Chapter 10
Pancreatic Cancer Stem Cells

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Abstract Various levels of evidence suggest that a small population of tumor cells known as cancer stem cells (CSCs) or tumor initiating stem like cells initiate and maintain tumors. CSCs have been identified in pancreatic cancer ductal adenocarcinomas (PDAC) and are known to self-renew and propagate the parental tumor. The lack of early symptoms, extensive metastasis and high resistance to chemotherapy and radiation render pancreatic cancer the fourth most common cause of cancer related death. Tumor initiating/propagating cells express cell surface markers such as CD133 and CD44 and show features of epithelial-mesenchymal transition (EMT) resulting in metastasis. In addition, densely glycosylated proteins known as mucins are found to be associated with pancreatic CSCs and play a role in EMT. Typically, activating mutations in the *Kras2* gene are detected in pancreatic tumors accompanied by inactivating mutations in tumor suppressor genes such as *Arf* or *P53*. The cell-of-origin of PDAC is still unknown, as both exocrine and endocrine cells can initiate tumors during chronic inflammation. Future studies investigating pancreatic stem cells and progenitor cells in more detail will help identify more precisely the cell-of-origin of PDAC. Understanding the underlying molecular pathways of the metastatic and drug resistant nature of these distinct cells will open up new avenues in targeting these cells. The highly heterogeneous pancreatic CSC pool is more resistant to standard chemotherapy than the more differentiated tumor cells, and therefore, strategies to specifically target PDAC CSCs will provide new therapeutic prospects for this devastating disease.

Keywords Pancreatic adenocarcinoma • Pancreatic progenitors • Pancreatic cancer stem cells • Metastasis-initiating cells • Chronic inflammation • Immunotherapy

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1 Pancreatic Cancer Biology and Pathology

1.1 The Normal Pancreas

The pancreas is an abdominal organ about 6 in. long and less than 2 in. wide in adults. The head of the pancreas is on the right side of the abdomen, posterior to the junction between the stomach and the duodenum. The body of the pancreas is located posterior to the stomach, and the tail of the pancreas is on the left side of the abdomen adjacent to the spleen. The pancreas contains both exocrine and endocrine glands. The exocrine pancreas contains acinar cells that produce zymogens for food digestion. Following activation of these zymogens, other digestive enzymes such as trypsin, amylase or carboxipeptidase are activated and reach the small bowel. Ductal cells produce digestive juices that are transported to the gut, while also secreting mucins and bicarbonate to raise the duodenal pH. More than 95 % of the cells in the pancreas are contained in the exocrine glands and ducts. The endocrine pancreas also produces insulin, glucagon, somatostatin, ghrelin and P peptide in the islets of Langerhans.

1.2 Pancreatic Cancer

In contrast to the decreasing mortality for other tumor types resulting from improved prevention and treatment options, pancreatic cancer shows a growing incidence and prevalence in defiance of increasing preclinical and clinical efforts (Ryan et al. 2014) (Cancer Facts & Figs. 2011. American Cancer Society, http://cancer.org). Pancreatic cancer has an overall 5-year survival rate of around 5 % and is the fourth most frequent cause for cancer-related deaths, (Hidalgo 2010). Only lung, colorectal and prostate cancers have a higher incidence. The disease has a poor prognosis due to a lack of early warning signs and typically presents with extensive metastasis upon initial diagnosis. Moreover, pancreatic cancer is highly resistant to conventional chemotherapy and radiotherapy. Surgery is generally not curative as there is generally extensive spread of the disease at presentation, and in some cases surgery is precluded by tumor invasion of adjacent large vessels.

Worldwide, the incidence of all types of pancreatic cancer (85 % of which are adenocarcinomas) ranges from 1 to 10 cases per 100,000 people, is generally higher in developed countries and among men, and has remained stable for the past 30 years relative to the incidence of other common solid tumors (Jemal et al. 2011). PDAC is rarely diagnosed in persons younger than 40 years of age, and the median age at diagnosis is 71 years. It is the eighth leading cause of death from cancer in men and the ninth leading cause of death from cancer in women throughout the world. In the United States, pancreatic cancer is expected to develop in 46,000 people and 40,000 people are expected to die from it per year (Siegel et al. 2014). Discrimination between exocrine and endocrine cancers of the pancreas is crucial,

as the different cell types of the pancreas form different types of tumors. The diverse pancreatic cancers have distinct risk factors and causes, different warning signs and symptoms, are diagnosed using different tests, have different treatment regimens, and show different prognosis.

1.3 Exocrine Tumors

Exocrine tumors are by far the most common type of pancreas cancer including:

– Pancreatic ductal adenocarcinoma (PDAC): These cancers usually begin in the ducts of the pancreas.
– Solid pseudopapillary neoplasms (SPNs): These are rare, slow-growing tumors that almost always occur in young women.
– Ampullary cancer (carcinoma of the ampulla of Vater): This cancer starts in the ampulla of Vater, which is where the bile duct and pancreatic duct come together and empty into the small intestine.
– Less common types of cancers: Other cancers of the exocrine pancreas include adenosquamous carcinomas, squamous cell carcinomas, signet ring cell carcinomas, undifferentiated carcinomas, and undifferentiated carcinomas with giant cells.

1.4 Endocrine Tumors

Tumors of the endocrine pancreas are uncommon, making up less than 4 % of all pancreatic cancers. As a group, they are sometimes known as *pancreatic neuroendocrine tumors (NETs)* or *islet cell tumors*. There are many types of pancreatic NETs:

– Functioning tumors: About half of pancreatic NETs make hormones that are released into the blood and cause symptoms. These are called *functioning* tumors and each one is named for the type of hormone-making cell it starts in.

- Gastrinomas come from cells that make gastrin. About half of gastrinomas are cancers.
- Insulinomas come from cells that make insulin. Most insulinomas are benign.
- Glucagonomas come from cells that make glucagon. Most glucagonomas are cancers.
- Somatostatinomas come from cells that make somatostatin. Most somatostatinomas are cancers.
- VIPomas come from cells that make vasoactive intestinal peptide (VIP). Most VIPomas are cancers.
- PPomas come from cells that make pancreatic polypeptide. Most PPomas are cancers.

The most common types of functioning NETs are gastrinomas and insulinomas. The other types occur very rarely.

– Non-functioning tumors: These tumors do not make enough excess hormones to cause symptoms. They are more likely to be cancer than functioning tumors. Because they do not make excess hormones that cause symptoms, they can often grow quite large before they are found.

1.5 Risk Factors and Biologic Features of PDAC

PDAC arising from the exocrine pancreas is the most common and lethal of these various pancreatic cancers, and causes for its development remain unknown. Several environmental factors have been implicated, but evidence of a causative role exists only for tobacco use. The risk of pancreatic cancer in smokers is 2.5–3.6 times higher than in non-smokers; the risk increases with greater tobacco use and longer exposure to smoke. The specific carcinogens in tobacco smoke are not well characterized, but cadmium is a likely candidate (Amaral et al. 2012). Interestingly, nicotine, although non-carcinogenic, accelerates *Kras*-initiated pancreatic cancer development and progression by altering the acinar cell compartment (i.e. dedifferentiation) and making it more susceptible to oncogenic transformation (Hermann et al. 2014).

For non-smokers, food intake is the major source of cadmium, which may derive from fertilizers or fossil fuel combustion (Luckett et al. 2012). Data are limited on the possible role of alcohol intake as a contributing factor. Some studies have shown an increased incidence of pancreatic cancer among patients with a history of diabetes or chronic pancreatitis. There is also evidence, though less conclusive, that chronic cirrhosis, a high-fat, high-cholesterol diet, and previous cholecystectomy are associated with an increased incidence of PDAC (Rebours et al. 2015; Sellam et al. 2015). More recently, an increased risk has been observed among patients with blood type A, B, or AB as compared with blood type O (Sun et al. 2015). According to recent studies, regular use of aspirin at a low dose may lower the risk of PDAC (Anderson et al. 2002; Streicher et al. 2014).

Although an estimated 5–10 % of pancreatic cancers have an inherited component, the genetic basis for familial aggregation has not been identified in most cases. A known family history of pancreatic cancer in a first-degree relative is associated with an increased risk of PDAC as compared with the general population, the relative risk being increased by a factor of 2, 6, and 30 in people with one, two, and three affected family members respectively. There is no effective screening tool to detect asymptomatic premalignant or early malignant tumors. Although there is consensus regarding the value of screening patients with an inherited predisposition for pancreatic cancer, there is no consensus on the most effective method of screening or the optimal interval between screenings.

Development of the disease involves an initial pre-invasive state termed pancreatic intraepithelial neoplasia (PanIN) classified into three stages based on increasing cellular atypia and mutations in key oncogenes and specific tumor suppressor genes. 90 % of all PDAC tumors contain mutations in the *KRas2* gene that result in permanently active Ras protein. Ras is a small GTPase involved in proliferation, survival, and differentiation, and is considered a master regulator of PDAC initiation and progression. In mouse models, PanINs and PDAC can be induced by activating mutations in the *Kras* gene (Hingorani et al. 2003; Morris et al. 2010). Other frequent genetic alterations in PDAC are inactivating mutations of *p16* (>95 % of cases) and *TP53* (>50 % of cases). Inactivating mutations of the tumor suppressors *SMAD4* and *BRCA2* are also frequently found in PDAC (Perez-Mancera et al. 2012). Reactivation of developmental embryonic signaling pathways such as Hedgehog and Notch suggests that tumor cells show regression to a dedifferentiated/progenitor-like state and may represent stem/precursor cells.

By far the most common cause of chronic pancreatitis is alcohol abuse, which is responsible for 60–90 % of cases. As with hereditary pancreatitis, the chronic inflammation seen in chronic pancreatitis is thought to predispose to development of PDAC. Inflammatory cytokines may induce cellular proliferation, reduce immunosurveillance and inhibit senescence, all of which enable the lesion to progress to PDAC. The organ-specific microbiome might be of special interest. Although the pancreas does not contain a known microbiome, the organ may be exposed to microorganism-associated molecular patterns and bacterial metabolites via anatomical links with the gut. Lipopolysaccharide derived from bacterial cell walls has been reported to increase pancreatic cancer development (Schwabe and Jobin 2013). Various animal models demonstrate that a germ-free environment or antibacterial treatment reduce tumor incidence.

Chronic pancreatitis may induce an increased level of plasticity in different pancreatic cell types, which favors malignant transformation. In addition, pancreatitis enhances proliferation and inflammatory response in the tissue associated with re-expression of *Pdx1* and reactivation of the embryonic Notch and Hedgehog signaling pathways. Pancreatitis-induced PanINs can be delayed by non-steroidal anti-inflammatory drugs (Guerra et al. 2011). Further study of the underlying mechanisms of chronic inflammation will provide more insights in understanding the link between the various genetic alterations in PDAC and malignant transformation. It is possible that *Kras* and *Trp53* might be mutated in pancreatic stem cells, which then become activated and proliferate due to secretion of inflammation-induced cytokines and growth factors. Therefore, genetically engineered mouse models utilizing specific promoters for tissue stem cells are required to obtain in-depth insights into tumor development and progression. The disease models also need to be investigated in the presence or absence of microorganism-associated molecular pattern and bacterial metabolites.

2 Pancreatic Stem Cells and Their Role in Pancreas Development

2.1 Pancreas Development and Precursors

During the embryonic developmental stage, the pancreas develops as dorsal and ventral evaginations from the foregut endoderm during the fifth week of gestation. Cells from the dorsal and ventral buds slowly undergo lineage commitment to the either endocrine or exocrine compartment. The endocrine compartment comprises the islets of Langerhans while the exocrine compartment is organized into acinar, ductal and centroacinar cells. In addition to these compartments, a novel gland like mucinous compartment known as the pancreatic ductal gland has been identified, and shown to possess a characteristic molecular signature. Much effort has been expended in efforts to identify which of these compartments give rise to PDAC progenitor cells.

The dorsal and ventral buds of embryonic pancreas are organized in a stratified epithelium comprising early multipotent pancreatic cells and early-differentiated endocrine cells, in particular glucagon$^+$ cells. At an early stage, these multipotent pancreatic cells still show a high level of plasticity and can be reprogrammed to an intestinal lineage. In contrast, at later stages of development, multipotent pancreatic cells become committed to the pancreatic lineage and express transcriptional factors for the pancreatic differentiation program (Pan and Wright 2011).

Extensive efforts have been made to identify pancreatic stem cells, which could be involved in the maintenance and/or regeneration of the pancreas in response to injury (e.g. chronic pancreatitis) and loss of β-cell mass, respectively. The characterization of such an elusive stem cell population could lead to the development of therapeutic strategies for the replacement of β-cells in patients with type I diabetes. Despite lacking a clear definition of postnatal pancreas stem cells for the different cell types within the pancreas, comprehensive knowledge has been accumulated regarding the characteristics of pancreatic stem cells during embryonic development. Thus, all pancreatic cells, both from exocrine and endocrine lineages, are believed to originate from an initial cell progenitor expressing the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) (Ahlgren et al. 1996).

The expression of this factor together with silencing of signaling mediated by Sonic hedgehog (Shh) in the surrounding mesenchymal tissue initiates embryonic pancreas development (Apelqvist et al. 1997). The implication of Shh in this process is supported by several observations, including a lack of Pdx1 expression in embryos with constitutively active hedgehog signaling (Hebrok et al. 1998). Thus, Pdx1 can be considered a critical transcription factor in pancreatic commitment, although there might be more actors implicated, since absence of this factor does not result in complete impairment of pancreas formation.

Another transcription factor was recently shown to play an important role in pancreas development in humans. Malfunctioning mutations of pancreas-specific transcription factor 1 (Ptf1) in humans result in impaired pancreas development,

while studies in mice showed that forced expression of Ptf1 induces pancreas development at ectopic locations. Ptf1 is activated in a subset of pancreatic stem cells expressing Pdx1, shortly after these cells acquired Pdx1 expression, but despite the apparent temporal sequence, the expression of Pdx1 and Ptf1 occurs in an independent manner. Ptf1 expression has been implicated in the commitment of precursor cells towards an exocrine phenotype because Ptf1 null mutant mice show impaired pancreas development but are still capable of developing endocrine cells (Krapp et al. 1998). In addition, commitment towards an exocrine fate seems to be potentiated through signaling of the surrounding mesenchyme on Pdx1 positive cells. Mesenchymal cells would enhance Notch signaling in progenitor cells via its downstream target hairy enhancer of split 1 (Hes1) and inhibit the expression of the pro-endocrine differentiation factor Neurogenin 3 (Ngn3) (Lee et al. 2001).

Determination of endocrine fate is induced by expression of the transcription factor Ngn3. In fact, Ngn3-positive cells represent the origin of all the heterogeneity of pancreatic endocrine cells. Both α- and β-cells can derive from Ngn3-positive cells, although they are generated at different ratios. In early pancreatic development during mouse embryogenesis, the vast majority of cells derived from Ngn3-positive cells are glucagon secreting α-cells, supporting a notion that Pdx1-Ngn3 forced expression primarily leads to the development of glucagon cells. The α-cells down-regulate Pdx1 expression and progress towards a non-epithelial phenotype through a process that strongly resembles Epithelial-to-Mesenchymal Transition (EMT).

On the other hand, β-cells retain Pdx1 expression and remain in low numbers as compared to glucagon secreting cells, until later in development when branching morphogenesis and acinar cell differentiation occur and require an amplification of the pool of β-cells. Commitment towards α- or β-cell fate seems to depend on the mutually exclusive action of the transcription factors, Aristalless related homeobox (Arx) and paired box gene 4 (Pax4) (Collombat et al. 2005; Collombat et al. 2003). Expression of Arx may induce the formation of α-cells, since deletion of this gene results in impaired generation of this cell type, whereas Pax4 appears to be responsible for β-cell formation.

The existence of different sequential progenitor cells raises the question of whether these cells can also be reverted to a less differentiated phenotype in order to give rise to a broader number of cell types. However, accumulating evidence suggests that β- cells are differentiated cells with very limited expansion capability. In fact, most β-cells seem to originate from a pool of already existing β-cell precursors rather than from expansion of ancient β-cells. Notch is not capable of compelling mature endocrine cells to revert towards a progenitor-like state. In contrast, Ngn3-positive cells demonstrate greater plasticity, since they can be reverted to a ductal progenitor phenotype. Therefore, while the pancreas lacks a clear hierarchical organization and a final definition of a putative pancreatic stem cell is still missing, it has been shown that a number of cellular compartments bear the potential to regenerate the different subsets of the pancreas and are putative targets for the cell-of-origin for PDAC.

2.2 Multipotent Stem Cells in Neonatal Pancreas

At the time of birth, there are still multipotent pancreatic stem cells, but multipotency is drastically decreased in adult cells. The hepatocyte growth factor receptor c-met identifies cells exhibiting colony-forming activity, while being negative for vascular markers. These c-met$^+$ cells can differentiate into the acinar, ductal and endocrine lineage in vitro and in vivo (Marsit et al. 2004). However, c-met$^+$ cells from adult pancreas lost this multi-lineage potential. Moreover, endocrine-committed CD133$^+$ and CD49f$^+$ pancreatic islet progenitors have been isolated from mouse fetal pancreas that are highly enriched for Ngn3, a consensus marker for progenitors (Sugiyama et al. 2007).

2.3 Multipotent Stem Cells in Adult Pancreas

As shown in other epithelial tissues, tissue resident stem cells with sphere forming capacity have been identified in adult murine pancreas that differentiate into pancreatic exocrine and endocrine lineages, but also into stellate cells and neuronal lineages (Seaberg et al. 2004). In addition, pancreas-derived insulin$^+$ multipotent precursors were isolated from Pdx1$^+$ progenitors in the islets. They are able to differentiate in vivo into β cells, other endocrine cell types, acinar cells, and neural cell types (Smukler et al. 2011). Interestingly, multipotent precursors can be also found in human pancreas suggesting that these multipotent precursors are quite conserved among species. Markers for a direct identification of these multipotent precursors would be helpful. So far, they have been isolated according to their functional characteristics.

Alternative sources of stem cells in the adult murine tissue are centroacinar cells (CACs) and terminal duct cells isolated based on their enhanced ALDH1 activity, increased stemness-associated genes, low levels of pancreatic differentiation markers and Pdx-1, and anchorage-independent cell growth in spheres (Rovira et al. 2010).

3 Cell of Origin of Pancreatic Ductal Adenocarcinoma

The specific cell type from which PDAC arises still remains elusive. A possible scenario for tumor initiation in solid organs is the malignant transformation of stem cells resident in normal tissue. Somatic stem cells are intrinsically endowed with the capacity of self-renewal and would therefore only need to accumulate sequential mutations to undergo malignant transformation and give rise to a tumor. Indeed, this hypothesis has just recently been validated for intestinal cancer. However, putative pancreatic stem cells in mice still cannot be genetically tracked due to their rather

vague description as mentioned above. This has hampered the field in providing definitive proof for this hypothesis. Until this stem cell model for the development of PDAC is either authenticated or disproved by the accumulation of more evidence, other models will need to be considered for a putative mechanism.

The generation of mouse models that closely recapitulate human disease has provided a unique platform for better understanding the cell types that are most susceptible for malignant transformation and may be candidates for the cell-of-origin for murine PDAC (Pérez-Mancera et al. 2012). Depending on the context, mature cells or common multipotent stem cells can undergo initial malignant transformation.

3.1 *Pdx1 Expressing Cells*

The KPC mouse (*Pdx1*Cre; LSL-*Kras*^{G12D}; LSL-*Trp53*^{R172H}) is one of the most frequently used pre-clinical models for human pancreatic cancer. To activate KrasG12D and produce the inactive mutant P53R172H in this model, expression of Cre recombinase occurs in Pdx1^{+} pancreatic progenitor cells during embryonic development resulting in removal of the stop cassette (LSL), and leading to metastatic and chemotherapy-resistant adenocarcinomas with features similar to human disease (Hingorani et al. 2005; Olive et al. 2009). However, this model also has some drawbacks, because the mutant alleles are induced from embryonic day 8.5 when pdx-1 is first expressed in multipotent pancreatic cells in the developing embryo. Moreover, the mutant alleles are activated in both exocrine and endocrine cells, including differentiated cells as well as local resident stem cells, which causes multiple lesions dissimilar to human tumors. Activation of *Kras*^{G12D} alone in Pdx1^{+} cell during embryonic development leads only to premalignant PanINs lesions and not to PDAC. Although theoretically all pancreatic cells can carry the transgene only a few lesions appear, indicating that only a small percentage of Pdx1^{+} cells are targeted.

An inducible *Pdx1*CreER^{T2} has been designed in which the mutant alleles can be switched on during adult life. Activation of *Kras*^{G12D} during adulthood in this model leads to PanIN lesions and acinar-ductal metaplasia. More specific promoters and temporally controlled Cre recombinase have recently been developed, and highlight diverse putative cell types as tumor initiating cells causing PanIN lesions or invasive PDAC.

3.2 *Ductal Cells*

Glandular ductal structures and expression of cytokeratin 19 (CK19) ductal gene are common features of invasive PDAC, and point to cells with ductal phenotype as cell-of-origin in pancreatic cancer. Other lines of evidence however appear to negate this; for instance, expression of *Kras*^{V12G} under the *CK19* promoter induces

inflammation, but not hyperplasia in the pancreas (Brembeck et al. 2003), while activation of *Kras*^{G12D} by CK19-CreERT2 induce PanIN lesions but not adenocarcinomas (Ray et al. 2011). More recent studies conclusively revealed that ductal and stem-like centroacinar cells were surprisingly refractory to oncogenic transformation, whereas acinar cells readily formed PDAC precursor lesions with ductal features (Kopp et al. 2012). It was shown that formation of acinar-derived premalignant lesions depends on ectopic induction of the ductal gene Sox9. Moreover, when concomitantly expressed with oncogenic *Kras*, *Sox9* accelerated formation of premalignant lesions. Although counterintuitive, these results suggest that its precursors arise via induction of a duct-like state in acinar cells.

3.3 Acinar and Centroacinar Cells

As suggested by above studies, acinar cells (including centroacinar cells) are more promising candidates as putative tumor-initiating cells of PDAC. In line with this hypothesis, ductal metaplasia inside acini together with PanIN lesions is a common event following *Kras*^{G12D} activation. If specific promoters for acinar cells are used such as elastase or proCPA1, Expression of mutated *Kras* alleles in combination with mutations in *Trp53* or *Arf* using specific promoters for acinar cells (such as *elastase* or *proCPA1*) lead to induction of PDAC in the presence of cerulean, an inducer of chronic pancreatitis (Gidekel Friedlander et al. 2009; Guerra et al. 2007).

3.4 β Cells

Adult β cells are refractory to transformation by *Kras*^{G12D} alone or in combination with additional mutations. However, in a context of chronic pancreatitis, activation of mutant *Kras* and elimination of tumor suppressor *p53* in insulin^{+} cells by treating *RipCreER*^{TM}; LSL-*Kras*^{G12D}; *Trp53*$^{flox/flox}$ with tamoxifen promote the development of poorly differentiated and undifferentiated adenocarcinomas (Gidekel Friedlander et al. 2009). These adenocarcinomas display a metastatic behavior similar to human tumors. Remarkably, *Kras*^{G12D} activation in adult *Pdx1*-expressing cells causes an early appearance of ductal and acinar structures inside the islets of Langerhans. These ductal lesions become elongated and produce mucin resembling PanIN lesions.

4 Pancreatic Cancer Stem Cells

Irrespective of the still-ongoing debate about the cell-of-origin in PDAC, increasing evidence suggests that cells with stemness features, also termed cancer stem cells (CSCs), exclusively drive pancreatic tumorigenesis in humans. The CSC hypothesis

is the subject of great interest within the field of PDAC as well as other malignancies, since it also provides a rationale for the phenomenon of high resistance to chemotherapy leading to relapse of disease after treatment. In this context, the biological characteristics of CSCs are consistent with findings from other solid tumors. Human PDAC CSCs are characterized by several biomarkers, are able to self-renew, and to propagate the parental tumor in transplantation assays using immunodeficient mice. Biomarkers for CSCs are crucial for their identification and their tracking during treatment, representing a novel measure of treatment response. Additionally, increased understanding of the biology of CSCs could lead to the development of new treatments specifically directed against these cells as the putative root of PDAC. Currently, pancreatic CSCs are mainly identified by flow cytometry using cell surface markers that are poorly defined and non-exclusively expressed on CSCs. To date, pancreatic CSCs have been identified and characterized using the surface markers Epithelial Cell Adhesion Molecule (EPCAM or CD326), CD44, CD24, CD133, CXCR4, and c-Met. More recently, other identification methods such as the side population assay or the ALdehyde DeHydrogenase-1a1 (ALDH1) activity assay have emerged. CSC can also be functionally enriched by their capability to form spheres in vitro.

It is important to note; however, that CSCs do not necessarily represent *bona fide* stem cells nor do they necessarily arise from tissue stem cells, but rather cancer stem cells have acquired certain traits of stem cells allowing them to indefinitely self-renew and give rise to their respective differentiated progenies. While cancer stem cells share several signaling pathways that are regularly operative in normal stem cells (Micalizzi et al. 2010), they are obviously distinct from normal stem cells in terms of their in vivo tumorigenicity defined as the generation of malignant lesions upon transplantation into secondary hosts (Alison et al. 2011). Still, while it has been shown conclusively that cancer stem cells 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 (Lonardo et al. 2012; Sainz et al. 2014). Thus, in order to advance our understanding of cancer stem cell biology and to develop clinically meaningful cancer stem cell-centered treatment strategies, these cells need to be studied in the context of their niche. Clinically it is of utmost importance that cancer stem cells have been proven to be highly resistant to current standard of care such as chemotherapy and radiotherapy, which makes them a probable cause of tumor recurrences after treatment (Noman et al. 2011). Consistently, primary tumors with a more prominent stem cell signature are associated with adverse outcome including higher rates of metastasis (Dalerba et al. 2011; Merlos-Suarez et al. 2011; Pece et al. 2010).

Identifying the most appropriate model systems for studying CSCs represents another important challenge for the field. The process of isolating putative CSC populations from resected tumors, whether for studying in vitro behavior or in order to obtain single cells for further analysis, is potentially prone to artifacts. Tumor digestion consists of mechanical and chemical disruption that can be harsh on the cells, impairing their viability. Therefore, the cells of interest may be lost and/or damaged during the isolation process. Moreover, modifications in cell behavior and

marker expression are to be expected due to changes in the CSC environment. Once isolated, CSCs may lose their properties due to lack of interaction with the stromal environment or with circulating stromal or endothelial cells. Furthermore, the low incidence of CSCs requires sensitive techniques for their identification and isolation. The development of comprehensive and corresponding in vitro and in vivo working models that recapitulate the whole heterogeneity of the resected tumor and mimic its complex network of relationships with the surrounding environment is thus of crucial importance for study of human CSCs. These models should correlate to the in vivo situation of the patient in order to develop and test efficient therapies targeting CSC populations. In this context, a great effort has been made on the development of primary tissue xenograft models and corresponding in vitro primary cell cultures as a platform for expansion of fresh tumor samples. These xenografts were proven highly relevant for several cancers as they accurately recapitulate the features of the patient tumor, including retaining the genetic features of the tumor, faithfully maintaining the heterogeneity of tumor cell composition, and its microenvironment including the stroma. Based on the outstanding clinical relevance of the original tumor composition, tissue xenografts have become important working models for the CSC field including pancreatic CSCs. In addition, in vivo imaging constitutes an important tool in the future working systems to study CSCs. Direct visualization of CSCs using reporter constructs provides a novel opportunity for a better understanding of tumor initiation and progression in their in vivo environment. This constitutes a crucial starting point for the evaluation of the treatment response to novel targeted therapies. Therefore, this model system provides important information on tumor biology and on the role on CSCs in the tumorigenic process, with minimal artifacts and alterations in comparison with the primary tissue.

The characteristics of the CSC population could determine the response to treatment or outcomes from cancer. To support this principle a CSC biomarker is required and has to be reproducible and measurable in patient samples. Ideal markers would be those that, while the cells remain viable, could be studied in a longitudinal fashion in order to correlate the presence of CSCs and disease outcome. The identification of CSC markers fulfilling these criteria would indeed represent a major breakthrough that could allow the development of a personalized therapeutic approach to the different types of CSCs that are resistant to chemo- and radiotherapy treatments. CSCs in PDAC have been identified by a variety of biomarkers, discussed below.

4.1 $CD44^+CD24^+EPCAM^+$ Tumor Cells

CSCs in human pancreatic tumors have been first reported in 2007. Administration of $CD44^+CD24^+ESA^+$ cells into immunodeficient mice led to tumors (Li et al. 2007), with as few as 10^2 $CD44^+CD24^+EPCAM^+$ cells initiating tumors in 50 % of transplanted mice. In contrast, up to 10^4 $CD44^-CD24^-EPCAM^-$ cells were required

to detect malignant growth. PDAC CSCs only represented 0.2–0.8 % of the whole tumor. Triple positive cells display typical cancer stem cell characteristics such as self-renewal and producing tumors of similar heterogeneity compared with the parental tumor. However, this could have been flawed by contaminating stromal cells that also include $EPCAM^-$ cells, thereby affecting the tumor formation capacity of this population. Of note, CD44 and CD24 have previously been used as cancer stem cell marker in other epithelial malignancies such as the breast or prostate cancer. Therefore, these surface markers might identify tumor-propagating cells regardless of the tumor type.

4.2 $CD133^+$ Tumor Cells

In 2007, our group demonstrated in a different study the tumorigenicity of $CD133^+$ cells isolated from fresh human PDAC. These PDAC CSCs represented 1–3 % of tumor cells (Hermann et al. 2007). As few as 5×10^2 $CD133^+$ cells induced tumor formation in immunodeficient mice, while 10^6 $CD133^-$ cells were needed for the same effect. The characteristics of the newly formed tumors were similar to the parental tumor. $CD133^+$ cells produced spheres in serum-free anchorage-independent conditions. In serial transplantations, $CD133^+$ cells exhibited self-renewal in vitro and in vivo. Interestingly, a study involving 80 PDAC patients showed that cytoplasmic CD133 expression significantly correlated with patients' outcome (Maeda et al. 2008). Finally, some $CD133^+$ cells also express $CD44^+CD24^+EPCAM^+$ (ranging from 10.3 % to 37.4 %), but no population of pure tumorigenic cells could be identified. Thus, the ideal combination of cell surface markers for the identification of a pure cancer stem cell population is still required.

4.3 Other CSC Markers

To enrich for CSC, ALDH1 has been described for isolating tumorigenic cells in the human pancreatic line L3.6pl (Kim et al. 2011). ALDH1 expression is linked to poor prognosis, and $ALDH1^+$ cells are reported to be more clonogenic with higher migratory and invasive potential (Rasheed et al. 2010). Another way to identify CSCs is a fluorescent reporter system to detect proteasome activity, in which low activity of the 26S proteasome indicates CSC features (Adikrisna et al. 2012). Moreover, the receptor for hepatocyte growth factor c-Met has also been used in PDAC to identify CSC (Li et al. 2011). Along with expression of CD133 or CD44, c-met expression strongly selected for CSC as demonstrated by enhanced in vivo tumorigenicity as compared to each of the single markers. For instance, $c\text{-}Met^+CD44^+$ cells show strong tumorigenic potential in generation of subcutaneous tumors.

As can already be conveyed from this rather large, diverse and ever growing panel of markers, the development of reliable cancer stem cells biomarker profiles

for accurately and prospectively isolating viable cells at high purity represents a daunting task. While numerous cell surface proteins have been positively evaluated in certain settings, the expression levels of many of these markers can drastically change based on environmental conditions (e.g. tumor digestion, cultivation in different conditions, xenografting), in response to treatment, and their expression is neither exclusively nor reproducibly linked to a functional cancer stem cell phenotype (Lonardo et al. 2010). Thus, alternative detection and isolation methods based on functional properties of cancer stem cells would not only avoid the use of such artifact-prone surface markers but should also provide novel insights into cancer stem cell biology. Towards this end, an intrinsic autofluorescent phenotype has been identified in cancer stem cells and was subsequently established as a novel and functionally relevant tool to isolate and characterize these cells down to single cell level (Miranda-Lorenzo et al. 2014). This distinct inherent cancer stem cell property represents a novel biological feature that is traceable in real time and provides unprecedented robustness and power for the identification and purification of cancer stem cells without the use of antibodies nor any kind of manipulation, thus drastically reducing experimental errors and artifacts. While surface marker panels are regularly tested for only certain cancer types, this novel marker has already been shown to reproducibility identify cancer stem cells across many tumor types including pancreatic, breast, lung, liver and colorectal cancer (Miranda-Lorenzo et al. 2014). Thus, it has now become possible to more accurately capture the dynamic complexity of cancer stem cells.

Functional assays such as sphere-formation capacity in vitro and particularly tumorigenicity in vivo still remain the gold standard for functionally validating CSCs. It is important to note that CSCs do not represent a homogenous clonal population of cells with equal capabilities but have undergone genetic evolution during the many years of tumor development and subsequent progression. While earlier studies in pancreatic cancer already pointed towards distinct populations of CSC with distinct features including the capability to metastasize, genetic evolution has now also been shown to occur in distant metastasis of pancreatic cancer. Genomic instability is a cause for different subclones of metastasis-initiating cells, although its relation to CSC subpopulations has not been determined yet. The issue of clonal heterogeneity of CSCs has recently been comprehensively addressed in acute lymphoblastic leukemia by studying DNA copy number alteration.

These studies demonstrate that different subclones of CSCs are present in individual patients suggesting that there is not a single CSC subset with a static phenotype, but rather that clonal evolution within CSCs is a common event. Genetic instability can cause the rise of different CSC subclones originating from a common progenitor (precursor), displing different proliferative properties and invasive features. Due to selection pressure, one or several subclones may play a dominant role in the tumorigenic and/or metastasis process. The evolving concept of CSC heterogeneity also indicates that therapeutic approaches need to be designed to target and eradicate all CSC subclones in order to be clinically efficient, as spared subclones

will lead to relapse of the disease. This multiclonality may at least in part rationalize eventual relapse of the disease even though the initiating oncogenic event has been clearly defined and targeted. This may be exemplified by the high recurrence rate in patients with chronic myeloid leukemia treated with Imatinib.

4.4 Migrating CSCs and Metastasis

Metastasizing cancer cells undergo a process called EMT involving genetic and epigenetic changes. The EMT program is required for tumor cell extravasation into the circulation, and to home and colonize remote sites of the host. A potential link between CSCs and metastasis and has been suggested previously (Brabletz et al. 2005). Moreover, stem cells are enriched in EMT genes as compared to their epithelial progeny (Mani et al. 2008), while migration to the vasculature is associated both with expression of EMT genes and other characteristics of pancreatic stem cells (Rhim et al. 2012). Interestingly, tumor cells still have the same tumorigenic potential compared with other bulk tumor cells after induction of EMT, while PanIN cells following EMT being more tumorigenic.

4.5 Heterogeneity of the CSC Pool

The CSC pool is not composed of identical CSCs but is heterogeneous due to genetic and epigenetic alterations in the CSC as well as the microenvironment. Therefore, CSCs can grow into different clones with distinct invasive properties, hypoxia resistance or susceptibility to chemotherapy. Our group has shown that while human PDAC $CD133^+$ cells show improved tumorigenicity, $CD133^+CXCR4^+$ cells display highly metastatic behavior. The specific ligand for the chemokine receptor CXCR4 is stromal derived factor-1 (SDF-1), which is secreted by the bone marrow stromal cells and is known to regulate stem cell homing to the bone marrow. Moreover, SDF-1/CXCR4 signaling promotes PDAC cell migration and invasion in vitro (Li et al. Cancer Lett 2012). Following depletion of $CD133^+CXCR4^+$ cells from the CSC pool, the metastatic behavior was abrogated while tumorigenicity was unaffected (Hermann et al. 2007). Consistent with these data, numbers of $CD133^+CXCR4^+$ cells were increased in patients with lymph node metastasis as compared to patients without metastatic disease.

Collectively, signaling pathways regulating stemness and EMT such as Hedgehog, Notch, and Wnt signaling pathways seem to be closely associated (Li et al. 2012). MicroRNAs also seem to be of importance, as the EMT-associated ZEB1 repressor has been reported to inhibit microRNAs such as miR-200c and miR-203, which in turn are involved in the inhibition of stemness (Wellner et al. 2009).

4.6 Pancreatic CSC Niche

Stem cells survive in a niche, which provides favorable conditions for it to self-renew. Similarly, a tumor is governed by its microenvironment/niche, which encompasses several components such as the cancer-associated fibroblasts, CSCs, immune cells, signaling molecules, blood vessels and the extracellular matrix. The tumor stroma is composed of pancreatic stellate cells which undergo paracrine Nodal/Activin signaling, thereby forming a paracrine niche for pancreatic CSCs. Pancreatic stellate cells secrete the embryonic morphogens Nodal/Activin, and thus support the in vitro sphere formation and invasiveness of pancreatic CSCs. Hamada et al. have shown that the presence of stellate cells improved the spheroid forming ability of cancer cells, while expression of CSC related genes such as *Nestin*, *ABCG2* and *LIN28* was induced. Hence, the cross talk between the niche and the CSCs remains pivotal.

4.7 Signaling Pathways Involved in the Maintenance of Pancreatic CSCs

Since self-renewal is a common feature of normal stem cells and CSCs, it is reasonable to believe that these cells share the same signaling pathways. The following signaling pathways such as Notch, Shh and Wnt play an important role in the pancreatic CSCs. In the normal pancreas, Notch signaling controls the balance between the self-renewal and differentiation processes. Additionally, Notch signaling is important for the pathogenesis of human cancers including PDAC. Studies showed that the overexpression of Notch-1 resulted in increased clonogenicity, migration, invasion and induction of EMT phenotype in AsPC-1, a pancreatic cancer cell line (Bao et al. 2011a). Moreover, overexpression of Notch-1 resulted in a significant increase in the pancreatosphere formation which concomitantly expressed higher levels of the CSC markers, EPCAM and CD44. Bao et al. have shown that Notch-1 signaling is crucial for the acquisition of EMT phenotype (Bao et al. 2011b). Likewise, Abel et al. have demonstrated that the Notch pathway is essential for the maintenance of the pancreatic CSC population (Abel et al. 2014). Knockdown of Hes1 using shRNA and inhibition of the Notch pathway components by gamma secretase resulted in the reduction of the self-renewal capacity of pancreatic CSCs. Altogether, these studies suggest that Notch signaling is important for the pancreatic CSC formation. Hedgehog signaling pathway is essential for cell differentiation and tissue patterning events during the embryonic development of the pancreas. Among the three hedgehog genes such as Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog homolog (Dhh), Shh shows the widest range of expression. One of these three ligands binds to the receptor Patched1, which relieves inhibition of the protein

smoothened (Smo), leading to the activation of downstream targets such as the GLI family of transcription factors and PTCH. A ninefold increase in *Shh* mRNA levels has been found in $CD44^+$ $CD24^+$ ESA^+ cells when compared to unsorted pancreatic cancer cells. Sonic hedgehog- Gli signaling is identified to be essential for the pancreatic CSCs. Sulforane (SFN), an active component in cruciferous vegetables, was found to inhibit the self-renewal capacity of pancreatic CSCs by blocking the hedgehog pathway.

In addition to the above-mentioned pathways, during embryonic development the Wnt-β-catenin signaling pathway plays an important role at different stages of pancreatic organogenesis. However, inhibition of this pathway is necessary for pancreatic specification during the early endoderm development. Canonical Wnt signaling is found to be important for the progression of pancreatic cancer. It has been reported that in colorectal cancer Wnt signaling is associated with EMT by activation of the transcription factor snail (Stemmer et al. 2008; Zhou and Hung 2005). Snail interacts with β-catenin, which is required for its activation. Since EMT is a process present in CSCs these findings suggest that b-catenin may have a role in pancreatic CSCs. However, in the future more studies are required to prove the role of β-catenin in pancreatic CSCs.

Apart from the three important signaling pathways, there are other pathways, which are involved in the maintenance of pancreatic CSCs. A recent study has reported that the inhibition of mTOR pathway by Rapamycin resulted in decreased viability of $CD133^+$ pancreatic cancer cells and reduced the sphere forming ability of pancreatic cancer cells (Matsubara et al. 2013). These results suggest that the mTOR pathway is essential for the self-renewal of pancreatic CSCs. Another study claims that the NF-kB pathway is highly activated in pancreatic CSCs, as treatment with NF-kB pathway inhibitors abrogates the stem cell-like properties (Sun et al. 2013). Altogether, several signaling pathways have been identified to play significant roles in conserving the cancer stem cell phenotype in pancreatic cancer.

5 Therapeutic Implications of Pancreatic Cancer Stem Cells

Despite great efforts, pancreatic cancer continues to be one of the deadliest cancer-related diseases in the world (Philip et al. 2009). Different treatment modalities exist for PDAC, among which surgery is the mainstay of treatment, but over 80 % of PDAC patients present with local invasion and distant metastasis upon first diagnosis. Extensive efforts have been made to improve the treatment outcome of PDAC including exploring drug combination and targeted drug chronic pancreatitis, with a 10–20 year lag between the incidences of pancreatitis and pancreatic cancer Still, progress to date has been modest (Hidalgo 2010). As a consequence, there is an urgent need to supplement current therapies and to develop novel, most likely multimodal therapeutic approaches.

5.1 Chemotherapy Against CSC Specific Features

Typically, $CD133^+$ CSC are resistant to standard chemotherapy for PDAC when compared to $CD133^-$ cells (Hermann et al. 2007). Following therapy with gemcitabine, the numbers of c-Met^+ CSCs may even increase. This resistance to gemcitabine could be abrogated by the c-Met inhibitor XL184 (Li et al. 2011). PDAC CSCs can also be specifically targeted by the embryonic Activin/Nodal signaling pathway that is reactivated in adult CSCs (Lonardo et al. 2011). The Activin/Nodal signaling pathway drives self-renewal of human pancreatic CSCs via Alk4/7, the TGFβ superfamily receptors for Activin/Nodal. Knockdown of this pathway decreased sphere formation in vitro and virtually abolished in vivo tumorigenicity. Combination therapy of the Nodal/Activin inhibitor SB431542 with gemcitabine and a Hedgehog pathway inhibitor targeting stromal cells succeeded in preventing relapses in human tumor xenografts in the long run.

HDAC inhibitors also represent an attractive approach to target CSCs. Here, 5-Aza-dC and SAHA reactivate miR-34a which is an effector of p53 that is downregulated in PDAC, and thereby block self-renewal and induce apoptosis (Nalls et al. 2011). Moreover, SAHA also blocks expression of EMT inducers such as Slug, Snail and ZEB1. Metformin, an oral anti-diabetic drug for type II diabetes therapy, has been reported to show anti-tumor activity in some cancers. In PDAC, metformin decreased tumor sphere formation, which was accompanied by downregulation of pluripotency-associated genes such as Oct4 and Nanog (Bao et al. 2012). Mechanistically, metformin seems to modulate microRNAs such as let-7a, miR-26a, miR-101 and miR-200b to target tumor CSCs. Additionally, inhibitors for Notch, Hegdehog, and CXCR4 have also been examined to treat pancreatic CSCs both in vitro and in vivo models (Xia et al. 2012).

5.2 Immunotherapy Against CSCs

To date, adoptive immunotherapies have undergone a revival due to immense success in hematological malignancies. Immunotherapy may also provide new therapeutic options for PDAC if directed against bulk tumor and CSCs. Due to the fact that PDAC is highly heterogeneous and contains a variety of different mutations (up to 60 different ones), single or combinational therapies targeting signaling pathways can most likely not cope with all the genetic or epigenetic changes found in PDAC. Therefore immunotherapies targeting CSC might eradicate the whole CSC as the root of the disease.

Recently, Visus et al. isolated CSCs, including PDAC CSCs, based on ALDH activity (Visus et al. 2011). In the next step, they generated in vitro ALDH1A1-specific $CD8^+$ T-cells to destroy $ALDH^{bright}$ CSCs in human tumor xenografts models and achieved reduced tumor growth and metastasis. A drawback of this strategy may be that ALDH1A1-specific $CD8^+$ T-cells might also kill normal $ALDH^{bright}$ stem cells such as hematopoietic stem cells.

Another study showed the effect of the bispecific antibody MT110 targeting the T-cell receptor CD3 complex and EPCAM, which is frequently overexpressed on the surface of PDAC cells, including CSC (Munz et al. 2009). A drawback of targeting EPCAM might be that this marker is lost when cells undergo EMT during metastasis, so that metastasized cells may not be targeted. MT110 significantly reduced the CSC population as evidenced by reduced sphere formation capacity and in vivo tumorigenicity. Currently, MT110 is investigated in a dose-escalating phase I clinical trial enrolling patients with different epithelial cancers (lung, colon, gastric). The first results appear to be promising, demonstrating low toxicity and early signs of biological activity, opening up new opportunities for patients with PDAC.

Adoptive immunotherapies using T-cells modified to express a chimeric antigen receptor (CAR-T) against a tumor cell surface antigen have shown promise in preclinical studies using murine models of PDAC (Abate-Daga et al. 2014; Anurathapan et al. 2014; Chmielewski et al. 2012; Maliar et al. 2012). CAR-T-cell therapies against human PDAC are currently being tested in clinical trials.

5.3 Other Strategies to Target CSCs

Due to the high expression of the RON receptor tyrosine kinase in $CD44^+CD24^+ESA^+$ CSC populations, doxorubicin-liposomes coated with a RON antibody improved internalization resulting in a clear decrease in CSC viability (Padhye et al. 2011). In addition, natural compounds from dietary sources represent strategies for eliminating CSCs. For example, curcumin present in curry powders and mustard or its analogue CDF has been reported to improve the sensitivity of PDAC cells to gemcitabine. In particular, increasing PTEN and miR-200 seem to mediate CDF reduced sphere-formation and tumor growth (Bao et al. 2011a). The polyphenol Resveratrol, e.g. found in red grapes, showed anti-tumoral properties in several cancers. Apart from its reported effects in glioblastoma and breast tumors CSCs, resveratrol also blocked self-renewal of pancreatic CSCs via activation of caspase 3/7 and inactivation of Bcl-2 (Xia et al. 2012). Taken together, targeting pancreatic CSCs, the bulk tumor, and the stroma will be critical to cure the disease in the soon future.

6 Conclusions and Future Perspectives

Extensive studies over the past several years revealed the importance of a small subset of cells that could sustain the tumor. Although there are several methods employed to isolate CSCs, there are limitations with each of the currently used methods. Therefore, there is a need to identify improved methods for isolating a pure CSC population. Markers such as EPCAM, CD44, CD133 and CXCR4 have been well established in pancreatic cancer but they serve as markers for other cancer cells as well. It is of utmost importance to identify specific markers, which aid in the

maintenance of pancreatic CSCs. In the past, the identification of circulating tumor cells opened a new chapter in the field of cancer. The recent identification and characterization of an intrinsic autofluorescent phenotype in CSCs in diverse epithelial cancers including pancreatic cancer may now also provide new avenues of research. It may even be employed for the detection of tumor cells circulating in the blood stream, for which new developments are also urgently needed. It may provide us with less invasive and repetitive access to CSCs, thereby hopefully facilitating the development of CSC-centered precision medicine approaches.

CSCs share some surface markers with their normal counterparts, the pancreatic tissue stem cells, but it is still unknown whether the cell-of–origin for PDAC is a normal tissue stem cell, a progenitor cell, or a differentiated exocrine or endocrine cell with acquired stem cell characteristics. In the future, specific CSC promoters will be used to drive activation of *Kras* oncogene to clarify which pancreatic cell subset derives the carcinogenic process. In contrast, the existence of pancreatic CSCs forming spheres in vitro and enhanced in vivo tumorigenicity of tumors identical to the parent cells has been confirmed.

The next milestone to deliver is the real-time observation of pancreatic CSCs in the native in vivo setting, as demonstrated for other solid tumors using clonal analysis after lineage tracing in mice (Chen et al. 2012; Driessens et al. 2012; Schepers et al. 2012). In addition, in vivo imaging of CSCs using abdominal windows might be helpful to get in-depth insights into the role of CSCs in their natural microenvironment. As evidence is now accumulating for novel therapeutic targets that are capable of eliminating CSC, newly emerging treatment regimens that include this knowledge arising from the CSC concept may eventually lead to a better outcome for patients suffering from this currently deadly disease.

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