Review article

The ever-changing landscape of pancreatic cancer stem cells

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ABSTRACT

Over the past decade, the cancer stem cell (CSC) concept in solid tumors has gained enormous momentum as an attractive model to explain tumor heterogeneity. The model proposes that tumors contain a subpopulation of rare cancer cells with stem-like properties that maintain the hierarchy of the tumor and drive tumor initiation, progression, metastasis, and chemoresistance. The identification and subsequent isolation of CSCs in pancreatic ductal adenocarcinoma (PDAC) in 2007 provided enormous insight into this extremely metastatic and chemoresistant tumor and renewed hope for developing more specific therapies against this disease. Unfortunately, we have made only marginal advances in applying the knowledge learned to the development of new and more effective treatments for pancreatic cancer. The latter has been partly due to the lack of adequate in vitro and in vivo systems compounded by the use of markers that do not reproducibly nor exclusively select for an enriched CSC population. Thus, attempts to define a pancreatic CSC-specific genetic, epigenetic or proteomic signature has been challenging. Fortunately recent advances in the CSC field have overcome many of these challenges and have opened up new opportunities for developing therapies that target the CSC population. In this review, we discuss these current advances, specifically new methods for the identification and isolation of pancreatic CSCs, new insights into the metabolic profile of CSCs at the level of mitochondrial respiration, and the utility of genetically engineered mouse models as surrogate systems to both study CSC biology and evaluate CSC-specific targeted therapies in vivo.

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Introduction

Over the last 10 years, new pancreatic cancer case and death rates have risen on average 0.8% and 0.4% each year, respectively (“SEER Stat Fact Sheets: Pancreas Cancer”, www.seer.cancer.gov).

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By the year 2030, pancreatic cancer [most frequently presenting as pancreatic ductal adenocarcinoma (PDAC)] is expected to surpass all other gastrointestinal cancers to become the second-leading cause of cancer-related deaths, trailing only lung cancer [1]. These alarming rates reflect the reality that while tremendous strides have been made in understanding and treating pancreatic cancer, we are still far from turning the tide on this incredibly deadly disease. The latter is believed to be multi-factorial but primarily due to the existence of a subpopulation of highly chemoresistant, slow cycling, “stem”-like cells within the tumor bulk known as cancer (stem) cells (CSCs).

The concept of CSCs is not new. It was first proposed by Rudolf Virchow over a century ago [2], and while numerous studies since then have alluded to the existence of CSCs in different tumors [3] it was not until the advent of FACS sorting combined with in vivo models of tumor growth in immunodeficient mice that allowed Dick and colleagues to formally prove their existence in hematological malignancies in 1994 [4]. The identification of CSCs in solid tumors, however, would not come until 2003, when Al-Hajj et al. identified and isolated tumorigenic cells from breast tumors and showed that these cells could form new tumors when transplanted in nude mice [5]. Since 2003, CSCs have been identified in the majority of solid tumors [6–9], including pancreatic cancer [10,11], and they are currently defined as a subpopulation of functionally distinct “stem”-like tumor cells with inherent self-renewal properties, multipotency and an exclusive ability to initiate and recapitulate the parental tumor upon serial passage in immunodeficient mice [12,13].

The CSC model assumes that only CSCs have exclusive tumorigenic potential, and these cells therefore drive tumor relapse and/or metastasis following chemotherapy. Thus, from a clinical perspective, only elimination of the CSC population would ensure tumor eradication. A handful of studies have suggested an association between PDAC tumors with “stem cell”-like signatures and poor treatment response or increased disease relapse [14,15]. More convincing, however, are data demonstrating that pancreatic CSCs (PaCSCs) isolated from primary tumors or established cell lines are more chemoresistant compared to their non-CSC counterparts [16–20], likely due to escape mechanisms shared with normal stem cells, such as “quiescence” and over-expression of multi-drug transporters [20,21]. Thus, the idea of eliminating PaCSCs as a therapeutic strategy for treating PDAC is not only gaining momentum, but CSC-specific treatment strategies are already being evaluated as potential future treatments for PDAC [22–32]. We refer the reader to several recently published reviews that discuss these treatment approaches more in depth [33,34]. This potential paradigm shift in pancreatic cancer treatment is partly due to our increasing ability to identify, isolate and study PaCSCs, which has afforded us a broader understanding of the role CSCs play in tumor maintenance, chemoresistance, relapse and metastasis. In this review, we examine the current advances made in the identification and isolation of PaCSCs and the systems available to study this unique subpopulation of cells. We also examine the concept of CSCs in genetically engineered mouse (GEM) models of PDAC as surrogate models for the development of PaCSC-specific therapeutics. Lastly, we critically discuss the evolving concept that PaCSCs can be targeted. For example, we review current evidence demonstrating that PaCSCs use mitochondrial respiration over glycolysis to meet their energy requirements and this difference can be therapeutically exploited.

Identification and isolation of PaCSCs

In order to understand and subsequently target CSCs, researchers have spent years identifying markers that can be used to isolate this extremely rare and small subpopulation of tumor cells. Over the past 10 years, PaCSCs have been identified in diverse in vitro and in vivo systems using a variety of different biomarkers. In 2007, Li et al. [10] and Hermann et al. [11] first demonstrated the existence of CSCs in PDAC using the cell surface markers CD44, CD24, and EpCAM, in combination, or CD133 alone, respectively. In both cases, they showed that these markers could discriminate for cells with “stem-like” properties, including exclusive in vivo tumorigenicity. Hermann et al. also showed the existence of metastatic CSCs at the invasive front of pancreatic tumors. Specifically, they showed that a distinct subpopulation of CSCs expressing both CD133 and CXCR4 were responsible for the metastatic phenotype of individual tumors and CD133+ CXCR4+ CSCs were preferentially found in patients with metastatic disease. While CD133, EpCAM, CD44 and CXCR4 continue to be widely used to isolate and study PaCSCs, other cell surface and functional markers have also been utilized to identify and isolate PaCSCs, although with varying specificity and reproducibility. These alternate CSC markers include, but are not limited to 26S proteasome activity [35], CD24 [10], hepatocyte growth factor receptor c-MET [30], CD90 [36], ALDH1 [37] and side population (SP) [38,39].

In 2014, we made a novel discovery in the field of CSC biology [32]. We showed that PDAC tumors contain a subpopulation of cells with discrete intracellular autofluorescent vesicles, and these autofluorescent vesicles could be used to efficiently isolate subsets of cells with robust CSC properties, including enhanced self-renewal, increased expression of pluripotency-associated genes, increased migration, pronounced chemoresistance and exclusive tumorigenic potential in vivo [20]. Importantly, we also observed this intrinsic autofluorescent marker in other tumor entities, including liver, lung, and colorectal cancers, and as such autofluorescence may represent a potential “universal” marker for identifying, isolating and studying human CSCs.

Subsequent studies determined that the source of the autofluorescence was the consequence of riboflavin accumulation in cytoplasmic ER-derived vesicles that over express the ATP-binding cassette (ABC) transporter ABCG2 [20,40]. Since riboflavin is a natural substrate for ABCG2 [41], its accumulation in these ABCG2-coated vesicles is not surprising. What remains unanswered, however, is why these vesicles form. ABCG2 is a well-recognized ABC transporter that is highly expressed on the surface of many cancer cells, functioning in large part to reduce the intracellular concentration of chemotherapeutic drugs [42]. In general, ABCG2 is translated in the endoplasmic reticulum (ER) and transported through the Golgi apparatus to the plasma membrane (PM). Over-expression of ABCG2, which is often observed in cancer cells, can lead to its misfolding and subsequent elimination via ER-associated degradation (ERAD). Interestingly, a study by Sugiyama T et al., showed that ABCG2 expression and trafficking is also regulated by the E3 ubiquitin–ligase co-factor Derlin-1. Specifically, they showed that over-expression of Derlin-1 can suppress ABCG2 ER to Golgi transport, resulting in its retention in the ER [43]. We have observed that ABCG2 is not only over expressed in PaCSC [20], but Derlin-1 is also over expressed (data not shown) and thus we hypothesize that ABCG2 is retained in the ER via a Derlin-1-mediated process thus driving the formation of cytoplasmic ABCG2-coated ER-derived vesicles in PaCSCs. These vesicles can then act as intracellular sinks for riboflavin, resulting in the formation of the CSC autofluorescent vesicle (Fig. 1).

More research is still needed to fully understand the potential of autofluorescence as a CSC marker, such as whether there exists a hierarchy within the autofluorescent CSC population or does the percentage of autofluorescent cells within a tumor correlate with clinical outcome data. In addition, it is tempting to speculate that autofluorescence provides a biological advantage to CSCs, similar to
the fungal vacuole \[44\], which also concentrates riboflavin, among other vitamins and basic amino acids. One could hypothesize that the "storage" of riboflavin in the CSC autofluorescent vesicles provides CSCs with a constant supply of the precursor vitamin necessary for the synthesis of flavin-dependent coenzymes, flavin mononucleotides, and flavin adenine dinucleotides, factors involved in many biological processes such as protection against radical oxygen species \[45\]. This reservoir of riboflavin could therefore provide CSCs with the necessary factors needed to survive and proliferate in inhospitable conditions, such as during nutrient deprivation or aggressive chemotherapy. While these hypotheses remain to be tested, evolution has taught us that cells seldomly create new cellular components with no innate biological advantage, thus it is highly unlikely that the autofluorescent CSC vesicles are merely decorative.

PaCSCs in genetically engineered mouse models (GEMMs)

GEMMs provide the unique opportunity to investigate the precise role of genetic alterations in a tightly controlled, genetically uniform background, without inter-patient variability. Their use to study and to characterize different human diseases and disorders is common practice and has led to the discovery and thorough investigation of genetic alterations in many genetic (hereditary) diseases, in particular in cancer research, occasionally translating into the development of new therapeutic approaches \[46\--\[48\].

Certainly the clinical translation of discoveries made in GEMMs is often difficult and depends on how "precisely" a GEMM recapitulates the corresponding human disease \[49\]. Three widely used GEMMs have been developed that allow for the successful study of pancreatic tumorigenesis ranging from normal pancreatic tissue to acinar-to-ductal metaplasia (ADM \[50\]), pancreatic intra-epithelial neoplasia (PanIN), and full-blown pancreatic ductal adenocarcinoma (PDAC). All typical models contain oncogenic K-Ras point mutations (G12D or G12V) \[51\], the most common genetic alteration found in more than 85% of pancreatic adenocarcinomas \[52,53\], and often additional mutations in p53, Smad4, or Brca2 \[54\] are combined. The three most popular models differ based on the promoter under which these mutations are regulated (Pdx-1, Ptf1a or Elastase). The expression of oncogenic Ras under the Pdx-1 or Ptf1a (also termed p48) promoters leads to reliable tumor formation in the murine pancreas \[55,56\] as these promoters are active in pancreatic progenitor cells \[55,57,58\]. With the Elastase promoter, the expression of oncogenic Ras (G12V) is restricted
GEMMs could be achieved [60]. Using Sca1 (stem cell antigen 1), an highly tumorigenic and metastatic cells with a CSC phenotype in cell surface markers, successful identification of pancreatic cancer and have been widely used. These models recapitulate many clinical, histopathological, and invasive characteristics of the human disease as well as chemo- and radio-resistance. Thus current PDAC GEMMs are well suited for the investigation of pancreatic cancer and have been widely used.

Recently, Ischenko et al. discovered that by using a combination of cell surface markers, successful identification and isolation of highly tumorigenic and metastatic cells with a CSC phenotype in GEMMs could be achieved [60]. Using Sca1+ (stem cell antigen 1), an antigen previously identified in hematopoietic stem cells [61], the authors were able to demonstrate that an EpCAM+CD24+CD44+Sca1+ (referred to as Sca1+) population displayed CSC properties in murine pancreatic cancer, while an EpCAM+CD24+Sca1− (referred to as Sca1−) population did not. Interestingly, the expression of Pdx1 inversely correlated with Sca1 and CD133 expression. Subcutaneous injection of Sca1+ cells resulted in fast tumorigenicity and high tumorigenic potential with subsequent formation of undifferentiated (anaplastic) carcinomas. Furthermore, only Sca1+ cells were able to initiate lung metastasis after tail vein injection. Analysis of both Sca1+ and Sca1− subpopulations in combination with CD133 showed that Sca1+ CD133− cells formed tumors much more rapidly than other cell populations [62].

In addition to using CSC-related surface markers, several studies have focused on genes that discriminate stem/progenitor cells in normal pancreas to identify CSC-like cells in tumor tissues. Apart from the four classical “Yamanaka factors” (i.e. Oct3/4, Klf4, Sox-2, and c-Myc) necessary for the reprogramming of differentiated cells into induced pluripotent stem cells (iPSCs) and the well-established stemness marker Nanog, several additional genes have been identified and implicated in the regulation of stemness in the pancreas. Using lineage tracing in a GEMM, Kopinke et al. identified the Notch1 target Hes1 to be restricted to pancreatic progenitor cells, and also to stem cells in the small and large intestine [62]. Rovira and colleagues were able to demonstrate that Aldefluor-positive E-cadherin-positive cells in the pancreas are highly enriched for markers associated with progenitor cells in the pancreas and other tissues [63], and are highly efficient at generating pancreatic sphere cultures, which in turn spontaneously differentiate into endocrine and exocrine cells, underlining their stemness. The fate of acinar cells is strongly regulated by Gata6 [64] and Mist1 [65], which seem to be key regulators of differentiation. Loss of Gata6 and its downstream target Mist1 has subsequently been correlated with acinar de-differentiation. Recent studies have shown a key role for the stemness-associated gene Sox9 in oncogenic K-Ras-dependent pathogenesis [66,67]. In normal acinar cells, Sox9 is not expressed; however, Sox9 expression in acinar cells seems to be one of the initial steps of malignant transformation of acinar cells and may be essential for PDAC development.

In a recent study, we investigated the effects of nicotine on pancreatic cancer tumorigenesis in GEMMs [67], and observed many of the regulatory steps and gene expression changes mentioned above. Nicotine in the drinking water of wild type mice initiated the loss of acinar differentiation, and thus paved the way for subsequent malignant transformation of the pancreas. Acinar de-differentiation resulted from ERK-mediated activation of c-Myc, subsequent repression of Gata6, which led to the subsequent repression of the downstream target Mist1 and the typical acinar cell genes such as Amylase, Elastase, CPA1, Chymotripsinogen, and p48. Importantly, cells also exhibited decreased acinar functionality as demonstrated by a significant loss of acinar granula. In the pancreas of two GEMMs of PDAC (either Pdx-1; K-ras; p53 (KPC) or Elastase; K-Ras), the effects were similar, with loss of acinar differentiation via Gata6 and Mist1 repression. The loss of acinar cell identity resulted in an increased number of Sox9+ acinar cells, corroborating the results from Kopp and colleagues [66]. Most importantly, however, the nicotine-induced loss of acinar differentiation resulted in highly accelerated formation of PanINs, and in more advanced neoplastic lesions and PDACs as compared to untreated mice. Moreover, in fully transformed tumor cells, nicotine induced a cancer stem cell phenotype by promoting stemness features, resulting in the upregulation of stemness-related genes in cancer cells, and promoting CSC features such as sphere formation, and tumorigenic potential. Finally, in a therapeutic approach to inhibit the CSC-promoting effects of nicotine on the pancreas, and with the aim to eliminate a key risk factor for the development of pancreatic cancer, we were able to demonstrate that treatment with the anti-diabetic drug metformin inhibits the de-differentiation, (re-) induces an acinar phenotype, and indeed serves as a preventive strategy against the development of PDAC in mouse models of pancreatic cancer (Fig. 3).

It is also important to note that PDAC GEMMs are excellent models to study the interaction between CSCs and the tumor microenvironment, which cannot be readily studied in vitro or in patient-derived xenograft (PDx) models. Current data supports an important role for tumor stroma cells [e.g. pancreatic stellate cells (PSCs) and tumor-associated macrophages (TAMs)] in PaCSC biology via paracrine-secreted factors that include Nodal/ActivinA, ISG15 and hCAP/LL-37 [68–71]. These factors, secreted by PSCs or TAMs, activate the CSC compartment and enhance CSC-mediated tumorigenesis (Fig. 3). Using the KPC mouse model described above, we recently showed that the effects of TAM secreted LL-37 on PaCSCs is not only recapitulated in the KPC PDAC mouse model but inhibitors targeting LL-37 signaling significantly reduced PDAC progression and tumor cell dissemination in vivo [70]. Thus, the sum of these studies not only highlight that PDAC GEMMs are an adequate surrogate model to study PaCSCs, but also underscore the use of these systems to test new anti-CSC-specific therapies (Fig. 2).

Metabolism of PaCSCs

One of the main features of pancreatic tumors is their pronounced desmoplasic microenvironment, composed of fibroblasts, PSCs and immune cells immersed in fibrict fibers [72]. This extremely dense stroma impedes tumor vascularization, generating a fundamentally hypoxic microenvironment in which nutrient availability is very limited [73]. In such challenging conditions, metabolic reprogramming through increased glucose metabolism via glycolysis (Warburg effect) may not fully cover the metabolic requirements of pancreatic tumors. Indeed, it has been demonstrated that K-Ras mutations drive a distinct metabolic program in pancreatic cancer cells, characterized by increased glucose and glutamine usage, lipid and protein scavenging, and reutilization of cellular components via autophagy [74–76].

Despite the recent efforts to decipher PDAC metabolic features, much less is known about the metabolic phenotype of PaCSCs. Very recently, we reported that, in contrast to their more differentiated progenies, pancreatic CSCs are highly dependent on mitochondrial oxidative phosphorylation (OXPHOS) [77]. This phenotype, characterized by elevated mitochondrial biogenesis and activity, was controlled by the transcription factor PGC-1α, and negatively regulated by c-Myc expression (Fig. 3A). Mitochondrial OXPHOS, coupled to the tricarboxylic acid (TCA) cycle, constitutes the main energy source for low proliferative and differentiated cells in adult tissues [78]. Interestingly, this pathway seems to be the preferred pathway for energy production in CSCs of various tumor types,
such as glioma and glioblastoma [79,80], lung cancer [81] and leukemia [82–84].

Although it involves a significantly larger number of biochemical reactions diminishing the overall rate of ATP production, mitochondrial OXPHOS is almost 10 times more efficient in terms of ATP generated per unit of glucose. With that in mind, one can hypothesize that the dependence of CSCs on OXPHOS may reflect an adaptation of these cells to the nutrient-poor microenvironment of pancreatic tumors. Indeed, PaCSCs would more efficiently use a range of different substrates either from the microenvironment or

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recycled via autophagy [85] (e.g. lipids, glutamine, and amino acids), all of them being able to feed the TCA cycle (Fig. 3B). Such metabolic efficacy would confer resistance to nutrient deprivation, as demonstrated for PaSCs upon glucose and glutamine depletion, conditions extremely toxic for their differentiated counterparts [77]. More importantly, oxidative CSCs would be able to metabolize diverse glycolysis end-products, such as lactate, very concentrated in the extracellular milieu since it is excreted by differentiated cancer cells and other cellular components of the stroma. While CSCs dependence on OXPHOS suggests that they would be located in more oxygenated areas of the tumor, it was estimated that O2 concentration in hypoxic microenvironments (from 8 to 57 mM) is ten times higher than the limiting oxygen concentration for functional mitochondrial respiration [86]. Thus, the dependence of PaSCs on OXPHOS should be mostly independent of their location within the tumor, and, indeed, the hypoxic environment favors stemness in pancreatic tumors [85,87]. Interestingly, dependence on OXPHOS and lack of metabolic plasticity in cells with tumor-initiating abilities may be the result of the loss of MYC expression in both human and murine PDAC models. Indeed, human PaSCs maintain low expression levels of this oncogene in order to keep their maximal stem-related abilities [77], and as mentioned above low levels of c-MYC favor a pro-OXPHOS phenotype in human PaSCs. Along these lines, Hayes et al. recently showed that Ras-dependent signaling shutdown could be inducing MYC proteasomal degradation due to the loss of phosphorylation [89], which may account for the phenotype observed by Viale et al. in the K-Ras-inducible murine system [88]. Despite the similarities between the human and murine models, K-Ras cannot be considered the absolute driver for the differential metabolic phenotype in the case of human PaSCs since K-Ras mutational status and activity would be shared by both CSCs and their more differentiated progenies. Instead, other downstream kinases may be differentially activated in both subpopulations or epigenetic differences may be controlling MYC protein stability and activity. In light of the findings by Hayes et al. [89], however, it would be interesting to determine the effect of Ras signaling ablation with ERK inhibitors on c-MYC expression and OXPHOS specifically in human PaSCs. Importantly, the inability of redirecting metabolic demands to alternative pathways when OXPHOS is not operative makes PaSCs virtually addicted to mitochondrial function, and as such, the mitochondria may represent an excellent cellular target for therapeutic purposes. In fact, prior to the identification of this phenotype in PaSCs, several reports already pointed out their enhanced sensitivity to mitochondrial inhibitors such as metformin [22,90–92]. Moreover, we have demonstrated that treatment with metformin and other mitochondrial inhibitors, with different mechanisms of action, effectively diminished the number and functionality of CSCs in vitro and in vivo by inducing apoptosis [77]. However, we found that the metabolic phenotype of PaSCs is heterogeneous, which has important consequences for the design of therapeutic strategies aimed at eliminating CSCs. Indeed, we found a small subset of PaSCs with a more glycolytic/plastic phenotype, conferred by increased MYC expression, which eventually became the dominant population in tumors treated with metformin and led to tumor growth and resistance to treatment. Altogether, current data suggests that the use of more potent mitochondrial inhibitors or combined strategies targeting MYC would be necessary for a successful implementation of this CSC-targeting therapy in the clinical setting.

Conclusions and perspectives

Although a growing number of therapeutic options for pancreatic cancer are becoming available [93], and our understanding of the pathogenic, cellular and molecular make-up of pancreatic cancer continues to grow, we are still challenged by an inability to translate our understanding of this disease into “actionable” and therapeutically relevant results. There is no doubt that we must change our drug development philosophy beyond that of classical chemotherapeutic agents that primarily target highly proliferating cancer cells. If we are to truly make a significant and clinically relevant impact on the future of pancreatic cancer, we can no longer ignore the CSC population. Given our ever increasing ability to identify and isolate these cells, our growing understanding of the biology that drives this highly tumorigenic subpopulation of cells and the in vivo models available to test new anti-CSCs therapeutics, there is more reason than ever to believe that one day pancreatic cancer may become a manageable illness rather than an unequivocal death sentence.

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