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REVIEW

Pancreatic cancer stem cells: features and detection methods

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with a high incidence of distant metastasis and recurrence. Cancer stem cells (CSCs), which are pluripotent, self-renewable, and capable of forming tumors, contribute to PDAC initiation and metastasis and are responsible for resistance to chemotherapy and radiation. Three types of experimental methods are commonly used to identify CSCs: CSC-specific marker detection, a sphere-formation assay that reveals cell proliferation under non-adherent conditions, and detection of side-population (SP) cells that possess high intracellular-to-extracellular pump functions. Several CSC-specific markers have been reported in PDACs, including CD133, CD24, CD44, CXCR4, EpCAM, ABCG2, c-Met, ALDH-1, and nestin. There remains controversy regarding which markers are specific to PDAC CSCs and which are expressed alone or in combination in CSCs. Examining characteristics of isolated CSCs and discovering CSC-specific treatment options are important to improve the prognosis of PDAC cases. This review summarizes CSC-detection methods for PDAC, including CSC-marker detection, the sphere-formation assay, and detection of SP cells.

Keywords Pancreatic cancer · Cancer stem cell/CSC · Sphere · Side population · Cancer stem cell marker

Introduction

Pancreatic cancer is among the most lethal types of malignant tumor, as ~338,000 people were diagnosed with the disease worldwide in 2012 [1] and with almost the same number dying as a result of it. Pancreatic ductal adenocarcinoma (PDAC), a major histological subtype comprising 90% of all pancreatic cancer, has a high mortality rate due to its aggressive growth and high metastatic rate [2]. Surgery offers the only possible cure for PDAC; however, 80% of PDAC patients are inoperable at diagnosis, and no curative treatment is available for advanced PDAC. This aggressiveness leads to an extremely poor prognosis, with the most recent overall survival rate for pancreatic cancer at 8% [3]. Even after surgery, the 5-year survival rate for PDAC remains low (15–20%), with most patients dying because of metastatic disease and local recurrence [4]. By the year 2030, pancreatic cancer is expected to surpass all gastrointestinal cancers to become the second-leading cause of cancer-related death in the United States, trailing only lung cancer [5].

The development of early detection methods and effective therapy for advanced PDAC patients is necessary to improve the poor prognosis. PDAC does not exhibit any characteristic symptoms in its early stages, and serum levels of CA19–9 and carcinoembryonic antigen are not useful for early diagnosis. Currently, chemotherapies or chemoradiotherapies are used to reduce tumor size and improve the prognosis of advanced PDAC patients; however, these treatments are not capable of fully eradicating PDAC cells.

The classical model of carcinogenesis can be described as "stochastic", where any cell in an organ, such as the pancreas, can be transformed by mutations [6]. This model assumes that

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tumors are biologically homogeneous. All or most cells in a fully developed PDAC are equally malignant, and a stochastic model implies that strategies focused on curing pancreatic cancer require the killing of all malignant cells. However, a recent genome-sequencing study found significant variability between individual PDACs and between primary and metastatic PDAC lesions [7]. A small number of cells possess stem-cell-like characteristics in various cancers, with such cells referred to as cancer stem cells (CSCs) [8–10]. A CSC is defined as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor" according to the 2006 symposium of the American Association of Cancer Research [11]. CSCs are also resistant to chemotherapy and radiation and are considered responsible for tumor recurrence after the completion of adjuvant therapy. In this review, we summarize the characteristics of and detection methods for pancreatic CSCs and the possibility of CSC-targeting therapy for PDAC.

Characterization of pancreatic CSCs

PDAC arises through multiple steps that include pancreatic intraepithelial neoplasias (PanINs) and culminate in invasive cancer [12]. Reports of CSCs in premalignant pancreatic lesions are limited. We previously reported that cells positive for the CSC markers cluster of differentiation (CD)24, CD44, C-X-C chemokine receptor type 4 (CXCR4), epithelial specific antigen (ESA), and nestin were enriched at higher PanIN grades, although CD133 did not increase with the malignancy grade [13].

The cell origin of pancreatic CSCs remains unknown [9]. Hypothesized sources include: 1) tissue stem cells or progenitor cells, 2) stem cells derived from bone marrow, and 3) de-differentiated cells resulting from genetic mutation. Pathways, such as Notch, Wnt/β-catenin, Sonic hedgehog (Shh), and B cell-specific Moloney murine leukemia virus-integration site (BMI)-1, might contribute to the development and progression of PDACs and to the biology of pancreatic CSCs [10]; however, specific genetic alterations or biomarkers for pancreatic CSCs within the population of pancreatic cancer cells remain to be elucidated. Several studies of genetically engineered mice suggested that pancreatic acinar cells, centroacinar cells [14], or acinar-ductal metaplasia [15] might represent the "cells of origin" in pancreatic cancer, whereas another study suggested that some pancreatic epithelial cells characterized by the expression of c-Met$^+$CD133$^+$CD34$^+$CD45$^-$Ter119$^-$ and Pdx1 are related to pancreatic carcinogenesis [16, 17]. Additionally, the effects of niche on normal stem cell function are reportedly critical [18], and tumor-associated stromal fibroblasts in PDAC regulate tumor-cell growth [19].

Identification and isolation of pancreatic CSCs

Three major methods are employed to identify CSCs from various organs: CSC-specific marker detection, the sphere-formation assay, and detection of side-population (SP) cells. In PDAC-tissue sections, CSCs are detected using CSC-specific markers and immunohistochemical or immunofluorescent staining. Pancreatic CSCs exhibit specific cell-membrane markers, including CD133, CD44, CD24, CXCR4, ATP-binding cassette sub-family G member 2 (ABCG2), and epithelial-cell-adhesion molecule (EpCAM; also known as ESA) [20–24]; however, the roles of these markers have not been studied in detail. We previously reported that nestin, an exocrine progenitor-cell marker in the pancreas [25], plays important roles in the migration, invasion, and metastasis of PDAC cells [26–29]. In cultured PDAC cells, CSC-marker-positive cells and SP cells, which contain abundant CSCs, have been detected and isolated using flow cytometry. In the sphere-formation assay, when PDACs are cultured in ultra-low attachment dishes, the CSCs form floating colonies. According to studies using these detection methods, CSCs account for only a small fraction in PDAC tissues or PDAC cell lines (Table 1).

CSC markers

Several cell-surface markers, including CD133 [28, 30], CXCR4 [23, 28], EpCAM, CD24 [21], CD44 [31, 32], ABCG2 [30], and c-Met [33], have been used for flow cytometric sorting of pancreatic CSCs. However, no unique marker has been identified for the isolation of CSCs from different tumor types; therefore, a combination of several markers might increase the purity of isolated CSCs [34, 35].

Putative pancreatic CSCs were first defined by the simultaneous expression of CD44, CD24, and EpCAM [32]. Li et al. [32] demonstrated that CD44$^+$CD24$^+$EpCAM$^+$ cells, which constituted only 0.2 to 0.8% of tumor cells, exhibited a 100-fold increase in tumorigenic potential as compared with CD44$^-$CD24$^-$EpCAM$^-$ cells. The CD44$^+$CD24$^+$EpCAM$^+$ subpopulation exhibited features typically observed in adult stem cells, including self-renewal, generation of differentiated progeny, and activated developmental signaling pathways, such as the Shh pathway [32]. Therefore, the CD44$^+$CD24$^+$EpCAM$^+$ subpopulation contains putative pancreatic CSCs, which fulfil the two functional criteria of self-renewal and differentiation into the full spectrum of tumor-cell progeny [36]. Because CD44 defends against reactive oxygen species [37], this might suggest a mechanism for correlation of CD44 with CSCs.

CD133, also known as prominin-1, was first discovered as a marker of normal hematopoietic stem cells [38] and later found to distinguish CSCs from a variety of tissues, including breast [39], brain [40], liver [41], colon [42, 43], prostate [44],

Table 1 Expression of CSC markers in human pancreatic ductal adenocarcinoma

CSC markers	IHC (%)	FCM (%)	Reference
ABCG2		0.4–7.33	46
ALDH		16.2	74
ALDH1	78.9		45
CD24	26	0.071–45.3	32
		3–28*	30
		57.8–70.1	51
CD24/CD44		2.1–3.5	51
CD44	15.1	46.1–100	32
		2–9*	30
		5.1–17.5	51
CD44/c-Met		0.5–5*	52
CD44/CD24/EpCAM		0.2–0.8*	30
CD133	14.5	0–1.61	32
		1.09–3.21	29
		1.98–69.85	46
CXCR4	35.6	0.274–38.2	32
EpCAM /ESA	60.6	1.36–93.7	32
		11–70*	30
Nestin	13.5	0.662–11.5	32

IHC: immunohistochemistry, FCM: flow cytometer, * Xenografted human pancreatic cancer cells

and pancreatic tumors [28, 45, 46]. CD133 is a glycosylated protein with five transmembrane domains and a ganglioside-binding motif present in the extracellular domain [47], with tyrosine phosphorylation capable of occurring in the cytoplasmic domain [48]. The possibility that CD133/Src signaling provides a regulatory switch from stemness properties to induce the epithelial-mesenchymal transition (EMT) has been reported [49]. In primary PDACs and PDAC cell lines, CD133 expression reflects high proliferation potential and tumorigenesis with chemotherapeutic resistance. Additionally the $CD133^+$ subpopulation of pancreatic cancers exhibits decreased apoptosis when treated with gemcitabine [28], and hypoxia induces tumor aggressiveness, with this process associated with the expansion of $CD133^+$ pancreatic cancer cells in a predominantly hypoxia-inducible factor-1α-dependent manner [50]. Moreover, CD133, nestin, and SRY-box-2 expression are elevated in CSCs under hypoxic conditions, and these cells also exhibit increased proliferation rates and self-renewal potential [51]. Hermann et al. [28] defined the $CD133^+$ subpopulation as CSCs from 11 primary human PDAC samples and PDAC cell lines, with this subpopulation capable of reconstituting pancreatic tumor growth with full tumor differentiation, similar to the $CD44^+CD24^+EpCAM^+$ subpopulation [28]. Additionally, an overlap of 10 to 40% between $CD44^+CD24^+EpCAM^+$ and $CD133^+$ PDAC cells has been reported [28]. Because of the different glycosylation patterns of CD133 in CSCs and differentiated tumor cells, only AC133 (a mouse monoclonal IgG antibody that recognizes epitope 1 of CD133) is reportedly capable of identifying CSCs [52].

CXCR4 is implicated in the invasion and metastasis of pancreatic cancer [53], with its expression initiated at the early stages of pancreatic carcinogenesis in PanINs and maintained during progression to invasive cancers and metastatic diseases. CXCR4-expressing cells also appear to mediate pancreatic cancer metastasis [28]. Regarding CD133, Hermann et al. [28] identified a distinct subpopulation of $CD133^+CXCR4^+$ CSCs that determine the metastatic phenotype of the individual pancreatic tumor. Stromal-cell-derived factor (SDF)-1, also known as CXCL12, is a specific ligand of the CXCR4 and induces the migration of $CD133^+$ cancer cells *in vitro*. *In vivo* experiments using sorted $CD133^+CXCR4^+$ cells demonstrated that co-expression of this receptor is essential for the generation of liver metastasis, indicating that targeting the SDF-1/CXCR4 axis might be a useful strategy for inhibiting PDAC metastasis.

Some other distinctive markers have been investigated, including aldehyde dehydrogenase-1 (ALDH-1) associated with tumorigenic cells in PDAC [24, 54–56]. $ALDH^+$ cells, detected using the Aldefluor assay, are also enriched for pancreatic tumor-initiating cells [56]. On the other hand, comprehensive investigations suggest abundant expression of ALDH-1 in normal pancreatic tissues, which would disqualify ALDH-1 as a suitable marker for CSCs in humans. Additionally, c-Met, the hepatocyte growth-factor receptor, plays important roles in pancreatic CSC biology [24], with expression of c-Met capable of identifying pancreatic CSCs along with high levels of CD44.

Recent studies show that doublecortin-like kinase (DclK)1 and leucine-rich repeat-containing G-protein-coupled receptor (Lgr)5 are putative novel CSC markers for pancreatic cancer [57]. DclK1 regulates several key oncogenes, including c-MYC, KRAS, and Notch, and EMT. DclK1 is expressed in the isolated normal pancreatic duct and islet cells, as well as in PanINs and PDAC [58]. DclK1-expressing normal pancreatic cells can possess progenitor-like function [59], and invasive and pre-invasive pancreatic cancer might depend upon DclK1-expressing cells with CSC capabilities. Lgr5 is the Wnt-target gene that marks Wnt-driven, actively diving stem cells [60]. Lgr5 is expressed in resected PDAC tissues [61], and patients harboring high levels of Lgr5-positive cells exhibit shorter median survival rates [62].

The clinicopathological roles of these CSC markers in PDAC tissues remain controversial. We performed an immunohistochemical analysis of the localization of CSC markers using tissues from 105 patients with conventional PDAC [13]. $CD24^+$, $CD44^+$, $CXCR4^+$, ESA-positive, and nestin-positive cells were detected in the following tissues listed in order of increasing percentage: normal ducts < low-grade PanINs <

high-grade PanINs < PDACs. Although CD133 did not increase with the malignancy grade, the expression of most CSC markers is related to carcinogenesis via the PanIN-to-PDAC sequence. The expression of CXCR4 and EpCAM is correlated with a well-differentiated histological type of PDAC, and venous invasion was positively associated with CD133 and inversely associated with EpCAM.

Sphere-formation assays

Cells from both normal and cancerous neural tissues have the ability to form colonies in spherical aggregates under non-adherent culture conditions, which indicates self-renewal capability [40]. PDAC cell lines can form spheres (Fig. 1a and b), and sphere-forming cells possess stem cell abilities [63–67]. Additionally, sphere-forming cells exhibit high tumor-formation rates relative to non-sphere-forming cells. Moreover, $CD44^{+}CD24^{+}$ fractions from pancreatic tumors are enriched in sphere-forming cells [66], and nestin, a pancreatic CSC marker, was more highly expressed in the spheres of three pancreatic cancer cell lines than in non-sphere cells [68]. Furthermore, pancreatic cancer cells derived from the metastatic foci of immunodeficient mice formed a greater number of spheres on low-attachment plates than their primary tumor counterparts [29]. On the other hand, it is possible that different culture media alter stemness characteristics, such as drug efflux ability, in spheres of PDAC cells. A previous study also reported that sphere-forming ability is not correlated with drug efflux ability [69].

SP cells

SP cells that can exclude the DNA dye Hoechst 33342 are considered to possess CSC-like features in several tumors [69–71]. Olempska et al. [30] demonstrated the existence of SP cells in PDACs [30]; however, it remains unclear whether the SP cells within PDACs are enriched for CSCs [72]. Furthermore, and the use of SP cells as CSCs in gastrointestinal cancers (including gastric and colorectal cancers) has generated conflicting data [73]. Zhou et al. [74] stained the human PDAC cell line PANC-1 with Hoechst 33342 dye and identified 2.1 to 8.7% (median 3.3%) of all viable cells as SP cells [74]. Additionally, SP cells exhibit enhanced capacities for efflux of gemcitabine and Hoechst 33342 dye, with the drug-efflux capacity of PANC-1 SP cells leading to a significant survival advantage. These results might support the hypothesis that SP cells within tumors use their self-renewal capacity to help maintain chemotherapy resistant cancer foci. In a previous study, we detected SP cells in PANC-1 cell populations from the metastatic tumors of immunodeficient mice at approximately twice the proportion of their occurrence in counterpart parental PANC-1 populations [29]. Therefore, targeting SP cells might diminish drug resistance, reduce metastasis, and improve patient survival.

To analyze SP cells, Hoechst 33342 is added to PDAC cells, followed by incubation. SP cells and major population (MP) cells are then separated by flow cytometry [75] (Fig. 2a). The injection of cells from the SP fraction resulted in a larger tumor volume than did the injection of the same number of cells from the MP fraction (Fig. 2b). Moreover, resected subcutaneous tumor tissues derived from SP and MP fractions exhibited similar histological features after 5 weeks. These histopathological findings might indicate the differentiation of CSCs to non-CSCs. A Hoechst 33342 SP assay requires an ultraviolet laser for maximum excitation, and this laser is expensive. A DNA-binding dye structurally similar to Hoechst 33342 and with an excitation spectrum shifted toward the violet range (DyeCycle Violet) has recently been used for SP analysis by flow cytometers equipped with violet laser diodes [76].

Several types of drug-efflux pumps are present in cancer cells. In the case of chemoresistance, several cancers

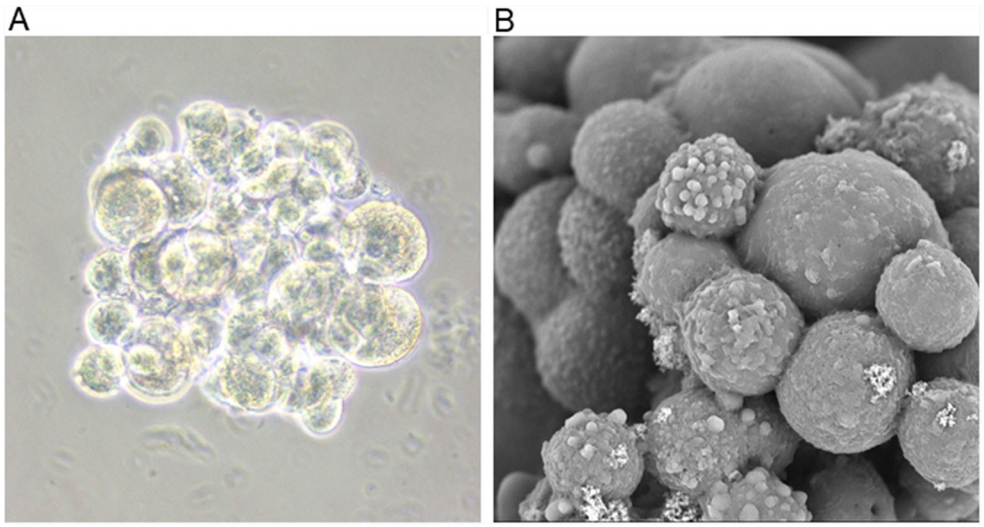

Fig. 1 Sphere of pancreatic cancer cells, with PANC-1 cells cultured in an ultra-low-attachment dish. Phase-contrast image of the sphere (A). Scanning electron microscope images of the sphere were obtained using the Phenom proX desktop scanning electron microscope (Phenom-World BV, Eindhoven, Netherlands) (B). Original magnification: 200× (A) and 7000× (B)

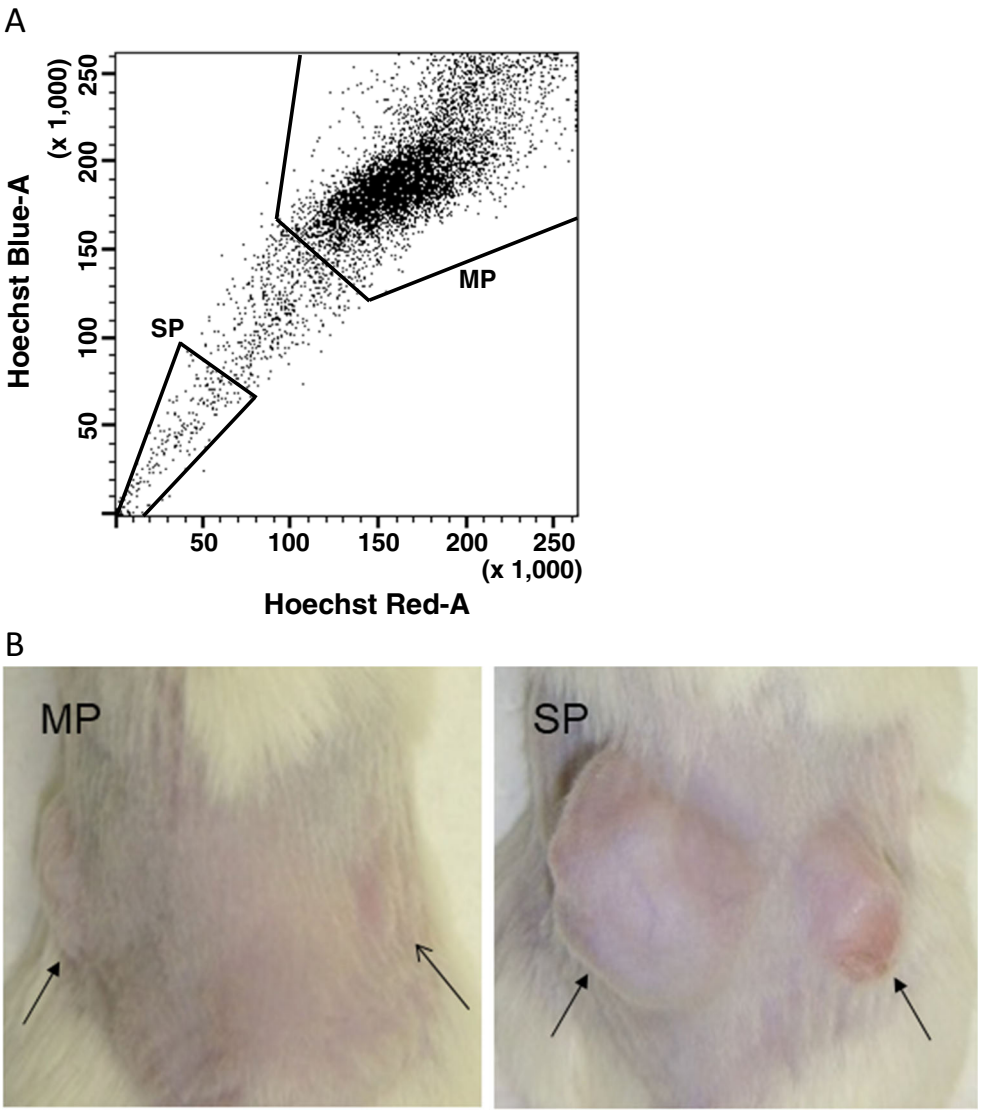

Fig. 2 SP and MP cells of KLM-1 pancreatic cancer cells. Hoechst-positive cells (upper right lesion) represent the MP fraction, and Hoechst-negative cells (lower left lesion) represent the SP fraction (A). Five weeks after subcutaneous injection of the same number of SP or MP cells (1×10^5 KLM-1 cells/mouse) into both sides of the back of NOD.CB17-Prkdc^{scid}/J (NOD/SCID) mice (B). Arrows indicate formed tumors

accumulate mutations or genetic changes, mainly in the multi-drug resistance (MDR)-1 transporter and to a lesser degree the MDR-3 pump, that increase pump activity, [77]. ABC transporters have also become a target for therapeutic development in cancer; however, it is considerably less clear whether the presence of this "SP" alone is sufficient to identify a CSC [69].

Future perspectives

There are three possible ways to deplete cancers of their CSC populations: 1) use therapeutic agents to selectively kill CSCs; 2) employ agents that drive CSCs to differentiate, thereby making them susceptible to standard therapy; and 3) regulate microenvironments.

One of the most promising approaches to CSC targeting involves inhibition of stem-cell-associated pathways, including those involving Shh, mammalian target of rapamycin (mTOR), Notch, BMI, and bone morphogenetic protein. CD133$^+$ PDAC cells exhibit high mTOR-signaling activity [78], and single-agent therapy with rapamycin alone resulted in a significant decrease in CD133$^+$ CSCs. Additionally, combined inhibition of the Shh and mTOR pathways by cyclopamine and rapamycin together with gemcitabine resulted in sufficiently efficacious targeting of CSCs. Furthermore, Shh blockade with cyclopamine led to a decrease in the population of CD133$^+$ CSCs in PDAC cell lines and the reduced metastatic potential of tumor cells. In conjunction with gemcitabine and rapamycin, blockage of this pathway leads to a decrease in overall *in vivo* tumorigenicity [52, 78].

Recent studies showed that the anti-death receptor 5 (anti-DR5) antibody drozitumab inhibits the growth of pancreatic cancer patient xenografts [79]. DR5 is expressed in 75 to 100% of pancreatic CSCs and 25% of bulk tumor cells. Additionally, the anti-diabetic drug metformin targets pancreatic CSCs, but not their differentiated progenies. Metformin

induces cell cycle arrest in the bulk of the more differentiated pancreatic cancer cells, but CSCs undergo rapid apoptotic cell death [80]. A recent report also demonstrated that non-CSCs were highly glycolytic, but CSCs were dependent upon oxidative metabolism, with very limited metabolic plasticity [81]. Therefore, mitochondrial inhibition by metformin translated into energy crisis and apoptosis in pancreatic CSCs. Furthermore, treatment with XL184, a c-Met inhibitor, leads to the depletion of pancreatic CSCs, decreased tumorsphere-forming capacity, and *in vivo* tumorigenicity [33].

Conclusion

Accumulating evidence suggests that a small number of CSCs exist within the population of PDAC cells. CSCs play key roles in the malignant behavior of PDAC, with SP cells, the sphere-formation assay, and CSC markers used to identify pancreatic CSCs. Analysis of CSC markers in surgical-tissue specimens is expected to identify reliable prognostic markers and estimate the effectiveness of anticancer therapy. Additional clinical and basic research is expected to continue the development of CSC-targeting therapies for PDAC.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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