Stem Cells In Pancreatic Cancer – From Concept To Translation

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Key words. Pancreatic ductal adenocarcinoma • cancer stem cells • stromal cells • therapeutics/intervention

ABSTRACT

Pancreatic cancer stem cells (CSC) have been first described in 2007 and since then have emerged as an intriguing entity of cancer cells with distinct functional features including self-renewal and exclusive in vivo tumorigenicity. The heterogeneous pancreatic CSC pool has been implicated in tumor propagation as well as metastatic spread. Clinically, the most important feature of CSCs is their strong resistance to standard chemotherapy, which results in fast disease relapse, even with today’s more advanced chemotherapeutic regimens. Therefore, novel therapeutic strategies to most efficiently target pancreatic CSCs are being developed and their careful clinical translation should provide new avenues to eradicate this deadly disease. Stem Cells 2015; 00:000–000

SIGNIFICANCE STATEMENT

Pancreatic cancer stem cells (CSC) have been first described in 2007 and since then have emerged as an intriguing entity of cancer cells with distinct functional features including self-renewal and exclusive in vivo tumorigenicity. The heterogeneous pancreatic CSC pool has been implicated in tumor propagation as well as metastatic spread. Clinically, the most important feature of CSCs is their strong resistance to standard chemotherapy, which results in fast disease relapse, even with today’s more advanced chemotherapeutic regimens. Therefore, novel therapeutic strategies to most efficiently target pancreatic CSCs are being developed and their careful clinical translation should provide new avenues to eradicate this deadly disease.

INTRODUCTION

I. Pancreatic cancer biology and pathology

Pancreatic cancer, most frequently presenting as pancreatic ductal adenocarcinoma (PDAC), is still a devastating diagnosis. The vast majority of clinical efforts to fight pancreatic cancer have not yet resulted in improved long-term survival. Among others, the following reasons have been accounted for the still poor outcome: i) missing early warning signs, ii) strong desmoplastic response impeding drug delivery, and iii) pronounced general resistance of pancreatic cancer cells to chemotherapy. Up to 90% of patients present with advanced disease and essentially all of them die within 12 months due to lack of effective maintenance treatments [1, 2]. Specifically, the more recent introduction of nab-paclitaxel (Abraxane) [3] and FOLFIRINOX [4] have improved initial response and subsequently extended median survival, but eventually the vast majority of patients still succumbs from progressive disease. Thus, while mortality for other tumor types is decreasing [1, 5], the incidence for pancreatic cancer (and thus related mortality) is still rising, presumably due to the growing prevalence of diabetes and metabolic syndrome as major risk factors [6]. This could make pancreatic cancer the 2nd most frequent cause of cancer related death by
2030, unless we succeed in developing more effective long-term treatments. The almost uniform occurrence of disease relapse has been attributed to the existence of pancreatic cancer stem cells (CSCs) and their distinct molecular features.

II. Pancreatic cancer stem cells

A growing body of evidence suggests that CSCs represent a subset of cancer cells with distinct stemness features that allow them to drive tumorigenesis and metastasis. In PDAC, as in other solid tumors [7, 8], CSCs are functionally defined by their ability to exclusively recapitulate the parental tumor upon transplantation into immunodeficient mice [9-16]. While CSCs and normal tissue stem cells share several signaling pathways, CSCs do not necessarily represent bona fide stem cells nor do they necessarily arise from tissue stem cells, but rather CSCs have acquired certain traits of stem cells allowing them to indefinitely self-renew and give rise to their respective differentiated progenies. While it has been shown conclusively that CSCs bear cell-intrinsic stemness features, they are also a product of their relationship with the tumor microenvironment affecting their aggressiveness, metastatic activity and drug resistance [17, 18]. Importantly, CSCs have proved to be particularly resistant to the current standard of care such as chemotherapy and radiotherapy, which renders them a primary source for tumor recurrences after or even during treatment [19]. Primary tumors with a more prominent stem cell signature are consistently associated with adverse outcomes and higher rates of metastases [20-22].

In order to now develop CSC-targeting therapies, it is therefore vital to further understand the biology of CSCs, and to study CSCs in the context of their niche. However, the low incidence of CSCs and their functional definition rendered their prospective identification and isolation a major challenge. CSC viability can be impaired by the mechanical and chemical tissue disruption during tumor processing, while the change in environment that occurs during cell culture may result in alteration or even loss of CSC properties. It is therefore vital to develop models that recapitulate the heterogeneity of primary tumors as well as the surrounding tumor environment, and increasing efforts are being made to design in vitro cell cultures and in vivo xenograft models from resected tumors. Such patient-derived xenograft (PDX) models have now been generated from a variety of cancers including PDAC [14], and have been shown to replicate characteristics of the primary patient tumor including genetic features and cellular heterogeneity. While the stromal microenvironment is also much richer than conventional cell line-based xenograft models, important species barriers between the rapidly arising mouse stroma and the human cancer cells represents a limitation, but could be overcome by frequently replenishing the human stroma cells including macrophages [17, 23].

Tracking and characterizing CSCs in cancer patients could provide information on the response to treatment, and may also allow the development of personalized therapeutic approaches based on the (epi-)genetic intra- and interpatient heterogeneity of CSCs. Over the past years, PDAC CSCs have been identified by a variety of biomarkers. In 2007, CD44+CD24+EPCAM+ cells [10] and CD133+ cells [12], respectively, were shown to be enriched for PDAC CSCs. Subsequently, other markers have also been used in an attempt to identify and isolate CSCs including ALDH1 [15], 26S proteasome activity [24], and hepatocyte growth factor receptor C-MET [9]. Though the list of CSC biomarkers is still growing, their expression is variably affected by isolation and culture conditions and response to treatment, and is moreover not exclusively linked to a CSC phenotype [25]. An intrinsic autofluorescent phenotype of PDAC CSCs has recently been identified and established to isolate and characterize these cells down to single cell level [11]. The source of the autofluorescence was found to be riboflavin actively sequestered in cytoplasmic vesicles by an ATP-dependent process. Interestingly, the autofluorescent population was detected in freshly digested tumors as well as early passage in vitro cultures from these tumors, but not in established tumor cell lines such as Panc-1. The autofluorescent phenotype is therefore a novel biological feature that appears to be robust, traceable in real time, and allows PDAC CSCs to be identified and purified, e.g. FACS and confocal microscopy, without the use of antibodies and independently of expression of cell surface markers (Figure 1). This phenotype could also be used to identify CSCs in several other carcinomas including breast, liver, lung and colorectal cancer [11] and thus could provide a unified platform for studying the complex dynamics of human CSCs.

Still, studying human CSCs bears the limitation that experiments need to be performed in an immunocompromised environment and controversies remain concerning the required level of immunodeficiency and the extend of supportive environment [13, 26-28]. Genetically engineered mouse models (GEMM) of PDAC faithfully resemble human disease and could provide a platform for studying CSCs in an immunocompetent environment without potential species barriers between the stroma and the cancer cells [29, 30]. Ischenko et al. recently reported an epithelial EPCAM+CD24+CD44+CD133+Sca1− population bearing CSC properties and metastatic potential [31]. While these studies provide first supportive evidence for a hierarchical organization of murine PDAC, they were mostly based on cultured primary cell lines [29]. Bailey et al. showed that DCLK1 marks a morphologically distinct subpopulation of cells with stem cell properties, but these studies focused on preinvasive pancreatic cancer [32]. Thus, more comprehensive studies of CSC phenotypes in murine PDAC are urgently needed.

III. Heterogeneity of the cancer stem cells

CSCs are not a homogeneous clonal population of cells, but undergo genetic evolution during tumor develop-
ment and progression (Figure 2). Subpopulations of CSCs, i.e. CD133+CXCR4+ [12] and C-MET+CD44+ cells [9], respectively, bear distinct features and have been found both in primary tumors as well as in distant metastases. Specifically, the chemokine receptor CXCR4 binds to stromal derived factor-1 (SDF-1), which is produced by bone marrow stromal cells to induce stem cell homing to the bone marrow, but also highly expressed in liver and lung. The invasiveness of CD133+CXCR4+ CSCs is markedly enhanced by SDF-1/CXCR4 signaling [12, 33]. Consistently, depletion of CD133+CXCR4+ cells from the heterogeneous CSC pool abolished metastasis without affecting tumorigenicity in vivo [12]. CD133+CXCR4+ CSCs were preferentially found in patients with metastatic disease. Thus, as different CSC subclones have different proliferative, invasive, metastatic, and resistance features, therapeutic approaches need to target all CSC subclones, but gaining access to metastatic CSC represented a huge challenge as biopsies are rarely obtained from patients with advanced disease.

Circulating tumor cells (CTCs) have been identified in patients with a variety of solid tumors[34, 35], and play an important role in seeding metastasis[36]. CTC levels in the bloodstream have been shown to predict disease outcome [37-39]. A number of techniques now exist to isolate CTCs from peripheral blood, but circulating CSCs are even more sparse [40-42]. Still, for metastatic breast and small cell lung cancer it has now been shown that a small subset of CTCs bears tumorigenic capacity in vivo, thus representing a putative CSC compartment within CTCs [43, 44]. Such circulating CSCs seem to bear unique features, distinct from other CSCs, that enable them to exit the primary tumor, evade immune surveillance, extravasate at distant sites, and drive metastatic growth [45, 46]. Several biomarkers have been used to track circulating CSCs. In breast cancer, ALDH1 and anthrax toxin receptor (ANTXR1) were found on CTCs exhibiting stem cell-like properties [47, 48]. Circulating CSCs in colorectal cancer patients were characterized by CD44v6 expression [49]. ABCG2 was present on circulating CSCs in lung cancer, PDAC, and retinoblastoma [50]. In addition, circulating CSCs are characterized by markers such as CD133, CXCR4, and CD44 that are also found on CSCs within the primary tumor [51, 52]. Due to the importance of this small subpopulation in driving drug resistance, metastasis and disease relapse, it will be essential to more fully phenotype circulating CSCs and prospectively isolated and thoroughly validate their tumorigenic/metastatic capacity.

The origin of circulating CSCs has not been established to date, and two non-exclusive hypotheses have been put forward (Figure 2). First, circulating and thus metastatic CSCs already arise in the primary tumor as CSCs with additional features rendering them capable of surviving in the blood stream and subsequently initiating metastatic spread [12]. Second, circulating CSCs may actually arise post hoc from disseminated tumor cells, e.g. out of a state of dormancy at a distant site after they already evaded from the primary tumor [53]. While both hypotheses are reasonable, none of them has been validated conclusively to date [54]. Consistent with the hypothesis that circulating CSCs are already present in primary tumors, stem cell marker positive cells isolated from primary tumors are able to form distant metastases when transplanted into secondary hosts [12, 55, 56]. Moreover, it has been clearly demonstrated that CSCs in the primary tumor display heterogeneous characteristics, which coincided, at least in PDAC with the expression of distinct surface markers [12].

Epithelial-to-mesenchymal transition (EMT) is a complex process leading to loss of epithelial traits via cellular de-differentiation, subsequent increased motility via rearrangements of cellular contact junctions and eventually the loss of cell adhesion. During this process, cells partially or fully transition from their epithelial phenotype into a mesenchymal one [57]. This transition enables the tumor cells to acquire migratory and invasive abilities, which facilitates their evasion from the primary tumor [58]. EMT is induced by several transcription factors, such as SNAIL, TWIST, ZEB1, ZEB2, SLUG, BMI-1, and others [59]. Importantly, EMT is thought to provide neoplastic epithelial cells not only with a mesenchymal and thus invasive phenotype, but may also induce stemness characteristics [60, 61]. Thus, EMT may propagate or, in some instance, even generate novo cells with exclusive tumorigenic and metastatic behavior [60]. As CSCs bear the functional plasticity for transitioning between mesenchymal-like and epithelial-like states, these cells are crucial for metastasis formation at distant sites [62]. Large-scale single cell studies in vitro showed that autofluorescent cells with CSC features could give rise to both autofluorescent and non-autofluorescent cells. By contrast, non-autofluorescent cells were never observed to give rise to autofluorescent cells, suggesting that CSCs cannot arise from non-CSCs. Furthermore, single non-autofluorescent cells formed no tumors in immunodeficient mice, while single autofluorescent cells produced tumors with similar features to the primary tumor, providing further evidence that the tumorigenic potential is restricted to the CSC subpopulation [11]. However, Chaffer et al. have shown that CSCs in breast cancer can arise from non-CSC cells, with the transcription factor ZEB1 playing a key role in this transition [63]. Further studies, including in vivo cell fate tracking experiments, are therefore needed to conclusively demonstrate whether pancreatic non-CSCs are capable of repleishing the CSC pool via EMT and therefore contribute to metastasis.

IV. Pancreatic cancer stem cell niche

Somatic stem cells reside in a niche providing optimal conditions for self-renewal [64, 65]. Over the past years we have begun to realize that dynamic interactions between malignant and stromal cells in the tumor microenvironment are also critical determinants for CSC fea-
tures. The pancreatic tumor microenvironment is composed of cancer-associated fibroblasts, pancreatic stellate cells, immune cells such as macrophages, blood vessels, and the extracellular matrix (Figure 3). Pancreatic stellate cells produce Nodal/Activin as pro-CSC factors [66]. The macrophage-derived IFN-stimulated factor ISG15 [17] and anti-microbial peptide cathelicidin LL-37 [23] also strongly promote stemness phenotypes of PDAC CSC including EMT. Interestingly, LL-37 was secreted by tumor-associated macrophages in response to TGF-β1 and particularly CSC-secreted Nodal/ActivinA. In return, LL-37 enhanced CSC features via formyl peptide receptor 2 (FPR2)- and P2X purinoceptor 7 receptor (P2X7R)-dependent mechanisms, which could be reversed by inhibiting these receptors. Importantly, in a GEMM of PDAC, the transformation process was inhibited by either reconstituting these mice with bone marrow from CRAMP (i.e. murine homolog of hCAP-18/LL-37) knockout mice or by pharmacologically inhibiting FPR2 and P2X7R [23]. Clinically even more important was the observation that LL-37 also enhanced chemoresistance of CSCs. Thus, in order to advance our understanding of CSC biology and to develop clinically meaningful CSC-centered treatment strategies, it will be essential to study drug response of CSCs in the context of their niche [17, 18].

The fibrous PDAC tissue is mostly made of cancer-associated fibroblasts, which impede drug delivery and worsen the prognosis for PDAC by directly and indirectly promoting tumor progression [67, 68]. Subsequently, the concept of targeting the stroma to enhance drug delivery was developed [30]. Hedgehog signaling is frequently up-regulated in fibrogenic components of the PDAC tumor microenvironment and inhibition of Hedgehog signaling indeed enhanced delivery of chemotherapy in a GEMM of PDAC [30]. However, a subsequent clinical phase I/II trial utilizing the same hedgehog inhibitor IPI-926 to deplete myofibroblasts in combination with gemcitabine had to be stopped, because patients in the hedgehog inhibitor arm were living shorter than patients in the control arms (ClinicalTrials.gov Identifier NCT01130142). In line with these findings, the small molecule inhibitor vismodegib also did not result in significant changes in CSCs content or clinical outcome in metastatic PDAC [69]. In subsequent preclinical studies, performed in the aftermath of the above negative clinical trials, myofibroblasts were genetically targeted in GEMM of PDAC and tumors also became more aggressive with enhanced immunosuppressive properties and increased CSC content [70].

Should inhibitors of hedgehog signaling thus be excluded from future translational studies in PDAC? Here it is important to note that several combination treatments including hedgehog inhibitors showed rather promising results. While stroma targeting alone may result in adverse outcome, its combination with CSC targeting agents could still be considered useful. Various treatments including inhibitors of Nodal/Activin signaling [71], mTOR inhibitors [72, 73] and the anti-diabetic drug metformin [74] were more effective when used in combination with hedgehog inhibitors. Interestingly, the antimalarial drug chloroquine was also shown to inhibit hedgehog signaling, but is also a potent inhibitor of CXCR4, and exerted lasting anti-tumor effects in combination with gemcitabine [75]. Clinical trials are currently ongoing (e.g. ClinicalTrials.gov Identifier NCT01777477). Thus, while hedgehog inhibitors as single adjuvant treatment did not fulfill clinical expectations in PDAC, their combination with CSC-targeting agents could still be considered useful.

In the meantime, however, new approaches for targeting the stroma have emerged. Enzymatic digestion of hyaluronic acid, a major component of the desmoplastic stroma, by hyaluronidase (PEGPH20) resulted in re-expansion of the tumor blood vessels, enhanced perfusion, and subsequently chemotherapeutics had improved vascular access to the tumor tissue [76, 77]. Clinical trials with PEGPH20 were rapidly initiated, but had to be put on clinical hold in early 2014 due to increased thromboembolic events in the PEGPH20 group. However, studies are now continuing under a revised protocol and results are expected to be available in 2016 (clinicaltrials.gov identifier NCT01839487). Sherman et al recently uncovered an important inhibitory role of the vitamin D receptor (VDR), which are highly expressed in pancreatic stellate cells and their engagement results in transcriptional expression of genes that maintain a quiescent stat. Continuous activation of VDR signaling by the vitamin D-like compound calcipotriol decreased stromal inflammation and fibrosis, and increased tumor sensitivity to gemcitabine [78]. A pilot clinical trial has already been initiated (ClinicalTrials.gov Identifier NCT02030860). Normalized blood flow in PDAC tumors could also be achieved by combined treatment with low-dose cilengitide and verapamil resulting in chemosensitization to gemcitabine with subsequently reduced tumor growth and spread [79]. Finally, genetic ablation of focal adhesion kinase (FAK) in tumor endothelial cells reduced doxorubicin-induced angiocrine signals, thus enhancing chemosensitization [80], which is an interesting strategy considering the current clinical testing of various FAK inhibitors.

V. Targeting of pancreatic cancer stem cells
Despite expanding research activities to develop more effective treatment modalities for patients with PDAC, there has been little therapeutic progress towards improving patients’ long-term survival and also above stroma-targeting strategies are unlikely to result in long-term survival of patients. Gemcitabine [81], FOLFIRINOX [4], and more recently the addition of nab-paclitaxel (Abraxane) [3] are able to moderately extend median survival, regularly in the range of a few months, but eventually the vast majority of patients will succumb from progressive disease. Although it is not a defining feature of CSCs, it has been conclusively shown that CD133+ CSCs in PDAC are more resistant to standard chemotherapy than their CD133− counterparts [12]. Consistently gemcitabine
therapy also led to a relative increase in the numbers of C-MET+ CSCs [9]. Survival of such highly resistant CSCs during chemotherapy despite initial tumor regression thus represents a plausible explanation for the later, mostly fatal relapse of the disease in patients with PDAC [3, 4].

**Targeting the regulatory machinery of CSCs.** Signaling pathways that are active in PDAC CSCs may be attractive therapeutic targets (Table 1). For example, the embryonic Activin/Nodal signaling pathway is silenced in postnatal live, but becomes reactivated in CSCs [71]. Interestingly, inhibition of Nodal/Activin (via blockade of their receptors Alk4/7) also inhibits activation of tumor-associated macrophages by TGFB family members, thus rendering this an intriguing double-target treatment strategy (Figure 3) [23]. In this context, mir-17-92 polycistronic cluster has recently been show to be downregulated in quiescent and thus more chemoresistant CSCs [82]. Induced overexpression of mir-17-92 reversed CSC quiescence and rendered them sensitive to gemcitabine, whereas knockdown of mir-17-92 in differentiated PDAC cells introduced CSC features. Indeed, mir-17-92 was found to target Nodal/Activin signaling by inhibiting expression of the receptor Alk4 and downstream targets p21, p57, and Tbx3. These findings therefore identify the miR-17-92 cluster as a functionally determining family of miRNAs in CSCs, and highlight the putative potential of developing modulators of this cluster to overcome drug resistance in pancreatic CSCs.

Inhibitors for Notch and CXCR4 have also shown promising activity against pancreatic CSCs [83]. The small molecule BBi608, identified by its ability to inhibit gene transcription driven by Stat3 and cancer stemness properties, efficiently blocked cancer relapse and metastasis in mice [84]. Integrin α(v)β3 was shown to be strongly expressed on pancreatic CSCs and, in the unliganded state, recruited KRAS to the cell membrane leading to resistance to erlotinib [85]. Inhibition of α(v)β3 reversed stemness and rendered the cells sensitive to erlotinib.

The anti-diabetic drug metformin demonstrated already anti-tumor activity in several cancer types. Intriguingly, pancreatic CSCs are highly vulnerable to metabolic reprogramming by metformin resulting in tumor regression and extended survival of preclinical mouse models [86]. Importantly, however, sensitivity to metformin showed strong inter-patient variability and, eventually, most of the tumors became resistant to metformin resulting in disease relapse. These findings suggest that the metabolic phenotype and/or plasticity of CSCs may vary between patients or even between different subpopulations of CSCs within each tumor. This could also explain the discouraging preliminary results obtained in two recent clinical trials using metformin in combination with standard of care for locally advanced and metastatic PDAC [87, 88]. Understanding the mechanism of metformin resistance could help to establish a revised protocol for the more effective use of this safe and cost-effective drug.

HDAC inhibitors may be an alternative approach by altering the CSC epigenome. The HDAC inhibitors 5-Aza-dC and SAHA blocked self-renewal, induced apoptosis by reactivating expression of miR-34a, an effector of p53 down-regulated in PDAC, and blocked expression of EMT transcription factors [89]. Very recently, the HDAC inhibitor mocetinostat was shown to be more effective than other HDAC inhibitors, such as SAHA, to interfere with ZEB1 function, restore miR-203 expression, repress stemness properties, and induce sensitivity against chemotherapy [90]. Salinomycin, an antibacterial drug, has been shown to block the multidrug resistance P-glycoprotein and thus inhibited proliferation of a number of cancer cell lines [91] and also blocked tumor growth and metastatic spread in a GEMM for PDAC [92]. FDA-approved antibiotics such as azithromycin have also been reported to eradicate CSCs in PDAC and other cancers [93]. Interestingly, azithromycin targets mitochondrial biogenesis as a ‘side effect’, further corroborating the notion that CSCs bear a distinct metabolic phenotype with strict dependence on mitochondrial biogenesis for clonal expansion and survival. Murine pancreatic cancer cells, which had survived Kras ablation and may also bear some features of CSCs, express high levels of genes regulating mitochondrial function, heavily rely on mitochondrial respiration, are sensitive to inhibitors of oxidative phosphorylation, and may therefore be susceptible to therapeutic approaches targeting mitochondrial function, respiration and biogenesis [94].

Natural compounds from dietary sources may also represent interesting strategies for eliminating CSCs. Resveratrol, a polyphenol found in red grapes, bears demonstrated anti-tumoral properties in several malignancies. Resveratrol showed efficacy against glioblastoma and breast CSCs, and also blocked self-renewal of pancreatic CSCs by activation of caspase 3/7 and inactivation of Bcl-2 [83]. Curcumin, a substance naturally present in curry powders and mustard, and its analogue Difluorinated-Curcumin (CDF) have been shown to improve sensitivity of PDAC cells to gemcitabine [95]. Enhancing PTEN signaling and miR-200 expression were shown to facilitate the effects of CDF to reduce sphere formation and in vivo tumor growth [96, 97]. Sulforaphane (SFN), an active component in cruciferous vegetables such as broccoli, was found to inhibit self-renewal of pancreatic CSCs by blocking the hedgehog pathway [98]. Preclinical studies have shown that vitamin D and respective analogues do not only revert pancreatic stellate cells into a quiescent phenotype, but also have direct anti-proliferative effects on pancreatic cancer cells [99, 100]. Engagement of VDR sensitized pancreatic cancer cells to gemcitabine, suppressed their stemness properties, and inhibited growth, invasion and metastases of pancreatic tumors [101, 102]. Further studies should now address whether appropriate doses and ideal combinations of any of these substances can

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intratumoral the does largely due to their recent success against hematological malignancies. The concept of immunotargeting is particularly intriguing for PDAC due to the strong inter- and intratumoral heterogeneity. As such, most single or combinational therapies targeting signaling pathways may not be able to cope with the underlying (epi)genetic diversity in PDAC, whereas immunotherapy does not depend on specific signaling pathways and thus could be more effective in eradicating the root of the disease. In this context, Visus et al. reported the isolation of CSCs from several tumors including PDAC based on ALDH activity [103]. In vitro ALDH1A1-specific CD8+ T cells were generated, and successfully deployed to destroy ALDHbright CSCs in human tumor xenografts models, leading to reduced tumor growth and metastasis. However, ALDH1A1-specific CD8+ T cells may also target normal ALDHbright stem cells such as hematopoietic stem cells. Dendritic cells loaded with CSC lysates could potently activate lymphocytes suggesting that vaccines based on CSC lysates may be a viable strategy for PDAC therapy [104].

The bispecific antibody MT110 targets the T-cell receptor CD3 complex and EPCAM and has shown preclinical efficacy in several PDX models [105, 106]. Unfortunately EPCAM expression may be lost in cells undergoing EMT, and metastasizing cells may therefore escape this treatment. In EPCAM+ tumors, however, MT110 efficiently eliminated the CSC population. MT110 is currently being investigated in a phase I clinical trial of different carcinomas (ClinicalTrials.gov identifier NCT00635596). A different approach would be to target tumor defenses against the host immune system. CD47 is a cell surface molecule that mediates an inhibitory signal to macrophages, thereby preventing phagocytosis of the tumor cells. CD47 is strongly expressed on PDAC CSCs, and targeting CD47 with a monoclonal antibody against CD47 efficiently enhanced phagocytosis and, equally important, long-term inhibition of CD47 also directly induced CSC apoptosis in the absence of macrophages [107]. CD47 targeting along with gemcitabine and even more so with nab-paclitaxel resulted in significant tumor regression in vivo. A CD47 monoclonal antibody (CC-90002) is currently being tested in a phase I study in patients with a variety of malignancies (clinicaltrials.gov identifier NCT02367196). Finally, adoptive immunotherapies using CAR-T cells (T cells modified to express a Chimeric Antigen Receptor against a tumor cell surface antigen) have also shown promise in GEMM for PDAC [108-111]. CAR-T cell therapies against human PDAC, targeting commonly expressed surface antigens such as CEA and mesothelin, are currently being tested in clinical trials (clinicaltrials.gov identifier NCT02394724 and NCT01583686), but their efficacy against CSCs remains to be determined.

CONCLUSIONS AND FUTURE PERSPECTIVES

Over the past years the importance of pancreatic CSCs for tumor progression and metastasis has been established. Markers such as EPCAM, CD44, CD133 and CXCR4 have been validated, but remain prone to artifacts. Here the recent identification and characterization of an intrinsic autofluorescent phenotype in CSCs could further enhance our ability to more robustly study the complex dynamics of human CSCs. The next milestone is to demonstrate that any of the various CSC-targeting approaches listed above actually harbor potential to ameliorate clinical outcome of patients with PDAC. At the same time we need to continue to further advance our understanding of PDAC CSC biology and their respective niche. This should lead to the identification of novel therapeutic targets capable of eliminating PDAC CSCs as well as the pro-CSC microenvironment. Together these studies should facilitate the development of CSC-centered multimodal precision medicine approaches for PDAC and thus may eventually improve the still miserable prognosis for PDAC patients.

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AUTHOR CONTRIBUTIONS

D.R.: Conception and design, manuscript writing; A.A.: Conception and design, manuscript writing; C.H.: Conception and design, manuscript writing, financial support, administrative support, final approval of manuscript.

REFERENCES

9 Li C, Wu JJ, Hynes M et al. c-Met is a marker of pancreatic cancer stem cells and
32 Bailey JM, Alisma J, Rasheed ZA et al. DCLK1 marks a morphologically distinct subpopulation of cells with stem cell properties in preinvasive pancreatic cancer. Gastroenterology. 2014;146:245-256.
52 Zhang SS, Han ZP, Jing YY et al. CD133(+)CXCR4(+) colon cancer cells exhibit metastatic potential and predict poor prognosis of patients. BMC medicine. 2012;10:85.
91 Meidhof S, Brabertz S, Lehmann W et al. ZEB1-associated drug resistance in cancer cells is reversed by the class I HDAC inhibitor mocetinostat. EMBO molecular medicine. 2015.

104 Yin T, Shi P, Gou S et al. Dendritic cells loaded with pancreatic Cancer Stem Cells (CSCs) lysates induce antitumor immune


Figure 1. Autofluorescence as an inherent CSC feature. (a) An autofluorescent subpopulation of cells was accidentally detected in human pancreatic cancer. This distinct inherent CSC property represents a novel biological feature that is traceable in real time and provides unprecedented robustness and power for the identification and purification of CSCs without the use of antibodies or any kind of manipulation, thus drastically reducing experimental errors and artifacts. (b) The autofluorescence was restricted to cytoplasmic vesicles, (c) and dependent on ABCG2 transporters expressed on the membrane of the autofluorescent vesicles. (d) The spectrum of autofluorescence was identified as originating from riboflavin, which is pumped into the vesicles by ABCG2. (e) Autofluorescence, ideally detected as the intersection with filters 530/40 and 580/30 upon excitation with a blue laser (488nm), is greatly enhanced when riboflavin content is increased to supra-physiological levels (30μM) and ideal for FACSorting and cell tracking by confocal microscopy.
Figure 2. Tumorigenesis and cancer progression. (Left) Intraclonal tumor heterogeneity is formed by cancer stem cells and their differentiated progenies. CSCs are capable of undergoing unlimited cell division while retaining their stem cell identity (self-renewal) and giving rise to non-CSCs with limited proliferative capacity (differentiation). CSCs evolve as the tumor progresses via (epi-)genetic alterations, but also in response to bilateral interactions with their niche, leading to diverse CSC subclones with distinct functionality. Following genetic or epigenetic changes, both cancer stem cells and non-cancer stem cells can display migratory behavior at the invasive front of primary tumors, which may be associated with EMT. (Center) Two hypotheses are proposed for the origination of circulating cancer stem cells: (1) circulating cancer stem cells may arise in the primary tumor as cancer stem cells with additional features rendering them capable of surviving in the blood stream and subsequently initiating metastatic spread, or, (2) after a period of dormancy, disseminated tumor cells may convert into circulating cancer stem cells through poorly understood processes yet to be elucidated. (Right) Circulating cancer cells must survive the hostile environment of the blood stream, evade immune surveillance and extravasate at a distant location to form metastatic lesions. Cancer stem cells can also recolonize their tumors of origin, in a process called “tumor reseeding”. This process selects for highly aggressive circulating tumor cells (CTCs), which are more efficient for metastasis than their parental populations.
Figure 3. The complex tumor stromal microenvironment provides a niche for CSCs, with cellular stromal compartments secreting factors essential for certain CSC features. Secretion of Nodal and Activin by pancreatic stellate cells (PSC) and cancer-associated fibroblasts (CAF) drives expression of CSC-specific genes and enhances invasiveness. Tumor associated macrophages (TAM) are activated by TGFβ and Nodal/Activin signaling, the former playing an important role in mediating local immunosuppression. Nodal is recognized by the Alk4 and Alk7 TGFβ serine/threonine kinase receptors and the Cripto-1 co-receptor. In turn, TAMs secrete ISG-15 and LL-37, which promote stemness features of CSCs, LL-37 being recognized by the receptors P2X7 and FPR2. The stroma-CSC interactions can be targeted with potential therapeutic relevance; for instance, TGFβ family signaling can be inhibited by the small molecule LY2157299, which also inhibits cell-autonomous signaling in cancer stem cells. LL-37 can be abrogated by the small molecule KN-62, and vitamin D analogues have inhibitory effects on both PSCs and CSCs.
### Table 1 – Strategies for direct or indirect therapeutic targeting of PDAC CSCs.

This may involve signaling pathways shown to be vital in CSCs, CSC metabolism, or the unique epigenetic state of CSCs. In addition, some antibiotics and certain natural compounds have also shown preclinical activity against PDAC. Finally, a variety of immunotherapeutic approaches have shown promise in preclinical models of PDAC, and some (such as CAR-T cell therapy) are currently being tested in Phase I clinical trials. Targeting the CSC niche may also result in enhanced vulnerability of the CSC compartment, but may require the combination with direct CSC targeting agent.

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<td>• Nodal/ActivinA</td>
<td>• Metformin</td>
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<td>• CICR4</td>
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<td>• Revesatrol (may induce CSC apoptosis)</td>
<td>• Dendritic cells primed with CSC lysates</td>
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<td>• Stat3</td>
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<td>• Cucurmin, CDF (chemosensitization)</td>
<td>• Monoclonal antibodies (CD47)</td>
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<td>• ovlβs</td>
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<td>• Sulforaphane (inhibits self-renewal)</td>
<td>• Bispecific antibodies (MT110)</td>
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<td></td>
<td></td>
<td></td>
<td>• Azithromycin (targets mitochondrial biogenesis)</td>
<td>• CAR-T cell therapy (CEA, mesothelin)</td>
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<td></td>
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<td></td>
<td>• Salinomycin (may induce CSC apoptosis)</td>
<td>• Hyaluronidase (PEGPH20; enhanced tumor perfusion)</td>
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<td></td>
<td></td>
<td></td>
<td>• Cucurmin, CDF (chemosensitization)</td>
<td>• A84, 5, 7 blockade (inhibits activation of macrophages towards M2)</td>
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<td></td>
<td>• Sulforaphane (inhibits self-renewal)</td>
<td>• Vitamin D analogue (e.g., calcipotriol; inhibition of stellite cell activation)</td>
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<tr>
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<td></td>
<td>• Azithromycin (targets mitochondrial biogenesis)</td>
<td>• FAK blockade (inhibition of angocrine factors leading to chemosensitization)</td>
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<td></td>
<td>• Salinomycin (may induce CSC apoptosis)</td>
<td>• Cilengitide plus verapamil (normalization of blood flow leading to chemosensitization)</td>
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