Cancer-associated fibroblasts: from spectators to protagonists in pancreatic cancer progression

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Running title: Fibroblasts play diverse roles in pancreatic cancer progression

Abstract

Our knowledge of the origins, heterogeneity and functions of cancer-associated fibroblasts (CAFs) in pancreatic ductal adenocarcinoma (PDAC) has exponentially increased over the last two decades. This has been facilitated by the implementation of new models and single-cell technologies. However, a few key studies preceded the current exciting times in CAF research and were fundamental in initiating the investigation of CAFs and of their roles in PDAC. With their study published in *Cancer Research* in 2008, Hwang and colleagues have been first to successfully isolate and immortalize human pancreatic stellate cells (HPSCs) from PDAC tissues. This new tool allowed them to probe the roles of CAFs in PDAC as never done before. By performing complementary *in vitro* and *in vivo* analyses, the authors demonstrated the involvement of HPSCs in PDAC malignant cell proliferation, invasion and therapy resistance. Here, we leverage that seminal study as a framework to discuss the advances made over the last 16 years in understanding the complexity and central roles of CAFs in PDAC progression.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies. We now appreciate that a major contributor to this lethality in PDAC is the extensive and desmoplastic tumor microenvironment (TME). Over the last two decades, multiple studies have shed light on the heterogeneous composition of this TME and have unveiled pivotal roles of non-malignant stromal cells, including cancer-associated fibroblasts (CAFs), in PDAC growth, metastasis formation, immunosuppression and therapy resistance. Consequently, CAFs went from being considered spectators to protagonists in PDAC progression. The work from Hwang and colleagues was one of the first to demonstrate pro-tumorigenic roles of CAFs. The authors achieved this by establishing immortalized human pancreatic stellate cells (HPSCs) from PDAC and leveraging them for *in vitro* and *in vivo* experiments (1).

Hwang and colleagues isolated these HPSCs from a PDAC patient sample by using an outgrowth method. Thus, the HPSCs described in their study likely represent a heterogenous population of CAFs.

Indeed, we now know that only a minority of PDAC CAFs are derived from PSCs (2), with CAF precursors also including mesothelial cells, resident fibroblasts, pericytes, fibrocytes, and mesenchymal stem cells, among others (3). Despite this, malignant cell-driven reprogramming of PSCs models several features of PDAC CAFs. Thus, PSC-derived models have been instrumental in expanding our understanding of CAF molecular and functional heterogeneity in PDAC. One of these models was established by us by culturing murine PSCs and PDAC organoids. This three-dimensional platform allowed for the first time to phenotypically characterize two different PDAC CAF populations (4). On the contrary, only homogeneous alpha smooth muscle actin (αSMA)-positive CAFs are observed in monolayer cultures, as also described in Hwang and colleagues (1). Follow-up single-cell analyses of murine and human tissues revealed further complexity of PDAC CAFs. To date, based on their protein markers and transcriptome, myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs) and antigen-presenting CAFs (apCAFs) are the three main populations that have been described across multiple studies (3). Whereas myCAFs are characterized by high αSMA expression and extracellular matrix (ECM) production, iCAFs are characterized by secretion of inflammatory cytokines and chemokines, including leukemia inhibitory factor (LIF) and interleukin-6 (IL6). Finally, apCAFs express major histocompatibility complex class II (MHCII) and can induce $CD4^+$ T cell activation (3). While iCAFs and myCAFs can originate from PSCs and can partially interconvert, apCAFs have a mesothelial cell of origin, which explains why they are not identified in PSC/organoid co-cultures (3,4). In addition to these three populations, other marker-defined CAF subsets have been more recently described. For example, Hutton and colleagues demonstrated the presence of two CAF lineages differing in CD105 expression and revealed that CD105-negative CAFs support an anti-tumor immune response (5). Thanks to this and other studies, we are now starting to understand the pleiotropic roles that distinct CAF populations have in regulating adaptive and innate immune responses in PDAC.

Although the impact of CAFs on the immune cells could not be properly explored with their human-derived transplantation mouse models, Hwang and colleagues focused on the ability of CAFs to promote PDAC primary tumor growth and metastasis via CAF-malignant cell crosstalk (1). By culturing human PDAC cell lines with conditioned media from HPSCs, the authors observed that paracrine signals induced mitogen-activated protein kinase (MAPK) and AKT pathways in the malignant cells, promoting cell proliferation, invasion, and migration. Consistent results were also obtained *in vivo*. Indeed, mice orthotopically co-injected with human PDAC cells and HPSCs had significantly bigger primary tumors and a higher metastatic burden compared to mice injected with only PDAC cells. While the authors did not further dissect the mechanistic crosstalk between HPSCs and PDAC malignant cells, they speculated that this could be driven by interleukin-1 β (IL-1β) and transforming growth factor β (TGF-β), which can induce MAPK and Akt signaling activation, respectively. Over the last few years, several studies have built from these initial findings and focused on the mechanisms underlying CAF roles in tumor progression and metastasis formation. For example, beside secreting ligands that directly support primary tumor growth, such as LIF and IL-6, CAFs release exosomes containing amino acids, lipids, lactate and intermediates of the tricarboxylic acid cycle that reprogram malignant cell metabolism to promote PDAC progression under nutrient-deprived conditions (5). Moreover, Vennin and colleagues showed that *Trp53*-mutant PDAC cells educate CAFs to secrete perlecan, which in turn promotes PDAC metastasis (6). Additionally, we recently demonstrated that an EGFR-activated myCAF subset promotes epithelial-mesenchymal transition (EMT) and metastasis of PDAC malignant cells (7). Of note, CAFs not only promote PDAC metastasis by directly acting on the malignant cells but also by shaping the pro-metastatic niche. Indeed, it has been shown that CAF-secreted IL-6 activates hepatocytes via STAT3 signaling and

triggers the release of serum amyloid A1 and A2 (SAA) (8). Consequentially, SAA promotes fibrosis and myeloid cell accumulation in the liver, resulting in the formation of a pro-metastatic microenvironment that facilitates PDAC cell seeding and metastatic outgrowth (8).

In addition to exploring CAF tumor-promoting roles, Hwang and colleagues also showed that HPSCs drive gemcitabine and radiotherapy resistance of PDAC cells (1), the mechanisms of which have been described in recent studies. For instance, CAFs can accumulate gemcitabine intracellularly, lowering its concentration within the TME and consequently reducing its effect on the malignant cells (3). Furthermore, CAF-produced ECM contributes to the formation of a TME with collapsed vessels and poor blood diffusion, which hinders drug delivery. Poor blood diffusion also leads to hypoxia, which reduces the production of cytotoxic reactive oxygen species, thus limiting the efficacy of radiotherapy (9). Moreover, CAF-mediated radioprotection of PDAC malignant cells can occur through β1-integrin/focal adhesion kinase signaling (9). In addition to radiotherapy and chemotherapy, over the last few years, immunotherapies have shown promise for the treatment of various malignancies. However, these strategies have not yet been effectively translated to PDAC as these tumors are characterized by a highly immunosuppressive TME. Several studies have demonstrated a role for CAFs in modulating the efficacy of immunotherapy strategies in transplantation models or genetically engineered mouse models (GEMMs). For example, TGF-β-dependent CAFs expressing leucine-rich repeat containing 15 (LRRC15) have been shown to limit PDAC response to immune checkpoint blockade (10).

Overall, the study published in 2008 by Hwang and colleagues identified CAFs as key drivers of critical steps of PDAC progression and therapy response (**Figure 1**). Subsequent studies revealed mechanisms behind these results and highlighted additional roles of CAFs, including their contribution to creating an immunosuppressive microenvironment (**Figure 1**). However, several questions remain to be addressed. For example, considering that CAFs can impact distant organs (3,8), whether they play roles in systemic conditions, such as cancer-associated cachexia, has yet to be determined. Furthermore, a better understanding of unique features of CAFs, as opposed to features also present in fibroblasts in inflammatory states, may help guide more effective therapeutic interventions. Moreover, given CAF heterogeneity and plasticity, it will be important to understand how treatments affect distinct PDAC CAF subtypes. Indeed, by knowing how the TME responds to treatment, we could be able to predict PDAC evolution and effectively counteract potential mechanisms of resistance in second-line treatments.

In the future, further advancements in multimodal single-cell and spatial technologies will allow researchers to further map PDAC CAF heterogeneity and the TME. However, it is now key to move away from phenotypical description alone. Ultimately, rigorous functional dissection of CAF heterogeneity at primary and secondary sites will be pivotal to develop effective combinatorial therapies for PDAC patients (**Figure 1**). Indeed, despite the exponential interest in CAF biology, to date, no CAF- or ECM-targeting strategy has been successfully translated to the clinic. The presence of tumor-restraining CAF subsets and ECM components and a limited understanding of the complex multi-cellular crosstalk within the TME have contributed to this failure (5). Development of new *in vitro* heterotypic co-culture models and *in vivo* CAF subtype-specific GEMMs will be required to deconvolute the complexity of the PDAC TME and design effective combination therapies for patient benefit. Finally, as CAF populations similar to those found in PDAC have been also found in other malignancies, a better understanding of PDAC CAF biology may have a wide impact in oncology.

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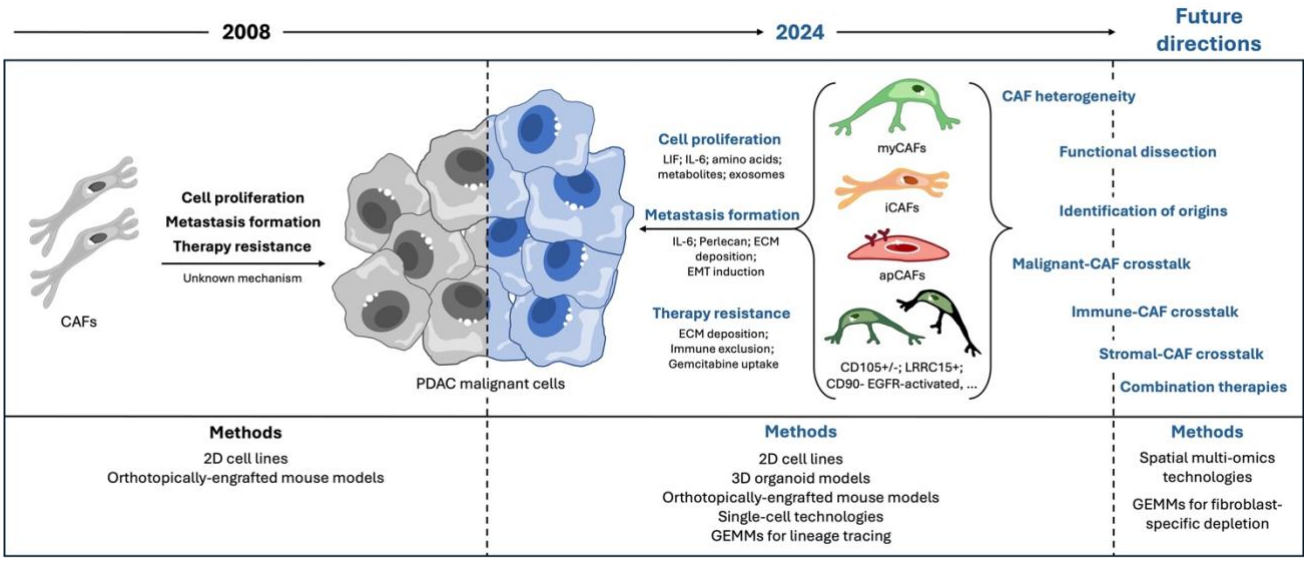

Figure 1. Progress in knowledge and future directions in understanding the roles of cancer-associated fibroblasts (CAFs) in pancreatic ductal adenocarcinoma (PDAC) since the publication by Hwang and colleagues in 2008. GEMMs, genetically engineered mouse models; TME, tumor microenvironment; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition.