It is better to light a candle than to curse the darkness: single-cell transcriptomics sheds new light on pancreas biology and disease

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ABSTRACT

Recent advances in single-cell RNA sequencing and bioinformatics have drastically increased our ability to interrogate the cellular composition of traditionally difficult to study organs, such as the pancreas. With the advent of these technologies and approaches, the field has grown, in just a few years, from profiling pancreas disease states to identifying molecular mechanisms of therapy resistance in pancreatic ductal adenocarcinoma, a particularly deadly cancer. Single-cell transcriptomics and related spatial approaches have identified previously undescribed epithelial and stromal cell types and states, how these populations change with disease progression, and potential mechanisms of action which will serve as the basis for designing new therapeutic strategies. Here, we review the recent literature on how single-cell transcriptomic approaches have changed our understanding of pancreas biology and disease progression.

Key messages

⇒ The exocrine pancreas has more epithelial heterogeneity under homoeostatic conditions than previously appreciated.
⇒ Pancreatic acinar cells demonstrate a remarkable level of plasticity under conditions of injury or oncogenesis and can transdifferentiate to cell types which direct disease progression.
⇒ Single-cell RNA sequencing (scRNA-seq) of murine models identifies epithelial and stromal changes along the metaplasia to preneoplasia to cancer transition.
⇒ Fibroblast taxonomy has rapidly expanded and may reflect new cell types or interconvertible states that modulate and/or reflect disease progression.
⇒ Tumourigenesis is accompanied by the replacement of a proinflammatory milieu with an immune suppressive infiltrate dominated by myeloid-derived suppressor cells and exhausted T cells.
⇒ scRNA-seq and spatial profiling of naïve and chemotherapy-treated patient samples identifies potential mechanisms of resistance and targetable states.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is currently the third-leading cause of cancer-related deaths in the USA. PDAC has an abysmal 5-year survival rate of only 11% due to late diagnosis and an exceptional recalcitrance to therapy.[1] These statistics demonstrate that a greater understanding of pancreas biology and disease progression is desperately needed to better treat patients. While PDAC is typically detected at a locally advanced or distant metastatic stage, microscopic lesions, including pancreatic intraepithelial neoplasia (PanINs) and cysts such as intraductal papillary mucinous neoplasms (IPMNs), are formed several years before cancer detection; the epithelial cells that associate with these lesions are suspected to transform and become cancerous. Constitutively, active mutant *KRAS* is a major driver of PDAC and is often expressed in cells that comprise premalignant lesions, affecting the epithelial cells themselves and their interactions with other cells in the tissue. Environmental signals (eg, diet, smoking and alcohol) or idiopathic stimuli can induce pancreatitis, an inflammatory condition of the pancreas. Acute pancreatitis is common and is a leading cause of gastrointestinal (GI)-related hospitalisations. Acute pancreatitis ranges from mild to severe, with high morbidity and mortality, however, few treatments exist.[2] In its chronic form, pancreatitis is a risk factor for PDAC.[3]

Although neoplastic cellularity can vary within and between tumours, on average, PDAC is composed of approximately 20% malignant and 80% stromal cells that can either support or inhibit progression and render the tumour recalcitrant to therapy.[4] Until recently, it was extremely challenging to investigate human PDAC and preinvasive lesions in molecular detail. Technological advances in single-cell RNA sequencing (scRNA-seq) and bioinformatics have provided biological research with its own 'information revolution' and have significantly enhanced our understanding of cellular heterogeneity within complex organs and disease states. Despite the relatively recent implementation of scRNA-seq, the field has rapidly expanded, and copious technical approaches have been developed extending this technology to tissues that have been historically challenging to study.

Recent scRNA-seq studies of the healthy and diseased pancreas in both murine models and patient samples have transformed our understanding of the

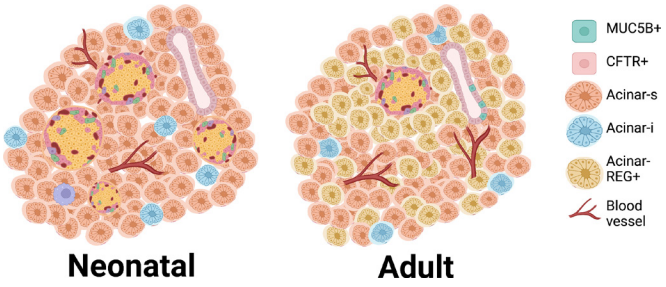

Figure 1 Previously unrecognised epithelial heterogeneity in the exocrine pancreas. Schematic of pancreas composition in both neonates (left) and adults (right). As humans mature, the exocrine compartment of the pancreas expands concomitant with angiogenesis. Additional cell types form including acinar-REG and MUC5B+ ductal populations.

cell types and polarisation states that function in pancreas physiology or drive disease. Herculean efforts have led to publicly available single-cell atlases, which are now guiding refined research directions in these fields.[5–10] Further, the application of scRNA-seq has been expanded beyond simple profiling and cell type discovery to stratifying patients for treatment selection and understanding mechanisms of therapy resistance. Here, we review how scRNA-seq has changed our understanding of exocrine pancreas physiology, plasticity, neoplasia and cancer.

Previously unrecognised epithelial heterogeneity in the exocrine pancreas

scRNA-seq has made its impact on the study of endocrine/islet pancreas physiology, dysregulation and diabetes.[11–13] The composition of the exocrine pancreas, however, has long been considered relatively simple, constituted by digestive enzyme-producing acinar cells and the bicarbonate-producing ductal cells that guide these secretions to the duodenum. Recent studies have demonstrated that these cell types are more heterogeneous under homoeostatic conditions than previously appreciated.[6 14–16] To profile the exocrine pancreas more comprehensively, Tosti *et al* recently employed single-nucleus RNA-sequencing (sNuc-seq), which allowed for analysis of acinar-dense human tissues at single-cell resolution.[5] Several novel observations were made, including the presence of at least two types of ductal cell (*CFTR*+, *MUC5B*+) and three states of acinar cell (acinar-s, acinar-i, acinar-REG) (figure 1) consistent with previous studies.[6 14 16] Based on gene expression analysis, digestive enzymes constitute ~50% of the 'acinar-s' transcriptome. 'Acinar-i' cells express less protein-encoding mRNA and are thought to be 'idling', while 'acinar-REG' cells, express regenerating protein (REG) family members (eg, *REG3A*, *REG1B*) and have been linked to pancreatic inflammation.[6] Interestingly, the authors found that the exocrine pancreas comprises ~50% of total volume in neonates, but 90% in adults. Further, acinar-REG and *MUC5B*+ populations were not found in neonates (figure 1). While many of these cell types/states have been validated by marker staining, further studies are required to establish functionality.

Lineage tracing identifies substantial acinar cell plasticity

Tosti *et al* also profiled the cellular composition of pancreatitis from patient samples (2726 nuclei).[5] In comparison to normal pancreas, the authors identified expansion of the MUC5B+ ductal population, significant changes in stromal populations reflecting inflammation and the formation of tuft cells.[5] Tuft cells are solitary chemosensory cells found throughout the hollow organs of the respiratory and digestive tracts.[17] Tuft cells are thought to act as sentinels, monitoring luminal contents and responding to noxious or infectious stimuli via inflammatory and neuronal type effectors.[18] Previous studies have shown that tuft cells transdifferentiate from acinar cells under conditions of chronic injury or oncogenic mutation during the process of acinar to ductal metaplasia.[19 20] ADM is a reparative programme in which acinar cells transdifferentiate to cells with ductal organisation to enable tissue reconstruction following injury. ADM, however, is also considered to be the first step in tumourigenesis.[21]

To comprehensively characterise the cell types/states that arise in ADM, Ma *et al* employed mouse models and lineage tracing (*Ptf1aCre*$^{ERT/+}$, *Rosa*$^{YFP/+}$) to follow the fate of adult acinar cells during injury.[22] scRNA-seq identified the formation of a mucinous ductal population, consistent with the Tosti *et al*'s study.[5] Application of several independent trajectory analysis algorithms predicted that a progenitor-like subset of the mucin/ductal population seeds tuft cell formation (*Pou2f3*+*Ptgs1*+) as well a previously undescribed heterogeneous population of hormone-producing enteroendocrine cells (*Sst*+, *Ppy*+, *Ghrl*+, *Ddc*+) as distinct lineages (figure 2). Bioinformatic analyses determined that the mucin/ductal population is highly enriched for classical markers of spasmolytic polypeptide expressing metaplasia (*Tff2*+*Muc6*+*Gkn3*+), which forms in the stomach in response to injury. The commonalities in marker expression between metaplasia in these organs and others in the GI tract strongly supports the use of the all-encompassing term 'pyloric metaplasia', which suggests that there is a reparative programme common to the GI tract which may be exploited to maintain homoeostasis.[23 24] Interestingly, regulon analysis, which predicts transcription factor activity by expression of known downstream target genes, identified factors which have been shown to serve as master regulators of cell type formation in other organs. For example, *Pou2f3* is enriched in tuft cells and *Neurog3*, the master regulator of endocrine cell formation in islets and intestines, is enriched in ADM-derived enteroendocrine cells.[25–27] Collectively, these data demonstrate a previously unrecognised level of plasticity in pancreatic acinar cells, which can co-opt cell differentiation programmes characteristic of other GI organs. The commonalities between these systems will allow for a deeper look into the function of these cell types/states in pancreas diseases.

Acinar to ductal metaplasia (ADM) also arises in the context of oncogenic *KRAS*, which has been shown to be mutated in over 90% of human PDAC.[28] To examine the cell types/states that arise in the context of *Kras*^{G12D}, Schlesinger *et al* employed genetically engineered mouse models (GEMMs) of pancreatic tumourigenesis and lineage tracing (*LSL-Kras*^{G12D}, *Ptf1aCre*$^{ERT/+}$, *LSL-tdTomato*).[29] scRNA-seq was conducted on mice of various ages to capture the changes that occur as ADM progresses to PanIN and PDAC. The authors identified increased expression of the aforementioned REG genes, which have been shown to drive ADM, in acinar cells (*Reg3b*).[30] Metaplasia-specific factors were identified including transcription factors such as *Onecut2*—previously identified as a master regulator of prostate cancer.[31] Six metaplastic cell types/states were characterised including gastric pit-like cells (*Gkn1*+*Tff1*+*Muc5ac*+) and chief-like cells (*Pga5*+*Pgc*+), tuft cells and enteroendocrine cells (consistent with prior studies,[20 32 33]), senescent cells and proliferative metaplastic cells (figure 2). Lineage trajectory analysis of this dataset suggested that the gastric-like population and tumour can arise through distinct cell states, but it is still not clear which metaplastic cell types become malignant. Interestingly, several *Kras*-induced gene signatures and populations were also identified in

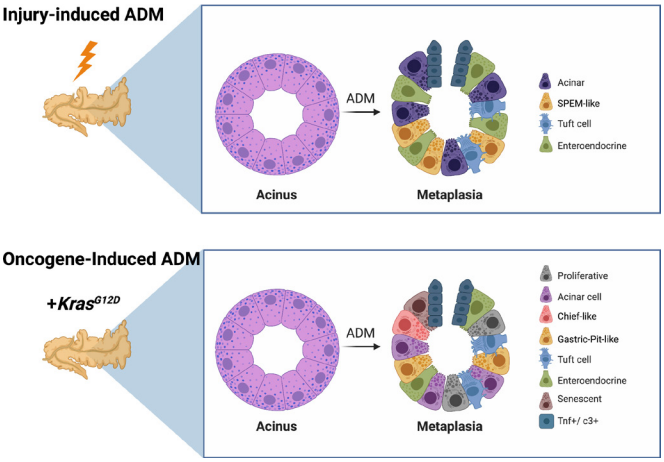

Figure 2 Lineage tracing identifies substantial acinar cell plasticity. In response to chronic injury (top) or oncogenic *Kras*^{G12D} expression (bottom), pancreatic acinar cells undergo metaplasia and form several cell types normally absent or rare in the pancreas. These populations include chemosensory tuft cells, hormone expressing enteroendocrine cells, gastric-like chief (SPEM) and pit cells, as well as proliferative and senescent populations. ADM, acinar to ductal metaplasia; SPEM, spasmolytic polypeptide expressing metaplasia.

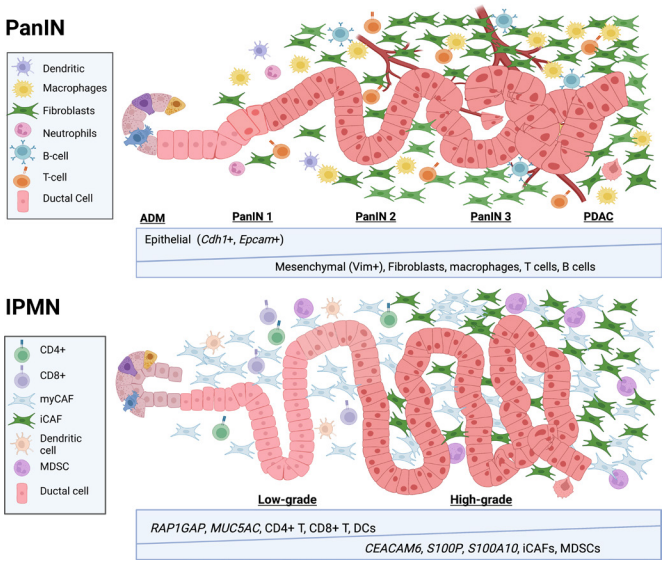

Figure 3 Precancer neoplasia and the transition to PDAC. PanIN (top) or IPMN (bottom) formation and progression to PDAC is associated with a loss of epithelial markers and a gain of mesenchymal markers. Stromal changes associated with both lesion types have been described, including changes in fibroblast populations and immune cell infiltration. iCAF, inflammatory cancer-associated fibroblasts; MDSC, myeloid-derived suppressor cells; myCAFs, myofibroblastic CAFs; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary mucinous neoplasms.

a scRNA-seq study by Chondronasiou *et al* where the authors used GEMMs overexpressing Yamanaka factors *Oct4*, *Sox2*, *Klf4* and *Myc*, demonstrating that *Kras*^{G12D} is sufficient to drive a common dedifferentiation programme.[34]

The occurrence of ADM in human tumourigenesis has been debated but challenging to evaluate overall due to the paucity of acinar and ADM cells sampled at single-cell resolution. Using a combination of snRNA-seq, scRNA-seq and immunostaining, Cui Zhou *et al* identified populations in human samples associated with PDAC expressing both acinar and ductal markers.[35] Lineage trajectory analysis predicted that some ADM populations are related to PanIN and can harbour genomic alterations (eg, CDKN2A aneuploidy). Altogether, these findings demonstrate that the acinar cell of origin for PDAC identified in mouse models likely recapitulates tumour progression in patients.

Temporal analyses identify epithelial changes in the transition from neoplasia to PDAC

Other groups have also combined scRNA-seq and time point sampling to identify epithelial and stromal changes that accompany disease progression. Hosein *et al* agnostically profiled phenotypic changes between early and late-stage pancreatic tumourigenesis using multiple GEMMs.[36] Pancreata from mice-expressing *Kras*^{G12D}, as well as biallelic deletions of the tumour suppressors *Ink4a/p16* or *Trp53*, were profiled by scRNA-seq at either an early time point consisting largely of PanIN or a late time point characterised by PDAC. Similar to the Schlesinger *et al*'s study, the authors found that early-stage disease is characterised by an expansion of ductal cells (*Krt18+Sox9+*) at the expense of acinar cells as well as an increase in total macrophages, fibroblasts and endothelial cells, as compared with the non-neoplastic pancreas.[29 36] In normal pancreas and PanIN, three distinct populations of fibroblasts were identified, only two of which persisted in late-stage PDAC. Late-stage disease was composed of two distinct cancer cell populations and an abundance of macrophages. Further, the transition from early-stage to late-stage disease was accompanied by a loss of epithelial markers (*Cdh1*, *EpCAM*) and a gain in mesenchymal markers (*Vim*, *S100a4*), possibly reflecting an epithelial-to-mesenchymal transition (EMT) (figure 3). These findings were recapitulated in mice harbouring the *p53*^{R172H} mutant allele. Thus, despite inter-mouse and model heterogeneity, late-stage disease in all three GEMMs is characterised by the same two populations of cancer cells (epithelial and mesenchymal), macrophages (inflammatory and MHC-II rich) and fibroblasts, illustrating a consistent pattern of intratumour cellular heterogeneity in GEMMs with distinct secondary driver mutations.

A similar approach was undertaken by Bernard *et al* to study disease progression in a second precursor lesion to PDAC, IPMN.[37] IPMNs are cystic tumours of the pancreas present in >5% of individuals over 60 years old.[38] Most IPMNs (90%) are diagnosed before cancer is present, providing a unique window of opportunity to prevent PDAC. To distinguish benign from aggressive IPMN, Bernard *et al* conducted scRNA-seq on low-grade or high-grade IPMN, as well as PDAC collected by surgical resection from patients.[37] Distinct epithelial changes were identified, such as a loss of putative tumour suppressor gene *RAP1GAP* and pit cell marker *MUC5AC* and a gain in *CEACAM6*, *S100P* and *S100A10* expression between low-grade and high-grade IPMN. Genes considered to be cancer specific (*TFF3*, *REG4*) were identified in low-grade IPMN demonstrating that these populations form early in disease progression. PDAC was more proliferative than high-grade or low-grade IPMN. Substantial stromal changes were also observed. A more proinflammatory milieu consisting of activated CD4+T cells, cytotoxic T cells and dendritic cells was identified in low-grade IPMN. PDAC, however, was characterised by a striking increase in myeloid-derived suppressor cells (MDSCs). Fibroblast composition changed as well, with *ACTA2+*populations in high-grade IPMN and PDAC, but *CXCL12+IL6+* populations restricted to PDAC alone (figure 3). Altogether, these studies demonstrate that

scRNA-seq can be used to identify epithelial and stromal changes associated with the neoplasia to cancer transition, which may be used to identify patients at risk of developing PDAC.

scRNA-seq identifies substantial intratumoural and intertumoural cellular heterogeneity in PDAC

To comprehensively profile intratumoural heterogeneity, Peng *et al* conducted scRNA-seq on 24 human PDAC samples and 11 normal pancreata.[39] The authors described two ductal cell types (disease associated/abnormal or malignant), both expressing ductal markers (*MMP7+SOX9+LCN2+*), with one additionally expressing PDAC-associated markers (*KRT19+CEACAM1/5/6+*) absent from normal pancreata. Neoplastic cells were confirmed by assessing the copy number variant landscape. Differential gene expression analysis was performed between abnormal, non-malignant ductal cells from PDAC and normal pancreata. Interestingly, 85% of identified genes were also upregulated in malignant ductal cells, suggesting that normal ductal cells take on dysregulation programmes when associated with malignancy. Within the malignant group, several subclusters expressing *KRT19* and *MUC5AC* were identified. All other clusters were relegated to only a fraction of patients, highlighting intertumoural heterogeneity. Few acinar cells were identified in PDAC patients, however, the authors identified substantial stromal heterogeneity, consistent with previously described studies.[29 36 37] Interestingly, proliferative PDAC samples were characterised by reduced expression of T cell marker expression (*CD3D*) both spatially and at the gene level, and T cell score, likely reflecting poorer prognosis. Altogether, this study represents a substantial dataset, which may serve as the foundation for mechanistic studies.

Other groups have also profiled PDAC patient samples and observed significant epithelial, stromal and tumourous heterogeneity.[10 35 40 41]

Several studies have used scRNA-seq to investigate how PDAC metastases form and to examine transcriptomic changes in comparison to primary tumours. Ting *et al* profiled primary and circulating tumour cells (CTCs) from murine models and patient samples.[42] The authors found that CTCs highly express stromal-derived extracellular matrix (ECM) proteins, including SPARC; knockdown of ECM proteins in cancer cells suppresses cell migration and invasiveness. Lin *et al* and Lee *et al* extended these studies to observe changes between the primary tumour and matched metastases in the same patients.[43 44] Intratumoural heterogeneity was observed, however, the authors described intertumoural heterogeneity in the tumour cells, with the stroma remaining relatively homogeneous between patients. Metastases were found to contain larger fractions of epithelial cells than primary tumours and to differ in immune composition, with metastases containing a distinct population of macrophages and more antigen-presenting cells. Larger cohort sizes may help to identify relevant subtle shifts in cell type or state abundance during the metastatic process, and further analyses of these differences may identify better therapeutic strategies to target local and distant sites.

Fibroblast heterogeneity reflects disease state and predicts progression

Fibroblasts are a mesenchymal stromal population that expand in pancreatitis and PDAC. They have been shown to aid in the survival and migration of cancer cells as well as to generate an ECM that physically interferes with drug delivery.[45 46] Several stroma depletion therapeutic strategies, however, have been shown to accelerate tumourigenesis, suggesting that cancer-associated fibroblasts (CAFs) may play a more complex role than previously appreciated.[47 48] Recent scRNA-seq-based studies of CAFs have identified transcriptionally and functionally distinct subpopulations.[49 50] To comprehensively catalogue CAF subtypes, Elyada *et al* conducted scRNA-seq on PDAC patient samples and *KPC* (*Kras*^{G12D}, *Trp53*^{R172H}, *Pdx1-Cre*) GEMM tumours.[49] Analysis of murine tumours revealed inflammatory CAFs (iCAFs, *Il6+Cxcl1+Has1+*) and myofibroblastic CAFs (myCAFs, *Acta2+Tagln+*), as well as a novel subtype expressing MHC class II family genes, denoted antigen presenting CAFs (apCAFs, *Cd74+H2Aa+H2-Ab1+*) (figure 4). Pathway analysis of differentially expressed genes between these three subtypes identified inflammatory signalling in iCAFs, ECM receptor interaction with myCAFs, and fatty-acid metabolism, MYC targets, and MTORC1 signalling in apCAFs. All three subtypes could be identified by RNA in situ hybridisation and flow cytometry and were identified in patient samples by scRNA-seq. Further, antigen presentation capacity was demonstrated for apCAFs in vitro, confirming that this newly described CAF subtype is functionally distinct.[49]

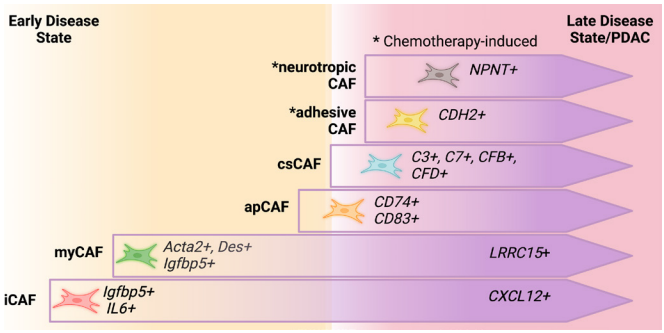

Figure 4 Fibroblast heterogeneity in diseases of the pancreas. Schematic of how cancer-associated fibroblast populations change throughout pancreas disease progression. Whether these populations represent distinct cell types or interconvertible states remain unknown. CAF, cancer-associated fibroblasts; iCAF, inflammatory CAFs; PDAC, pancreatic ductal adenocarcinoma. *, chemotherapy-induced CAF population.

The markers identified in the Elyada *et al*'s study have now become standard for phenotyping GEMMs and patient samples. In the aforementioned studies, Schlesinger *et al* identified *Igfbp5+* (also identified in Hosein *et al*[36]) and *Il6+* (iCAF) CAF populations associated with PanIN in GEMMs, with iCAFs expressing higher levels of cytokines and secreted proteins later in disease progression.[29] At later time points in disease progression, myCAFs (*Acta2+Des+Igfbp5+*), apCAFs (*CD74+CD83+*) and proliferating CAFs were identified. Hosein *et al* identified three types of CAFs present early in tumour formation in GEMMs, with only two, corresponding to iCAFs and myCAFs, persisting late in PDAC. A subcluster of CAFs expressing MHC-II-associated genes was identified.[36] Despite the consistent identification of apCAFs in murine models, their detection in human disease has been variable. Bernard *et al* identified both myCAFs and iCAFs, but not apCAFs, in their study on precursor lesions. The myCAF population was associated with low-grade and high-grade human IPMN as well as PDAC, but iCAFs (*CXCL12+*) were associated only with PDAC.[37] Conversely, Cui Zhou *et al* found an increase in iCAF abundance in chemotherapy-treated patient samples as well as apCAFs.[35] Peng *et al* described eight distinct fibroblast clusters in human samples, which may include

additional, newly identified CAF populations (figure 4).[39] Chen *et al* conducted scRNA-seq on patient samples spanning PDAC stages I–III and identified complement secreting CAFs (csCAFs) which were found adjacent to malignant cells only in early PDAC.[40] Dominguez *et al* conducted scRNA-seq on 22 PDAC patient samples (>80k cells) and identified TGFβ-programmed *LRRC15*+CAFs, which surround tumour islets and are absent from the normal pancreas.[51] Analysis of data from immunotherapy clinical trials has revealed that the *LRRC15*+ CAF signature correlates with poor response to anti-PD-L1 therapy, demonstrating prognostic value. The contribution of scRNA-seq studies to the identification of CAF heterogeneity in cancer was recently reviewed in detail by Lavie *et al*.[52] Collectively, the abundance of recently described CAF subtypes and states has led to the field coalescing around the viewpoint that these various CAF phenotypes are not stable over time and that there may be some overlapping features between CAF subtypes.[53 54] Thus, one way to approach this conundrum might be to consider CAFs in the context of 'tumour restraining' and 'tumour promoting' rather than multiple distinct categories.

The immune landscape evolves during pancreatic tumourigenesis

scRNA-seq studies have captured snapshots of the immune landscape and how it evolves with disease progression. A recent study characterised pancreatic tissue from chronic pancreatitis patients and control donors.[55] In addition to T cell receptor profiling, the authors reported on potential interactions between myeloid cells and T cells and highlighted a possible role for the CCR6-CCL20 signalling axis. In mouse models of tumourigenesis, Schlesinger *et al* found that PanIN formation was accompanied by immune infiltration and expression of proinflammatory genes in the epithelium and stroma.[29] While several populations were identified, macrophages dominated the immune infiltrate and *CD206*, a marker of suppressive macrophages, was enriched in tumour-associated macrophages (TAMs). CD4+T cells expressed both *Gata3* and *Foxp3*, a marker of suppressive T cells, even at the precursor PanIN stage. Hosein *et al* found that tumourigenesis in GEMMs was accompanied by a marked increase in TAMs; TAMs being the dominant cell population in late-stage disease. Early tumourigenesis was characterised by populations of TAMs distinguished by IL-1 receptor ligand (eg, *Il1b*), chemokine and complement-associated gene expression, and genes associated with antigen processing.[36] Late-stage TAMs were characterised by elevated chemokine expression or MHC-II antigen presentation genes. In human IPMN, Bernard *et al* found that low-grade lesions were accompanied by a proinflammatory milieu of cytotoxic T cells, activated T-helper cells and dendritic cells, which were progressively replaced with MDSCs (table 1).[37] Collectively, these studies highlight the replacement of a proinflammatory infiltrate with a dominant immune suppressive myeloid/macrophage population during pancreatic tumourigenesis. The signals that mediate this transition and its overall effect on tumour progression are not fully understood.

In human PDAC, Elyada *et al* identified myeloid and lymphoid populations in tumours and adjacent normal tissue. The myeloid population was largely composed of monocytes and resident macrophages (96%); less than 4% were dendritic cells and neutrophil markers were detected.[49] Conventional and Langerhans-like dendritic cells were identified by *IRF8* and *CD1A* expression, respectively. The T cell and NK cell clusters contained CD8+ and CD4+ T cells along with proliferating T regulatory cells and NK cells. CD8+T cells expressed markers of cytotoxic activity at low levels, and IFNγ and exhaustion markers were expressed exclusively by CD8+T cells residing in PDAC samples. Peng *et al* additionally identified B cells.[39] Lin *et*

Table 1 Inflammatory cell infiltration in diseases of the pancreas

Study	Model/source	Early disease state (Early PanIN, low-grade IPMN)	Late disease state (PanIN3, high-grade IPMN)	PDAC
Schlesinger *et al*[29]	*Prf1aCre*$^{ER/+}$, *Kras*$^{LSL-G12D}$, *tdTomato*	Macrophages	Macrophages, plasmacytoid, conventional and cDC3 dendritic cells	CD4+T and CD8+T, NK, macrophages, neutrophils, plasma B, dendritic and B cells
Raghavan *et al*[62]	Primary and metastatic human samples	N/A	N/A	scBasal:C1QC+TAMs
Hosein *et al*[36]	Kras$^{LSL-G12D/+}$, Ink4a$^{fl/fl}$, Ptf1a$^{cre/+}$	Macrophages (*IL1b*+, complement and chemokine expressing)	Lymphocytes, macrophage (antigen-presenting MHC II molecules)	Macrophages
Bernard *et al*[37]	Human low-grade/high-grade IPMN, PDAC	T cells (CD4+ and CD8+); B cells (CD20+, CD19+); MDSCs (cDC2-type dendritic cells)	B cells (CD20+, CD19+); MDSCs (cDC2-type dendritic cells)	MDSCs (cDC2-type dendritic cells)
Peng *et al*[39]	Primary human PDAC tumours	N/A	N/A	Macrophages, T and B cells
Lin *et al*[43]	PDAC primary and metastatic samples	N/A	N/A	BDCA-1+ dendritic cells, CD14+/CD68+macrophages and T cells, TILs, TAMs
Elyada *et al*[49]	Primary and metastatic human PDAC samples	N/A	N/A	Myeloid: dendritic cells, macrophages, monocytes Lymphoid: T (CD4+, CD8+), CD4+Treg, NK
Hwang *et al*[56]	Primary human PDAC samples	N/A	N/A	Neuro-like lineage: CD8+T and cDC2↑ Mesenchymal, basaloid and squamoid lineages: CD8+T↓

Description of inflammatory cell populations and their association with different stages of pancreas disease progression.
IPMNs, intraductal papillary mucinous neoplasms; MDSCs, myeloid-derived suppressor cells; N/A, not available; NK, natural killer cells; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; TAMs, tumour-associated macrophages; TILs, tumour-infiltrating lymphocytes; Treg, T regulatory cells.

al identified dendritic cells by *BDCA1* expression and found that TAMs differed between primary tumours and metastases, with the former expressing genes associated with ECM deposition and wound healing and the latter expressing MHCI/II, *CD74* and genes associated with antigen presentation (table 1).[43] In a comprehensive study combining scRNA-seq with CyTOF and multiplex immunohistochemistry on patient samples, Steele *et al* identified an inverse correlation between myeloid cells and CD8+T cell infiltration in PDAC.[41] As compared with T cells associated with the normal pancreas, PDAC CD8+T cells expressed higher levels of both activation, trafficking (*GZMB*, *GZMA*, *KLF2*) and exhaustion markers (*EOMES* and *GZMK*). In particular, the authors identified high expression of checkpoint receptor *TIGIT*. Protein expression of TIGIT in the blood correlated to that in the tumours of individual patients, suggesting that it may serve as a non-invasive biomarker of disease progression. Cui Zhou *et al* also identified *TIGIT* expression in CD8+T cells as well as expression of receptor *NECTIN* ligands in tumour cells, suggesting a mechanism by which cancer cells can directly inactivate T cell effector function.[35] Altogether, these studies describe the PDAC microenvironment as immunosuppressive, with T cell populations shifted from cytotoxic to exhausted. scRNA-seq studies of chemotherapy-treated PDAC samples, however, suggest that T cell populations can be shifted back towards cytotoxic with antitumour activity. Hwang *et al* found that PDAC samples from patients treated with neoadjuvant chemotherapy and radiotherapy (CRT) plus losartan had greater expression of CD8+T cells expressing effector markers as compared with neoadjuvant CRT alone.[56]

Tumour-associated endothelial cell changes identified by scRNA-seq

Endothelial cells play an important role in immune cell recruitment, supplying nutrients to tumours and in mediating metastases. Schlesinger *et al* found that endothelial cells in murine premalignant lesions express high levels of selectins, adhesion molecules and cytokines, and may assist in recruiting immune cells to the tumour.[29] Shiau *et al* performed snRNA-seq on ~15 000 endothelial cells from chemotherapy treated or treatment naïve PDAC patients and identified signatures associated with poor clinical outcomes.[57] Zhang *et al* recently performed a computational analysis of scRNA-seq of endothelial cells from six different cancer types, including PDAC.[58] They identified six endothelial subtypes and found that tip-like endothelial cells (*CXCR4+ESM1+ANGPT2+*) were more abundant in tumours than normal tissue and were positively correlated with reduced patient survival. Altogether, these studies identify significant changes in endothelial cells in response to tumour development and chemotherapy, however, functional studies are required to identify the consequences of these changes on disease progression.

Translational use of scRNA-seq to identify PDAC vulnerabilities and mechanisms of therapeutic resistance

Transcriptional subtypes/states serve as important biomarkers for stratifying response to therapy and prognosis, but the full potential of this approach has not been realised due to limitations in resolution and cell-type specificity. For many years, the pancreatic cancer field was limited to a dichotomy of bulk transcriptomic subtypes with contributions from an unknown mixture of cancer and stromal cells: (1) classical, encompassing a spectrum of pancreatic lineage precursors and (2) basal-like, exhibiting loss of endodermal identity and genetic aberrations in chromatin modifiers.[59 60] Most tumours harbour both classical and basal-like cancer cells, leading to a bulk transcriptional continuum that includes a 'hybrid' state.[61]

To explore the relationship between clinically relevant cancer transcriptional subtypes, their tumour microenvironments (TMEs), and therapeutic response, Raghavan *et al* conducted scRNA-seq on metastatic biopsies and matched organoids from PDAC patients.[62] In addition to metastatic cells scoring highly for either classical-like (*LGALS4+CTSE+TFF1+*) or basal-like signatures (*KRT17+S100A2+KRT6A+*) that coexisted intratumourally, the authors uncovered a new intermediate that coexpresses both signatures at the single-cell level (termed an 'intermediate coexpressor' or 'IC'), which was validated in situ, and suggests a potential transition state. The TME associated with the frequencies of these three transcriptional programmes was found to be distinct. In matched organoids, there was a strong selection against the basal and IC programmes under standard media conditions. Contrasting patient and model, they nominated critical factors missing in vitro; by adding them back, they restored in vivo cell state heterogeneity, demonstrating that microenvironmental signals are key regulators of PDAC state. Most crucially, they found that non-genetic modulation of cell state can significantly influence drug responses. This study highlights the importance of faithful avatars when testing potential therapeutic agents and the exciting possibility of rationally controlling transcriptional plasticity, either directly or via the TME.

Recent clinical trials support the use of neoadjuvant chemotherapy with or without radiotherapy for resectable and borderline resectable PDAC[63]; nevertheless, residual disease is almost always present, so capturing cell-type specific treatment-resistant gene expression states is critical to improve therapeutic strategies. To harness a large frozen biobank and concurrently improve cell recovery and RNA integrity, Hwang *et al* used snRNA-seq to capture high-quality nuclei across 43 treated and untreated PDAC patient tumours.[56] Using this high-resolution dataset, Hwang *et al* reported an expanded cell taxonomy of malignant cells and CAFs in PDAC, including (1) partitioning of the aggregate bulk basal-like/squamous/quasi-mesenchymal subtype into discrete basaloid, squamoid and mesenchymal programmes; (2) identifying neuroendocrine-like and acinar-like programmes that support the cancer-intrinsic existence of the aberrantly differentiated endocrine exocrine subtype[59 60]; and (3) discovering a novel neural-like progenitor (NRP) malignant programme that featured pathways and genes associated with neuronal development and stem-like state. NRP was strongly enriched after treatment and was associated with the worst prognosis in a multivariable analysis of two large independent cohorts demonstrating clinical relevance. Identified CAF populations include neurotropic CAFs (*NPNT1+*), adhesive CAFs (*CDH2+*), immunomodulatory CAFs (*CCL21+IL15+*) and myofibroblastic CAFs (*ACTA2+*) (figure 4).

In another study, Cui Zhou *et al* spatially sampled 31 PDAC patient tumours (11 untreated, 20 chemotherapy treated) and profiled them with scRNA-seq/nucleus RNA-sequencing and bulk proteogenomics.[35] Distinct subpopulations of cancer cells exhibiting signatures of *KRAS* signalling, EMT, proliferation and cytotoxic stress were identified, and chemoresistant specimens exhibited a threefold enrichment in inflammatory CAFs. Overall, these studies provide a high-resolution examination of PDAC, which deepens our understanding of its molecular underpinnings and establishes a new paradigm for translating single-cell transcriptomics to clinical oncology.

Table 2 Feature comparison of modern spatial-omics approaches

Category	Examples	Spatial resolution	Transcriptome coverage	Detection efficiency	Tissue area	Multiomics	Sample diversity
Selecting ROIs	Physical: LCM,[72] Tomo-seq,[73] STRP-seq[74] Optical: NICHE-seq,[75] SPACECAT,[76] ZipSeq,[77] NanoString GeoMx DSP[78]	Multicellular	Up to whole transcriptome	Moderate	Large	RNA, protein	FFPE optimised: LCM, NanoString GeoMx DSP
Single-molecule FISH	seqFISH,[79] Vizgen MERSCOPE/MERFISH,[80] ACDBio RNAscope, NanoString CosMx SMI[81]	Single-cell/ subcellular	Up to 1000s of transcripts	High	Small	RNA, protein, DNA, chromatin structure	FFPE compatible: Vizgen MERSCOPE/ MERFISH, ACDBio RNAscope FFPE optimised: (NanoString CosMx
In situ sequencing	Cartana ISS,[82] FISSEQ,[83] 10× Xenium, BAR-seq[84]	Single-cell/ subcellular	Up to 1000s of transcripts	Low	Small	RNA, protein, DNA	FFPE compatible: 10× Xenium
NGS with spatial barcoding	Spatial transcriptomics/10× Visium,[85] DBiT-seq,[86] Slide-seq,[87] PIXEL-seq,[88] BGI Stereo-seq[89]	Multicellular	Up to whole transcriptome	Low	Large	RNA, DNA	FFPE compatible: 10× Visium, DBiT-seq, BGI Stereo-seq

Partially adapted from Moses and Pachter.[67]
DSP, digital spatial profiling; FISH, fluorescence in situ hybridization; FISSEQ, fluorescence in situ sequencing; LCM, laser capture microscopy; NGS, next generation sequencing; ROI, regions of interest.

Emerging single-cell technologies

In the scRNA-seq studies mentioned above, the relative position of the profiled cells in the tissue is lost during processing and analysis. Emerging spatial proteotranscriptomic technologies enable the analysis of cellular phenotypes while preserving the in situ tissue architecture, specifically the localisation and interrelationships among cells. Several groups have recently published studies using scRNA-seq or snRNA-seq in combination with spatial transcriptomics on chemotherapy-treated or treatment-naïve PDAC patients.[35 56 64] These studies demonstrate that integrating single-cell and spatial transcriptomics enables cross-validation and additional novel insights not possible from either approach alone.[65] Researchers may explore the interactions (eg, ligand-receptor) between different cells in the TME, the factors that dampen chemotherapy efficacy, and the signals that induce an immunosuppressive TME. Recently developed spatial methods are summarised in table 2.[66 67] Multiomic studies that assess genetics, gene expression, chromatin accessibility and DNA methylation from the same cells will shed light on genetic aberrations and epigenetic modifications that govern the malignant processes and states of associated TME cells.[68]

DISCUSSION

scRNA-seq is a powerful technique that allows for the investigation of complex multicellular organs and tumours. Using scRNA-seq and bioinformatics techniques, it is possible to explore epithelial heterogeneity, predict the trajectory of cell state changes throughout tumourigenesis and characterise potential interactions between stromal and immune populations. scRNA-seq experiments done in GEMMs of pancreatic tumourigenesis support detailed profiling of early events that lead to the formation of premalignant lesions and cancer. In the presence of injury and inflammation and/or on constitutive activation of oncogenic *Kras*, metaplastic populations can form microscopic (PanIN) or macroscopic (IPMN) lesions. Interestingly, metaplastic cell types derived from acinar cells in the setting of pancreatitis and mutant $Kras^{G12D}$ are similar and suggest that these new transcriptional programmes are beneficial and help to resolve or mitigate stress. Consistent with this possibility, Del Poggetto *et al* showed that pancreatic injury induces epigenetic memory, which helps to mitigate successive insults, quickly restoring organ function and reducing cell death.[69]

While current scRNA-seq methods allow for detailed profiling of cell types/states that occupy premalignant lesions in the pancreas, additional work is needed to understand if these populations are preferentially derived from the multiple acinar populations characterised by Tosti *et al*, to better understand the response/contribution of ductal cell heterogeneity to exocrine disease progression and to determine the relative contribution of different metaplastic cell types to malignancy.[5] Further, functional experiments are needed to determine if the identified polarisation states of different cell types are relevant to organ function or disease progression and to determine the functional role of different metaplastic cell types in disease progression. For example, recent studies conducted in GEMMs of pancreatic tumourigenesis showed that tuft cells inhibit disease progression, in part through the production of eicosanoids, and that MUC5AC+pit like cells drive disease progression through STAT3 signalling.[70 71]

Limitations and future directions

The aforementioned studies largely use tissue dissociation protocols to recover single-cell suspensions. Acinar cells, however, are much more sensitive to chemical and mechanical stresses than other populations and tend to lyse during sample preparation. This issue is reflected in the data in several manners: (1) fewer acinar cells detected in scRNA-seq data than in histological sections from matched samples, (2) acinar cell abundance reflecting the dissociation protocol used and (3) acinar cell lysates producing low-quality cDNA libraries due to large concentrations of RNAases and proteases contaminating the cell supernatant. Further, acinar cells express a small number of genes at very high levels. Therefore, acinar mRNA can be detected in other cell types, and it is important to account for this during data analysis.

Another critical limitation of most scRNA-seq studies is that the data reflect only a snapshot of that tissue or disease state. Orthogonal experiments can be done to track tissue dynamics,

but the order of events that lead to the observed cell states in the premalignant lesions, tumours or metastasis can only be indirectly inferred. Computational trajectory methods have been developed to order cells based on gene expression levels; however, the outcome of this analysis depends on the subsets of cells included. Thus, complementary experimental studies using lineage tracing are needed to infer the correct order of events during disease progression.

In summary, we anticipate that scRNA-seq technologies will improve in coming years, allowing for analysis of a greater number of cells at reduced cost, thereby supporting higher resolution and improved detection of cell types and states. In parallel, spatial transcriptomics and other multiomic methods that correlate gene expression, protein expression, the DNA mutational landscape, DNA methylation and chromatin accessibility will provide a deep mechanistic understanding of PDAC formation, progression and avenues for treatment.

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