

## Cancer stem cell in the progression and therapy of pancreatic cancer

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### 1. ABSTRACT

Pancreatic cancer (PC) is an aggressive malignancy with a high incidence of distant metastasis and mortality. Emerging evidence has demonstrated that pancreatic cancer stem cells (CSCs), which have the potential to self-renew and are pluripotent, are crucially important in the progression and therapy of PC. The origin of pancreatic CSCs was suggested to be pancreatic acinar cells, centroacinar cells, or acinar-ductal metaplasia. And several CSC-specific markers for pancreatic cancer have been reported, including CD133, CD24, CD44 and CXCR4. Several studies reported the molecular mechanisms regulating human pancreatic CSCs characteristics. In the progression of PC, CSCs are linked with the aggressiveness of PC with association of epithelial to mesenchymal transition (EMT). In the therapy of PC, especially chemotherapy, CSCs offer new insight into PC therapy, especially the mechanism of drug resistance. Therefore, strategies for modulating and treating CSCs can lead to novel targeted therapies for pancreatic cancer.

### 2. INTRODUCTION

Pancreatic cancer (PC) is one of the most lethal cancers of the gastrointestinal tract characterized by late diagnosis and treatment resistance. PC is responsible for more than 266,000 deaths each year in the world (1). It was also estimated that 37,000 patients will be newly diagnosed with PC, and 34,000 patients will die in the United States, and thus PC remains the fourth leading cause of cancer-related deaths in the United States (2). Despite recent improvements in surgical and chemotherapeutic approaches, PC continues to have a dismal prognosis with an average overall median survival of 4–6 months, due to the lack of early symptoms, the vast majority of patients present with metastatic disease, rendering their malignancy inoperable (3,4). Consequently, the overall 5-year survival is less than 5% (5). The identification of new molecular mechanism for PC to overcome the dismal prognosis is therefore necessary.

Recent studies have shown that a small number of cells possess stem cell-like characters in various cancers,

and such cells have been called cancer stem cells (CSC) (6-8). CSCs have similar features as tissue stem cells, including pluripotency and the ability to self-renew. CSCs mainly remain in the G0 phase of the cell cycle and are less sensitive to radiation and chemotherapy than proliferating cells (9). CSCs are plausible as the best candidate for the seed responsible for tumorigenesis, metastasis, and chemo- and radio-resistance (10). Several researchers recently paid close attention to the therapeutic approach targeting CSCs as a new strategy for cancer treatment. In this review, we will summarize the role of CSCs in the progression and therapy of PC.

### 3. AN OVERVIEW OF PANCREATIC CSCS

The origin of pancreatic CSCs has been controversial. Some studies of genetically engineered mouse models have suggested that pancreatic acinar cells, centroacinar cells (11), or acinar-ductal metaplasia (12) may be the "cell of origin" in pancreatic cancer. The other study suggested that some of the pancreatic epithelial cells characterized by the expression of c-Met, CD133+CD34+CD45-Ter119- and Pdx1 are related to pancreatic carcinogenesis (13,14). However, additional studies are needed to clarify the origin of pancreatic CSCs. CD44+CD24+ESA+ (15) and CD133 (16) are well known as pancreatic CSC markers based on xenograft tumorigenesis. But the detailed function of these markers remains uncertain.

When compared with CD133- cells, CD133+ pancreatic CSCs showed more aggressive behavior, such as increased cell proliferation, migration, and invasion, especially in the presence of pancreatic stromal cells (17). This may be because they underwent epithelial-mesenchymal transition (EMT) more readily (18). CD133+ cells also have higher tumorigenic and metastatic potential than CD44 and CD24 positive cells (19). These findings suggest that CD133 might be a meaningful cell surface marker of pancreatic cancer stem cells.

CXCR4 has been implicated in mediating pancreatic cancer invasion and metastases (20). Hermann and colleagues (21) found that a subpopulation of migrating CD133+CXCR4+ CSCs is essential for PC metastasis. SDF-1, or CXCL12, a specific ligand of the CXCR4 receptor, was shown to be the strongest inducer of migration for CD133+ cancer cells *in vitro*. Thus, they further suggested that strategies aimed at modulating the SDF-1/CXCR4 axis may have important clinical applications to inhibit metastasis of CSCs (21). However, whether CXCR4 expression can be a pancreatic CSC marker is still unconfirmed.

The mechanisms maintaining the "stemness" of CSC are complex. Recently, microRNAs have been found to participate in this process. microRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts, usually resulting in translational repression or target degradation and gene silencing (22,23). microRNA (miR)-34 may play an important role in pancreatic cancer stem cell self-renewal

and/or cell fate determination, potentially via the direct modulation of downstream targets Bcl-2 and Notch (24). In addition, 210 miRNAs including miR-99a, miR-100, miR-125b, miR-192 and miR-429, and 258 stem cell-associated miRNAs that were differentially expressed in the pancreatic CSCs. These differentially expressed microRNAs in pancreatic CSCs provide insights into possible linkages between clusters of miRNAs and clusters of stem cell-associated mRNAs in CSCs, which may be benefit for an understanding of CSCs and CSC-targeted cancer therapy (25).

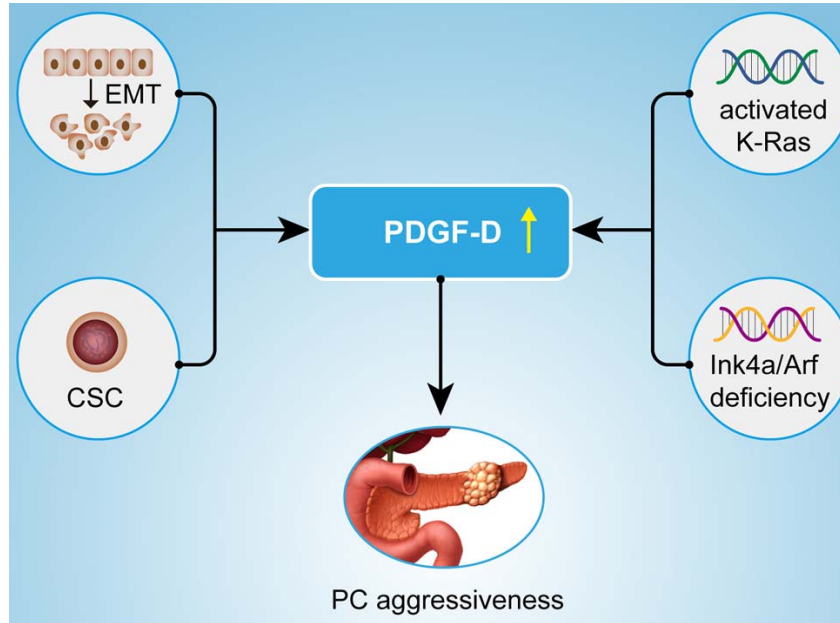
Furthermore, *in vitro* study investigated the molecular mechanisms by which GDC-0449 regulates human pancreatic CSCs characteristics (26). The Sonic Hedgehog (SHH) signaling pathway (27) is aberrantly reactivated and recognized as one of the mediators in the majority of PCs from *in vitro* and *in vivo* studies. SHH signaling system plays a key role also in pancreatic CSC biology including in the regulation of CSCs self-renewal, differentiation; and tumorigenic potential (28). SHH signaling pathway at the level of Gli genes has a critical role in normal pancreas development, and dysregulated SHH signaling plays some role in pancreatic cancer (29). Human pancreatic cancers over express Gli (28,30). GDC-0449 inhibited cell viability and induced apoptosis in pancreatic CSCs. This inhibitor also suppressed cell viability, Gli-DNA binding and transcriptional activities, and induced apoptosis through caspase-3 activation and PARP cleavage in pancreatic CSCs (26). Thus, activated Gli genes repress DRs and Fas expressions, up-regulate the expressions of Bcl-2 and PDGFR $\alpha$  and facilitate pancreatic CSCs survival.

In addition to their own elements of CSCs, the niche of CSCs also can impact the stemness of this stem cells (31). Hamada and colleagues (31) found that indirect co-culture of pancreatic cancer cells with pancreatic stellate cells (PSCs) enhanced the spheroid-forming ability of cancer cells and induced the expression of cancer stem cell-related genes ABCG2, Nestin and LIN28. In addition, co-injection of PSCs enhanced tumorigenicity of pancreatic cancer cells *in vivo*. These results suggested a novel role of PSCs as a part of the cancer stem cell niche.

### 4. CSCS IN EMT AND PC PROGRESSION

Epithelial to mesenchymal transition (EMT) and the reverse process, mesenchymal epithelial transition (MET) are important biological transformation processes not only for morphogenesis and embryogenesis, but also in cancer progression, contributing to invasion and metastasis (32). A CSC enriched fraction from pancreatic cancer cell line, called side population (SP) cells, possess superior potentials of phenotypic switch, including EMT or MET, micro-invasion, and *in vivo* metastasis, compared to main population cells (33).

On the other hand, EMT and CSC phenotype can be regulated through the molecular mechanism by which Notch-1 contributes to the acquisition of EMT phenotype and CSC self-renewal capacity (34-37). Furthermore, the



**Figure 1.** EMT and CSC in PC aggressiveness. Acquisition of the EMT phenotype and induction of CSC characteristics associated with the aggressiveness of PC, which could be mediated in part through activation of the PDGF-D signaling pathway. By stimulating PDGF-D expression and its signaling, Activated K-Ras and Ink4a/Arf deficiency could promote aggressiveness of PC.

activation of Notch-1 signaling contributes to the acquisition of EMT phenotype, which is in part mediated through the regulation of miR-200b and CSC self-renewal capacity, and these processes could be attenuated by genistein treatment (38). Thus, Notch-1 induces epithelial-mesenchymal transition consistent with CSC phenotype in pancreatic cancer cells. Except for Notch-1, over-expression of FoxM1 leads to EMT and CSC phenotype in pancreatic cancer cells, which is in part mediated through the regulation of miR-200b and these processes, could also be attenuated by genistein (39). Moreover, the acquisition of EMT phenotype and induction of CSC characteristics could be linked with the aggressiveness of PC mediated in part through the activation of PDGF-D signaling pathway (40) (Figure 1). And by induction of EMT consistent with CSC phenotype, activated K-Ras and INK4a/Arf deficiency promote aggressiveness of PC, since the deletion of Ink4a/Arf in K-Ras(G12D) expressing mice led to high expression of PDGF-D signaling pathway in the tumor and tumor-derived cell line (RInk-1 cells) (40).

## 5. CSCS IN PC THERAPY

At diagnosis, most PC patients are inoperable, and no curable treatment is available for advanced-stage PC. The CSC hypothesis offers new insight into PC therapy, especially the mechanism of drug resistance (41). The existence of CSCs producing alpha-fetoprotein (AFP) in human PC has been identified, which expresses ABCA12 transporter at a level more than twice as high as that in the non-AFP-producing cells. And the expression of ABCA12 is associated with drug resistance (42). In the CD44<sup>+</sup>CD24<sup>+</sup> CSCs, embryonic stem-related genes

Oct4 and Nanog were up regulated, which was correlated with the multi-potency and a higher drug-resistance of pancreatic CSCs (43).

### 5.1. CSCs in EMT-related tumor therapy

By targeting pancreatic CSCs, sorafenib (SO) is promising for treatment of PC. And sulforaphane (SF), an isothiocyanate enriched in broccoli, may be suited to increase targeting of CSCs by SO. It's due to induction of apoptosis, inhibition of proliferation and angiogenesis, and downregulation of SO-induced expression of proteins involved in EMT that SF completely eradicated SO-induced NF-kappaB binding, which was associated with abrogated clonogenicity, spheroid formation, ALDH1 activity, migratory capacity, and induction of apoptosis on the combination treatment with SF and SO. At the same time, combination therapy reduced the tumor size in a synergistic manner *in vivo* (44). By inhibiting EMT and pluripotency maintaining factors, resveratrol can inhibit pancreatic CSCs characteristics in human and Kras(G12D) transgenic mice (45). In addition, SF inhibited expression of proteins involved in the EMT (beta-catenin, vimentin, twist-1, and ZEB1), suggesting the blockade of signaling involved in early metastasis (46). Moreover, inhibition of Nanog by lentiviral-mediated shRNA expression enhanced the inhibitory effects of SF on self-renewal capacity of CSCs. SF induced apoptosis by inhibiting the expression of Bcl-2 and XIAP, phosphorylation of FKHR, and activating caspase-3 (46).

The homeobox gene MSX2 is an inducer of EMT. The functional cooperation of MSX2 and SP1 has been identified in the transcriptional regulation of ABCG2

via the SP1 binding elements within the ABCG2 promoter. These findings clarified the intriguing regulatory mechanism of the CSC-related gene, and will delineate a novel therapeutic target in PC (47).

### 5.2. CSCs in the efficacy of quercetin therapy

Quercetin is a major polyphenol and flavonoid commonly detected in many fruits and vegetables. Both *in vitro* and *in vivo* models of pancreatic CSCs, quercetin could mediate the reduction of pancreatic CSCs self-renewal (48,46). And quercetin could diminish ALDH1 activity and reverted apoptosis resistance of CSCs (48). The combination of quercetin with SF had synergistic effects on self-renewal capacity of pancreatic CSCs (48,46). Although quercetin led to enhanced binding of the survival factor NF- $\kappa$ B, co-incubation with SF completely eliminated this pro-proliferative feature. Moreover, quercetin prevented expression of proteins involved in the EMT, which was even stronger in presence of SF, suggesting the blockade of signaling involved in early metastasis. *In vivo*, quercetin inhibited growth of CSC-enriched xenografts associated with reduced proliferation, angiogenesis, CSC-marker expression and induction of apoptosis (48).

Quercetin combined with (-)-epigallocatechin-3-gallate (EGCG), an active compound in green tea, had synergistic inhibitory effects on self-renewal capacity of CSCs through attenuation of TCF/LEF and Gli activities (49). In addition, EGCG single could inhibit the expression of pluripotency maintaining transcription factors (Nanog, c-Myc and Oct-4) and self-renewal capacity of pancreatic CSCs. Inhibition of Nanog by shRNA enhanced the inhibitory effects of EGCG on self-renewal capacity of CSCs. EGCG inhibited cell proliferation and induced apoptosis by inhibiting the expression of Bcl-2 and XIAP and activating caspase-3. Interestingly, EGCG also inhibited the components of SHH pathway (smoothened, patched, Gli1 and Gli2) and Gli transcriptional activity. Furthermore, EGCG inhibited EMT by inhibiting the expression of Snail, Slug and ZEB1, and TCF/LEF transcriptional activity (49).

These findings suggest that food ingredients complement each other in the elimination of CSC-characteristics. Since carcinogenesis is a complex process, combination of bioactive dietary agents with complementary activities may be most effective.

### 5.3. CSCs in the efficacy of gemcitabine therapy

Currently, gemcitabine is the standard chemotherapeutic agent used in patients with pancreatic cancer (50). However, the clinical impact of gemcitabine remains modest (51). This limitation in conventional treatments is mainly due to the profound resistance of cancer cells to anti-cancer drugs, which can be inherent and/or acquired (52, 53). An and colleagues (54) established a gemcitabine-resistant PC cell line SW1990/GZ to explore the relationship between drug-resistant cell line SW1990/GZ and pancreatic CSCs, and they found that gemcitabine-resistant cell line SW1990/GZ has a higher proportion of pancreatic CSCs compared to its parental cell

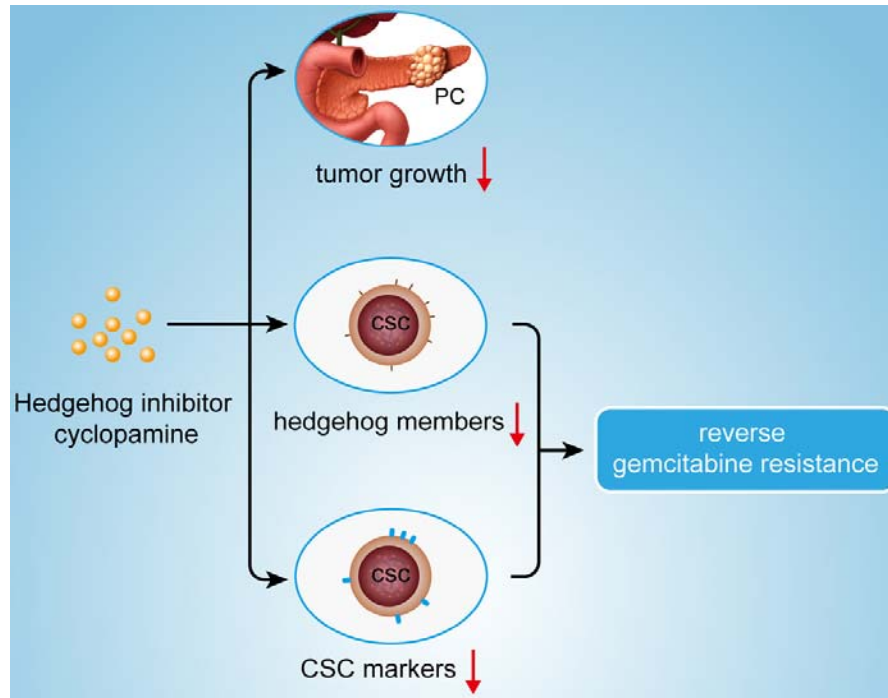
line SW1990. The gemcitabine resistance of PC cells is associated with a cancer stem cell-like phenotype (55). Therefore, CSCs is involved into the drug resistance of PC, and would be a promising target in the drug resistant PC.

In a direct xenograft model of PC, targeting CSC could increase the efficacy of gemcitabine. Combined treatment with gemcitabine and cyclopamine induced tumor regression and decrease in CSC markers and hedgehog signaling. Misregulated hedgehog signalling, which is normally an essential pathway during embryonic pancreatic development, has been implicated in several forms of cancer, including human pancreatic carcinoma (56–58). It plays an important role in maintaining the CSCs pool. Hedgehog inhibitors as part of a dual compartment therapeutic approach were able to further reduce tumor growth and decreased both static and dynamic markers of CSC (59). Furthermore, since gemcitabine-resistant pancreatic cancer cells highly express CSCs markers and some of the hedgehog members, inhibition of hedgehog by cyclopamine even could effectively reverse gemcitabine resistance in pancreatic cancer (60) (Figure 2). The Nodal and Activin could regulate self-renewal of pancreatic CSCs and would be a target of drug resistant PC. Knockdown or pharmacological inhibition of the Nodal/Activin receptor Alk4/7 in CSCs can virtually abrogate their self-renewal capacity and *in vivo* tumorigenicity, and reverse the resistance of orthotopically engrafted cancer stem cells to gemcitabine. The stroma-targeting hedgehog pathway inhibitor can enhance delivery of the Nodal/Activin inhibitor and translated into long-term, progression-free survival (61).

In addition to reverse gemcitabine resistance of PC cells, enhancing the antiproliferative activity of gemcitabine is another choice to improve the efficacy of gemcitabine in PC therapy. Zeste homolog-2 (EZH2) plays a pivotal role in cancer stem cell (CSC) self-renewal through methylation of histone H3 lysine-27 (H3K27me3). The EZH2 inhibitor 3-deazaneplanocin A (DZNep) modulated EZH2 and H3K27me3 protein expression and synergistically enhanced the antiproliferative activity of gemcitabine. Furthermore, the drug combination reduced the percentages of cells in G(2)-M phase and significantly increased apoptosis compared with gemcitabine alone. DZNep and DZNep/gemcitabine combination significantly reduced the volume of PDAC spheroids growing in CSC-selective medium and decreased the proportion of CD133+ cells (6). Venkatesha and colleagues (62) also found that Chk1 inhibition can selectively sensitize pancreatic CSCs to gemcitabine. Chk1 inhibition in combination with gemcitabine can reduce both the percentage and the tumor-initiating capacity of pancreatic CSCs.

## 6. CONCLUSIONS

Though the origin, detection, and characteristics of pancreatic CSCs have not been fully clarified, accumulating evidence suggests that CSCs would have an



**Figure 2.** Hedgehog inhibitor in PC. Hedgehog inhibitor cyclopamine can reduce tumor growth and decrease both static and dynamic markers of CSC. Since gemcitabine-resistant PC cells highly express CSC markers and some of the hedgehog members, inhibition of hedgehog by cyclopamine is able to effectively reverse gemcitabine resistance in PC.

important impact on the malignant behavior of PC and the design of novel therapies. Therefore, it is critical to improve our understanding of the detailed mechanism CSCs in the progression and therapy, especially chemotherapy. Strategies for modulating and treating CSCs can lead to novel targeted therapies for pancreatic cancer.

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**Abbreviations:** PC: Pancreatic cancer; CSCs: cancer stem cells; EMT: mesenchymal transition; microRNA: miR; SHH: Sonic Hedgehog; PSCs: pancreatic stellate cells; SP: side population; AFP: alpha-fetoprotein; SO: sorafenib; SF: sulforaphane; EGCG: epigallocatechin-3-gallate; DZNeP: 3-deazaneplanocin A

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