

Heterogeneity and Targeting of Pancreatic Cancer Stem Cells

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Abstract

Cancer stem cells (CSC) have been identified in an ever-increasing number of human malignancies on the basis of their ability to recapitulate tumors in the ectopic setting and maintain long-term tumorigenic potential. In addition, in pancreatic adenocarcinoma, CSCs may display additional properties, such as relative drug resistance and enhanced invasive and migratory potential that implicate a role in disease pathogenesis spanning initial tumor formation to metastatic disease progression. Importantly, these findings also indicate that the development of novel therapeutic strategies capable of inhibiting or eliminating CSCs will improve clinical outcomes. Preclinical studies have already described a wide array of potential approaches that target CSC-specific surface antigens and cellular pathways involved in cell survival, adhesion, self-renewal, and differentiation. Further, progress in this area should continue to move forward as the unique biology of CSCs is better understood. All preclinical studies to date have focused on targeting specific and phenotypically defined CSCs, but multiple cell populations with the ability to form tumors and self-renew have been identified in pancreatic carcinoma. As the clinical efficacy of CSC-directed therapies will depend on the inhibition of all sources of tumor self-renewal, better understanding of how specific CSC populations are related to one another and whether each possesses specific functional properties will be critical. In this *CCR Focus* article, we discuss the potential relationships between different pancreatic CSC populations and strategies to identify novel targeting approaches. *Clin Cancer Res*; 18(16): 4277–84. ©2012 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) carries one of the worst prognoses of any malignancy and is the fourth leading cause of cancer-related deaths in the United States (1). Despite advances in understanding of the basic biology of PDAC, survival rates have not significantly improved over the past 30 years, and less than 5% of patients are alive 5 years after diagnosis. Therefore, new treatments are needed for PDAC, and cancer stem cells (CSC) have emerged as potential therapeutic targets.

CSCs represent phenotypically distinct cells that possess enhanced tumor-initiating potential, self-renewal, and the ability to recapitulate the cellular heterogeneity of the original tumor (2). Following these initial findings, additional features, including their rarity, relative chemoresistance, and metastatic potential have been described, and these properties have allowed them to be referred to by

more precise terms, such as tumor-initiating cells. However, due to the heterogeneous properties exhibited by CSCs, it has been difficult to provide a label capable of encompassing all of these attributes. Therefore, we refer to these specialized cell populations by the general term "CSC" throughout this review. Although the identification of CSCs was limited to myeloid leukemias in the 1990s (3, 4), they have been described in an increasing number of solid tumors over the past decade, including multiple reports in PDAC (5–7). Several aspects of the CSC hypothesis have been hotly debated (8–10), but their clinical significance is the aspect that is most relevant. In PDAC, early data have indicated that the identification of CSCs in primary tumors is associated with shorter overall survival (6), and it is likely that additional functional properties, including relative resistance to the standard cytotoxic agent gemcitabine and enhanced metastatic potential are, in part, responsible for these findings (7, 11).

The identification and characterization of CSCs has generated novel hypotheses with regard to the mechanisms involved in PDAC growth and dissemination, but several critical questions remain. We initially review studies identifying pancreatic CSCs and hypothesize how these distinct cell populations may be related to one another. Thereafter, we discuss potential strategies to target pancreatic CSCs.

Identification of pancreatic CSCs

At the most basic level, the CSC hypothesis links phenotypically defined tumor cells with specific functional

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Table 1. Phenotype and functional properties of pancreatic CSC populations

Study	Phenotype	Properties
Li et al. (5)	CD44 ⁺ CD24 ⁺ ESA ⁺	Increased Sonic Hedgehog expression
Hermann et al. (7)	CD133 ⁺	Chemoresistant; CD133 ⁺ CXCR4 ⁺ cells responsible for metastasis
Rasheed et al. (6)	ALDH ⁺	CSCs associated with overall survival; CSCs exhibit mesenchymal features and are frequently found in metastatic lesions
Ishizawa et al. (25)		ALDH ⁺ and CD44 ⁺ CD24 ⁺ cells are equally tumorigenic
Li et al. (22)	CD44 ⁺ c-Met ⁺	Highly metastatic

properties, and CSCs have been stringently defined by their ability to differentiate and self-renew (12). The differentiation of CSCs gives rise to the full range of malignant cell types and histologic recapitulation of the original tumor, whereas self-renewal is responsible for maintaining long-term growth potential. In most diseases, the ability of putative CSCs to form tumors has been evaluated using immunodeficient mice (e.g., NOD/SCID and NSG) followed by histologic examination and serial transplantation to show self-renewal (13). Although these mouse models remain the gold standard to evaluate CSCs, *in vitro* assays have been developed to assess the clonogenic potential of CSCs, including colony formation in semi-solid media or tumor sphere formation in liquid culture. Moreover, these *in vitro* assays may quantify the number of cells with self-renewal and long-term growth potential through serial rounds of plating.

Candidate CSC markers have largely consisted of differentially expressed cell-surface antigens or drug-resistance pathways. One approach to identify novel CSC populations has been the use of surface antigens expressed by normal stem cells in the tissue of origin, such as CD34 in myeloid leukemias (3, 14). Alternatively, antigens or enzymes capable of identifying normal stem cells in multiple tissues, such as CD133 and aldehyde dehydrogenase (ALDH), have been used to isolate CSCs in several diseases (15–19). Finally, specific antigens associated with poor prognosis, such as CD44 or c-Met, have served as CSC markers (5, 20–22).

The initial identification of pancreatic CSCs extended ground-breaking work in breast cancer and investigated the expression of CD44, CD24, and epithelial-specific antigen (ESA; Table 1; ref. 5). Relative to unsorted cells, CD44⁺CD24⁺ESA⁺ cells isolated from low-passage PDAC xenografts were highly tumorigenic and recapitulated the

histology and cellular heterogeneity of the original tumor. Further, the functional differences between CD44⁺CD24⁺ESA⁺ and CD44⁻CD24⁻ESA⁻ cells were maintained following subcutaneous or orthotopic injection, indicating that tumorigenic potential was cell autonomous and independent of local environmental factors. A second report showed that CD133 could also identify pancreatic CSCs (7). In addition to being highly tumorigenic, CD133⁺ pancreatic cancer cells were found to be relatively resistant to gemcitabine treatment compared with CD133⁻ cells.

In addition, cellular markers associated with drug resistance have been used to identify CSCs. ALDH, specifically ALDH1A1, is required for the synthesis of all-*trans*-retinoic acid, and high enzyme activity marks normal mouse pancreatic progenitor cells and normal human stem cells in several organ systems (23, 24). Moreover, ALDH may play a role in drug resistance as it can metabolize and neutralize cytotoxic alkylators, such as cyclophosphamide. We studied ALDH in PDAC and found that ALDH⁺ cells are highly tumorigenic compared with bulk tumor cells (6, 25). Moreover, ALDH⁺ cells appear to be relatively resistant to gemcitabine *in vivo* and have increased invasive potential, indicating a role in disease progression (6, 11).

Despite the importance of CD44, CD133, and ALDH in identifying pancreatic CSCs, it is unclear whether these antigens are involved in regulating CSC function or merely serve as phenotypic markers. However, other pancreatic CSC markers have been identified that may be functionally relevant. For example, CXCR4 serves as the chemokine receptor for stromal cell-derived factor-1 (SDF-1, CXCL12) and is expressed by a subset of CD133⁺ CSCs that have enhanced metastatic capacity (7). In addition, recent studies have shown that c-Met can identify and regulate pancreatic CSCs in a manner similar to findings in glioblastoma (22, 26). Thus, several strategies have been used to identify pancreatic CSCs, and some of these may provide insights into regulatory factors and potential targeting strategies.

The relationship between distinct pancreatic CSC populations

In most normal organ systems, such as the blood, central nervous system (CNS), and skin, cells are functionally and phenotypically organized according to a strict cellular hierarchy in which self-renewing stem cells give rise to short-lived progenitors and then terminally differentiated effector cells. The earliest studies in acute myeloid leukemia (AML) showed that tumor cells resembling normal hematopoietic stem cells can self-renew and give rise to relatively differentiated and nontumorigenic blasts (4). Therefore, it has been generally assumed that cancers are organized in a hierarchical manner similar to normal tissues. However, several CSC populations have been identified in PDAC, and it is not clear how each of these populations fits into a specific hierarchy or how they are related to one another. One possibility is that all of the current markers recognize

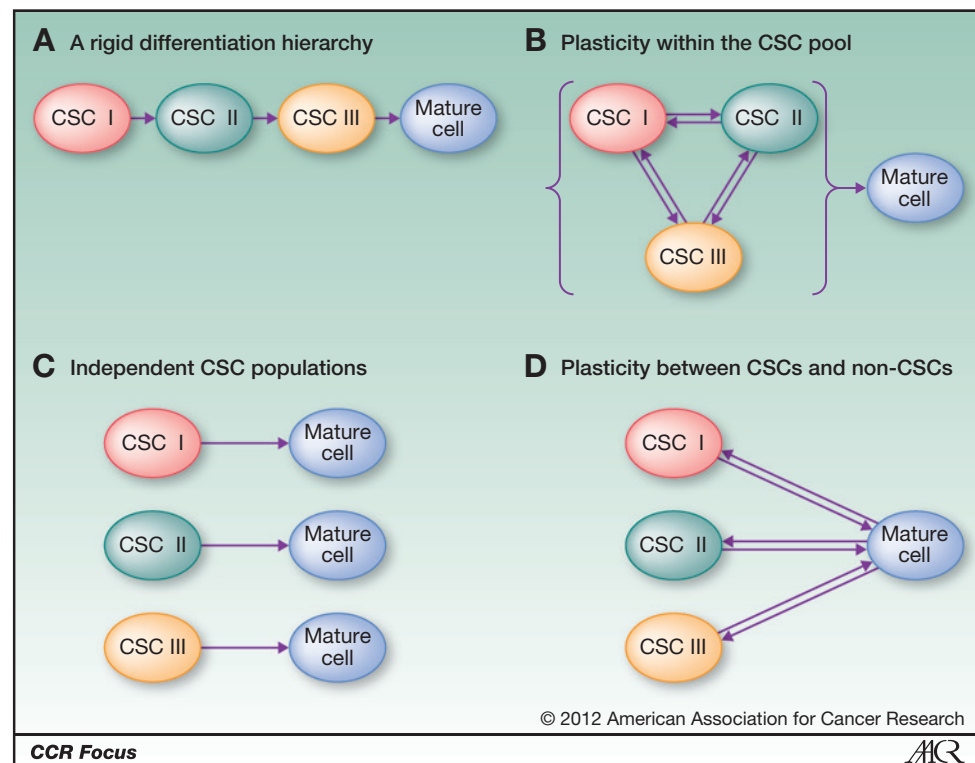
the same cell, but the vast majority of ALDH⁺ pancreatic tumor cells appear to lack CD44 and CD133. Therefore, it is likely that these antigens identify at least 2, or even 3, unique cell populations (6, 27). Alternatively, because each putative CSC marker enriches for cells with increased tumorigenic potential but fails to isolate pure populations of CSCs (i.e., every cell expressing a specific marker is not tumorigenic), it is possible that combining antigens will greatly increase the purity of CSCs. However, this does not appear to be the case as the tumor-initiating-cell frequency of rare PDAC cells coexpressing CD44, CD24, and ALDH is not significantly greater than that of either ALDH⁺ or CD44⁺CD24⁺ cells (25). Moreover, c-Met is expressed, albeit at variable levels, on CD44⁺, CD133⁺, or ALDH⁺ cells, but increased tumorigenic potential is limited to CD44⁺c-Met^{high} cells (22).

The significance of the various pancreatic CSC markers and the cells they identify clearly requires further clarification. If multiple CSC populations actually exist, an understanding of how they are related to one another will be important because clinically effective targeting possibly requires the elimination of all self-renewing cells within the tumor. One possibility is that PDAC cells are organized in a hierarchical and linear manner with a single, phenotypically distinct CSC at the apex giving rise to the other CSC populations and ultimately nonclonogenic mature tumor cells (Fig. 1). In addition, it is possible that each phenotypically distinct CSC population represents a specific cellular state of the same clonogenic cell that gives rise to

mature tumor cells. Another possibility is that each CSC population is unrelated to another and parallel lines of mature tumor cell production exist. Finally, it is conceivable that a rigid hierarchy of unidirectional differentiation does not exist, but that the system is plastic with nonclonogenic cells giving rise to tumorigenic CSCs displaying a variety of phenotypes. To better understand how different CSCs are related to one another, studies examining the overlap between putative CSC populations and the cell types that arise from each specific CSC are needed.

Beyond the organization of phenotypically defined CSC populations, it is also unclear whether the various CSCs are functionally similar or distinct. Although tumor formation, histologic recapitulation, and self-renewal define CSCs, other properties, including relative drug resistance, invasion, migration, and metastatic potential have been ascribed to CSCs and may contribute to their clinical impact (28). It is possible that certain CSC populations could be primarily responsible for tumor initiation and maintenance at the primary site of disease, whereas others could be responsible for tumor dissemination and growth at metastatic sites, such as the subpopulation of CD133⁺ CSCs expressing CXCR4 (7). In addition, it is possible that different organs, such as the liver and lung, harbor different microenvironments with distinct endothelial or stromal cell types or extracellular matrix components that promote or inhibit tumor growth (Fig. 2; ref. 29). Therefore, if metastatic dissemination depends on the interaction of CSCs with a particular niche, then different niches

Figure 1. Potential relationships between CSCs and mature tumor cells. A, a linear organization with a single phenotypically distinct CSC giving rise to the other CSC populations and ultimately nonclonogenic mature cells. B, each phenotypic CSC represents a distinct state of the same clonogenic cell that gives rise to the mature tumor cell. C, each CSC population is unrelated to another and parallel lines of mature tumor cell production exist. D, a plastic system in which nonclonogenic mature cells give rise to CSC displaying a variety of phenotypes.



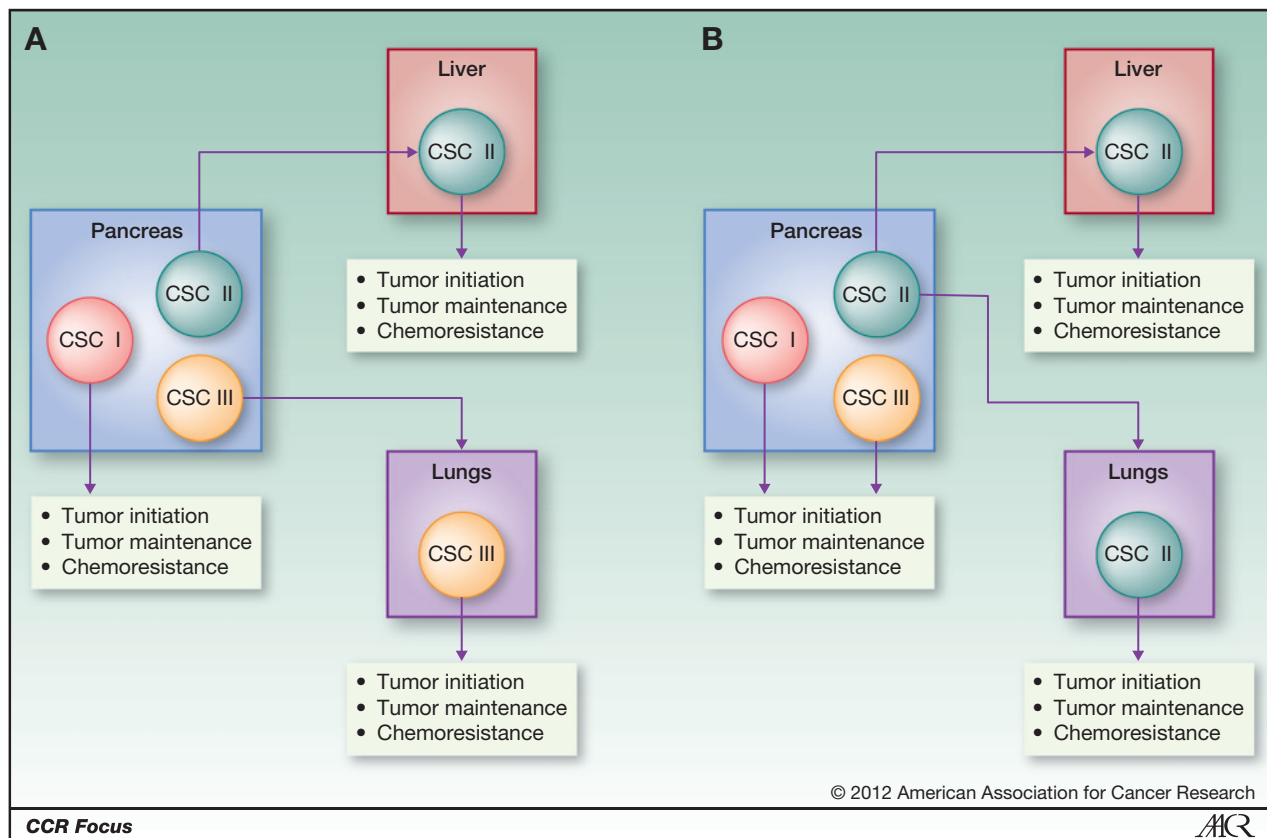


Figure 2. Potential functional relationships between CSCs. A, distinct CSCs may give rise to macroscopic tumors in distinct anatomic locations. Each population is responsible for tumor growth and resistance to therapy in different organs. B, a primary CSC population is responsible for tumor initiation and growth at the primary site. Additional populations are responsible for initiation and maintenance at metastatic sites and for resistance to chemotherapy.

might call for unique CSCs. An evaluation of the tumor-forming potential of specific CSCs at orthotopic and different metastatic sites may determine whether certain populations are better suited to grow within particular locations.

Moreover, it is possible that the phenotypes exhibited by CSCs are dictated by the external microenvironment. For example, pancreatic tumors are characterized by desmoplasia and dense fibrosis that may expose cells to relative hypoxia, and the hypoxic state has been found to alter the expression of the CSC marker CD133 in brain tumors (30). In addition, several markers used to identify CSCs, such as ALDH and the side population assay, are indicative of drug-resistance mechanisms, and it is possible that their expression is induced in response to cellular damage. Finally, it is possible that the adaptive metabolic changes undertaken by tumor cells also modify the expression of CSC makers, although such findings have yet to be reported (31).

Recent studies have shown a clear link between CSCs and the epithelial-to-mesenchymal transition (EMT) in solid tumors. Therefore, it is possible that CSCs represent a specific cellular state expressing multiple phenotypes. Reports using breast cancer models have shown that the

induction of EMT by TGF- β or the modulation of specific gene expression (e.g., induction of *Twist* or repression of *E-cadherin*) results in increased expression of CD44 and tumorigenic potential (32, 33). In pancreatic cancer, ALDH⁺ cells appear to have a gene expression profile consistent with EMT and increased invasive and migratory potential as compared with bulk tumor cells and CD44⁺ CD24⁺ cells (6). Moreover, studies examining ZEB1, an inducer of EMT, in pancreatic cancer cells have identified a direct link among EMT, increased tumorigenicity, and drug resistance (34). Therefore, it is possible that a more "epithelial" or "mesenchymal" state is important in determining the functional properties of CSCs. The specific functional properties of different pancreatic CSCs are unclear, and the quantification of tumor formation, metastatic potential, and drug resistance is needed.

Inter- and inpatient diversity of pancreatic CSCs

Interpatient heterogeneity may contribute to the existence of multiple pancreatic CSCs. Recurrent genetic alterations are a hallmark of cancer, and mutations in *KRAs* are present in the vast majority of PDACs (35, 36). On the other hand, mutations in other genes, such as *p53* and *Smad4/DPC4*, can be identified in some, but not all

Table 2. Pancreatic CSC-specific targeting strategies and agents

Study	Target receptor/ pathway	Target population	Agent(s)
Li et al. (22)	c-Met	c-Met ^{high} CD44 ⁺ CD44 ⁺ CD24 ⁺ ESA ⁺	XL184
Rajeshkumar et al. (51)	DR5	ALDH ⁺ CD44 ⁺ CD24 ⁺ ESA ⁺	DR5 Agonistic monoclonal antibody
Lonardo et al. (52)	ALK4	CD133 ⁺	SB431542
Jimeno et al. (11)	Hedgehog	ALDH ⁺	Cyclopamine, IPI269609
Feldmann et al. (53, 54)		CD44 ⁺ CD24 ⁺ ESA ⁺	
Mullendore et al. (57)	Notch	ALDH ⁺	GSI-18
Zhang et al. (58)	EMT	CD133 ⁺	Salinomycin

tumors (37, 38). Therefore, pancreatic cancers are not genetically homogeneous but rather vary from patient to patient (39). If alterations in specific genes are prognostic and CSCs truly dictate the natural history of PDACs, given their potential roles in tumor formation, drug resistance, and metastatic progression, then, it is likely that specific mutations influence both the phenotype and function of CSCs. Currently, it is unclear whether phenotypically identical CSCs from different patients have the same functional attributes and contribute to disease progression in similar ways. However, such a finding would imply that personalized and individualized CSC-targeting therapies are needed. To examine inter-patient diversity, the functional properties of different CSCs derived from human tumors with distinct genotypes will need to be determined. In addition, the examination of CSC phenotypes and functional properties in tumors derived from transgenic animal models of pancreatic cancer may be particularly helpful because specific genetic lesions can be modulated in these systems (40).

To further complicate matters, increasing evidence indicates that human cancers can be genetically heterogeneous within the same individual (41–44). Therefore, intrapatient genetic heterogeneity may also drive the phenotypic and functional diversification of CSCs. In many cancers, including PDAC, specific genetic alterations may accumulate in an orderly fashion during disease progression (45, 46); thus, it is possible that different CSCs are responsible for relapse and progression over the course of the disease. In PDAC, metastatic lesions may be genetically distinct from one another and the primary tumor (47). Moreover, primary tumors are composed of geographically and genetically distinct subclones. The role of genetic evolution and diversification in the emergence of distinct CSCs, or conversely, the impact of CSCs on the clonal composition of an individual tumor is not entirely clear, but it is likely that these 2 processes interact at some level. A systematic investigation of genetic lesions within CSCs, their phenotypes, and functional properties, such as tumorigenic potential, metastasis, and drug resistance, within primary tumors and metastatic lesions

derived from the same patient may address this possibility.

Targeting of pancreatic CSCs

The self-renewal potential and resistance to traditional cytotoxic agents indicate that successful CSC-targeting strategies will improve clinical outcomes. One potential approach is targeting the cell-surface antigens that characterize pancreatic CSCs using monoclonal antibodies. For example, a bispecific antibody recognizing both ESA and CD3 has been found to eliminate pancreatic CSCs by redirecting cytotoxic T lymphocytes (48). CD44 is another surface protein expressed by CSCs in multiple diseases (49), and a specific monoclonal antibody against CD44 can eliminate AML stem cells by inducing terminal differentiation (50). In addition, the functional activities of specific pancreatic CSC markers may serve as potential targets (Table 2). The hepatocyte growth factor (HGF) receptor c-Met identifies highly tumorigenic CSCs in combination with CD44, and the pharmacologic inhibition of its activity has been found to inhibit tumor growth and metastasis (22). Another functionally relevant marker is CXCR4, which plays an important role in the homing of hematopoietic stem cells to the bone marrow. CXCR4 has been identified on a subset of CD133⁺ pancreatic CSCs with enhanced metastatic capacity, and CXCR4 antagonists may prevent tumor dissemination (7). Another potential cell-surface target is the Death receptor 5 (DR5), which induces apoptosis following binding to TRAIL. A recent study found that ALDH⁺ and CD44⁺CD24⁺ pancreatic CSCs express relatively increased levels of DR5, and receptor engagement using an agonistic monoclonal antibody markedly reduced CSC frequency and tumor growth *in vivo* (51).

Several cellular signaling pathways have been identified that regulate the self-renewal of normal stem cells and may serve as targets against CSCs. These include pathways required for normal embryonic development, and the Hedgehog (Hh), Notch, and Nodal/Activin pathways may be active in pancreatic CSCs. Nodal and Activin are ligands of the TGF- β superfamily, and a recent study showed that

these ligands and their receptor ALK4 are overexpressed in pancreatic CSCs (52). The pharmacologic inhibition or knockdown of ALK4 abrogated self-renewal and tumorigenicity and also sensitized CSCs to gemcitabine. Another series of studies has examined the Hh signaling pathway in pancreatic CSCs and found that pharmacologic pathway inhibition reduced the frequency of CSCs and decreased tumor formation and metastasis (11, 53, 54). Notably, a recent phase II clinical trial compared gemcitabine alone or in combination with the novel Hh inhibitor saridegib (IPI-926) in patients with metastatic pancreatic cancer on the basis of preclinical data showing enhanced responses to cytotoxic chemotherapy (55). A higher rate of progressive disease was observed at an interim analysis in patients receiving saridegib (56). Although the precise reasons for these results are unclear, it is possible that the Hh pathway regulates the development, rather than maintenance, of metastatic lesions, and other ongoing trials of Hh inhibitors in the neoadjuvant setting may provide a better scenario to detect these potential anti-CSC effects. Finally, the inhibition of Notch signaling has been found to inhibit EMT and cellular invasion as well as to decrease the frequency of ALDH⁺ CSCs (57). Thus, cellular pathways involved in regulating the self-renewal of normal stem cells may represent pancreatic CSC targets.

In addition, the association of EMT and CSCs may form the basis for identifying novel targeting agents. High-throughput strategies to screen for novel anti-CSC compounds have been difficult to carry out because of the lack of pure CSC populations and the complex nature of the assays used to assess their functions, but several methods may induce EMT and increase the frequency of CSCs. This approach was ingeniously used by Gupta and colleagues, who genetically engineered human breast cancer cell lines to induce EMT and screened for compounds that could induce cell death (32). The ionophore salinomycin was identified as a potential CSC targeting agent and then subsequently found to block tumor formation and metastasis *in vivo*. Shortly thereafter, salinomycin was shown to inhibit the growth of pancreatic CSCs, indicating that it

may represent a potential CSC-targeting agent in multiple malignancies (58). Therefore, similar strategies based on EMT may identify novel agents that inhibit pancreatic CSCs.

Conclusions

The CSC hypothesis may provide novel insights into the pathogenesis of PDAC and the mechanisms that regulate clinical chemoresistance and the propensity to develop metastatic disease. Moreover, it may lead to a better understanding of self-renewal that allows tumors to persist over time and to the development of novel therapeutic strategies targeting the regulatory pathways involved. With the increased recognition of cellular heterogeneity in PDAC and identification of tumor cells with enhanced tumorigenic potential and self-renewal, questions have emerged regarding the diversity of pancreatic CSCs and their relationships to one another that will need to be addressed. However, these findings are likely to provide a framework to better understand advancements in many of the other fields that are the subjects of this *CCR Focus* section, including alterations in genetics, tumor metabolism, and the microenvironment. Ultimately, merging these distinct aspects of PDAC biology may provide the basis for truly novel and effective therapies with a positive impact clinical outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest have been disclosed.

Authors' Contributions

Conception and design: V.R. Penchev, Z.A. Rasheed, A. Maitra, W. Matsui
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