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# Pancreatic Cancer: Advances and Challenges

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## Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest cancers. Significant efforts have largely defined major genetic factors driving PDAC pathogenesis and progression. Pancreatic tumors are characterized by a complex microenvironment that orchestrates metabolic alterations and supports a milieu of interactions among various cell types within this niche. In this review, we highlight the foundational studies that have driven our understanding of these processes. We further discuss the recent technological advances that continue to expand our understanding of PDAC complexity. We posit that the clinical translation of these research endeavors will enhance the dismal survival of this recalcitrant disease.

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## eTOC

Enabled by technological development on various fronts, our understanding of the complexity in the biology of pancreatic ductal adenocarcinoma deepens, laying the foundation for promising translation of the biological insights into clinically actionable advances.

## Introduction:

Pancreatic ductal adenocarcinoma (PDAC) has surpassed breast cancer to become the third leading cause of cancer-related death in the United States[1,2]. More concerningly, PDAC is projected to overtake colorectal cancer before 2040, moving only behind lung cancer as a leading cause of cancer-related mortality. The factors driving the lethality of PDAC are numerous, centered on an inability to detect the disease until late in progression, often after distant metastasis[3]. Moreover, outside of the minority (10–15%) of cases ascribed to germline mutations or known risk factors, such as mucinous cystic lesions and chronic pancreatitis, there is no single attributable risk factor for most patients[4]. Further complicating diagnosis, localized disease is largely asymptomatic or accompanied by ill-defined symptoms, there is a paucity of diagnostic biomarkers for earlier stage tumors, and the difficult to access anatomical location of the pancreas prevents routine office-based screening. The steadily increasing incidence of disease[5], driven by a combination of factors, including the obesity epidemic and greater life expectancy, underscores the urgent need to identify and deploy new therapeutic strategies to achieve benefit for most patients.

As most patients present with advanced, unresectable disease, only about 12% are predicted to survive beyond 5 years. While these numbers are dismal at first glance, some reasons for optimism exist. PDAC survival statistics have doubled over the past decade through a combination of improved clinical care and understanding of the biology of pancreatic cancer[6,7]. This progress coincides with a newfound appreciation for the complexity of PDAC, which occurs not only across patients but also can vary dramatically within individual tumors. Growing understanding of these variances in genetics, treatment response, and aspects of tumor composition are clarifying the scope of the problem that needs to be solved to accelerate the progress in extending patient survival.

In this review, we will briefly introduce the current treatment paradigms for PDAC, and then expand upon the biological underpinnings driving its pathogenesis and progression. We will also discuss the complexity of the pancreatic tumor microenvironment (TME) and interactions among the numerous cell types within this niche. The translational implications of research advances, such as novel therapeutic strategies and technologies, will be highlighted within pertinent sections. Finally, we will discuss how the clinical trial landscape of PDAC has evolved over the past decade, including lessons learned from pivotal trials that were unsuccessful in prolonging survival and the adoption of innovative trial designs to improve outcomes in this recalcitrant disease.

### Treatment Strategies for Pancreatic Ductal Adenocarcinoma

Chemotherapy and surgery are the primary treatment options for PDAC. However, only 15–20% of patients are eligible for surgery at diagnosis[3]. Most PDAC patients present

with distant metastasis at diagnosis, where removal of the primary lesion through a major surgical procedure is unlikely to impact the outcome. Second, the pancreas is close to critical vasculature. Even in patients without clinical evidence of extra-pancreatic spread, significant involvement or occlusion of these vessels by the primary tumor can prevent eligibility for surgery. Finally, partial removal of the pancreas, often along with a portion of the duodenum and the creation of *de novo* anastomoses, is a major surgery with lasting impacts on digestion and systemic metabolism. Accordingly, patients must be in robust health to withstand the operation and recover to benefit from the procedure.

The clinical care for most patients, regardless of surgical eligibility, includes systemic chemotherapy. In the past decade, two new combination regimens have emerged as the first-line therapy in patients with advanced PDAC. The first is a combination of 5-fluorouracil (5-FU), leucovorin, irinotecan and oxaliplatin, which carries the acronym FOLFIRINOX. The second is the combination of gemcitabine and an albumin nanoparticle conjugate of paclitaxel (nab-paclitaxel)[8,9]. Patients progressing on first-line regimens can be offered the possibility of switching to the other regimen, or a second-line regimen that includes a liposomal formulation of irinotecan in combination with 5-FU[10], provided they have not received either of these agents previously. Overall, there are no universal standards for second-line regimens and beyond in PDAC, and the course of therapy is often determined by the patient's performance status, presence of "actionable" targets, and the availability of appropriate clinical trials.

Unfortunately, even in cases where surgical resection can be performed, nearly 3 in 4 of patients will develop recurrence within two years, suggesting patients undergoing resection also harbor micro-metastatic disease[11]. Supporting this, pre-clinical studies have demonstrated that disseminated PDAC cells can be observed in circulation before localized invasion is readily visualized in primary tumors[12]. Accordingly, to enhance the possibility of eliminating PDAC cells that have escaped the primary tumor, prior to or during resection, patients who undergo surgery are typically treated with adjuvant chemotherapy in the post-operative setting. Based on the patient's performance status, this can be either a modification of the original FOLFIRINOX regimen, or one containing gemcitabine, such as gemcitabine and cisplatin or gemcitabine and nab-paclitaxel[13–15]. Finally, one of the practices that has become increasingly adopted in the context of PDAC therapy over the past decade, albeit not uniformly around the globe, is that of systemic pre-operative (neoadjuvant) therapy[16]. Neoadjuvant therapy has the potential to shrink "borderline" resectable PDAC and enable the tumor to undergo resection. Indeed, patients whose PDAC exhibits a major or complete pathological response to neoadjuvant therapy demonstrate significantly improved overall survival[17], although the biological underpinnings of this response remain to be discerned. In recently completed randomized clinical trials[18], neoadjuvant chemoradiation in conjunction with adjuvant chemotherapy shows significantly improved five-year overall survival rates compared to patients who received upfront surgery with adjuvant therapy (20.5% versus 6.5%).

While the aforementioned data describe advances in standard of care therapy of PDAC, they also highlight the significant challenges with achieving cures in this recalcitrant disease. There is reasonable evidence that earlier diagnosis and surgical resection of PDAC provides

the best opportunity for long-term survival. In retrospective analyses of a national cancer surveillance database in the United States, a remarkable 80% of patients who present with the lowest stages of PDAC (Stage IA or IB disease) were alive at five years, compared to less than 10% of patients with advanced disease[19]. Unfortunately, patients with such low stage tumors are typically diagnosed incidentally and comprise no more than 3–5% of cases. This has led to a burgeoning interest at enabling earlier diagnosis of PDAC in cohorts that are higher than average risk for this disease[20]. This approach is effective in individuals or families who undergo prospective surveillance for PDAC due to an inherited lifetime risk, such as a germline pathogenic mutation[4]. In one large multi-center international trial performed in high-risk cohorts, almost three-fourths of patients who were diagnosed with PDAC on active surveillance had Stage I disease, and an equal proportion were still alive at five years, reiterating that earlier detection can translate into improved outcomes[21].

While surgery and chemotherapy remain the mainstay of PDAC clinical management, there have been several compelling developments with the application of targeted therapies in this disease, and these will be discussed in the context of selected genomic drivers in the section below.

### Precursor Lesions of Pancreatic Cancer: An Opportunity for Early Detection

Among the histological subtypes of cancer arising in the pancreas, PDAC is both the most frequent exocrine neoplasm and the most frequent cancer overall. PDAC accounts for greater than 90% of pancreas cancers and has a distinct molecular profile and natural history from pancreatic neuroendocrine tumors, the second most common neoplasm in this organ[22]. Early molecular profiling coupled with histology allowed for the development of a putative pathological progression model; analogous to the adenoma-carcinoma model in the colon[23] (Fig. 1). Here, intraductal precursors are postulated to undergo a progression from low to high-grade lesions, acquiring increasing cytological atypia and genetic aberrations, culminating in invasive adenocarcinomas. At least two pathways to invasive cancer in the pancreas have been recognized, each with somewhat distinct frequency, natural history, and genetics. Pancreatic Intraepithelial Neoplasia (PanINs) are the most frequently observed precursor lesions and are, by definition, microscopic lesions that cannot be observed on abdominal imaging scans[24]. In contrast, cystic mucinous neoplasms of the pancreas, of which intraductal papillary mucinous neoplasms (IPMNs) are the most common histological subtype, are macroscopic precursors that arise within the main pancreatic duct or one of its branches. These can be observed and followed for progression by imaging techniques[25]. Approximately 85–90% of PDAC cases are ascribed to originate on the backdrop of PanIN lesions, the remaining 10–15% in cystic precursors[20]. Recent phylogenetic analyses of precursor lesions from the pancreata of patients with established cancer have confirmed the stepwise progression model and accumulation of genetic alterations in the transition from PanINs to PDAC postulated using more rudimentary techniques[26]. Further, modeling studies performed on the mutational data have estimated that the timeline of multistep progression to invasive neoplasia in the pancreas likely spans multiple years and provides a theoretically wide window of opportunity for early detection[27].

The plausibility of multistep progression of PanIN and IPMN has also been validated using genetically engineered mouse models (GEMMs). For example, conditional expression of oncogenic mutant *Kras* in the pancreas, using *Cre*-recombinase driven by pancreatic transcription factor promoters activated during embryonic development, leads to development of murine PanIN lesions histologically reminiscent of human disease[28]. Only a fraction of these mice will go on to develop invasive cancer, and those which do show a concurrent loss of a tumor suppressor gene. However, combined expression of oncogenic *Kras* coupled with the deletion or loss of function of *Trp53*, *Cdkn2a*, or *Smad4* in the pancreas of mice readily leads to invasive PDAC[29–32]. Early studies in these models demonstrated that the combined haploinsufficiency of *Smad4*, coupled with oncogenic KRAS expression, leads to the development of cystic neoplasms, linking *Kras* mutations to other putative PDAC precursor lesions[33]. More recent IPMN models have been generated that genocopy recurrent driver alterations observed in cognate human lesions (i.e. *Kras*, *Gnas*, *Rnf43*) (Fig 1)[34–36], and these models demonstrate the comparable stepwise progression observed in patient derived IPMNs.

Importantly, the understanding that PDAC arises through a multistep series of precursor lesions, with a prolonged timeline for progression to invasive neoplasia, has generated substantial interest in discovery of circulating biomarkers for early detection, especially in high-risk cohorts[37]. The carbohydrate antigen 19–9 (CA19–9), also known as sialyl-Lewis[a], is currently the most widely used diagnostic biomarker for PDAC, although it has a suboptimal sensitivity in early-stage disease. Nonetheless, CA19–9 elevations can be observed in plasma samples obtained in the pre-diagnostic setting, suggesting a potential window of opportunity for early detection[38]. While an in-depth discussion of ongoing biomarker research in PDAC is beyond the scope of this review, a multitude of circulating tumor-derived analytes (proteins, autoantibodies, metabolites, DNA, non-coding RNAs and extracellular vesicles, among others) have been assessed in the context of PDAC early detection[39–41]. For patients with cystic lesions, aspirated cyst fluid (obtained using endoscopic ultrasound guided biopsy) is an attractive proximal biospecimen for assessment of analytes[42]. There is burgeoning enthusiasm for the potential of "multi-cancer early detection" (MCED) tests, which can interrogate for the presence of multiple cancer types, typically using aberrant tumor-derived DNA signals (mutation, methylation or fragments) in circulation[43]. One cautionary note worth reiterating with any biomarker endeavors in PDAC is the potential for false positives in the average-risk population, mandating exceptionally superlative performance characteristics (high specificity and high sensitivity). Therefore, current guidelines advise against screening the general population and instead emphasize biomarker studies be focused on well-defined high-risk cohorts[44].

### Genomic Drivers of Pancreatic Cancer

In contrast to the absence of a single attributable cause in most patients, the somatic genetic drivers of pancreatic cancer are well characterized (Fig. 1). Mutations in the v-Kiras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) are found in over 90% of patient samples[45]. *KRAS* mutations are observed in most low-grade PanINs and IPMNs, suggesting *KRAS* is the initiating genetic event driving exocrine neoplasia[46,47]. In addition to *KRAS* mutations, the genomic landscape of PDAC is also dominated by loss of function alterations

of *TP53*, *SMAD4* (also known as *DPC4*), and *CDKN2A*[45,48–50]. The temporal appearance of these alterations during multistep progression suggests that *TP53* and *SMAD4* typically occur late, usually in the setting of high-grade lesions or cancer in both PanINs and IPMNs[23,51].

Ras has pleiotropic cell autonomous and non-cell autonomous effects in PDAC. Each of the other major drivers adds one or more unique facets the phenotype, natural history and therapeutic vulnerability of PDAC. For example, *CDKN2A* is often lost by homozygous deletions at the chromosomal 9p21 locus, which is associated with codeletions of a neighboring interferon cluster in half of cases. Interferon cluster loss creates a "cold" tumor immune milieu and resistance to immunotherapy[52], a phenotype that is likely to be generalized across other solid cancers with chromosomal 9p21 deletions[53]. *SMAD4* loss has a profound deleterious effect on prognosis[54], and is associated with higher rates of metastatic dissemination during neoadjuvant therapy and terminal disease[55,56]. *TP53* is the most commonly mutated tumor suppressor gene in PDAC, and altered p53 function has a multitude of tumor-promoting effects on neoplastic cells, including increased genomic instability[30], reprogrammed cellular metabolism[57,58], and enhanced metastatic propensity[59]. PDAC mouse models have suggested *TP53* mutations promote a patterned loss of genomic integrity as opposed to a chaotic process[60]. Finally, it is important to note that "hotspot" *TP53* missense mutations might have a distinct impact on the pathophysiology of cancer cells from that of truncating mutations or homozygous deletions which lead to absence of functional protein[59,61], underscoring an additional layer of nuance when considering the role of tumor suppressor genes in PDAC.

Given the near ubiquitous presence in pancreatic tumorigenesis, it was postulated that PDAC might be "addicted" to oncogenic Ras signaling. Providing an early clue toward the complex heterogeneity of PDAC, RNAi-mediated gene silencing of oncogenic *KRAS* revealed a significant portion of PDAC cell lines demonstrate no reliance on KRAS expression for viability[62]. Further insights into the requirement of KRAS signaling in pancreatic tumor maintenance was made through the development of GEMMs with inducible and reversable expression of mutant *Kras* in the pancreas. Withdrawal of oncogenic *Kras* resulted in regression of invasive neoplasia, including regression of metastatic disease[63–65]. Notably, in such an inducible system, PanINs can be observed to re-differentiate back to acinar cells in the absence of tumor suppressor loss[66]. However, withdrawal of mutant Ras signaling in preclinical models that include loss of tumor suppressor function leads to tumors that can re-occur in a Ras-independent fashion[67]. While "Ras-escaper" cells have orthogonal targetable vulnerabilities[68], the ability of PDAC to survive Ras inhibition highlights a likely challenge of newly developed small molecule inhibitors of mutant KRAS.

The advent of allele-specific (targeting KRAS[G12C] and KRAS[G12D])[69,70], and a broader cache of allele agnostic ("pan-RAS") small molecule inhibitors[71], has generated considerable enthusiasm for their application in *KRAS*-mutated cancers, including PDAC. Covalent inhibitors of the KRAS[G12C] allele were the first to be evaluated in the clinic[72,73], and two agents have recently been approved by the US FDA for use in KRAS[G12C]-mutant non-small cell lung cancer. However, the KRAS[G12C] allele occurs in only 1.6% of PDAC cases, whereas the KRAS[G12D] allele is present in greater than 40%, followed in

frequency by KRAS[G12V] and KRAS[G12R] mutations[74]. In the first published clinical series of KRAS[G12C] inhibitor monotherapy in advanced PDAC, responses occurred in a minority of patients (~21%) and were generally transient[75]. This is not surprising, as correlative studies conducted in other KRAS[G12C]-mutant tumor types treated with these inhibitors have demonstrated the emergence of pleiotropic resistance through both cell autonomous and non-cell autonomous mechanisms[76–78]. Recently, data from pre-clinical PDAC models has shown promising responses to a small molecule inhibitor targeting KRAS[G12D] mutations common in PDAC[79]. However, given the observed resistance to KRAS[G12C] inhibitors and observations from KRAS extinction models, it is probable these drugs will face similar challenges as they enter the clinic. Accordingly, significant activity is currently focused on identifying combinations that would lead to more sustained responses in patients[80].

While much of the excitement on therapeutic targeting of the erstwhile "undruggable" Ras oncoprotein in PDAC has focused on small molecule inhibitors, additional modalities of direct *KRAS* inhibition have been developed. For example, a directly binding protein "degrader" that forms a ternary complex with mutant Ras and E3 ubiquitin ligase is currently being evaluated in the clinic[81]. Similarly, genetic extinction of mutant KRAS[G12D] allele using exosome delivered synthetic siRNAs is currently undergoing early phase trials in advanced PDAC[82]. Finally, adoptive T cell therapy using autologous T cells expressing an engineered T cell receptor targeting HLA-restricted mutant *KRAS* neoantigen has shown promising early results and is currently being expanded to other epitopes and HLA contexts[83].

The most frequently encountered driver mutations in PDAC were identified prior to the advent of next generation sequencing (NGS) by targeted PCR, immunohistochemistry, or through their association with hereditary syndromes. The widespread adoption of NGS has greatly expanded the genomic landscape of PDAC and identified a large compendium of less frequently altered genes (10% or lower) that play a role in selected tumor subsets[45,48–50]. These less-frequent mutations largely cluster around central themes, including DNA maintenance and DNA damage response, epigenetic modifying proteins, and axon guidance pathway components. However, despite its aggressive nature, the overall mutational load of PDAC is less than many common solid tumors, such as lung cancers and melanoma[84].

Approximately 5–7% of patients with PDAC harbor a germline mutation in one of the Fanconi anemia family of genes (*BRCA1*, *BRCA2*, and *PALB2*) encoding for proteins engaged in homologous recombination repair[85]. It is postulated that somatic loss of the remaining wild-type allele in the tumor cells would lead to a homologous repair deficient (HRD) phenotype, rendering such tumors exquisitely sensitive to agents that induce double strand DNA breaks (e.g., platinum agents like cisplatin and oxaliplatin), or to inhibitors of the DNA repair enzyme PARP, which would result in synthetic lethality given the pre-existing compromise in DNA repair function[86]. Indeed, a pivotal clinical trial in patients with PDAC and germline *BRCA1/2* mutations established the benefit of administering an orally bioavailable PARP inhibitor on improved progression free survival (PFS). This led to the approval of targeted therapy in this setting[87]. Unfortunately, longer follow up data did not demonstrate a similar improvement in overall survival (OS) compared to patients who received placebo alone following platinum-based therapy[88]. Nonetheless, PARP inhibitors

continue to be embraced in the clinic, largely due to the impact of a "chemo-free" regimen on the quality of life for patients.

More recent studies have demonstrated that, in addition to patients with germline mutations, those harboring *de novo* somatic mutations of *BRCA1/2* and *PALB2* also show benefit from PARP inhibitors, provided there is bi-allelic loss and established homologous repair deficiency in the tumors[89]. Ongoing trials are also PARP inhibitors in additional scenarios, such as in the postoperative (adjuvant) setting and in combination with immunotherapy[90]. Mutations of *ATM* are the second most frequent known cause of germline mutations in PDAC, after *BRCA2*, conferring a comparable lifetime increased risk of PDAC in mutation carriers[91]. While the ATM protein is a serine/threonine kinase that is recruited to sites of DNA double strand breaks and is involved in DNA repair, bi-allelic mutations of *ATM* have not been shown to result in susceptibility to PARP inhibitor monotherapy like *BRCA1/2*[92,93].

Chromatin remodeling and SWI/SNF complex genes are one of the most frequently altered class of genes in PDAC[45,48–50], and can occur via somatic deletions of the associated chromosomal loci[94]. Perturbation of gene function in GEMMs has strongly suggested a tumor suppressor role for many of these altered genes. For example, deletion of *KDM6A* results in accelerated tumorigenesis and acquisition of an aggressive transcriptional subtype[95–97]. Similarly, deletion of SWI/SNF factors *Arid1a* or *Brg1* results in the acceleration of pancreatic tumorigenesis on the backdrop of cystic neoplasms resembling human IPMNs[98–100]. In contrast to prototypal tumor suppressor genes like *TP53* and *CDKN2A*, chromatin regulatory factors may have a more context-specific and biphasic function in PDAC, with studies in autochthonous models suggesting both tumor suppressor and oncogenic functions during distinct stages of tumorigenesis[101,102]. Given the cumulative frequency of PDAC cases that exhibit alterations in this class of genes, there is significant promise of leveraging therapeutic vulnerabilities that arise within these patients[97,103].

Finally, there are also so-called "alternative drivers" found in the 8–10% of cases that are *KRAS* wild type[104,105]. These are enriched in patients who have younger onset PDAC (<50 years) and can manifest with variant histological features. The alternative drivers (e.g., *ALK, TRK, RET, NRG1, BRAF, EGFR*) activate the MAP kinase signaling pathway, reiterating the primacy of this downstream Ras effector to PDAC pathogenesis. Importantly, many alternative drivers are activated by fusions, rather than point mutations. Given the availability of inhibitors approved in a tumor-agnostic manner for such "actionable" targets, every attempt should be made to identify the presence of these drivers, particularly in younger onset, *KRAS*-wild type tumors[106–109].

## Pancreatic Cancer Cell-of-Origin: An Unsettled Debate

Given the ductal histological appearance of PDAC, it was widely assumed that precursor lesions arise from mutations in the normal pancreatic ductal epithelial cells (Fig. 1). However, numerous studies have demonstrated that murine acinar cells can de-differentiate to a progenitor-like state with a ductal appearance. This has been termed acinar-to-ductal-metaplasia (ADM)[110,111], and lineage tracing studies in mice confirmed that acinar cells can repopulate damaged tissue[112]. The early demonstration of the oncogenic transformation of

the pancreas in mice utilized embryonic *Cre*-drivers that enable recombination in the entire exocrine pancreas[28–31]. Further studies using *Cre*-drivers specific to adult acinar cells in mice demonstrated ADM and PanIN formation by expression of oncogenic KRAS[113–117]; however, these models did not eliminate the potential of ductal transformation.

The first direct comparison using adult acinar vs. ductal expression of oncogenic KRAS demonstrated that KRAS promotes acinar cells to generate neoplastic lesions at a frequency over a hundred-fold higher than ductal cells[118]. This study also supported key aspects of the ADM to PanIN tumorigenesis model. Deletion of the ductal progenitor gene Sox9 prevented KRAS-driven transformation, while Sox9 overexpression accelerated PanIN development. Further studies have demonstrated the heterozygous or homozygous deletion of factors associated with terminal acinar differentiation such as *Ptf1a*, *Mist1*, and *Nr5a2* accelerate KRAS-driven tumorigenesis[119–122]. Conversely, stabilized expression of PTF1A and MIST1 inhibit PanIN formation in the context of mutant KRAS expression[122,123].

Subsequent studies have demonstrated that, while the plasticity of acinar cells allows them to be more readily transformed by oncogenic KRAS, their ability to serve as a sole cell-of-origin for neoplasia has proven context dependent. Combining oncogenic KRAS expression in the adult-ductal lineage with *Pten* loss leads to the formation of IPMNs[124], and the deletion of *Trp53* leads to the rapid development of invasive carcinoma[125–127]. Further, the induction of pancreatic damage by pancreatic ductal ligation is sufficient to promote KRAS-mediated ductal-lineage tumorigenesis[128]. This was not observed in the context of damage through cerulein-induced pancreatitis[118], which drives inflammation through damage of acinar cells. Taken together, these observations directly link the ability of oncogenic KRAS to promote tumorigenesis to lineage-specific regenerative responses.

Overall, the accumulation of evidence in murine models has shown that, in the right context, either acinar or ductal cells can serve as a progenitor population for PDAC (Fig. 1). These studies have largely leaned on lineage tracing approaches that are only possible in mouse models, and conclusively connecting observations from the different cells of origin to human disease remains a work in progress. However, recent advances in our understanding of potential subtypes of patient disease, discussed in detail in following section, have begun to allow interpretation of the cell-of-origin models derived in mice to human disease[127], as well as potential phenotypes[129].

## Transcriptional Subtypes of Human Pancreatic Cancer

Approaches to comprehensively interrogate the transcriptional landscape, as opposed to DNA mutations, has identified several PDAC subtypes across patients (Fig. 1). Collisson, Sadanandam and colleagues led one of the first major efforts to subtype pancreatic cancers, utilizing a combination of patient microarray datasets and PDAC cell-lines. They identified three subtypes, termed "classical", "quasimesenchymal", and "exocrine-like"[130]. Importantly, these subtypes could predict drug response in cell culture models, and patient outcomes were significantly different across their subtypes. Similarly, using an RNA-sequencing approach to assess PDAC patient tumors and low passage cell lines, Bailey and colleagues identified four subtypes of PDAC, which they termed "squamous", "pancreatic

progenitor", "immunogenic", and "aberrantly differentiated endocrine exocrine" (ADEX) type tumors[50].

These efforts to subtype patient samples were confounded by the characteristic histology of PDAC, in which cancer cells frequently dwarfed by the immune and stromal cells populations. To ensure the genomic and transcriptomic signatures represented those of the cancer cells, samples were screened for high levels of epithelial content, resulting in a drop out of a significant number of samples[49,50]. To address this shortcoming, Moffit and colleagues devised a "virtual dissection strategy" to computationally subtract the transcriptional background imposed by stromal and immune cells from bulk-RNA sequencing signatures[131]. Using this approach, they identified two subtypes of PDAC tumors, termed "classical" and "basal-like", in addition to two stromal subtypes termed "normal stroma" and "activated stroma".

Taking an alternative approach to the constraints imposed by the low percentage of the PDAC cells within the TME, several efforts have been made using laser capture microdissection (LCM) on PDAC sections to collect epithelial or stromal enriched areas of tissues[132,133]. Using this approach, Puleo et al. identified five subtypes, termed "pure basal like", "stroma activated", "desmoplastic", "pure classical", and "immune classical"[133]. They also noted overlaps with their subtypes, the Moffitt subtypes, and most of the Bailey subtypes. However, they suggested that their findings demonstrate that the Bailey ADEX subtype is likely contamination of pancreatic acinar cells.

Similarly, Mauer et al., found isolated epithelial tissues supported the Moffitt "classical" or "basal-like" subtypes, while they found no evidence of the Bailey "ADEX" subtype or Collisson "exocrine-like" subtypes in epithelial enriched tumor samples[132]. The lack of "ADEX" or "exocrine-like" subtype in these two studies fits with an observation from the Cancer Genome Atlas (TGCA). Namely, samples "ADEX" or "exocrine-like" signatures are associated with low PDAC cellularity[45], suggesting these signals derive from stromal/immune/non-cancerous elements of tissues. Finally, Maurer stromal areas of PDAC tumors revealed two prominent subtypes, which they postulated represented "ECM-rich" or "Immune-rich" phenotypes[132].

Following these studies, another major sequencing effort was made by Chan-Seng-Yu and colleagues. Here, the authors performed LCM enrichment of epithelial tissue from a large cohort of advanced PDAC patients combined with whole-genome sequencing and transcriptomic sequencing[134]. They identified five different subtypes of PDAC tumors: "basal-like-A", "basal-like-B", "hybrid", "classical-A" and "classical-B". They reported best overlap with the Moffitt subtypes, and their resolution allowed for subclassification of both, while their hybrid subtype did not consistently fall into previously reported classes. Importantly, correlating their subtypes with single-cell RNA sequencing data, they demonstrated that different subtypes can co-exist within individual tumors.

Overall, as our understanding of transcriptional subtypes of PDAC has evolved, consensus has best coalesced around two major subtypes, classical and basal-like, with some populations showing mixed features[135] (Fig. 1). There is potential to differentiate these into

further subclasses, and multiple types have potential to co-exist within induvial tumors[134]. The cell state of the classical and basal-like subtypes is potentially plastic and several candidate regulators such as TP63[136], which can be induced by *KDM6A* loss[97], can promote more aggressive subtypes. Further, the transcriptional landscape of PDAC cells can also be influenced by microenvironmental factors[137]. However, single cell sequencing, as discussed in detail later, has showcased how much more of the transcriptional states of PDAC cells remains to be uncovered.

A related question is how to exploit this concept therapeutically: the recent COMPASS trial provided proof of principle validation of subtype-based treatment of patients. Patients with classical subtype of PDAC had superior responses to first line 5-flurouracil-basd regimens than those with "basal-like" cancers[138]. This initial observation is now being further validated with a multi-center trial that is also examining whether contemporaneously established *ex vivo* organoid cultures from patient biopsies can provide phenotypic data on a broader range of drug sensitivities.

### The Pancreatic Cancer Tumor Microenvironment

One of the most fascinating and challenging features of pancreatic cancer is the tumor microenvironment (TME). The pancreatic TME is composed of numerous populations of fibroblasts, dense extracellular matrix, a poorly formed vascular system, and a diverse and largely suppressive populations of immune cells. The neoplastic cells in PDAC often constitute a minority of the overall cellularity within the tumor. Indeed, the aggressive biology, resistance to therapy, and heterogeneity of the disease is driven nearly as much by the non-cancerous components of the tumor as the PDAC cells. However, the oncogenic KRAS signaling in the PDAC cells initiates and coordinates the development of this growth supportive niche[139], and the vast network of interactions between and among these populations is only beginning to be understood.

**Cancer Associated Fibroblasts—**A significant effort to understand the pancreatic TME has focused on understanding stromal fibroblast populations (Fig. 2). Fibroblasts represent the most abundant cell type in the TME and are largely responsible for the deposition of the extracellular matrix driving the characteristic physiological properties of PDAC[140]. However, the extent of the diversity of these cancer-associated fibroblasts (CAFs), and their specific progenitor populations remain unknown and is a major area of ongoing investigation.

The normal pancreas contains heterogeneous fibroblasts populations (Fig. 2) including pancreatic stellate cells —similar to liver stellate cells and containing vitamin A-rich lipid droplets[141]— perivascular fibroblasts, and other parenchymal fibroblasts. Another potential origin of CAFs is from bone marrow derived progenitors. While stellate cells were long believed to be the only source of stromal fibroblasts, recent lineage tracing studies targeting Fabp4^{+} stellate cells revealed that they give rise to a minority of CAFs[142,143]. While not abundant, stellate cell derived CAFs have a tumor promoting role supported by unique gene expression and metabolic profiles. Conversely, lineage tracing analysis of perivascular fibroblasts, expressing the transcription factor *Gli1*, showed this population expands to

form nearly half of pre-CAFs in PanINs and CAFs in cancer; in contrast, parenchymal fibroblasts, expressing *Hoxb6* do not expand during carcinogenesis[144]. Finally, mesothelial cells, expressing *Wilms Tumor 1* (*Wt1*) also contribute to a sub-population of CAFs[145]. Thus, among resident pancreatic fibroblasts, different populations have vastly different abilities to expand during carcinogenesis. A recent study took a step further back in tracing the origins of CAFs in PDAC. First, a combination of dual labeling approaches and bone marrow transplants showed the contribution of bone marrow progenitors and cancer epithelial cells to the CAF population is minimal[146]. Conversely, tracing the embryonic origin of tissue resident fibroblasts through the splanchnic mesenchyme expressing *Isl Lim homeodomain 1 (Isl1)* labeled most tissue resident fibroblasts and pancreatic CAFs[146]. A key area of further research is whether different cellular origins account for the phenotypic and functional differences of CAFs, as addressed below.

The heterogeneity of CAFs within PDAC tumors is an actively evolving area, in part due to the advent of single cell technologies (Fig. 2). Early efforts to identify and understand different CAF populations identified a myofibroblast-like population (myCAF) and an inflammatory fibroblast population (iCAF)[147]. MyCAFs are localized close to the neoplastic cells and thought to be responsible for deposition of extracellular matrix, whereas iCAFs tend to occupy spaces between islands of the PDAC cells and release IL6 and other anti-inflammatory cytokines. Interestingly, mechanistic investigation into myCAF and iCAF biology *in vitro* suggested potential plasticity between the two populations[148], dependent on extracellular signaling molecules present.

Beyond myCAF and iCAFs, several additional populations of CAFs have been characterized (Fig. 2). One population expresses several components of the antigen presentation machinery typically found on myeloid populations. Given this feature, these have been termed apCAFs, although whether those CAFs prime a functional immune response is unclear[149]. Of note, the transcriptional profiles of apCAFs overlap with mesothelial cells[150], and based on lineage tracing studies in mice have been postulated to derive from a mesothelial origin[145].

Other classifications of CAFs are based on their expression of specific factors. For example, CAFs expressing Fibroblast Activation Protein (FAP) also express high levels of CXCL12 and inhibit tumor T cell infiltration. Depletion of these fibroblasts, or targeting of CXCL12, sensitizes PDAC to immune checkpoint blockade in preclinical models[151]. As another example, single-cell mass cytometric analysis has identified that the cell surface marker CD105 can be used to identify two functionally distinct and non-interconvertible populations of CAFs, namely, tumor permissive $CD105^+$ CAFs, and tumor suppressive $CD105^-$ CAFs[152]. Recently, fibroblasts expressing leucine-rich-repeat-containing protein 15 (LRRC15) were identified in mouse PDAC but not in the normal pancreas, and these fibroblasts are postulated to promote tumor growth and inhibit anti-tumor immune responses[153].

In addition to heterogeneity among CAF populations, fibroblast status throughout tumorigenesis and progression can be influenced by genetic mutations present in the PDAC cells[154,155], suggesting the overall characteristics of the TME might be specific to each

patient. Further complexity of PDAC TME has been observed spatially, as CAF populations and their organization of tumor architecture can vary across different regions of tumors creating "sub-tumor microenvironments" within patients[156]. Interestingly, the specific nature of the sub-TME in an individual tumor did not predict outcome, but higher sub-TME heterogeneity was a poor prognostic sign, possibly because heterogeneous systems are inherently more resilient. CAF biology in pancreatic cancer is an area of active investigation. The functional role of specific CAF populations is not fully clear; whether CAFs exist as distinct, stable populations, or if their status is plastic is not known, and, finally, whether it would be beneficial to eliminate or, conversely, reprogram, CAFs is an area of debate.

**Extracellular Matrix—**CAFs are largely responsible for deposition of the dense extracellular matrix found in PDAC, largely consisting of collagen and hyaluronic acid. These matrix molecules create a physical barrier, presence of which is debated as a hinderance to chemotherapy delivery[140,157]. Early studies suggested that targeting the sonic hedgehog pathway, important for epithelial-fibroblast paracrine signaling, would improve chemotherapy by reducing this matrix barrier[158]. However, clinical trials showed a worsened survival with combined hedgehog inhibition and chemotherapy. Subsequent studies suggested depletion of stromal fibroblasts through hedgehog inhibition, epithelial sonic hedgehog deletion, stromal ablation, or inhibition of lysyl oxidase like-2 increases tumor access to the blood supply and facilitates metastasis, having deleterious consequences on natural history of the disease[159–162].

Collagen is the most abundant matrix molecule found in PDAC[163]. The majority of collagen is type I collagen[164], largely generated by CAFs[147,165]. Targeted deletion of type I collagen from SMA-expressing fibroblasts increases immune suppressive myeloid infiltration, accelerating tumor progression[166]. Beyond physiological heterotrimeric collagen I produced by fibroblasts, PDAC cells produce "tonic" (low) levels of a distinct oncogenic homo-trimeric collagen that binds to $\alpha 3\beta 1$ integrin, thereby mediating immune suppression and reprogramming the tumor-associated microbiome[167]. Collagen also acts as a signaling molecule, activating discoidin domain receptor signaling that shape multiple roles in PDAC pathology[168–171]. Accordingly, the role of collagens in the pancreatic TME extends beyond a structural role, and the potential of targeting collagen-mediated crosstalk is only beginning to be understood.

Hyaluronic acid is a glycosaminoglycan polymer, thought to be released by predominantly by CAFs, that avidly retains water and promotes the characteristically high interstitial fluid pressure found in pancreatic tumors[140]. Targeting hyaluronic acid in the TME was hoped to be a more effective way to improve penetration of chemotherapy, releasing pressure on collapsed blood vessels while leaving cancer restraining fibrosis intact. Initial preclinical data in mouse models suggested that use of hyaluronidase combined with chemotherapy showed great promise[140,157]. Nonetheless, randomized clinical trials combining a pegylated hyaluronic acid degrading enzyme, PEG-hyaluronidase, with standard-of-care chemotherapy for advanced PDAC did not show an increase in overall survival regardless of chemotherapy backbone or selection of patients with high levels of hyaluronic acid by immunostaining[172,173].

Alternative methods to target stromal matrix have also demonstrated preclinical promise. Focal adhesion kinase (FAK), hyperactivated in pancreatic cancer cells, is an important driver of fibrosis and immune suppression in the TME[174]. FAK inhibition was shown to markedly reduced fibrosis and decreased the infiltration of tumor-promoting immune cells in mice. Further, combined treatment of FAK inhibition with immune checkpoint blockade demonstrated tumor regression in an aggressive mouse model of PDAC, suggesting this approach will translate to the clinic. More recently, FAK inhibition-mediated reprogramming of the stroma was shown to overcome radiation resistance and sensitize the tumors to immunotherapy[175]. Taken together, it has become clear that targeting the non-cellular component of the TME represents a challenging prospect. However, there is optimism that new approaches are poised to overcome the shortcomings of previous approaches to target the PDAC tumor matrix.

**Innate Immune Cells—**Myeloid cell populations make up the largest immune cell population in pancreatic tumors (Fig. 3). This has prompted investigation into the roles of various myeloid populations in PDAC biology, particularly related to immune suppression. Oncogenic KRAS signaling drives expression of granulocyte colony stimulating factor (GM-CSF) by PDAC cells, promoting recruitment of immunosuppressive myeloid-derived suppressor cells (MDSCs)[176,177]. Tumor-associated macrophages (TAMs) arising from both tissue resident macrophages or circulating monocytes appear to play important but potentially different roles in shaping the TME, and suppression of the adaptive immune response[178]. Importantly, targeting MDSCs and/or TAMs improves adaptive immune responses in mouse models of pancreatic cancer and can potentiate checkpoint inhibition approaches[176,177,179,180] (Fig. 3). Among these, novel agonists developed in PDAC models to target the CD11b/CD18 receptor present on most tumor-infiltrating myeloid subsets have shown promising pre-clinical data, currently being tested in the clinic[181,182]. A different approach uses CD40 agonists to reprogram macrophages to a less immunosuppressive status, leading to CD8 T cell infiltration that would drive the conversion from a "cold" to a "hot tumor"[183]. Two distinct CD40 agonists have recently been evaluated in clinical trials in the advanced and neoadjuvant settings in PDAC. Although the response was modest, extensive tissue and blood based correlative studies confirmed CD40 agonism enhanced T cell infiltration in PDAC[184,185].

Beyond immune-suppression by myeloid cells, a major barrier to mounting an anti-cancer adaptive immune response to PDAC appears to be a lack of adequate antigenpresentation. Dendritic cells (DCs) are found in low numbers in PDAC, as compared to other tumor types, and the DCs present are of poor quality for antigen presentation[186] (Fig. 3). These mechanistic data lend additional weight to efforts to increase DC licensing as part of enhancing PDAC patient response to checkpoint inhibition[186,187].

**Adaptive Immune Cells—**One of the most notable features of the PDAC TME is the lack of infiltration by cytotoxic CD8 T cells. Other T cell populations within the TME have all been noted to play protumorigenic roles, including Th17, Th22, CD4, and γδ T cells[188–191] (Fig. 3). Interestingly, even though CD4 T cell ablation enabled CD8 mediated regression of PDAC tumors in mice, depletion of FoxP3+ T regulatory cells (Tregs) accelerated

tumorigenesis due to compensatory myeloid infiltration[192]. This shift in immune infiltration follows a loss of myCAFs upon Treg depletion, as Tregs proved a significant source of TGF-beta.

This is only one example of the complicated interplay between the immune and stromal compartments in PDAC, illustrating why efforts to enable a sustained immune response have proven difficult in this complex TME. Currently, several combinatorial innovative approaches to overcome these barriers are being tested in the clinic, and some of these principles are revisited in the last section of the review.

## Pancreatic Cancer Metabolism: Adaptations Present Therapeutic Opportunities

The physical barriers imposed within PDAC tumors function to limit access to blood-derived nutrients and oxygen, leading to dysregulate nutrient profiles and promote regional hypoxia. Direct measurement of the metabolites in human PDAC tumors revealed classes of metabolites are in low supply for cells within the tumors, and others are increased in abundance[193]. Additionally, analysis of the nutrients available within murine tumor interstitial fluid demonstrates a starkly different profile within pancreatic tumors vs. serum levels[194]. PDAC cells exhibit numerous adaptations to not only survive but thrive under the nutrient constraints imposed by the TME. These center around two themes: oncogenic KRAS mediated reprogramming of cellular metabolism to mediate survival and enable proliferation, and adaptations to enhance nutrient acquisition and/or scavenging pathways (Figure 4a).

**Intermediary Metabolism and Potential Targets in PDAC**—The metabolic reprogramming of central carbon metabolism in PDAC occurs in both glucose and glutamine metabolism. Mutant KRAS drives upregulated glycolysis, not only increasing production of lactate, but also providing key carbon inputs for synthesis of molecules required for proliferation. Examples include the non-oxidative pentose phosphate pathway, which enables nucleotide biosynthesis, and the hexosamine biosynthesis pathway, which generates GlcNAc required for posttranslational modifications[64,195] (Figure 4a). Directly targeting glycolysis through lactate dehydrogenase inhibition has shown mixed results depending on p53 status[58], and this approach can be potentially overcome through compensatory upregulation of oxidative phosphorylation[196]. Further, combining inhibition of glycolysis with blockade of KRAS effector signaling pathways has shown efficacy in culture and xenograft models[197], suggesting utility for paired oncogenic metabolism and signaling combination treatments.

Oncogenic Ras also drives glutamine anaplerosis to provide a significant source of carbon for TCA cycle, biosynthesis of glutathione necessary to detoxify reactive oxygen species (ROS), and enables a non-canonical malate-aspartate shuttle to generate NADPH for redox balance[198] (Figure 4a). Given the importance of glutamine metabolism in pancreatic cancer, glutaminase (GLS), which drives the hydrolysis of glutamine to glutamate and ammonia, has drawn significant interest as a drug target. However, despite showing potent growth inhibitory effects in short-term PDAC cell proliferation assays, GLS inhibition was found to be ineffective in pancreatic tumor models through remodeling of compensatory metabolic

pathways[199]. Further, PDAC can engage the biosynthesis of glutamine through glutamate ammonia ligase in response to glutamine withdrawal, further demonstrating metabolic plasticity by pancreatic cancer cells[200].

However, there appears to be promise in targeting glutamine metabolism using inhibitors with less substrate specificity. 6-Diazo-5-oxo-l-norleucine (DON) was identified as a potential anti-cancer glutamine antagonist more than a half century ago[201], but its lack of substrate specificity provided too much off-target toxicity[202]. However, recent advances have optimized DON as a pro-drug with pharmacokinetic properties that increase tumor targeting[203], including an orally available analog with promising pre-clinical results in murine PDAC models[204]. Interestingly, recent studies have shown that the use of DON or analogs in murine PDAC models also appear to have immunomodulatory effects[204,205], highlighting the potential of targeting metabolism in combination with immunotherapy.

**Nutrient Recycling and Scavenging**—Beyond alterations in central carbon metabolism, pancreatic cancer cells exhibit dramatic changes in organelle programming and function. One of the best studied examples of this centers on the increased biogenesis of lysosomes and constitutive engagement of the autophagy "self-eating" recycling program[206] (Figure 4a). Autophagy is critical for cancer cell growth and tumorigenesis in mouse models of PDAC[207–209], and genetic inhibition of critical autophagy proteins in mice leads to regression of autochthonous PDAC tumors[210]. Mitophagy, the selective engulfment and degradation of mitochondria through autophagy, has also been shown to be important in PDAC tumorigenesis in mice and organoid models[211]. Importantly, clinical trials have shown that hydroxychloroquine, which inhibits autophagic flux by preventing lysosomal acidification, enhances PDAC patient response to chemotherapy[212,213].

In addition to organelle maintenance, autophagy plays a critical role in maintaining iron homeostasis in PDAC cells[214]. Iron is a necessary co-factor for proteins in metabolism and other critical cellular processes[215]; however, free intracellular iron must be stored within the protein ferritin to prevent generation of free radicals. In PDAC cells, ferritin is trafficked to autophagosomes via nuclear receptor coactivator 4 (NCOA4), and free iron is subsequently released by autophagy through a process termed "ferritinophagy"[214]. Increased expression of NCOA4 accelerates tumorigenesis in mice, and genetic ablation leads to delayed tumor growth that is eventually overcome through development of compensatory iron acquisition pathways[216]. Importantly, MEK-inhibition in PDAC increased both lysosome biosynthesis and ferritinophagy, necessary to support increased iron-sulfur cluster proteins for respiration[217] (Figure 4a). Labile iron can also potentiate cell death via the lipid oxidation process known as "ferroptosis"[218], which is currently an active and exciting area of research in PDAC[219–223].

Autophagy has also been shown to play important roles in resistance to targeted therapies. Inhibition of the mutant KRAS-MEK-MAPK signaling cascade using ERK inhibitors drives increased autophagic flux in PDAC cells to facilitate survival[224,225]. Combined pharmacological targeting of MEK or ERK with hydroxychloroquine potently inhibits xenograft tumor growth and has shown efficacy in clinical treatment of PDAC, with additional trials on-going. Beyond direct consequences on intercellular metabolism,

autophagy also facilitate tumor survival by influencing cellular immunoreactivity. Major histocompatibility complex I (MHC-I) down-regulation is a major mechanism by which cancer cells escape detection by the immune system[226]. Indeed, MHC-I expression is low at the PDAC cell surface, instead found predominantly localized within autophagosomes and lysosomes[227]. Genetic or pharmacological inhibition of autophagy in mice bearing PDAC tumors promotes enhanced cell-surface MHC-I expression and antigen presentation, leading to an increased response to immune checkpoint blockade therapy. The autophagy-MHC-I relationship provides mechanistic insight to functional genomics studies in mice, which have also linked autophagy and immune evasion in PDAC[228].

Autophagy drives degradation/recycling of components already within cancer cells; however, deregulated activation of nutrient uptake processes also fuel pancreatic cancer metabolism. For example, macropinocytosis is a non-substrate specific fluid phase uptake process engaged by oncogenic KRAS signaling[229]. Numerous studies have now demonstrated that PDAC cells utilize macropinocytosis to obtain amino acids through the uptake and degradation of albumin and collagen[230–232] (Figure 4a). Macropinocytosis has also been shown to utilize necrotic cell debris as a fuel source, which can provide sugars, fatty acids, nucleotides, and amino acids to fuel cancer cell metabolism[233,234]. Macropinocytosis was initially thought to be a ubiquitous feature of oncogenic *KRAS* mutations[231]. However, only a subset of PDAC cell lines with mutant *KRAS* demonstrate a constitutive macropinocytosis program, whereas others engage this process only when challenged with glutamine deprivation[235].

Collectively, these studies highlight the heterogeneity of metabolic features and behaviors utilized by PDAC cells extend beyond simple re-wiring of anabolic and catabolic pathways. These combine to enable PDAC cells to survive nutrient challenging conditions in the primary tumor and provide metabolic flexibility to enable metastasis. However, the unique metabolic features of PDAC cells also present numerous therapeutic opportunities to eliminate cancer cells, while sparing normal tissues.

### Metabolic Heterogeneity in Pancreatic Cancer

As discussed above, the metabolic milieu in pancreatic tumors is deregulated, with some nutrients being enriched and others depleted. The differential metabolism of these regions can be readily observed through imaging techniques[236,237], including spatial heterogeneity of glycolysis. Recognition of regional metabolic heterogeneity within pancreatic tumors has inspired efforts to classify PDAC cells in a manner reminiscent of the transcriptional subtypes. PDAC cell lines classified by bioenergetic preferences and response to metabolic inhibitors revealed "glycolytic", "lipogenic", and "slow proliferating" metabolic subtypes[238]. These states also correspond to transcriptional subtypes. The glycolytic metabolic subtype aligns with a "quasimesenchymal" transcriptional subtype and lipogenic metabolism state with a "classical" signature[130]. More recently, metabolic characterization of clonal lines isolated from tumors revealed that two distinct populations can co-exist within the same tumor[239]. Accordingly, the levels of intratumoral metabolic heterogeneity are only beginning to be appreciated.

The disparity between nutrient availability imposed by the pancreatic TME, the bloodstream and other routes of dissemination, and secondary organs colonized in metastasis allow for varied metabolic states for PDAC cells[194]. Glutamine deprivation promotes an epithelial-to-mesenchymal transition (EMT) through the upregulation of the master EMT regulator Slug[240], enabling PDAC metastasis to the lung. Comparison of the epigenetic and metabolic state of primary vs. metastatic PDAC patient tumors revealed the upregulation of anabolic glucose metabolism with a concurrent dependence on the oxidative pentose phosphate pathway during the evolution of metastasis[241]. This re-wired metabolic pathway revealed a dependence on a unique pentose conversion pathway, centered on the upregulated activity of phosphogluconate dehydrogenase (PGD)[242]. High glycolytic flux is required to maintain upregulated PGD metabolism, and this in maintained in part through suppression of thioredoxin-interacting protein (TXNIP) transcription[243]. Targeting TXNIP can reverse the metabolic and epigenetic programming identified to be unique in metastatic PDAC.

Several other links between PDAC metabolism and cell state have also been reported. PDAC cells that survive withdrawal of oncogenic KRAS signaling exhibit a targetable dependence on mitochondrial metabolism[68]. Similarly, pancreatic tumor-initiating cells have been found to preferentially utilize mitochondrial metabolism, as compared to glycolytic metabolism preferred by bulk tumor cells[244]. Differential activation of the integrated stress response can also be used to define sub-populations within individual tumors, which drives differential sensitivity to metabolic inhibitors[239].

Given the breadth of metabolic diversity that can exist within tumors, there is growing interest in developing techniques to simultaneously inhibit multiple pathways to combat heterogeneity[239,245]. For example, extracellular asparagine from serum and produced by a constitutively active integrated stress response in subpopulations of PDAC cells can be targeted using asparaginase, potently sensitizing PDAC tumors to mitochondrial inhibition in pre-clinical models[239,246]. Further, metabolic inhibition approaches combined with RAS pathway inhibitors have shown strong pre-clinical data and are currently in clinical trials[197,224,225]. These signaling/metabolic inhibitor combinations have even more potential to grow as mutant KRAS inhibitors are established in the clinic.

### Metabolic Interactions in the Pancreatic Tumor Immune Microenvironment

As previously discussed, the neoplastic epithelial cells within PDAC tumors are often vastly outnumbered by the stromal and immune cell populations. The varied localization, motility, and function of these different cell populations translate to differential nutrient requirements and can lead to tumor-supporting metabolic interactions. These symbiotic interactions fuel PDAC metabolism, promote resistance to chemotherapy, and drive mechanisms of immune suppression (Figure 4b).

**Stromal Metabolic Crosstalk—**Pancreatic CAFs are in close proximity to PDAC cells[147]. CAFs provide signals that promote epigenetic and metabolic re-wiring of cancer cell metabolism[247]. Examination of metabolic exchange between pancreatic CAFs and cancer cells revealed that alanine is selectively released by fibroblasts and consumed by PDAC cells[248]. The exchange of alanine and other non-essential amino acids from

fibroblasts to promote PDAC metabolism is linked to both autophagy and macropinocytosis in the fibroblasts[248,249] (Figure 4b). Further, the exchange of metabolites such as pyruvate can be used by PDAC cells to maintain redox balance[250].

Multiple mechanisms have been identified that promote exchange of amino acids from fibroblasts to cancer cells. Stellate cell-derived exosomes have been shown to carry numerous metabolite and protein cargo to pancreatic cancer cells, including amino acids[251,252]. Differential expression of solute carrier proteins (SLCs) can also function to promote a one-way fibroblast-SLC1A4 / PDAC-SLC38A2 exchange axis, enabling the exchange of alanine[253].

Fibroblasts have also been shown to engage in reciprocal reprogramming to drive metabolite exchange axes. Activation of CAFs from quiescent stellate cells engages a metabolic program supporting the secretion of several lipid species. Among these lysophosphatidylcholines are hydrolyzed by autotaxin released from cancer cells to provide the potent signaling molecule lysophosphatidic acid, which promotes PDAC proliferation and migration[254] (Figure 4b). Similarly, PDAC program CAFs to engage a BCAT1-driven metabolic program, leading to CAF production of branched-chain α-ketoacid precursors reciprocally exchanged to fuel PDAC metabolism[255].

In addition to direct metabolic exchange, extracellular matrix produced by stromal fibroblasts and PDAC cells can serve as a fuel for PDAC metabolism. As previously noted, PDAC cells can utilize macropinocytosis to obtain proline from CAF produced collagen[230]. PDAC cells can also obtain GlcNAC through N-acetylglucosamine kinase salvage from matrix components[256], including hyaluronic acid[257] (Figure 4b).

**Tumor-Associated Macrophage Metabolic Crosstalk**—Myeloid cells, including TAMs, provide another potential source of metabolic support to fuel PDAC metabolism. TAMs and other anti-inflammatory macrophages release numerous pyrimidine species that are avidly consumed by PDAC cells[258]. Beyond a potential role in supporting PDAC metabolism, the deoxycytidine released among these pyrimidines inhibits the efficacy of the standard of care chemotherapy gemcitabine, and depletion of myeloid cells in mice sensitizes PDAC tumors to gemcitabine treatment. Deoxycytidine can also be released from CAFs[259], and both TAM and CAF-released deoxycytidine act to inhibit the efficacy of gemcitabine through molecular competition at deoxycytidine kinase[258,259] (Figure 4b).

The programming of TAMs is mediated by oncogenic-KRAS reprogramming of PDAC cells. Lactate produced from upregulated glycolysis in PDAC cells combines with GM-CSF, released in a KRAS-dependent fashion, to stimulate macrophage polarization[260]. The polarization of TAMs occurs through a PI3K-AKT pathway, activating ACLY signaling and the expression of the arginine catabolic enzyme Arginase 1 (Arg1). Arginase-mediated arginine depletion by myeloid cells is a metabolic mediator of immune suppression through limitation of arginine to cytotoxic T cells, which impairs their activity[261]. In addition, anti-inflammatory macrophages and pancreatic TAMs utilize a predominantly mitochondrial bioenergetic metabolic programming, fueled by fatty acid metabolism[258]. Accordingly, there are multiple avenues macrophage metabolism can be targeted to relieve immune suppression

in pancreatic cancer, and ongoing studies in this area aim to exploit these metabolic vulnerabilities to facilitate anti-tumor immunity.

**Other Metabolic Interactions in PDAC**—Neural invasion is a well-recognized feature of pancreatic tumors, with the severity negatively correlating to prognosis[262]. Neurons can also provide serine that functions to support mRNA translation in PDAC[263] (Figure 4b). Beyond host cells supporting the neoplastic cells within the tumors, bacterial and fungal pathogens can infiltrate into PDAC[264–266]. Among these, gammaproteobacterial PDAC have been shown to directly detoxify gemcitabine through expression of cytidine deaminase[267].

Heterogeneity between PDAC cells also supports multiple axes of metabolic symbiosis. For example, lactate produced by cancer cells in hypoxic regions can be used to fuel PDAC cells in well oxygenated areas[195]. Additionally, asparagine produced by subpopulations of PDAC cells that have a constitutively active integrated stress response support the mitochondrial metabolism of other PDAC cells during limited respiration[239], providing a barrier to metabolic targeting.

Taken together, these studies demonstrate that targeting metabolism is not as simple as defining metabolic vulnerabilities of the cancer cells, and the metabolic state of PDAC cells is heterogenous within individual tumors. However, our increased understanding of the symbiotic support mechanisms of nutrients within the pancreatic TME have already identified several therapeutic opportunities. These targets will only expand as our understanding of the PDAC metabolic crosstalk network is further defined.

## New Technologies Driving New Insights

The advancement of single-cell analysis technologies and high-dimensional spatial analysis techniques have rapidly broadened our understanding of PDAC. A common thread emerging from these cutting-edge studies is that the levels of heterogeneity that can exist within PDAC tumors extend far beyond what has previously been described. This underappreciated level of complexity spans populations of stromal cells, immune cells, and distinct populations of cancer cells that co-exist within individual tumors. Here, we will highlight studies that illustrate examples of several new technologies, and how they can be combined to bring new insights to clinical therapy.

**Mass cytometry**—Mass cytometry by time-of-flight (CyTOF) combines the use of heavy metal conjugated antibodies with flow-cytometry coupled to a mass spectrometer. This broadly expands the number of antigens that can be run simultaneously for single cell analysis, allowing high-dimensional profiling to better define cell types in the TME. For example, stromal heterogeneity in PDAC is widely recognized, but the relationships between different lineages and a consensus of markers to identify fibroblast behaviors within tumor remains less defined. The use of mass cytometry comparing millions of cells isolated from 19 murine PDAC tumors against library of 40+ known fibroblast and mesenchymal cell markers was integral to the discovery of CD105 as a key indicator of tumor promoting/tumor suppressive fibroblast behavior[152]. The extended number of unique markers that can be used in parallel with mass cytometry has also proven to be a powerful tool for immunophenotyping. Illustrating this, a 36 marker CyTOF characterization of peripheral

blood from PDAC patients revealed neoadjuvant FOLFIRINOX promotes an increase in effector T cells and concurrent decrease in suppressive immune populations[268]. The data gained through this approach suggests neoadjuvant treated patients would potentially respond better to adjuvant immunotherapy.

**Single-cell RNA Sequencing**—The advancement of single-cell RNA sequencing (scRNA-seq) technology has completely re-shaped our view of the pancreatic TME. scRNA-seq data confirmed the existence of multiple CAF populations within human and murine pancreatic tumors and allowed the discovery of apCAFs[149]. Integration of multiple scRNA-seq datasets combined with lineage tracing studies demonstrated that ap-CAFs can arise from a mesothelial origin and regulate the expansion of regulatory T cells and immune suppression[145]. scRNA-seq datasets from human and murine tumors have identified multiple unique neoplastic epithelial populations within individual tumors[149,269], which represent multiple PDAC subtypes within the same tumor[270]. In fact, PDAC cell lines and patient-derived organoid cultures demonstrate multiple, distinct, sub-populations by scRNA-seq[271], highlighting epithelial cell heterogeneity even in *ex vivo* models. Further, patient-derived organoid transcriptional landscape and sub-population proportions shift during passaging[137,271], suggesting that representation of the *in vivo* biology of the original tumor is not stable in culture. However, addition of contextual factors lacking in such *ex vivo* culture models can potentially rescue the heterogeneity of the original tumor models and the retain the ability to predict response to therapy[137]. Additional iterations of single cell profiling approaches – such as single cell T-cell receptor sequencing (scTCR-seq) of tumor infiltrating lymphocytes – have now been developed and have provided insights into antigenic targets of immune response in this disease[272].

**Spatial Analysis Techniques**—CyTOF and scRNA-seq of isolated cell populations from PDAC tissue has provided an unprecedented depth of knowledge on the complexity and heterogeneity of the TME. However, the spatial organization of cell populations also has a central role in shaping their relationships and phenotypes. Fortunately, advances in multiplexed immunohistochemical staining and imaging technology have enabled the expansion of markers that can be used in spatial assessment of cancer cell number, function and evolutionary trajectories[273,274]. Illustrating the utility of this approach, multiplexed myeloid, lymphoid, and functional antibody panels were able to spatially define the relationship between myeloid and T cell populations in PDAC patient tissue[275]. Of note, while many differences in immune infiltration and behavior were found between different patients, this ability to spatially assess these data allowed the observation of widespread heterogeneity in individual patients. The extrapolation of spatial profiling technologies into the threedimensional (3D) space has the potential to further expand our understanding into the heterogeneity of PDAC and its constituent elements[276].

**Synergistic Applications of New Techniques**—Parallel use of spatial and isolated single-cell analysis techniques has proven adept at identification of interactions between cell types in the TME. For example, combined scRNA-seq, multiplex IHC, and CyTOF immune profiling demonstrated an inverse correlation between suppressive myeloid populations and CD8 effector T cell infiltrations[277]. These further identified that CD8 T cells show

indications of systemic exhaustion, and a network analysis of potential checkpoint receptor-ligand pairing suggested *TIGIT* as a key node of immune-suppression. In line with these observations, subsequent studies using murine PDAC models have further implicated the CD155/TIGIT axis as a potential target to move forward to clinical trials to enhance immunotherapy[278]. Similarly, the combined use of spatial profiling with single-nucleus RNA sequencing has enabled the interrogation of the response of PDAC to neoadjuvant therapy and identified a treatment-refractory "neural-like" progenitor cell type that escapes standard of care regimens[279]. Recent advances in spatial profiling technologies have also allowed the newer platforms to approach true single cell, and even subcellular, resolution[280]. Given the increasingly facile extension of these technologies to archival samples and limited biospecimens typically obtained from clinical trials, there is strong optimism that the correlative studies enabled by these platforms will lead to the insights needed to develop lasting and durable approaches to treat PDAC.

## Clinical Trials: Setting Expectations for Future Success

Increased understanding of the biology of PDAC has led to a plethora of clinical trials to improve treatment; however, most have fallen short of expectations. In fact, PDAC suffers from one of the highest rates of Phase 3 failures amongst common solid cancers. This has led to calls to revamp how drug development is furthered for this disease, and to redefine the thresholds needed for agents to move forward to larger phase trials[281,282].

The indelible lessons we continue to learn are that the past is prologue and that the underlying biology remains one of the most pivotal arbitrators of meaningful success in the clinic. For example, it is now evident that simply combining two "off the shelf" immune checkpoint inhibitors to enhance effector T cell function – a strategy that has worked in several other solid cancers – has minimal to no impact in a disease where a multiplicity of immune defects co-exist[283,284]. In a recent preclinical study, dual therapy against CD8 T cell checkpoints using a 41-BB agonist and LAG-3 inhibitor by itself was ineffectual, and yet when administered with a myeloid cell targeted CXCR1/2 antagonist, the triple regimen led to sustained responses in biologically relevant models[285]. Not surprisingly, therefore, the first clinical hints of success at deploying immunotherapy in PDAC have thus come from regimens that combine chemotherapy with targeting of one or more additional nodes within the dysfunctional TME (myeloid cells, Tregs) in combination with immune checkpoint inhibitors[286,287]. This has the dual benefit of tumor shrinkage and acts as an *in situ* antigen release mechanism to allow for an immune response.

Many of the recent promising clinical signals in PDAC have arisen through rigorous preclinical analyses in syngeneic and autochthonous models that predated the trial and informed its design[288]. While preclinical models remain far from perfect, they do offer valuable insights into potential mechanisms of failure when trials are unsuccessful. One notable example of an ultimately unsuccessful but informative clinical paradigm came by way of a series of pre-clinical studies proposing the efficacy of systemic therapy would improve through efforts to penetrate the dense fibrosis and high interstitial pressure promoted by the CAFs in pancreatic tumors[140,158]. Subsequently, attempts to inactivate

CAFs through sonic hedgehog inhibition proved ineffective in patients[289], and preclinical studies that were conducted after the fact pinpointed reasons for this failure[159,290].

Even rigorously conducted preclinical studies are not predictive of success upon subsequent clinical translation if they deviate significantly from the preclinical "recipe". An unfortunate example of this pertains to the trials of hyaluronic acid targeted therapies in patients with advanced PADC, which used dosages that were three orders of magnitude lower than what garnered improved survival in animal models[291]. Moving forward, it is imperative that clinical trials are only implemented when preceded by rigorous and efficacious preclinical studies in biologically relevant models. Further, trials should recapitulate methodologies and read outs that generated success in model systems. Finally, trials should be designed in a manner where we "learn from every patient" through the implementation of a multitude of tissue and blood-based correlative analyses. In fact, partly driven by the challenging trajectory of "conventional" drug development in PDAC, there has been an evolving shift from large, empirical trials towards those that are focused on fewer patient numbers, but which feature in-depth correlative studies that provide insights into mechanisms of response and resistance[184,292,293].

In many instances, these "team science" efforts are funded by philanthropic foundations such as Stand Up to Cancer (SU2C), the Lustgarten Foundation for Pancreatic Cancer Research, the Parker Institute for Cancer Immunotherapy (PICI), and Break Through Cancer, amongst others. Pairing the most promising early phase trials with larger, multi-institutional registration efforts that feature an innovative adaptive design platform, such as PanCAN's Precision Promise[294], has the potential to completely transform the path to accelerated therapeutic approval in PDAC.

## Summary:

Significant progress has been made in understanding the biology of pancreatic cancer over the past several decades, unfortunately, these have not translated into a breakthrough in clinical care for the majority of patients. We have presented several themes within this review that we believe can serve as "*Hallmarks of Pancreatic Cancer Therapy*" (Figure 5). These center on genomic alterations, metabolism, the tumor microenvironment, immunotherapy, and innovative clinical trial design. Importantly, approaches that encompass as many of these features as possible present the highest likelihood of success. Examples of these can be found across the spectrum of investigational compounds and clinically deployed approaches to target the genetic and immunological features of PDAC[295,296], cancer metabolism[297], and chemoresistance[298] There are also other exciting avenues of new therapeutic development, such as cancer vaccines and antibody-drug conjugates, that we were not able to cover within the scope of this review. Overall, we are optimistic that these collective efforts will soon transform PDAC from recalcitrant to a manageable disease.

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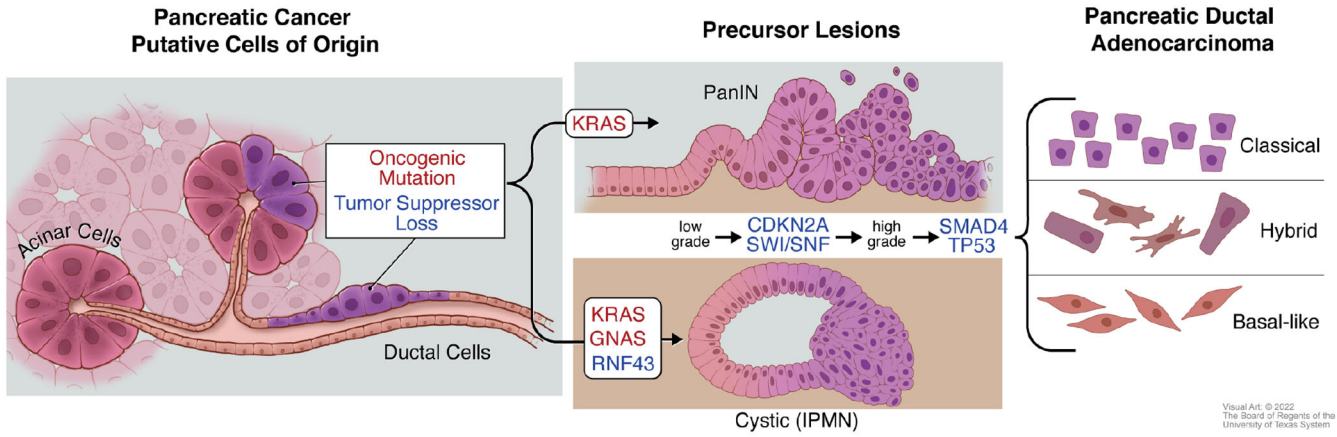
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**Figure 1: Initiation and Progression of Pancreatic Cancer.**
Pancreatic ductal adenocarcinoma (PDAC) forms from the exocrine tissue of the pancreas. Acinar and ductal cells have both shown the potential to serve as cells of origin for PDAC upon acquisition of oncogenic mutations and/or loss of tumor suppressor function. Activating mutations in the oncogene *KRAS* are found in the two most commonly observed PDAC precursor lesions: pancreatic intraepithelial neoplasia (PanINs) and cystic lesions termed intraductal papillary mucinous neoplasm (IPMNs). In addition to *KRAS* mutations, activating mutations in the gene encoding for the G-protein alpha subunit Gαs (*GNAS*) and loss of function of the tumor suppressor gene RING-type E3 ubiquitin ligase (*RNF43*) are associated with IPMNs. As these precursor lesions progress from low grade to high grade lesions, loss of the tumor suppressor *CDKN2A* or components of the SWI/SNF chromatin-remodeling complexes are observed. Further deletion or inactivating mutations of tumor suppressor genes *SMAD4* or *TP53* accompany the advancement of precursor lesions to PDAC. PDAC has also been classified into several RNA-based transcriptomic subtypes. "Classic" and "Basal-like" have emerged as two consensus groups, with a third "Hybrid" capturing those with overlapping features.

## CAF Lineages and CAF Heterogeneity



Visual Art: © 2022
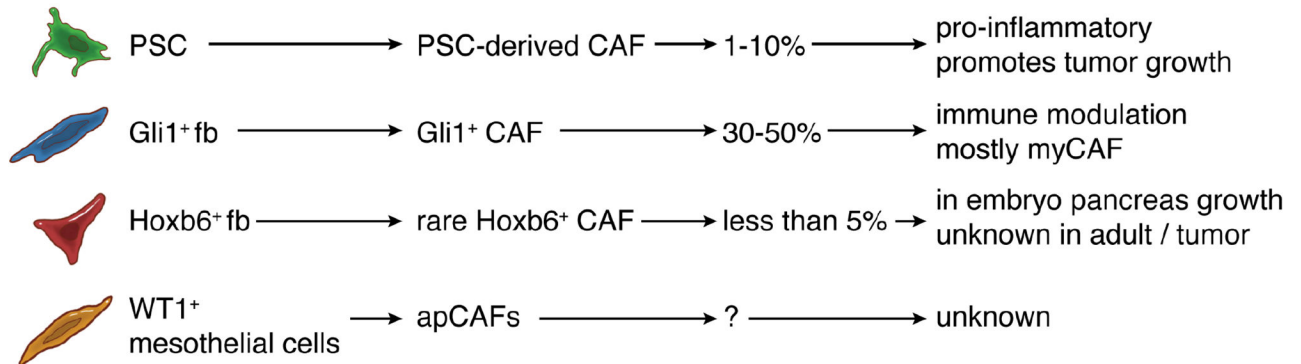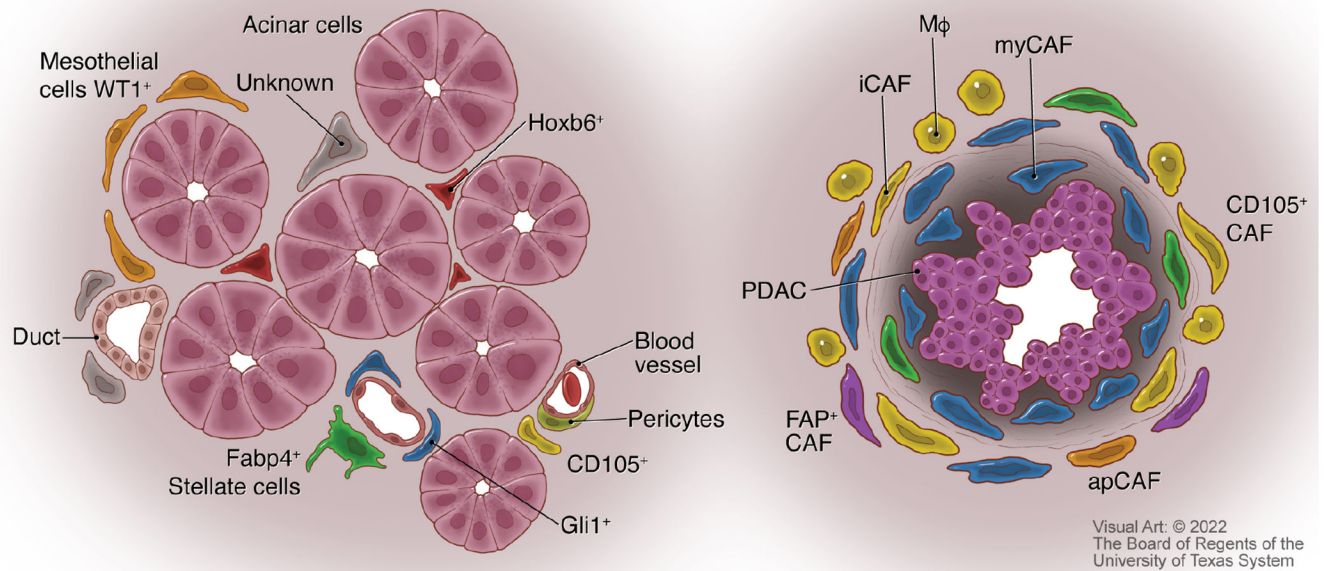The Board of Regents of the
University of Texas System

**Figure 2: Cancer-associated Fibroblast Origin and Heterogeneity.**
Several types of fibroblasts are present in both the normal pancreas (left) and PDAC (right). Normal pancreatic fibroblast populations include pancreatic stellate cells (PSCs), Gli1^{+} fibroblasts, Hoxb6^{+} fibroblasts, Fabp4^{+} fibroblasts, CD105^{+} fibroblasts, pericytes, and WT1^{+} mesothelial cells that encase the organ. Within PDAC, cancer-associated fibroblast (CAFs) populations include myofibroblastic CAFs (myCAFs) associated with collagen deposition, inflammatory CAFs (iCAFs) that associate with macrophages (MΦ), antigen-presenting CAFs (apCAFs), CD105^{+} CAFs, and fibroblast activation protein containing (FAP^{+}) fibroblasts. Lineage tracing has demonstrated that PSC-derived CAFs make up less than 10% of the overall number of CAFs and have roles in promoting tumor growth and inflammation. Gli1^{+} CAFs can account for nearly half of all the fibroblasts in pancreatic cancer, with roles in depositing matrix and modulating immune response. Hoxb6-derived CAFs are infrequent and have no known function akin to their role in embryonic pancreas growth. The number of mesothelial-derived CAFs is unknown, as is their function outside of a potential antigen presentation role.
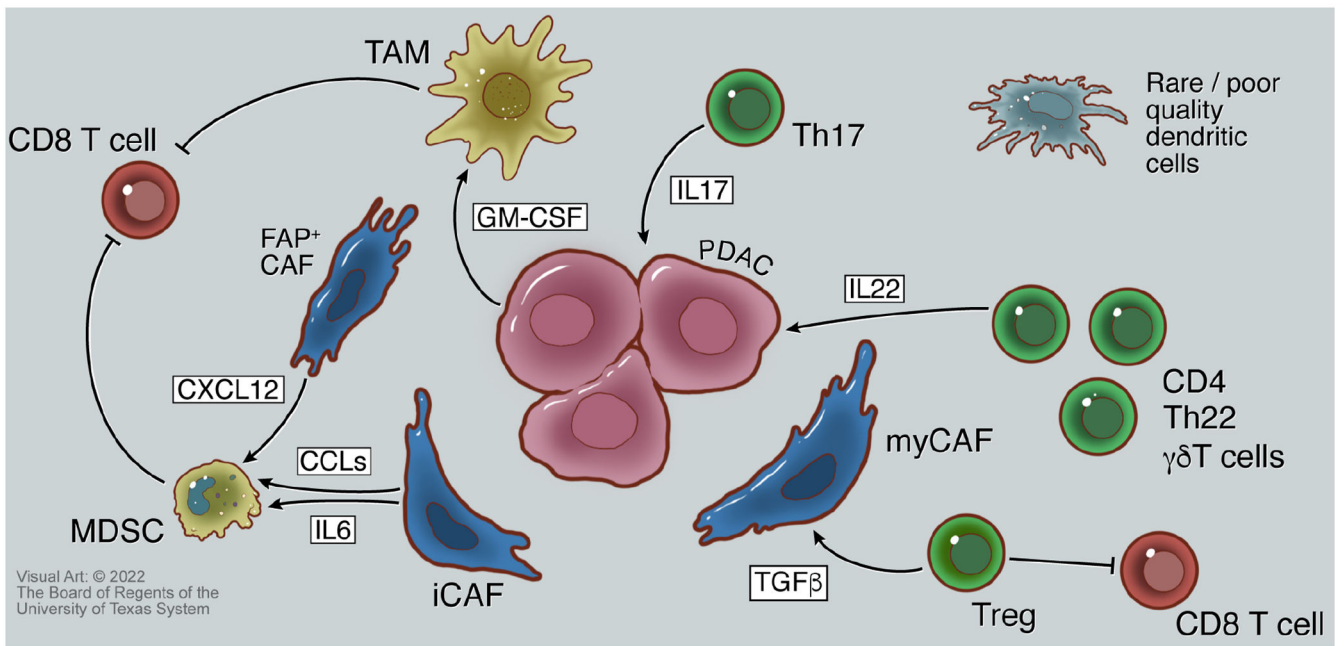
**Figure 3: Immune Interactions in Pancreatic Cancer.**
A network of interactions between fibroblasts, cancer cells, and immune cells create an immune-suppressive tumor microenvironment. Monocyte-derived suppressor cells (MDSCs) that inhibit CD8 cytotoxic T cells are polarized by chemokine ligands (CCLs) and interleukin 6 (IL6) from inflammatory cancer-associated fibroblasts (iCAFs), and by CXCL12 from fibroblast activation protein containing (FAP$^+$) fibroblasts. Granulocyte macrophage colony-stimulating factor (GM-CSF) released by neoplastic cells also polarizes tumor-associated macrophages that inhibit CD8 T cells. Further, T regulatory cells (Tregs) inhibit CD8 T cells, and supply TGF-β that allows for myofibroblastic cancer-associated fibroblast (myCAF) activation. IL17 and IL22 released by Th17 and Th22 cells, respectively, provide pro-growth signals to cells to PDAC cells via binding to cognate cell surface receptors. Dendritic cells are sparse in pancreatic cancers, and those present are of low quality, further contributing to the lack of an effective anti-tumor adaptive immune response to PDAC.
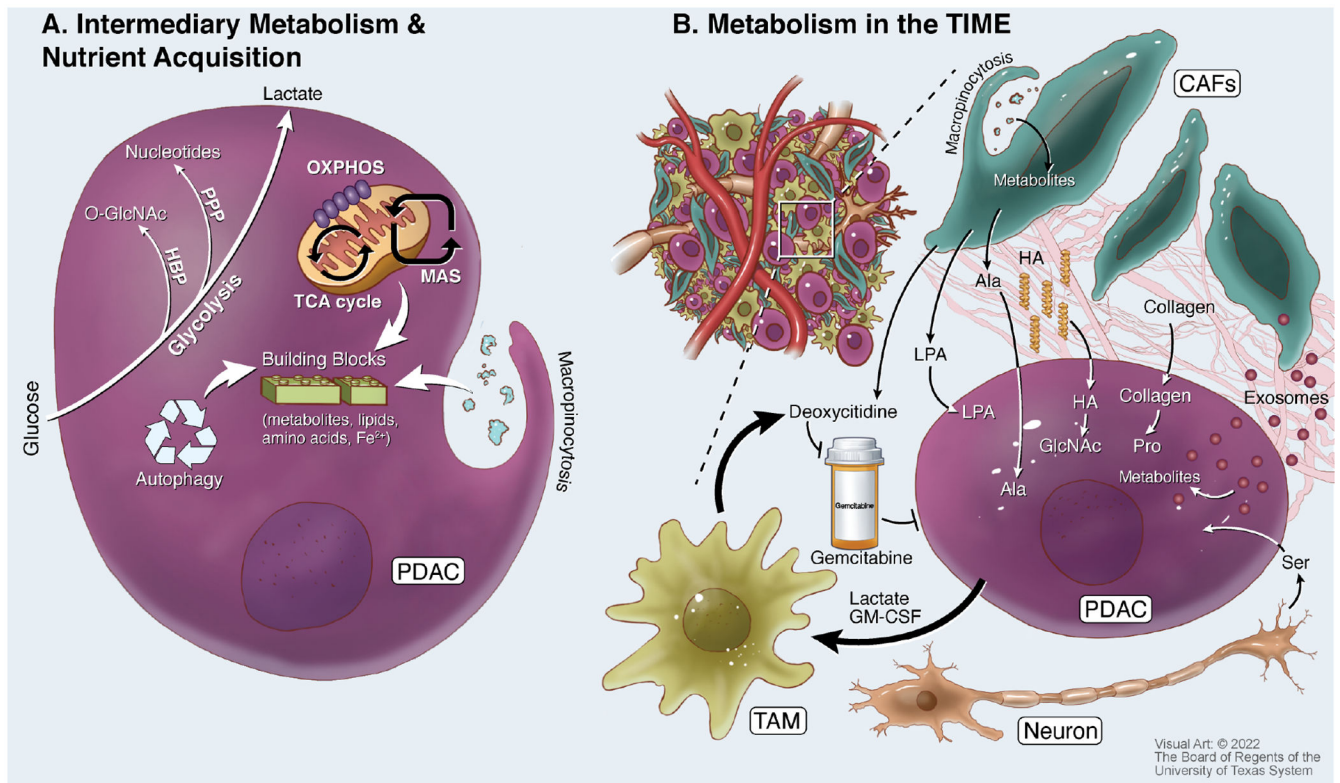
**Figure 4: Altered Metabolism and Metabolic Interactions of Pancreatic Tumors.**
**A**. Pancreatic ductal adenocarcinoma (PDAC) cells have increased glucose consumption to fuel glycolysis and anabolic metabolism from glycolytic intermediates. These include upregulation of the hexosamine biosynthetic pathway (HBP) to produce O-GlcNAc needed for protein glycosylation, and the pentose phosphate pathway (PPP) to produce nucleotides. PDAC cells also take advantage of a re-wired malate-aspartate shuttle (MAS) to fuel mitochondrial metabolism and oxidative phosphorylation (OXPHOS), and intermediates from the tricarboxylic acid (TCA) cycle provide synthetic metabolic building blocks. In addition to creating new biosynthetic material, PDAC cells recycle proteins and organelles through autophagy, and obtain extracellular materials through a non-specific fluid uptake process known as macropinocytosis. **B**. Many metabolic interactions shape the tumor microenvironment (TME). Cancer-associated fibroblasts (CAFs) provide alanine (Ala), lysophosphatidic acid (LPA), and exosomes loaded with metabolites to PDAC cells. The nutrient support of CAFs is at least in part fueled by macropinocytotic uptake of extracellular materials. PDAC cells metabolize extracellular matrix deposited in the TME to obtain hyaluronic acid (HA) to fuel GlcNAc pools and collagen to support proline (Pro) pools. Tumor-associated macrophages (TAMs) are polarized by signals including lactate and granulocyte macrophage colony-stimulating factor (GM-CSF) from PDAC cells. TAMs release deoxycytidine, also released by CAFs, that directly competes with the anti-metabolite chemotherapy gemcitabine. In addition, neurons in PDAC tumors can share serine (Ser), which is used to support mRNA translation.
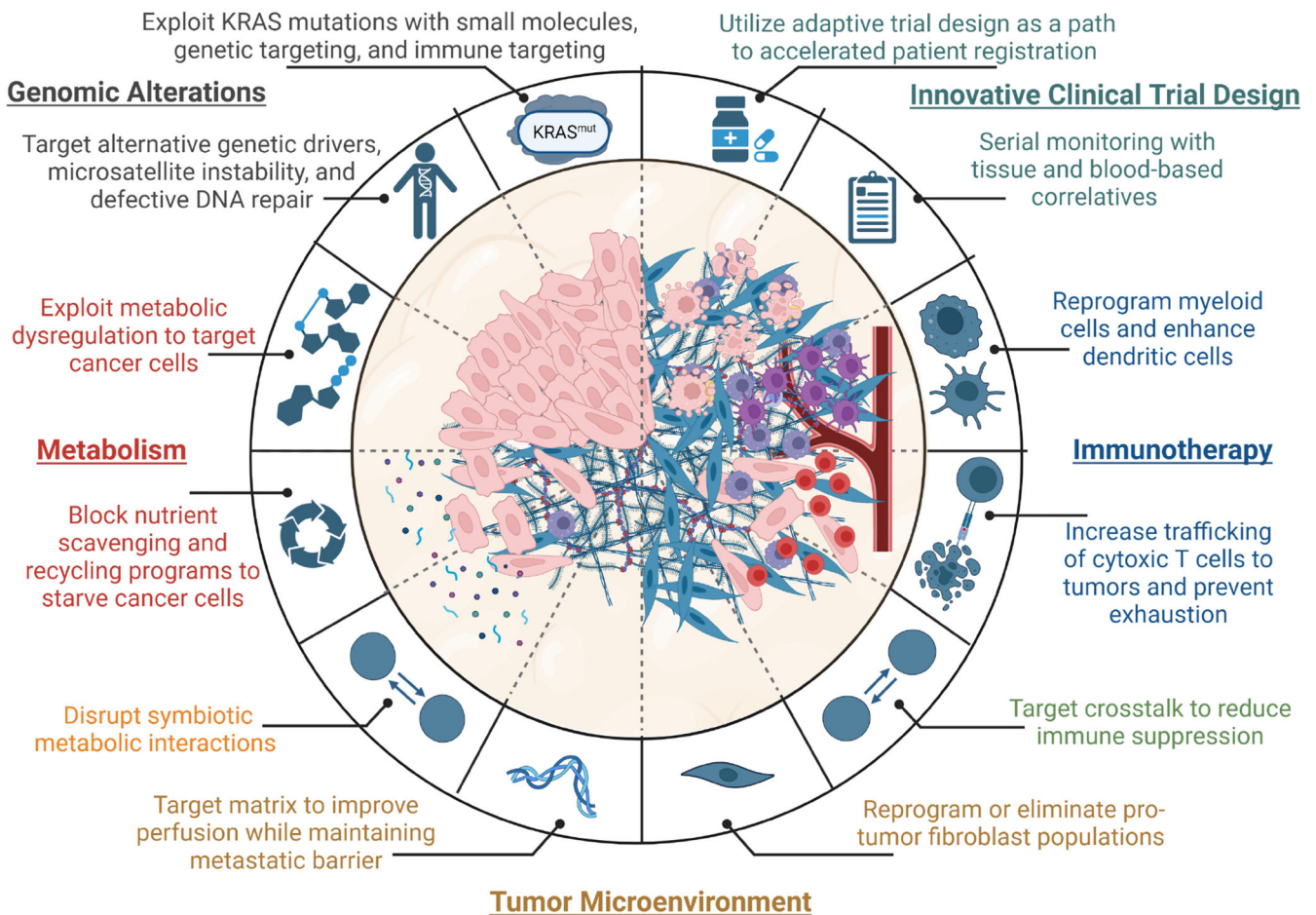
**Figure 5: Hallmarks of Pancreatic Cancer Therapy.**
Five major themes have emerged as priorities to improve pancreatic cancer treatment.
**Genomic Alterations**: Numerous approaches to directly target mutant RAS are entering
clinical care and sequencing can identify alternative genetic drivers that can be exploited
to effectively treat smaller subsets of patients. **Metabolism**: The rewired metabolism
of pancreatic cancer cells presents opportunities to selectively target neoplastic cells.
Scavenging, recycling, and metabolic crosstalk programs engaged to deal with nutrient
dysregulation in pancreatic tumors can be blocked to starve cancer cells and prevent
therapy resistance. **Tumor Microenvironment**: The characteristic pancreatic tumor
microenvironment can be remodeled to increase drug perfusion by targeting stromal
fibroblasts or the matrix directly, however, these strategies must not remove the barrier to
cancer cell migration. **Immunotherapy**: Disruption of interactions between cell populations
and reprogramming of myeloid cells in pancreatic tumors can relieve immune suppression.
Decreases in immune suppression will likely need to be coupled with efforts to increase
antigen presentation, increase cytotoxic T cell infiltration into tumors, and prevent their
exhaustion. **Innovative Clinical Trial Design**: Clinical studies testing need to prioritize
approaches most likely to succeed, continuously reassess patients on the trials using
molecular correlatives, and quickly transition patients who are not responding onto different

treatments. Importantly, these priorities are not mutually exclusive, and best probability of success lies in methods that will address multiple themes described above.