

## Human Pancreatic Cancer Stem Cells: Implications for How We Treat Pancreatic Cancer

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### Abstract

Pancreatic cancer has the worst prognosis of any major malignancy, with an annual death rate that approximates the annual incidence rate. Delayed diagnosis, relative chemotherapy and radiation resistance and an intrinsic biologic aggressiveness all contribute to the abysmal prognosis associated with pancreatic cancer. Answers to the frustrating effort to find effective therapies for pancreatic cancer may be gained through a renewed perspective on tumorigenesis as a process governed by a select population of cells, termed cancer stem cells (CSCs). Cancer stem cells, like their normal counterparts, have the properties of self-renewal and multilineage differentiation and possess inherently heightened DNA damage response and repair mechanisms that make them difficult to eradicate. Initially discovered in leukemias, researchers have identified CSCs in several solid-organ malignancies including breast, brain, prostate, and colon cancers. We have recently identified a CSC population in human pancreatic cancers. These pancreatic CSC represent 0.5% to 1.0% of all pancreatic cancer cells and express the cell surface markers CD44, CD24, and epithelial-specific antigen. Pancreatic CSCs have been shown to be resistant to standard chemotherapy and radiation, and devising specific therapies to target this distinct cell population is likely needed to identify effective therapies to treat this dismal disease.

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### Introduction

Pancreatic cancer is a highly lethal disease, which is usually diagnosed in an advanced state for which there are little or no effective therapies. Attempts to better understand the molecular characteristics of pancreatic cancer have focused on studying gene and protein expression profiles of samples of pancreatic cancer. However, these studies have not taken into account the heterogeneity of cancer cells present within a particular tumor. Emerging data have shown that malignant tumors are quite heterogeneous and that they are composed of a small subset of distinct cancer cells (usually defined by cell surface marker expression) that are responsible for tumor initiation and propagation, termed cancer stem cells (CSCs), and of more differentiated cancer cells, which have very limited proliferative potential. These cells are called CSCs because, like their normal counterparts, they possess the ability to self-renew and produce differentiated progeny. Cancer stem cells have now been identified in several solid tumor types, including breast, brain, prostate, and colon cancers [1–5].

### Isolation of Pancreatic CSCs

The existence of CSCs was first observed in hematopoietic malignancies. More than a decade ago, John Dick's group at the University

of Toronto observed that rare subpopulations of cells within leukemia are able to generate leukemia in nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice [6]. These cells were discriminated by the surface marker expression CD34<sup>+</sup>CD38<sup>-</sup> and displayed properties of self-renewal and multilineage differentiation along with potent proliferative capacity. The technique of sorting cells based on cell surface marker expression and dilutional tumorigenesis assays assessing the ability of subsets of cancer cells to produce tumor xenografts in immunocompromised NOD-SCID mice was also applied to investigate the possible existence of solid-organ CSCs. Using these techniques, the first solid-organ CSC was isolated from breast cancers by Al-Hajj et al. [1]. Using the markers CD44, CD24, and epithelial-specific antigen (ESA), which are known to be

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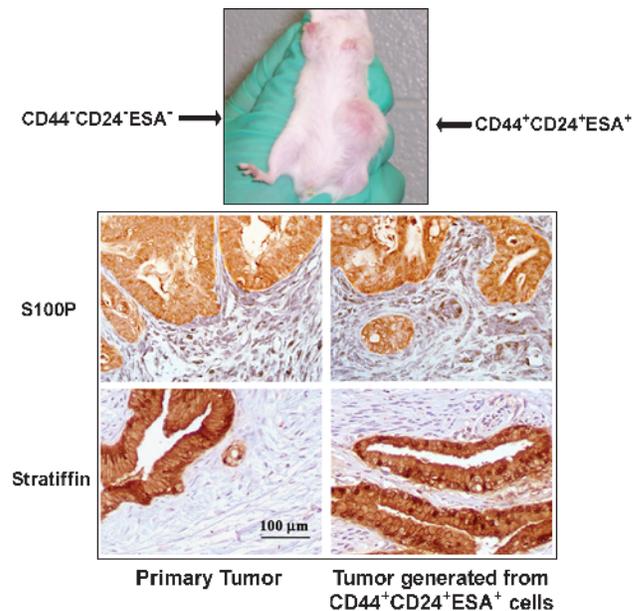
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intimately involved in cell–cell adhesion with multiple signaling functions [7–9], the investigators determined that the cancer cell population expressing the markers CD44<sup>+</sup>ESA<sup>+</sup>CD24<sup>-low</sup> was capable of re-creating a breast cancer with all its *in vivo* complexity with as few as 100 cells. Unsorted breast cancer cells and CD44<sup>+</sup>CD24<sup>+</sup> cells from breast cancer specimens did not contribute to *de novo* tumorigenesis [2]. Further studies of these breast CSCs showed activation of pathways that govern self-renewal in normal stem cells, including the hedgehog signaling pathway and the polycomb gene family member (BMI-1) [10].

As in breast cancer, FACS analysis revealed heterogeneous surface marker expression for CD44, CD24, and ESA among pancreatic tumor cells. To prove that distinct subpopulations were responsible for tumor initiation in pancreatic cancer, FACS-sorted pancreatic cancer cells were derived directly from patient’s primary tumors or primary tumors established as low-passage xenografts in NOD-SCID mice. Cells were sorted for the markers CD44, CD24, and ESA, both individually or in combination, and were injected into mice, and their tumorigenic potential was assessed. In a dose–response analysis of unsorted pancreatic cancer cells (10<sup>2</sup>–10<sup>4</sup>) injected per mouse, no tumor growth was evident at 16 weeks unless at least 10<sup>4</sup> cells were injected, where four of six mice developed tumors. For cancer cells sorted for the markers CD44, CD24, and ESA, the expression of these individual markers identified cell populations with enhanced tumorigenic potential (Table 1). Injection of cancer cells expressing dual marker combinations (CD44<sup>+</sup>ESA<sup>+</sup>, CD24<sup>+</sup>ESA<sup>+</sup>, and CD44<sup>+</sup>CD24<sup>+</sup>) resulted in further enhanced tumorigenic potential compared with single marker–sorted cells, with more tumors forming with injection of as few as 100 cells, and no tumors forming in marker-negative cells until at least 10<sup>3</sup> cells were injected. The sorted cell population with the highest tumorigenic potential expressed all of the markers CD44, CD24, and ESA, with CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells comprising only 0.2% to 0.8% of all pancreatic cancer cells. Six of 12 animals injected with 100 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells formed tumors, whereas cells negative for expression of these cell surface markers did not develop any tumors until 10<sup>4</sup> CD44<sup>-</sup>CD24<sup>-</sup>ESA<sup>-</sup> cells were injected, when only 1 of 12 animals developed a tumor (Table 1). Thus, pancreatic cancer cells expressing the cell surface markers CD44, CD24, and ESA had at least a 100-fold increased tumorigenic potential compared with nontumorigenic cells [11]. The percentage of pancreatic cancer cells expressing these cell surface markers in individual tumors was maintained on passaging in NOD-SCID mice.

CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells’ ability to recapitulate the primary tumor of origin in its entirety was verified by histology. Tumors derived from CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells were remarkably similar in appearance to the patient’s primary tumor and also had similar patterns of expression of the pancreatic adenocarcinoma markers S100P and stratifin (Figure 1). The FACS surface marker expression pattern of xenografts derived from CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells paralleled the phenotypic diversity characterized in the



**Figure 1.** Tumor formation in NOD-SCID mice injected with pancreatic cancer stem cells. Implantation of 500 CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic CSCs results in tumor formation (top panel). Expression of the proteins S100P (middle two panels) and stratifin (bottom two panels) is similar in the tumor generated from CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> cells and the patient’s primary tumor.

original tumor, further verifying that CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells have the capacity to both self-renew and produce differentiated progeny [11].

Another discriminating cell surface marker that enriches for populations of CSCs in both brain and colon cancer is CD133 [3–5]. In a study by Hermann et al. [12], they found that a small but distinct population of pancreatic cancer cells expresses CD133 and enriches for cells with potent tumor-initiating capacity. CD133<sup>+</sup> cells comprised 1% to 3% of pancreatic adenocarcinoma cells analyzed in their study. In comparison to CD133<sup>-</sup> and bulk pancreatic cancer cells, as few as 500 CD133<sup>+</sup> cells injected in immunocompromised mice generated xenografts with the phenotype of the primary pancreatic cancer of origin. Interestingly, their report states that there was approximately 14% overlap between CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> and CD133<sup>+</sup> cells [12]. These findings suggest that more than one set of specific cell surface markers may enrich for pancreatic CSC populations and that a more distinguishing expression marker or set of markers to identify pancreatic CSCs may yet to be discovered (Table 2).

### Self-renewal Pathways in Pancreatic CSCs and Therapeutic Implications

Self-renewal and differentiation potential are features of stem cells. Self-renewal allows normal stem cells to persist during the lifetime of the organism, and differentiation of stem cells provides the progenitors and mature cells for tissue genesis, maintenance, and regeneration after stress or injury. Pathways that are important for self-renewal in normal stem cells are often found to be dysregulated in human cancers [13].

**Table 1.** Tumor Formation Ability of Sorted Pancreatic Cancer Cells Using Cell Surface Markers.

Marker/Cell Number	10 <sup>4</sup>	10 <sup>3</sup>	500	100
Unsorted	4/6	0/6	0/3	0/3
CD24 <sup>+</sup> ESA <sup>+</sup>	6/8	5/8	5/8	2/8
CD24 <sup>-</sup> ESA <sup>-</sup>	2/8	1/8	0/8	0/8
CD44 <sup>+</sup> /CD24 <sup>+</sup> /ESA <sup>+</sup>	10/12	10/12	7/12	6/12
CD44 <sup>-</sup> /CD24 <sup>-</sup> /ESA <sup>-</sup>	1/12	0/12	0/12	0/12

**Table 2.** Cell Surface Markers Used to Identify Pancreatic CSCs.

1. CD44 <sup>+</sup> /CD24 <sup>+</sup> /ESA	Li et al. [11]
2. CD133 <sup>+</sup>	Hermann et al. [12]

Self-renewal signaling pathways that have been implicated in solid-organ malignancies include Notch, Wnt/ $\beta$ -catenin, phosphatase and tensin homologue deleted from chromosome 10, sonic hedgehog (SHH), and BMI-1 [10,14–18]. To assess whether upregulation of molecules important in self-renewal pathways occurred in pancreatic cancer cells, we performed real-time reverse transcription–polymerase chain reaction to assess the expression of developmental signaling molecules. We observed that the SHH transcript was elevated 4-fold in CD44<sup>−</sup>CD24<sup>−</sup>ESA<sup>−</sup> nontumorigenic pancreatic cancer cells and 46-fold in CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic cancer cells when compared with normal pancreatic epithelial cells (Figure 2). These data suggested that there is significant upregulation of SHH in pancreatic CSC that persists, albeit at a much lower level, in the differentiated progeny [11].

It has been shown in other studies that human pancreatic adenocarcinomas display increased activation of the hedgehog pathway [18,19]. Transgenic overexpression of SHH within the pancreas results in the development of pancreatic cancer precursor lesions, termed PanIN lesions, accumulation of genetic mutations commonly seen in pancreatic cancer, including *K-ras* mutations, and upregulation of *Her2/neu*. Inhibition of hedgehog signaling by cyclopamine inhibited pancreatic cancer growth both *in vitro* and *in vivo*, suggesting that this signaling pathway has an early and critical role in pancreatic cancer development.

Overexpression of the polycomb gene family member protein BMI-1 has been shown to contribute to the invasive potential of epithelial malignancies including breast, brain, colon, and oral cancers [20–23]. An important recent revelation was the finding that BMI-1 is crucial for the maintenance and self-renewal in both normal stem cells [24–26] and CSCs [10,27]. There are no published reports investigating the functional role of BMI-1 in pancreatic cancer. We observed that BMI-1 is significantly elevated in CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic CSCs compared to their differentiated CD44<sup>−</sup>CD24<sup>−</sup>ESA<sup>−</sup> pancreatic cancer cell counterparts, suggesting that BMI-1 may play a role in the maintenance of self-renewal in this pancreatic CSC population (Figure 2).

### The Role of Pancreatic CSCs in Tumor Metastasis

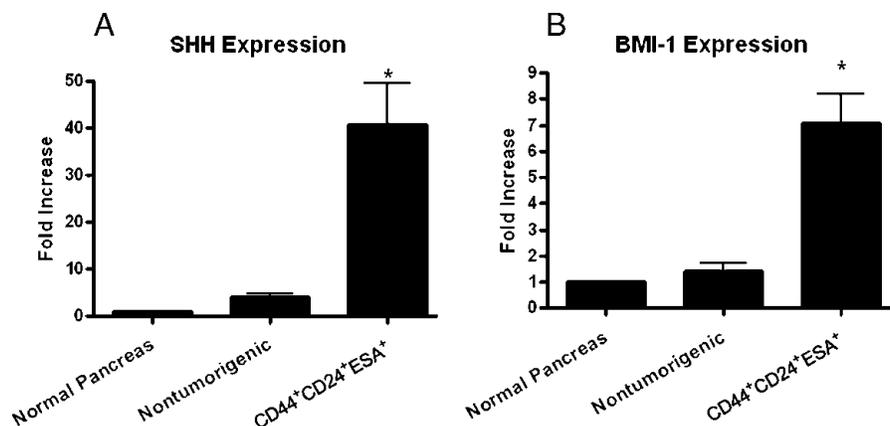
The cytokine receptor CXCR4 is expressed in hematopoietic stem cells and interacts with the ligand stromal cell–derived factor 1 (SDF-1) that is secreted by bone marrow stromal cells. This chemotactic signal-

ing cascade is responsible for the homing of hematopoietic cells to the bone marrow. The CXCR4 cytokine is also overexpressed in advanced and metastatic breast cancers and may play a key role in cancer cell mobility [28,29]. CXCR4 has also been found to be overexpressed in advanced pancreatic cancer samples [30]. Hermann et al. [12] recently explored the relationship between pancreatic CSCs and CXCR4 and found that CD133<sup>+</sup> pancreatic CSC present at the invading front of pancreatic cancers showed high expression levels of CXCR4. Interestingly, whereas CD133<sup>+</sup>CXCR4<sup>−</sup> and CD133<sup>+</sup>CXCR4<sup>+</sup> cells were able to form primary tumors equally, only CD133<sup>+</sup>CXCR4<sup>+</sup> cells were able to metastasize [12]. The results of this study suggest that there are stationary and migratory forms of pancreatic CSCs. Importantly, abrogation of CXCR4 signaling prevented metastasis in this tumor model. These findings may have clinical implications when considering strategies to inhibit metastasis of pancreatic CSCs.

### Therapeutic Consequences of Pancreatic CSCs

A practical consequence of CSC heterogeneity is that strategies to induce cell death to treat cancer must address the unique survival mechanisms of the CSC within the cancer cell population. Most traditional cancer treatments have been developed and assayed based on their ability to kill most of the cancer cell population and result in tumor shrinkage. These treatments likely miss the CSCs, which have been shown in several cancer types to be quite resistant to standard chemotherapy and radiation. A prediction of the CSC model is that treatments that target the CSC will be required to result in an effective cure of cancer. As such, tumor shrinkage is not going to be a useful parameter to measure effectiveness of CSC therapies, and approaches to measure CSC burden will need to be devised.

Studies in CD34<sup>−</sup>CD38<sup>−</sup> leukemic stem cells showed that these leukemic CSCs were significantly less sensitive to daunorubicin or cytarabine than bulk leukemic cells [31]. In glioblastoma, it was determined that the CSC population expressing CD133<sup>+</sup> in both primary tumors and xenografts was enriched two- to four-fold following ionizing radiation [32]. Enrichment of the CD133<sup>+</sup> brain CSC occurred due to inherent resistance of this distinct population to radiation-induced apoptosis. The ability of the CSC to resist apoptosis-induced radiation was conferred by the preferential activation of DNA damage response



**Figure 2.** Upregulation of developmental signaling molecules in pancreatic CSCs. mRNA expression of SHH (A) and BMI-1 (B) in normal pancreas, nontumorigenic CD44<sup>−</sup>CD24<sup>−</sup>ESA<sup>−</sup> pancreatic cancer cells, and highly tumorigenic CD44<sup>+</sup>CD24<sup>+</sup>ESA<sup>+</sup> pancreatic CSCs. Total RNA was quantitated by real-time reverse transcription–polymerase chain reaction. Data are expressed as the mean  $\pm$  SE. \* $P$  < .05 versus normal pancreas.

in these cells [32]. In a recent report, Hermann et al. [12] showed that CD133<sup>+</sup> pancreatic CSCs are resistant to standard chemotherapy.

Some studies suggest that Wnt/ $\beta$ -catenin signaling leads to increased tolerance of DNA damage, thus conferring radiation resistance of CSCs [33–35]. Wnt signaling activates the DNA damage response, and one transcriptional target of  $\beta$ -catenin signaling is survivin, which is known to promote cellular survival in pancreatic cancer in response to apoptotic stimuli such as ionizing radiation [36]. The complex nature of CSC survival mechanisms extends beyond Wnt/ $\beta$ -catenin signaling. Notch activity has also been implicated in breast cancer's response to radiation injury [37], and targeting this pathway has shown effective antitumor response in preclinical trials [38]. Alternatively, some studies suggest that the level of compaction of chromatin dictates accessibility to genomic DNA and subsequent mediation of DNA damage responses and that a looser configuration of chromatin in stem cells leads to accelerated DNA repair following injury. Such has been shown in embryonic stem cells that have lower levels of the chromatin structural protein histone-1. In a study by Murga et al. [39], embryonic stem cells with lower levels of histone-1, which results in less chromatin compaction, had enhanced recovery from DNA damage in comparison to differentiated cells. Pancreatic CSCs likely share some of the signaling cascades involved in cellular responses to DNA damage present in other stem cell systems; however, the specific responses and mechanisms involved in the chemotherapy and radiation resistance of pancreatic CSCs remain to be elucidated.

### Microenvironmental Cues

The stromal epithelial interactions that occur in the stem cell niche of cancers have profound implications in understanding the complex processes involved in tumor initiation, propagation, and metastasis. Pancreatic cancer is noted for its profound desmoplasia. Although CSCs have inherent defense mechanisms to radiation and chemotherapy, the robust desmoplasia in pancreatic cancer likely provides a milieu for additive resistance. Supporting this possibility is a recent report that demonstrated that the addition of conditioned media of pancreatic cancer-associated fibroblasts to cultures of pancreatic cancer cells inhibited the response of the cancer cells to chemotherapy and radiation study [40].

Pancreatic cancers have been demonstrated to be quite hypoxic, and CSCs may be enriched in areas of hypoxia, which promotes stem cell maintenance and blocks differentiation [41]. Hypoxia induces chemokines such as hypoxia-inducible factor 1, which upregulates vascular endothelial growth factor and thus promotes tumor angiogenesis [42]. Hypoxia-inducible factor 1 is also implicated in tumor responses to radiation injury through induction of metabolic pathways, activation of p53 signaling, and induction of tumor proliferation [43]. The expression of hypoxia-inducible factor 1 and vascular endothelial growth factor was observed to be significantly upregulated in pancreatic cancer [44]. The interaction of pancreatic CSC and the hypoxic tumor microenvironment is a fertile area for future investigation.

Understanding microenvironmental cues will be important when considering CSC therapeutics. It is likely that the best preclinical model system to test CSC therapeutics will be primary pancreatic cancers established in the orthotopic location, that is, the pancreatic niche. This model system will not only mimic the heterogeneity of tumors seen in actual patients but will also allow the contribution of the tumor microenvironment, thus will allow assessment of the therapeutic responses in a clinically relevant setting.

### Conclusions

Evidence suggests that pancreatic cancer is a CSC-driven disease. Isolation and characterization of pancreatic CSCs reveal that these tumor-initiating cells share important molecular pathways seen in other solid-organ CSCs and contribute to resistance to conventional chemotherapy and radiation. A better understanding of the pathways governing the key properties of self-renewal, differentiation, and interactions between pancreatic CSCs and the tumor microenvironment will ultimately aid in the development of more effective therapeutics against pancreatic cancer.

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