unselected and differential cell subsets, including CD133+/−, ALDHhigh/low, and Sca-1+/− were sorted, plated, and exposed to increasing doses of each drug. We were unable to detect a significant difference in dose response curves between the sorted subpopulations irrespective of marker and agent (Fig. 5).

**Discussion**

The development of effective therapies to treat pancreatic cancer hinges upon our ability to replicate the complex
heterogeneity found in human tumors in preclinical mouse model systems to better understand why current treatment regimens fail. Compelling evidence from studies with GEMMs of pancreatic cancer, specifically the KPC model, suggests that these animals replicate the desmoplasia, poor tissue perfusion, and resistance to chemotherapy that is characteristic of human PDAC (15). This has led to the development of drug trials using KPC animals to stage and simulate human clinical trials for evaluating treatments that either stop the advancement of premalignant disease or treat established tumors (39). Staging for these trials requires careful ultrasound imaging techniques, and although these procedures are effective, they are often time consuming (40). The present study was undertaken to incorporate a bioluminescence reporter into the KPC background to facilitate tumor staging and improve throughput. A predicted drawback of this system is the likelihood that Pdx-1-Cre recombination initiates

Figure 3.
Marker and histologic analysis of secondary tumors from CD133 and ALDH subpopulations. A and C, flow cytometry analysis of KPC-4053 and KPCL-3887 allograft cells stained for mouse anti-CD133 or Aldefluor activity and sorted for CD133^+/ or ALDH^{high/low} cells for secondary tumor implantation. H&E staining displays the histologic phenotype of secondary tumors resulting from either CD133^+/ or ALDH^{high/low} cells (×20). B and D, flow cytometry analysis of secondary tumors resulting from CD133^+/ or ALDH^{high/low} implanted cells. Representative dot blots are shown. Dead, hematopoietic, and vascular cells were excluded by DAPI incorporation, staining for lineage markers and CD31, respectively.
expression of luciferase in the progenitor cells that give rise to the pancreatic epithelium, even if those cells do not contribute to tumorigenesis, thus resulting in high background noise. Although we did observe a rise in bioluminescence that was significantly greater than background in KPCL animals, these changes were often nonlinear with time and occurred shortly before the need to humanely euthanize the animals. This coupled with previously established variations in tumor latency (22), and the observation of cystic tumors, a phenotype observed by another laboratory (K.P. Olive; personal communication), does not support use of the KPCL model system as an improvement over the application of ultrasound imaging for disease staging. Nonetheless, the development of several independent luciferase-tagged PDAC tumors and matching cell lines should be immensely useful in facilitating preclinical experiments to probe the extent of heterogeneity that exists in KPC tumors arising in syngeneic littermates.

The present study was designed to focus on the heterogeneity of tumor-initiating cells, an area not well studied in this GEMM. Critical to the evaluation of tumorigenic potential in distinct cell subsets is their transplantation into recipient mice, which are often immunocompromised. There are several caveats to these experiments, including differences in microenvironment, injection conditions, and the level of immune defects in the mouse strain that can have significant effects on the read-out of tumorigenic potential (12). In the case of human PDAC, reports of tumor-initiating cells range from 1/11,217 (0.0089%) to between 1/2,500 (0.04%) and 1/18,000 (0.0055%) depending on the patient tumor and whether NOD/SCID or NSG mice are used for the implantation of tumor cells (6, 18). These studies suggest that cells with the capacity to reinitiate the tumor in human PDAC are infrequent. Our data indicate that bulk, unselected tumor cells in the KPC background are highly tumorigenic, with as few as 10 cells needed to recapitulate the parent tumor upon syngeneic engraftment. In addition, tumorigenic potential was not significantly affected by orthotopic reinjection of KPCL cells directly into the pancreas as opposed to subcutaneous

expression of luciferase in the progenitor cells that give rise to the pancreatic epithelium, even if those cells do not contribute to tumorigenesis, thus resulting in high background noise. Although we did observe a rise in bioluminescence that was significantly greater than background in KPCL animals, these changes were often nonlinear with time and occurred shortly before the need to humanely euthanize the animals. This coupled with previously established variations in tumor latency (22), and the observation of cystic tumors, a phenotype observed by another laboratory (K.P. Olive; personal communication), does not support use of the KPCL model system as an improvement over the application of ultrasound imaging for disease staging. Nonetheless, the development of several independent luciferase-tagged PDAC tumors and matching cell lines should be immensely useful in facilitating preclinical experiments to probe the extent of heterogeneity that exists in KPC tumors arising in syngeneic littermates.

The present study was designed to focus on the heterogeneity of tumor-initiating cells, an area not well studied in this GEMM. Critical to the evaluation of tumorigenic potential in distinct cell subsets is their transplantation into recipient mice, which are often immunocompromised. There are several caveats to these experiments, including differences in microenvironment, injection conditions, and the level of immune defects in the mouse strain that can have significant effects on the read-out of tumorigenic potential (12). In the case of human PDAC, reports of tumor-initiating cells range from 1/11,217 (0.0089%) to between 1/2,500 (0.04%) and 1/18,000 (0.0055%) depending on the patient tumor and whether NOD/SCID or NSG mice are used for the implantation of tumor cells (6, 18). These studies suggest that cells with the capacity to reinitiate the tumor in human PDAC are infrequent. Our data indicate that bulk, unselected tumor cells in the KPC background are highly tumorigenic, with as few as 10 cells needed to recapitulate the parent tumor upon syngeneic engraftment. In addition, tumorigenic potential was not significantly affected by orthotopic reinjection of KPCL cells directly into the pancreas as opposed to subcutaneous
Figure 5.
Drug sensitivity of KPC tumor cell subpopulations. CD133, Aldefluor, and Sca-1-positive or -negative cells or unsorted KPC cells were cultured in the presence of increasing concentrations of gemcitabine or salinomycin. After 72 hours, cell viability was assessed using CellTiter Glo reagent, and percent viability was normalized to vehicle-treated controls. Pair-wise multiple comparisons were performed with one-way ANOVA with Bonferroni adjustment. P values < 0.05 were considered statistically significant.

implantation. This suggests that factors driving tumor-forming potential are intrinsic to KPCL cells and not the injection location. Our findings are in line with observations in GEMMs of other cancers, including melanoma, leukemia, and malignant neural sheath tumors, demonstrating that syngeneic engraftment of tumor cells may reveal higher rates of tumorigenic potential (11, 13, 14). In addition, a recent report using cells isolated from an inducible KrasG12D/p53fl/fl/WT mouse model of PDAC indicated that bulk tumor cells are also highly tumorigenic (1:100) in agreement with our findings (32).

It is important to point out that the limiting dilution assays described in the present study were carried out on KPCL tumor cells derived from passaged tumor fragments and not directly from the spontaneous mouse PDAC tumor. Although this procedure was adopted to eliminate any remnant normal mouse pancreatic epithelial cells, we cannot rule out that the initial tumor establishment in syngeneic allografts may select for the most tumorigenic cells, thereby leading to higher rates of tumor-initiating cells in our model system. In addition, the KPC model differs from the development of human PDAC in some potentially important ways when considering tumorigenic potential. The expression of G12D mutant Trp53R172H is initiated at embryonic stages, while the development of human PDAC is thought to cover a span greater than 20 years as activating mutations to KRAS, silencing of p16/CDKN2A, and inactivation of TP53 and SMAD4 are accumulated (41, 42). Genetic sequencing of multiple geographic regions of primary human PDAC and metastatic sites suggests that heterogeneity may follow a clonal evolution model for the progression of the disease and even require different treatment strategies depending on the clone (41, 43, 44). Conversely, observations in mouse PDAC tumors following genetic ablation of mutant KrasG12D expression indicate that there are CD133+ tumor cells that survive with cancer stem–like properties, but are not driven by genetic clonal selection (32). Therefore, it may not be surprising to see significant variations in the frequency of tumor-initiating cells between human pancreatic tumors and mouse models of the disease due to complex differences in the origination of the tumor and the different selective pressures that influence tumor heterogeneity in each system. Nonetheless, we were unable to discriminate between tumorigenic and nontumorigenic KPCL tumor cells using cell surface and functional markers including CD133 that have been indicated for enriching CSCs in other reports (31, 32). It is possible that timing of mutant KrasG12D expression, different cell isolation conditions, or intercolony tumor heterogeneity may influence the expression of CSC markers, all adding to the difficulties encountered when comparing results among different laboratories.

In lieu of specific markers, we focused on testing functional characteristics attributed to CSCs, including the formation of clonal tumorsphere colonies and resistance to drug therapy. The ability of cells to grow as tumorspheres is widely used as a measure of CSC numbers and used to enrich for cells with increased tumorigenic capacity in the absence of specific markers; however, many question the validity of this technique in several tumor types (45). Our results indicate KPCL cells cultured as tumorspheres are not significantly more tumorigenic than the same cells grown in two-dimensional monolayer culture. These observations suggest that tumorsphere formation alone is not a reliable read-out of CSC activity in the KPC1 tumor model. We were further unable to identify a CSC marker subpopulation that responded differently to chemotherapy (gemcitabine) or a CSC-targeted therapy (salinomycin). Collectively, our data indicate that most KPCL tumor cells are highly tumorigenic, and by the measures done in the experiments described here, are functionally homogeneous.

In summary, we have observed that the incidence of tumorigenic cells is significantly more common in KPCL tumors than in human tumors. This does not mean that rare CSCs do not exist in human pancreatic tumors, but it identifies key differences between the model systems that should be taken into account when assessing therapeutic responses of CSC populations in GEMMs of this disease. The KPC model has indisputably proven invaluable in recapitulating many aspects of human disease, including histopathology, occurrence of
metastasis to clinically relevant sites, and genomic instability (46). However, it is important to note the differences in tumor hierarchy between human and mouse models as efforts to disrupt the tumor hierarchy in human PDAC may not be mirrored in the KPC animal model. Our findings offer important insights into the phenotypic and functional heterogeneity of tumor cells in this GEMM that should prove informative when utilizing this model to design studies directed against cancer stem–like cells in human pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J.S. Dosch, A. Rehemtulla, J.S. Sebolt-Leopold
Development of methodology: J.S. Dosch, J.S. Sebolt-Leopold
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.S. Dosch, E.K. Ziemke, A. Shettigar, J.S. Sebolt-Leopold
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.S. Dosch, E.K. Ziemke, A. Shettigar, J.S. Sebolt-Leopold
Writing, review, and/or revision of the manuscript: J.S. Dosch, J.S. Sebolt-Leopold

References


Tumor-Initiating Cells are Common in KPC Mouse Model

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.K. Ziemke, J.S. Sebolt-Leopold
Study supervision: J.S. Sebolt-Leopold

Acknowledgments

The authors thank Drs. Andrew Rhim, Eric Fearon, Kathy Luker, and Filip Bednar for critical review of the article. They also thank Dr. Kathleen Cho for the Rosa26LSL-Luciferase mice as well as Dr. Marina Pasca di Magliano for the KrasG12D mice. They are grateful to Michael Pihlaja and the UM Core Facility for their assistance with flow cytometry experiments. The expert technical assistance of Dr. Stephanie Galban for advice in animal breeding, along with Paul Keller and Nicole Allen for carrying out animal genotyping is also gratefully acknowledged.

Grant Support

This work was supported by a grant from the NIH (R01 CA155198; J.S. Sebolt-Leopold). J.S. Dosch was supported by University of Michigan Department of Radiology Training Grant (NIH T32 EB005172-03). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 19, 2014; revised July 2, 2015; accepted July 25, 2015. Published OnlineFirst September 10, 2015.
Cancer Stem Cell Marker Phenotypes Are Reversible and Functionally Homogeneous in a Preclinical Model of Pancreatic Cancer

Joseph S. Dosch, Elizabeth K. Ziemke, Amrith Shettigar, et al.


Updated version Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-2793

Cited articles This article cites 46 articles, 21 of which you can access for free at: http://cancerres.aacrjournals.org/content/75/21/4582.full#ref-list-1

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/75/21/4582. Click on “Request Permissions” which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.