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REVIEW ARTICLE

Genetics and Genomics

The application of single-cell sequencing in pancreatic neoplasm: analysis, diagnosis and treatment

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Pancreatic neoplasms, including pancreatic ductal adenocarcinoma (PDAC), intraductal papillary mucinous neoplasm (IPMN) and pancreatic cystic neoplasms (PCNs), are the most puzzling diseases. Numerous studies have not brought significant improvements in prognosis and diagnosis, especially in PDAC. One important reason is that previous studies only focused on differences between patients and healthy individuals but ignored intratumoral heterogeneity. In recent years, single-cell sequencing techniques, represented by single-cell RNA sequencing (scRNA-seq), have emerged by which researchers can analyse each cell in tumours instead of their average levels. Herein, we summarise the new current knowledge of single-cell sequencing in pancreatic neoplasms with respect to techniques, tumour heterogeneities and treatments.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant cancers, with a 5-year survival rate of 9% and nearly no improvement over recent decades [1]. PDAC is predicted to become the second major cause of cancer-related deaths in developed countries by 2030 [2]. The dominant cause of the poor survival rate is tumour heterogeneity, leading to delayed disease detection and limited effectiveness of systemic therapies [3]. High intratumor cell variability is commonly observed in PDACs, which results from a combination of genetic, epigenetic, and macro-environmental factors [4]. The loss of specific molecular markers and various genetic mutations in tumour cells make early diagnosis and standardised treatment very difficult. Another feature of PDAC is a complex matrix and stromal cells, including cancer-associated fibroblasts (CAFs) and immune cells. As a major component of the extracellular matrix, the deletion of CAFs shows dramatically opposite effects on PDAC progression; in some models, it accelerate tumour cell growth, while other models slow it [5]. Pancreatic cancer is an evolutionary disease that develops from precancerous lesions to carcinoma in situ and finally to metastatic disease. Noninvasive precursor lesions include pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN), and patients can achieve a favourable prognosis compared to PDAC if the disease is detected early enough. At present, because of the low incidence in an unselected population, screening for pancreatic cancer in an early stage is still difficult and impractical through existing techniques such as multidetector CT angiography using a dual-phase pancreatic protocol, MRI, endoscopic ultrasound, endoscopic retrograde cholangiopancreatography, serum biomarkers CA19-9, CEA, CA125 [6]. In sum, uncovering each subset cell type in pancreatic neoplasms will understand their heterogeneity from premalignant lesions to cancer, and clarifying the interaction between neoplastic or cancer cells with the tumour stroma and tumour microenvironment may bring us to a new era of pancreatic neoplasm diagnosis and treatment.

Traditionally, the most widely used techniques for gene-expression and molecular profiling analysis include quantitative PCR, microarrays, and bulk RNA sequencing, which influence the average transcriptome in all cells from whole bulk tissue. Thus, huge differences in gene-expression mutations and dysregulation between different subtypes of tumour cells can be covered, especially in specimens like PDAC with a high degree of cellular and transcriptomic heterogeneity [7]. The advent of single- cell sequencing has solved these limitations by revealing the transcriptome of every cell in the given sample at a high resolution and throughput [8, 9]. Single-cell sequencing provides the analysis of cellular heterogeneity, funding new subtypes of cells and cellular states, and elucidation of dynamic cellular transitions during tumour evolution and differentiation [10]. Thus, single-cell sequencing has had widespread application in the field of pancreatic neoplasm research.

In this review, we describe the single-cell sequencing workflow and techniques that have been using in pancreatic neoplasm research. Then, we summarise findings of cancer cellular heterogeneity, circulating tumour cells, cancer-associated fibroblasts, immune microenvironment, and pancreatic precancerous lesions

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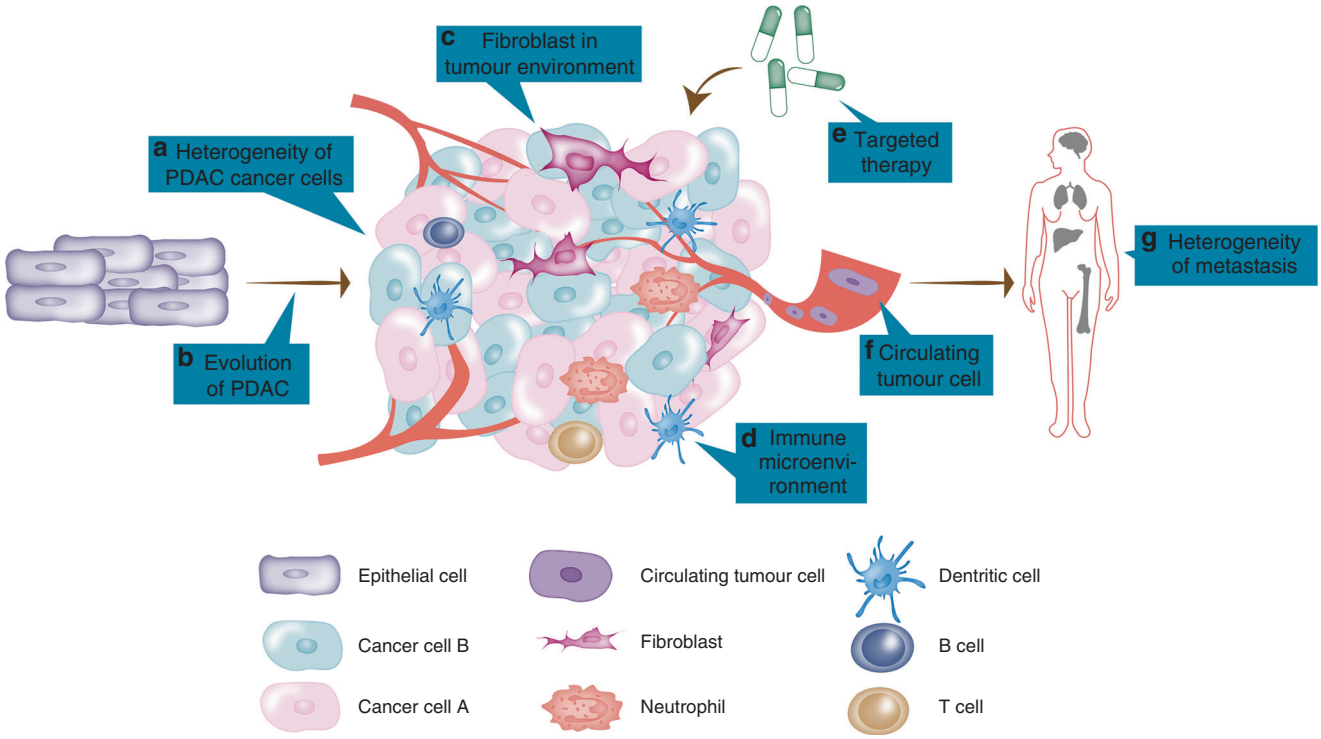

Fig. 1 Application of single-cell sequencing in PDAC studies. a Heterogeneity of PDAC cancer cells. **b** The evolutionary process from precursor lesion to PDAC. **c**, **d** Tumour microenvironment contains fibroblast and immune microenvironment. **e** Targeted therapy is becoming more and more important because of the poor prognosis of surgery resection. **f** The heterogeneity and process from CTCs to metastasis. **g** Heterogeneity of metastasis is related to lesion location and prognosis.

explored by single-cell sequencing, which are expected to increase our knowledge about pancreatic neoplasms and improve their diagnosis and prognosis (Fig. 1).

SINGLE-CELL SEQUENCING TECHNIQUES: THE PRINCIPLE AND PROGRESS

History and basic workflow

The first single-cell RNA sequencing was performed with only a single-mouse blastomere in 2009, and the researchers detected the expression of 75% (5270) more genes than microarray techniques and identified 1753 new splice junctions, which showed remarkable sensitivity compared with previous methods [11]. Since then, various single-cell sequencing techniques have been developed and widely used, particularly in cancer-associated research. Recently, single-cell sequencing has provided DNA, mRNA, and protein analysis, although each method has several differences in detail, all techniques have a common workflow: preparation of single-cell suspensions, single-cell capture and lysis, reverse transcription and amplification, library preparation, sequencing, and analysis [10] (Fig. 2). Despite single-cell RNA sequencing (scRNA-seq) is the fastest-developing and most widely used single-cell resolution method, the growing number of analysis methods (nearly 600 as of July 2022) become a barrier for novices to choose correct algorithms. In most cases, soft packages, such as Seurat, scanpy and SINCERA, can provide whole clustering processes, meanwhile, drawing on the methodology in high-quality papers is a viable approach. However, a further challenge is how to validate a correct method could be copied from one class dataset to other category datasets. In other words, datasets used to test a method are quite small, which means that the method may lack universality. This problem appears to be more prominent in PDAC analysis because the extremely complicated heterogeneity of PDAC acquires more accurate algorithms and parameters. We summarise a general and practical approach as a novice tutorial for scRNA-seq, and then we introduce several analysis techniques, which have been validated in PDAC research.

Single-cell capture and sequencing methods

The method of how to prepare single-cell suspension has already well reviewed [12], and the following important issue is choosing an appropriate capture method. Droplet-based and plate-based methods are two main strategies. The plate-based technology isolates cells into wells, while the droplet-based technology captures each cell through microfluidic droplet. The droplet-based technology is a high-throughput method but has more technical noise than the plate-based technology because of its low-sequencing depth [13]. Therefore, the plate-based method is preferable when samples have rare cell types.

Based on the strategy of sequencing methods, scRNA-seq protocols can be divided into full-length protocols (e.g., Smart-seq [14] and Smart-seq2 [15]), which attempt to sequence full-length for each transcript, and tag-based protocols (e.g., Drop-seq [16], MARS-seq [17] and CEL-seq2 [18]), which only sequence 3′-end or 5′-end transcripts and combine with unique molecular identifiers (UMIs) to reduce the technical noise. At present, the study of cell atlas is based on the method of UMI methods (e.g., drop-seq) because of the measurement of a large number of cells (>10,000). The sequencing depth is recommended about 1 million reads/cells. Cell atlas usually focus on cells classification and identification of marker genes, so the information provided by UMIs is sufficient. While if we are interested in more biological information to do transcriptome annotation, different splice forms quantification or sequence variants identification, adopting full-length protocols, especially the Smart-seq2, is more appropriate [19].

In conclusion, choosing strategies of different single-cell capture and sequencing methods depends on research targets and the number of cells.

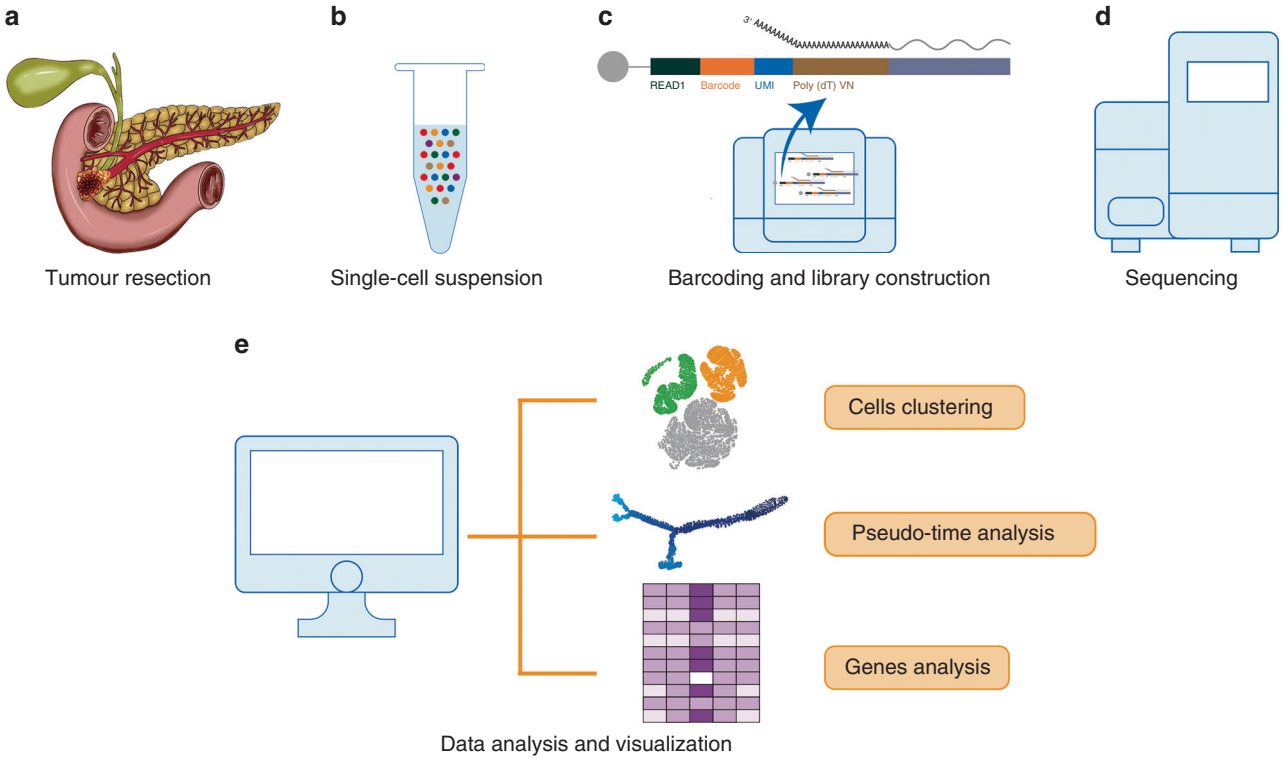

Fig. 2 The basic workflow of single-cell sequencing. a Gaining bulk fresh tumour samples through surgical resection. **b**–**d** First, we should prepare samples into single-cell suspensions, and next barcoding and library construction is the key step, finally sequencing on the computer. We can build the platform ourselves or choose the finished products that have been commercialised like 10× Genomics, Smart-seq2, Drop-seq and so on. **e** After sequencing, we have huge data to do all kinds of analysis, among which cells clustering, pseudo-time analysis, and genes analysis are the most basic. Meanwhile, a lot of new algorithms have been developed and single-cell analysis is the most active area.

Quality control and normalisation

After acquiring gene-expression matrix, the consequent step is quality control (QC), which applies a filter for removing low-quality cells and ensure that the data is sufficient for downstream analysis. However, adjusting the parameters repeatedly may be necessary when analysing data, because the judgement of whether data quality is good or not is just based on results of downstream analysis. Thus, it is beneficial to start with permissive QC. To remove the effect of count sampling and reduce technical and biological bias, normalisation and imputation is important. The most commonly used normalisation method is the linear, global scaling method, in which counts per million (CPM) is the simplest method, while Scran [20] has been confirmed to perform better for batch correction than other tested methods [21, 22]. The next type of technical bias is dropout. Several tools have been developed recently to reduce this noise, such as MAGIC [23], scVI [24], SAVER [25]. The batch effect is also the most common variation, which results from multiple collection and processing of samples in one experiment. This phenomenon is prominent in the 10× protocol or when merging data from different studies. The best way to circumvent batch effects is reasonable designs and consistent operations, while it is often difficult. Therefore, bioinformatics techniques including Harmony [26], seurat3 [27], BBKNN [28] are used for post-sequencing remediation.

Clustering and annotation

Clustering and annotation are the typically steps of any single-cell analysis. Before clustering, the first step is dimensionality reduction (DR). Although the gene-expression matrix normally contains more than 20,000 genes, many of these genes lack analytical value, for they have zero counts or similar expression values in all cells. Hence, selecting the top 1000 to 5000 highly variable genes for subsequent analysis is suitable for most of cases [29]. Then, the selected expression matrix changes into a low-dimensional space by performing specialised dimensionality reduction algorithms. Principal component analysis (PCA) is a typically liner approach, which is commonly used to investigate the performance of previous steps (e.g., QC, feature selection and normalisation). For visualisation purpose, the non-linear dimensionality reduction, including t-SNE [30] and UMAP [31], are the standard method.

Cell clustering is generally considered as a classical unsupervised machine learning problem. The recommended practice is that using κ-Nearest Neighbour approach to obtain a KNN graph, and then performing the Louvain algorithm [32] to cluster. The above steps can be implemented via the Seurat package. After a successful cell clustering, the upregulated genes were used for cluster annotation.

Although there is a standard process for cell clustering and annotation, it is still too difficult for novices to understand and complete it. First, the DR and resultant clustering are model- and parameter- depend, which means different models and parameters could gain different clustering results, and the annotation depends on the researcher's knowledge of molecular markers [33–35]. Therefore, cell types, especially rare but critical cell types, may be ignored by investigators without sufficient background knowledge. Second, the cell types are not actually characterised by single-cell but cluster, so re- or sub-clustering and re-annotation are very common but can be time-consuming.

To overcome the above problems, Peng Xie et al. [36] developed the first scRNA-seq analytical framework named "SuperCT", which employed artificial neural-network (ANN) structures as one of the options of SC and the learning algorithm. Super CT is more accurate and efficient than other algorithms. For example, when analysed a KPC mice dataset, the Seurat yielded 12 putative cell clusters, while SuperCT characterised a total of 17 cell

types and the primary epithelial tumour cells had been assigned to the 'Epithelial' type successfully. SuperCT has a continual learning mechanism, and its prediction is independent of the result of clustering or human interpretation. Meanwhile, insufficiency of cells and batch effect cannot impede the accurate characterisation of the cell types. However, SuperCT still has several drawbacks. The different cell-types identified by the UC method may be merged as one cell-types and some obvious clusters were categorised as 'unknown' by SC.

Recently, automated cluster annotation has become popular. The most common issue for automated cluster annotation is that none of them can provide an exactly correct annotation. For example, Scamp [37], a widely used approach, can assign new cells to a known type or reject them into a "unknown" type, while it is powerless to identify subclusters within this "unknown" category. Targeting these problems, Zanini et al. [38] developed an algorithm called 'northstar' to classify single-cell transcriptomes and discover new cell types. When analysed 1622 single cells from 11 pancreatic tumours, northstar annotated cell types differently and these differences were consistent with the biological characteristics of the samples as expected. scVI [24] is another popular package for single-cell analysis. scVI is around 100 times slower than northstar, and often splits the atlas clusters into different subcluster or mix them, when performed in the face of an incomplete atlas. On the contrary, clusters will never be spilt or merged by northstar. However, northstar may get inaccurate annotations when similar cell types separate insufficiently. For instance, the author found that northstar assigned NK cells to T cells. Therefore, although northstar shows a superior ability to assign cell types and higher accuracy than scVI and scmap, manual annotation adjustments are still required after automatic annotations.

However, it is sometimes difficult to distinguish normal cells from tumour cells by biomarkers and gene-expression profiles alone because they often express the same epithelial markers. The identification of aneuploid copy number profiles is another effective approach that can be observed in most human tumours (88%) [39]. Previous methods to calculate copy number profiles, including inferCNV [40] and HoneyBadger [41], involved low throughput and higher coverage depth, which was not suitable for single-cell sequencing data. To solve this problem, Ruli Gao et al. [42] developed an integrative Bayesian segmentation approach called copy number karyotyping of aneuploid tumours (CopyKAT), which can obtain a genomic copy number profile at a resolution of 5 Mb in high-throughput scRNA-seq data and delineate the clonal substructure. In this study, CopyKAT successfully identified aneuploid tumour cell clusters in 9717 single-cell transcriptomes from five individuals with PDAC. A remarkable limitation of CopyKAT is that some paediatric cancers and hematopoietic cancers have few aneuploid copy number events, which means CopyKAT may not be suitable. Meanwhile, CopyKAT cannot provide reliable copy number information due to the technical variability. This makes CopyKAT incorrect when analysing extremely rare subpopulations.

In summary, tons of algorithms have been developed for scRNA-seq, while all of them still face several challenges, including technical, biological and computational challenges [43]. Except for most common noise like dropouts, doublets and batch effect, technical confounders also contain mitochondrial RNA, ribosomal genes, sequencing depth and so on. This noise can mistakenly identify rare cell subclusters that have no biological significance. Sometimes, however, these differences may represent the actual state of cells. For example, dropouts usually result from low-sequencing depth, which leads to failure report of transcripts; on the other hand, the cell may not be transcribed and zero is the true state. Similarly, the biological signature such as cell-cycle phase, overall RNA content and cell size can confound clustering analysis. Although many algorithms are developed to handle these problems, which one should be chosen, how to adjust parameters and how to interpret analysis results still require the experience and subjective judgement of the researcher. In other words, there is no perfect programme, which is suitable for every task, while there still exists a universally applicable protocol, and some methods perform better for personality analysis of PDAC.

HETEROGENEITY OF PDAC CANCER CELLS

PDAC has obvious differences in resectable rates and chemotherapy resistance due to the intratumoral heterogeneity of PDAC cancer cells. Recently, two main PDAC types have been identified: one is the classical subtype, which has a higher resectable rate because of better differentiation, and the other is the basal-like subtype, which presents worse prognosis and loss of differentiation [44]. Although this binary classification was widely accepted, people developed more accurate classifications with the application of single-cell analysis.

Cancer cells in PDAC in situ

Genomic and transcriptomic studies revealed that various gene mutations occur in the PDAC progression, including KRAS, TP53, SMAD4, CDKN2A and other novel recurrent mutations [45]. These studies suggested that cancer cells in PDAC are not homogeneous but can be divided into different subclusters. Recently, Peng et al. [46] generated single-cell RNA-seq profiles from 24 PDAC tumour samples and 11 normal pancreases, and then identified 10 main clusters including ductal, acinar, fibroblast, and immune cells. They clustered two types of ductal cells named type 1 and type 2 cells. Type 1 ductal cells were demonstrated to be relatively normal ductal cells, whereas type 2 cells expended in PDAC only were malignant. Notably, type 2 ductal cells were highly heterogeneous and were further clustered into 7 subgroups through t-SNE analysis. Subgroups 3 and 7 could be detected in most patients. Subgroup 3 was the major and shared population in PDAC patients, while subgroup 7 was a low population but had important functions related to cell proliferation and the cell cycle. Juiz et al. [47] utilised three-dimensional ex-vivo culture [48] to obtain biopsy-derived pancreatic cancer organoids (BDPCOs) that only had pure epithelial transformed cells without the presence of stromal and immune cells. Four different cell clusters were identified and named C0 to C3 and the aggressiveness of the four clusters can be ordered from the most to the least as follows: C1, C0, C3, and C2.

Almost all studies have simply presented static atlas of tumour, but it is not satisfactory because dynamic evolutions of cancers may have more biological significance. Recently, Hosein et al. [49] demonstrated that phenotypic cancer cell heterogeneity is a late event and an ongoing acinar-to-ductal metaplasia state exists during the progression of PDAC. Specifically, early neoplastic KIC cells highly expressed normal pancreatic acinar genes, and cancer cells of late KIC downregulated normal pancreatic function genes and upregulated genes associated with ribosome, glycolysis/gluconeogenesis, and amino acid biosynthesis. This study provides more evidence from an evolution perspective, while there is still a lack of studies to uncover heterogeneity changes by comparing PDAC originating in different sites and before and after drug therapy [46].

Table 1 presents recent studies of the heterogeneity of primary PDAC cancer cells [46].

Heterogeneity of metastasis tumour

Multiple omics have validated that epithelial-mesenchymal transition (EMT) promotes tumour metastasis and therapy resistance both in PDAC [50] and other malignant tumours [51]. The high percentage of EMT^{+} tumour cells and basal-like or quasi-mesenchymal gene-expression subtypes in PDAC is associated with a worse clinical prognosis [52]. Lin et al. [53] was the first to

Table 1. Heterogeneity of primary PDAC cancer cells.

Reference	Samples	Number of clusters	Specific genes in each cluster	Gene functions
Peng et al. (2019) [46]	24 Human tumours	7	C1: Not listed	Detoxification
			C2: Not listed	Epithelial cell differentiation
			C3: Not listed	Translation
			C4: Not listed	Migration-related terms
			C5: Not listed d	Neutrophil activation
			C6: Not listed	Migration-related terms and GO terms
			C7: *CCNB1 CCNB2 MK167 TOP2A*	Cell cycle Cell proliferation
Moncada et al. (2020) [84]	2 Human tumours	2	C1: *TM4SF1*	Marker
			C2: *S100A4*	Marker
Juiz et al. [47] (2020)	20 Biopsy-derived pancreatic cancer organoids	4	C0: Low expression of other clusters' marker genes	
			C1: High expression: *PDE3A* *HFM1 DLG2* *SLCO5A1* Low expression: *INO80* *CSMD1*	Phosphodiesterase Helicase Encoding a solute carrier organic anion transporter. Encoding a component of the telomere nucleoprotein complex. A potential tumour suppression gene.
			C2: *NEAT1*	Regulating transcription of genes involved in cancer progression.
			C3: *ANKRD36/36C/36B*	Encoding cell cycle-regulated kinases involved in microtubule formation or stabilisation at the spindle pole during chromosome segregation.

This table summarises recent studies of the heterogeneity of primary PDAC cancer cells.

perform scRNA-seq on fresh biopsies from PDAC metastasis and primary tumours. Despite interpatient differences, tumour cells can be clustered into two major subclusters: epithelial cells, which expressed epithelial cell adhesion molecule (EpCAM) and cytokeratin 19, and cells with EMT characteristics. Based on the same dataset, Xu et al. [54] clustered five types of ductal cells, in which type 5 ductal cells were significantly related to EMT. The type 5 cells expressed marker genes REG4 and CEACAM 5. Through cell-cell communication and EMT, these cells had a better metastasis and therapy resistance capacity. These researches provided robust evidence again that patients with a high number of EMT^{+} cells have a poorer survival rate. However, previous studies have showed that the majority of pancreatic metastasis cancer are epithelial [55], and it has no effect on PDAC metastasis in mice with the depletion of EMT driver [56]. Considering EMT is not a simple binary process, but instead a plastic continuum of partial EMT (pEMT), Carstens et al. [57] found that the human and KPC mice PDAC cancer cells was a continuum of over 50 different EMT phenotypes though scRNA-seq. Then, they demonstrated that inhibition of EMT leads to epithelial stabilisation, which results in promotion of tumour metastasis. There are two explanations for this paradox, one is the metastasis goes through the EMT and TME processes successively [58, 59], the other is cancer cells at the metastasis sites have never undergone EMT [56].

Studies on the bulk tissue and cell lines suggested that MEG3, a LincRNA, was lowly expressed in PDAC and it can inhibit tumour progression. Nonetheless, Pan et al. [60] demonstrated that MEG3 was highly expressed in a distinct metastatic cancer cell cluster, and MEG3 enhanced PDAC metastasis. The authors considered that PDAC tissues have complex cell components while cell lines cannot fully mimic the PDAC features. The advantages of single-cell sequencing will be better demonstrated.

IMMUNE MICROENVIRONMENT OF PDAC

Neither traditional surgical approaches nor multidisciplinary can contribute to long-term prognosis [61]. One of the major reasons is that the complex immune microenvironment contains macrophages, T cells, B cells, natural killer cells, dendritic cells and so on. Similar to cancer cells, heterogeneous immune cells play a role in tumour occurrence and development, which is a potential therapeutic breakthrough point. Table 2 shows the heterogeneity of immune cells of PDAC.

T cell

Chen et al. [62] identified 11 distinct T-cell types based on specific cell markers, including CD8 + T cell, CD4 + T cell, Th1/2 cells, cytotoxic T cells, effector T cells, Tregs, exhausted T cells, and memory T cells. DUSP4 (a member of the dual specificity protein phosphatase subfamily) is a significant marker for the diagnosis and prognosis of PDAC that shows a unique expression pattern in Tregs and exhausted T cells. Analysed by TCGA database revealed that the patients with high expression levels of DUSP4 had obviously worse survival. The major dynamic changes in PDAC were that the proportion of cytotoxic T cells, effector T cells, Tregs, exhausted T cells, and memory T cells increased notably with the progression of PDAC. Moreover, pseudo-time analysis showed that the order in which T-cell subtypes evolved was naive T cells, effector T cells, Tregs, and exhausted T cells, which demonstrated that the T-cell state transition from activation to suppression and exhaustion gradually with PDAC worsen. Peng et al. [46] also

Table 2. Heterogeneity of immune cells of PDAC.

Reference	Samples	Cell type	Number of clusters	Specific genes in each cluster	Gene functions
Peng et al. (2019) [46]	24 Human tumours	T cell	5	C1-CD8: *GZMA GZMH*	Cytotoxic markers
				C2-CD4: *CCR7 SELC*	Homing markers and representing naive CD4 T cells.
				C3-CD4: *FOXP3 IL2RA*	Treg signature genes
				C4-CD4: *SELC IC7R*	Representing central memory T cells.
				C5-CD8: *GZMA GZMH CENPA CENPE*	Cytotoxic markers Cell-cycle related genes
		Macrophage	5	C1: *HLA CD74*	ClassIIantigen presentation
				C2: *COL1A*	ECM deposition and remodelling
				C3: *MDSC*	Chemokine gene for the migration of myeloid- derived
				C4: *CXCL9 CXC10*	Chemokine genes for infiltration of cytotoxic T cells.
				C5: *S100A8 S100A9*	Generating pro-inflammatory cytokines
Moncada et al. (2020) [84]	2 Human tumours	Macrophage	2	C1: *IL1B*	Corresponding to inflammatory M1 state
				C2: *CD163 MS4A4A*	Resembling the M2alternatively activated state.
Lin et al. [53] (2020)	10 Human tumours	Lymphocyte	2	C0: *TIGIT CTLA4 PDCD1 HAVCR2 LAG3 LAYN*	Exhaustion markers
				C1: Not listed	Representing naive-like T cells.
Chen et al. (2021) [62]	21 Human tumours	T cell	11	*Tregs*: *FOXP3 TNFRSF18 CTLA4 TIGIF LANY*	Regulating T cells activation, negatively regulating cytokine production and inflammatory response.
		B cell	2	plasmocyte and memory B cells: *CD27*	Markers
				Naive B cell: *CD27*	Lacking of *CD27* expression
Elyada et al. (2019) [73]	6 Human tumours	T cell	5	Tregs: *CTLA4 TIGIT*	immune-inhibitory molecules
				tumour-derived T cells: *LAG3 EOMES*	Suggesting that tumour-derived T cells had become exhausted after an initial activation phase.
		Macrophage	3	Alternatively activated macrophages: *PP1 LY6E MARCO*	Associated with a non-inflammatory, immune-suppressive phenotype of macrophage activation
		Dendritic cells	3	"Langerhans-like" DCs: *CD1A CD207*	Markers
				conventional DCs: *IDO1*	Catalyzeing tryptophan depletion and kynurenine production

In this table are summarised the heterogeneity of immune cells of PDAC.

observed two clusters of $CD8^+$ T cells and three clusters of $CD4^+$ T cells. In contrast to the high level of proliferative ductal markers, the expression level of T-cell markers was low, and both the existence of proliferative ductal cells and loss of T-cell activation resulted in poor prognosis. However, C5-$CD8^+$ cells, which highly expressed cell-cycle related genes still had proliferating capacities. As the PDAC progresses, both the proportion of Treg [62] and the expression of CTLA4 in Tregs [63] are increased. The number of intratumoral T cells in PDAC, which were treated with anti-CTLA4 antibody was 5 times that of the control group [63]. Compared with primary cancers, there was no specific T-cell subcluster with unique function in metastasis cancers, because T cells from primary cancers and metastasis cancers were mixed together in study by Wei Lin et al. [53].

B cell

In the early stage of PDAC, no infiltration of B cells was found, while six distinct subclusters were identified in the PDAC progression stage. This discovery suggested that B cells infiltrated the tumour microenvironment gradually and evolved into different subtypes to play protumor or antitumor functions, respectively. Interestingly, regulatory B cells were absent and two main forms of B cells in six populations were plasmacytes and memory B cells, which highly expressed CD27 [62]. In the KRC mice, Hosein et al. [63] observed that a small number of B cells presented in early tumours and a large amount of plasma cells in KRC moribund stage, while there were only a few plasma cells in moribund KPC mice. These data indicate that the expansion and maturation of an adaptive B cell response is diverse in patients and different mouse models.

Macrophage

The canonical binary classification of macrophages includes classically activated macrophages (M1) and alternatively activated macrophages (M2) [64]. However, more subpopulations of macrophages have been uncovered by a variety of techniques, especially single-cell sequencing. Kai Chen et al. [62] revealed 4 distinguishable macrophage subpopulations, including M1/M2 macrophages, tumour-associated macrophages (TAMs), $CD169^+$ macrophages and TCR^+ macrophages. The proportion of M1 macrophages, M2 macrophages and TAMs increased with the development of PDAC; M1 macrophages were antitumor components, while, M2 macrophages and TAM are protumor components. This phenomenon suggests that PDAC has a complex and contradictory immune microenvironment. In contrast to tumour-infiltrating lymphocytes (TILs), Wei Lin et al. [53] found that macrophages from primary and metastatic tumours clustered separately. Macrophages from primary tumours exhibit characteristics of M2-like macrophages, which highly express genes related to the cellular matrix and late stage of the wound healing-related process. Meanwhile, macrophages in the metastasis expressed genes related to antigen-presenting function, such as CD74, FCER1G and MHC I/II-related genes.

Myeloid-derived suppressor cell

Myeloid-derived suppressor cells (MDSCs) are a group of immune cells that originate from the bone marrow and suppress immune response in various diseases including PDAC [65]. Previous studies demonstrated that over expression of CXCL5, which is a pivotal chemokine to mediate the cellular composition [66], was associated with a poor prognosis in PDAC. Hosein et al. [63] identified that MDSCs were unique population of expressing CXCL5 receptor and CXCR2 in both the KRC and KPC GEEMs. Inhibition of CXCR2 increased T-cell infiltration and sensitisation to ICB. Chen et al. [67] found a specific MDSC subtype, which expressed high levels of CD206, F4/80, arginase-1, CCL2, and interleukin-18. These MDSCs were recruited in the low stromal Col1 microenvironment and induced the expression of CXCL5 and finally contributed to immunosuppressive microenvironment in PDAC. Meanwhile, Lee et al. [68] uncovered a type of macrophages marked by migration inhibitory factor (MIF). These macrophages promoted myeloid-derived suppressor cell-mediated immunosuppression. These findings establish possible logical links between macrophages infiltration and MDSCs in PDAC.

FIBROBLASTS IN TUMOUR MICROENVIRONMENT

There is a consensus that fibroblasts especially cancer-associated fibroblasts (CAFs) play an important role in tumour aggressiveness and resistance to chemotherapy. CAFs are involved in the formation of dense stroma in the tumour microenvironment (TME) [69], and release cytokines and growth factors that regulate the growth of pancreatic cancer cells and infiltration of immune cells into the TME [70]. However, with more in-depth researches, the exact functions of CAFs in PDAC continue to be puzzling. In some PDAC models, depletion of a specific CAF subpopulation improves antitumor immunity and suppresses PDAC progression [71], whereas in other models, depletion of CAFs accelerates PDAC progression [72]. The major reason is the heterogeneity of mesenchymal cells; therefore, researchers applied scRNA-seq to address this tissue.

In previous studies, researchers demonstrated two distinct subtypes of CAFs in PDAC: myofibroblastic CAFs (myCAFs) that expressed high levels of αSMA, and inflammatory CAFs (iCAFs) that expressed high levels of cytokines and chemokines. Elyada et al. [73] found a new subcluster named "antigen-presenting CAFs" (apCAFs), which expressed genes belonging to the MHC class II family. Functions of apCAFs were enriched in the pathways of antigen presentation and processing, fatty acid metabolism, MYC targets and MTORC1 signalling. Hosein et al. [63] also observed this unique fibroblast population in KIC mice. In the latest research, Huang et al. [74] demonstrated that apCAFs cannot induce full CD4 + T cells activation and clonal expansion, but induce naive CD4 + T cells into Tregs through IL1 and TGF-β signalling pathways, because apCAFs lack co-stimulatory molecules. Therefore, it is reasonable to infer that apCAFs can promote tumour growth. New evidence revealed that apCAFs originate from mesothelial cells, and wound-associated tumour paracrine signals from the tumour niche induce the transition from mesothelial cells to apCAFs, while this transition can be inhibited by an anti-mesothelin antibody [74]. In addition, when cultured in a two-dimensional monolayer, apCAFs lost their MHC II expression and upregulated myCAFs markers [73]. These findings indicate that apCAFs are a dynamic fibroblast population and require environmental cues to be maintained as a subpopulation. Similarly, there is also a debate whether fibroblast activation protein $(FAP)^+$ CAFs and $αSMA^+$ CAFs are the same population. McAndrews et al. [75] identified that they were distinct subpopulations in both human PDAC samples and transgenic mouse models. They found the $αSMA^+$ CAFs restrained PDAC, while FAP^+ CAFs promoted PDAC. In addition to the heterogeneity of cell types, different extents of desmoplasia also affect prognosis and response to therapy. In previous studies, low desmoplasia has been shown to contribute to poor prognosis [76], while others found low density of stroma benefited patients [77]. Wang et al. [78] observed a novel subtype of CAFs with a highly activated metabolic state (meCAFs) in loose type PDAC. meCAFs was a double-edged sword, which led to poor clinical outcomes in PDAC patients but improved immunotherapy responses. Dominguez et al. [79] also identified a specifical TGFβ-driven CAFs, which specifically expressed $LRRC15^+$, and $LRRC15^+$ CAFs next to the tumour islets were observed to promote tumour growth. This phenomenon is consistent with a previous study showing that TGFβ-associated stroma is correlated with poor prognosis [80, 81].

Table 3 summarises the heterogeneity of fibroblasts in the tumour microenvironment.

Table 3. Heterogeneity of fibroblasts in tumour microenvironment.

Reference	Samples	Number of clusters	Specific genes in each cluster	Gene functions
Chen et al. (2021) [62]	11 Human tumours	3	Classical CAFs: *COL1A1 LUN1 MMP11 FAP SFRP2*	Involving in extracellular matrix deposition.
			csCAFs: *C3 C7 CFB CFD CFH CFI*	Regulating immune and inflammation response.
			Pancreatic stellate cells: *RGS5 ADIRF CRIP1 NDUFA4L2 NOTCH3 PDGFA*	Markers
Elyada et al. (2019) [73]	6 Human tumours	2	ICAFs: *IL6 IL8 CXCL1 CXCL2 CCL2 CXCL12*	Markers
			myCAFs: *aSMA*	Markers
	4 KPC tumours	3	iCAF: *Col14a1*	Encoding hyaluronan synthase Has1 and specific collagens
			myCAFs: *Acta2 Tagln Igfbp3 Thy1 Col12a1 Thbs2*	Smooth muscle genes Markers
			apCAFs: *H2-Aa H2-Ab1 Cd74 Saa3 Slpi*	Belonging to the Major Histocompatibility Complex (MHC) class II family. implicated as a pro-tumorigenic factor in pancreatic. identified as a pro-inflammatory gene.
Lin et al. (2020) [53]	10 Human tumours	3	C0: *POSTN MMP11*	Representing myofibroblasts.
			C1: not listed	Associated with quiescent CAFs.
			C2: *RGS5 NOTCH3 CSRP2*	Resembling smooth muscles cells.

This table summarises the heterogeneity of fibroblasts in the tumour microenvironment.

SCRNA-SEQ COMBINES WITH SPATIAL TRANSCRIPTOME

Although single-cell sequencing has unprecedented resolution, the disintegration of tissue before sequencing results in the loss of spatial information, which is critical to our understanding of cellular interactions. More recently, the spatial transcriptomics (ST) method [82] can provide an unbiased map of expressed transcripts in a given cryosection. However, ST also has a main limitation in that ST has a low resolution: each ST spot contains ~10–200 cells depending on the different tissues [83]. Aiming to address each approach's limitation, Reuben Moncada et al. [84] developed a key method "multimodal intersection analysis (MIA)" to integrate scRNA-seq and ST datasets. MIA computed the overlap between each pair of cell type-specific and tissue region specific gene sets, and then, a hypergeometric test was performed to assess significant enrichment or depletion. Finally, an MIA map was generated with robust utility to provide spatial and functional annotations for the scRNA-seq-clustered cell populations. MIA was further applied in the identification and mapping of cell type subpopulations across tissue regions. Four ductal subpopulations were found in PDAC; however, only the hypoxic and terminal ductal cell populations were significantly enriched in the cancer region. By mapping distinct malignant populations across PDAC tissue sections and deconvolving each cell state relationship in the tumour microenvironment, MIA ultimately uncovered a relationship between cancer cells that express a stress-response gene module with inflammatory fibroblasts. Thus, MIA can provide a more comprehensive characterisation of cell types regarding their native environment and mutual relations.

Intersecting scRNA-seq with ST capture sites is sufficient to identify normal or cancerous areas and infer cell types; however, it still lacks the resolution of single cells and capture locations are cellular heterogeneous in many times. To address these limitations, Elosua-Bayes et al. [85] developed SPOTlight, a deconvolution algorithm that utilises a nonnegative matrix factorisation (NMF) regression algorithm and adds prior information to the model to improve sensitivity and robustness. Moreover, SPOTlight can unveil cell composition at each capture location by using nonnegative least squares (NNLS), and a unit-variance normalisation step enables it to analyse both paired and unmatched ST and scRNA-seq data. Performing SPOTlight on the Peng's [46] dataset, they also detected the hybridisation of two types of tumour cells in a cancer region and enrichment of ductal cells with a hypoxia gene signature. Moreover, SPOTlight shed light on the distribution of immune cells in the tumour sections and clearly showed that PDAC also had striking segmentation of immune cell states similar to the regional distribution of normal and cancer cells. Preexhausted CD8 + T cells and activated CD4 + T as well as pro-inflammatory TAMs and proliferative CD8 + T cells were significantly increased. The above example illustrates the beneficial effect of SPOTlight and that can be used to detect the cell type location and composition of ST spots.

CIRCULATING TUMOUR CELLS

Compared with canonical screening tests such as fine-needle aspiration biopsy (EUS-FNA) and imaging diagnosis, liquid biopsy including circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) has huge potential for early diagnosis of pancreatic cancer. CTCs escape from the primary tumour into the bloodstream ultimately resulting in distinct metastasis. Consistent with primary or metastatic cancer cells, CTCs show great heterogeneity. However, CTCs are extremely rare in the peripheral circulation, with an estimated one to ten CTCs per 10^8 normal blood cells in a millilitre of peripheral blood [86]. Meanwhile, CTCs have low expression of EPCAM, which makes it difficult to isolate and identify this rare population. To overcome these limitations, scientists developed single-cell analysis and other supporting techniques to investigated CTCs of PDAC.

To better gather and isolate circulating tumour cells from KPC mice, David et al. [87] applied an inertial focusing-enhanced microfluidic device (CTC-iChip), which effectively eliminated normal blood cells, and enhanced the CTC concentration to 118 CTCs/ml. Finally, they generated a total of 75 CTCs of sufficient quality for further analysis and found that they formed three major CTC clusters named "classical CTC" (CTC-c), CTC-plt and CTC-pro. CTC-c had highest percentage (55%), while CTC-plt (32%) and CTC-pro (13%) were characterised by platelet derived markers and proliferative signatures, respectively. Further analysis of CTC-c showed that two sets of genes were highly expressed compared with primary tumours; one set was stem cell-associated genes such as Aldh1a2, Klf4, Igfbp5 and Dcn, and the other was extracellular matrix (ECM) genes, especially core matrisome protein (SPARC) genes. In addition, CTC-c cells lost the epithelial markers E-cadherin (Cdh1) and Muc1. These transcript changes contributed to CTC generation, survival, and metastasis. Similarly, Morten Lapin et al. [88] used a multimarker negative depletion strategy [89] to analyse CTCs from human peripheral blood and generated epithelial-like CTC clusters (CTC-E) and mesenchymal-like CTC clusters (CTC-M). High SPARC expression was also detected in all CTCs, and notably, CSC markers, including CD24, CD44, and ALH1A1, were highly expressed and related to high metastatic potential. After combining and analysing publicly available datasets of CTCs, Lei Zhu et al. [90] found the novel biomarker GAS2L1. In the murine pancreatic CTC dataset GSE51372-75, GAS2L1 successfully identified 70 cells from a total of 73 cells, and the sensitivity was 93.3%. Meanwhile, in the human pancreatic CTC dataset GSE60407-7, 3 cells were identified from 7 cells. This result indicated that, similar to other known CTC biomarkers, GAS2L1 can identify only a subset of CTCs. However, when combined with another marker, EPCAM, the sensitivity distinctly increased to 97.3% and 100%, respectively. Thus, GAS2L1 combined with EPCAM may be a potential strategy in identifying pancreatic CTCs. Dimitrov-Markov et al. [91] observed high expression of antiapoptotic genes, including BIRC5 (survivin), which plays an important role in the early stage of cancer cell culture. Further studies found that both BIRC5 knockdown and the BIRC5 inhibitor YM155 can induce cell death, decrease tumour burden, and improve prognosis.

PRECURSOR LESIONS OF PDAC

Surgical resection remains the only curative option for pancreatic cancer. However, only 10-15% of newly diagnosed patients have opportunities for surgery [92]. PDAC is an evolutionary disease that takes ~11 years for PDAC precursor lesions to evolve into infiltrating cancer and an additional 6.8 years to evolve into distant metastasis [93]. Therefore, the patient has enough time to be operated on before the disease becomes malignant and gains a prominently enhanced prognosis. scRNA-seq is a powerful method that can be used to profile the complex evolution processes for precursor lesions in PDAC and find new potential markers and therapeutic targets.

Intraductal papillary mucinous neoplasms (IPMNs)

IPMNs, the most common cystic neoplasms, are a bona fide precursor to PDAC [94]. Most IPMNs harbour low-grade dysplasia (LGD) and high-grade dysplasia (HGD), and can even harbour invasive components (PDAC). Bernard et al. [95] analysed pancreatic tumours in different stages, including 2 PDACs, 2 HGD-IPMNs, and 2 LGD-IPMNs. LGD lesions present with a small population of cells with high expression of proliferation-related genes; 8.9% of epithelial cells had the same transcript and phenocopy features as HGD-IPMNs, and 1.2% of cells presented with features of pancreatic cancer. As the disease became more malignant, the proportion of cytotoxic T cells, $CD4^+$ T cells, and B cells decreased, while the proportion of myeloid-derived suppressor cells increased. This study indicated that not only neoplastic cells but also the microenvironment play a role in pancreatic tumour evolution. Yuko Kuboki et al. [96] observed that IPMNs had significant intratumoral heterogeneity after analysing 10 IPMNs. Seven IPMNs shared KRAS and/or GNAAS mutations, while 2 IPMNs had two different KRAS mutations, which suggests that IPMNs had multiple independent origins and genetic heterogeneity. Meanwhile, in addition to KRAS and GNAS mutations, three IPMNs presented with RNF43 mutations, and one IPMN harboured three distinct ARID1 mutations. Therefore, the mutation of KRAS and GNAS is an early event resulting in IPMNs, and other gene mutations, such as RNF43 and ARID1A, induce IPMNs to transform into malignancies.

Acinar to ductal metaplasia

A previous study demonstrated that acinar to ductal metaplasia leads to the formation of pancreatic intraepithelial neoplasia (PanIN) and is a principal mechanism for PDAC formation [97]. Schlesinger et al. [98] created posttamoxifen injection (PTI) mice at six different time points by injecting tamoxifen into PRT mice. They defined mice after 3 months PTI or later as later-stage samples because clear PanINs can be observed. In later-stage pancreases, three distinct epithelial cell types were clustered, including acinar cells, ductal cells, and metaplastic cells. Metaplastic cells expressed high levels of acinar cell markers, such as tdTomato, but expressed low levels of classical acinar enzymes. Compared with acinar and ductal cells, transcription factors (TFs) correlated with tumour growth were significantly and highly expressed in metaplastic cells, including Id1, Id3, Runx1, Onecut2, and Foxq1 [98]. Notably, Onecut2 and Foxq1 were only expressed in late metaplastic cells and correlated with worse outcomes in PDAC patients. Finally, compared with each stage of cells, researchers found that Marcksl1, Mmp7, and Igfbp7 were potential biomarkers for early PDAC diagnosis.

THE ROLE OF SCRNA-SEQ IN TUMOUR THERAPY

Although surgical resection is the only cure for pancreatic cancer, the rate of resection and prognosis of PDAC are disappointing. To identify new therapeutic targets, scRNA-seq has been widely used to uncover mechanisms for the development and metastasis of PDAC.

Combination with ICPis

Previous studies have demonstrated that immune checkpoint inhibitors (ICPis) have extensive efficacy in many malignancies; however, they have not shown activity in PDAC [99, 100] and may be related to the tumour macroenvironment (TME), especially immune cells, in PDAC [101, 102]. Pan et al. [103] discovered that PDAC patients with high tumour-infiltrating macrophages and high tumour expression of CD47 had poor clinical outcomes. Researchers applied anti-treatment in syngenetic mouse models and found that CD47 blockade alone suppressed tumour growth in Panc02 cells but not MPC-83 cells. Interestingly, when combined with anti-PD-L1, the tumour burden decreased in MPC-83 cells. Their scRNA-seq data demonstrated that intratumoral macrophages and lymphocytes were obviously remodelled by anti-CD47 treatment. In all models, pro-inflammatory macrophages were increased, and anti-inflammatory macrophages were reduced, while several key immune activating genes that were highly expressed in MPC-83 only included *Arg1, Pdcd1, Gzmb, Nos2, and Ifit3*.

Similarly, PDAC models are resistant to CDK4/6 inhibitors, although KRAS, which dominates pancreatic cancer gene mutations, influences the cell cycle through the activation of CDK4/6 kinases [104–106]. Knudsen et al. [107] found that the combination of MEK inhibitors enhanced the response to CDK4/6 inhibitors

in PDAC. Through scRNA-seq, they uncovered that this combination had an obvious effect on altering myeloid populations and inducing T-cell infiltration. This finding suggests that this combination treatment potently cooperates with ICPis in RAS-driven PDAC.

Controlling metastasis

Dimitrov-Markov et al. [91] used scRNA-seq and found that CTCs highly expressed *BIRC5* (survivin), which is a member of the inhibitor of apoptosis protein (IAP) family. Then, they demonstrated that the survivin inhibitor YM155 or survivin knockdown decreased the metastatic burden in PDX models. In addition, cancer cells highly expressed REG4 and CEACAM5 may respond to TGF-β inhibitors [54], since TGF-β molecular blockaders have been demonstrated to reprogramme the contexture of TME and reshape the anti-cancer immunology [81, 108].

Radiofrequency ablation

Radiofrequency ablation (RFA) is an effective therapy for metastasis because RFA may release tumour antigens that elicit a systemic adaptive immune response against tumours [109].

However, the changes in the non-RFA sides after RFA treatment are still unknown. Fei et al. [110] found that the formation of immune cells in non-RFA tumours changed sharply, leading to decreased immunosuppressive cells and increased functional DCs, CD4 +, and CD8 + T cells. Unfortunately, the infiltration of CD8 + PD-1 + T cells increased, and the expression of PD-1 and LAG3 was upregulated at the same time. These data suggested that RFA combined with immune checkpoint inhibitors may overcome T-cell exhaustion and enhance the efficiency of RFA in distant tumours.

CONCLUSION

Pancreatic neoplasms, especially PDAC, remain the most complex and malignant disease due to their high heterogeneity. Single-cell sequencing benefits from its single-cell resolution and has become the most appropriate approach to uncover the underlying mechanisms of PDAC. Several frameworks and algorithms of single-cell sequencing have been applied, such as CopyKAT, MIA, and SPOTlight, among which scRNA-seq combined with the spatial transcriptome has presented more obvious advantages. scRNA-seq enables rapid determination each cellular gene-expression patterns of thousands of individual cells. By using this emerging sequencing technology, scientists have revealed high heterogeneities in cancer cells, the tumour environment, circulating tumour cells, and the progression from precancerous lesions to PDAC. Immune checkpoint inhibitors (ICPis) have shown cooperates in many cancers; however, PDAC is still resistant to them. Recent studies found that a few new targets and inhibitors had an obvious ability to suppress PDAC growth when they were combined with ICPis. In conclusion, single-cell sequencing provides us with novel insights into intratumoral heterogeneity, subclusters identification, unique genes mutations, and dynamic evolution in PDAC. However, some shortcomings limit its large-scale use. As a high-throughput protocol, scRNA-seq only captures a fraction of molecules, ranging from 5% to 20% RNA physically present in cells. Meanwhile, enzymes used to obtain single-cell suspensions can degrade RNA, which is more pronounced in PDAC. The batch effect is frequently observed as samples are collected at different periods. These technical noises mentioned above generate bias and make it more difficult to differentiate rare cell types. Meanwhile, there are many algorithms and paraments for single-cell analysis that make novices too difficult to choose a correct combination. Another limitation is the expensive cost of experiments. Ziegenhain et al. [19] summarised a clear list of cost of each scRNA-seq protocol.

DATA AVAILABILITY

Not applicable.

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AUTHOR CONTRIBUTIONS

All authors were involved in acquired data, interpreted the results, reviewed literature, drafted and revised the manuscript and agrees to be accountable for all aspects of the work.

COMPETING INTERESTS

The authors declare no competing interests.

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Not applicable.

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ADDITIONAL INFORMATION

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