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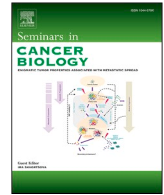

Organoids, tissue slices and organotypic cultures: Advancing our understanding of pancreatic ductal adenocarcinoma through in vitro and ex vivo models

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) has one of the worst prognoses of all common solid cancers. For the large majority of PDAC patients, only systemic therapies with very limited efficacy are indicated. In addition, immunotherapies have not brought the advances seen in other cancer types. Several key characteristics of PDAC contribute to poor treatment outcomes, and in this review, we will discuss how these characteristics are best captured in currently available *ex vivo* or *in vitro* model systems. For instance, PDAC is hallmarked by a highly desmoplastic and immune-suppressed tumor microenvironment that impacts disease progression and therapy resistance. Also, large differences in tumor biology exist between and within tumors, complicating treatment decisions. Furthermore, PDAC has a very high propensity for locally invasive and metastatic growth. The use of animal models is often not desirable or feasible and several *in vitro* and *ex vivo* model systems have been developed, such as organotypic cocultures and tissue slices, among others. However, the absence of a full host organism impacts the ability of these models to accurately capture the characteristics that contribute to poor outcomes in PDAC. We will discuss the caveats and advantages of these model systems in the context of PDAC's key characteristics and provide recommendations on model choice and the possibilities for optimization. These considerations should be of use to researchers aiming to study PDAC in the *in vitro* setting.

1. Modelling pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death, with similar incidence and mortality rates [1]. Overall survival is low around 8–13 % and surgical resection of the primary tumor is the only hope for cure [2,3]. However, this is only possible in about 15–20 % of cases, and most patients have advanced disease at diagnosis [4]. In addition to a typically late diagnosis, PDAC is hallmarked by several key characteristics that further contribute to poor treatment outcome. A large majority of PDAC tissue is composed of non-tumor cells collectively known as the tumor microenvironment (TME) [5]. The cellular composition of the pancreatic TME is diverse, comprising cell populations such as cancer-associated fibroblasts (CAFs), endothelial cells, adipocytes, cancer stem cells (CSCs), immune cells, and neurons. This desmoplastic microenvironment with unique mechanical and immunosuppressive features, also has a high degree of

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intra- and intertumoral heterogeneity. Therefore, new therapeutic strategies are needed to address or circumvent these features and to improve survival rates. To experimentally address these factors, it is important that the experimental models used in translational research accurately reflect the characteristics of the primary tumor (Table 1). *In vitro* models are established from isolated cells or tissues, while *ex vivo* models use intact tissues or organs that preserve their natural function. Both are maintained in an artificial environment with controlled temperature, pH, and nutrients to support growth [6–8]. This review will discuss the advantages and limitations of *in vitro* model systems in relation to the key aspects of PDAC and provide recommendations for model selection and optimization, which overcome the ethical implications of using vertebrate models in research, whilst providing an excellent alternative or complementary system to these models [9].

1.1. Adherent cell lines and spheroids

Two-dimensional (2D) cell cultures, including both immortalized and primary cell lines, have been widely utilized in cancer research. Immortalized cell lines offer several advantages, such as homogeneity, unlimited proliferative capacity, and ease of reproducibility, making them ideal for high-throughput drug screening assays [10]. On the other hand, primary cell lines (not grown for many passages after isolation) more accurately reflect the original tumor heterogeneity observed between patients and do not present the genetic drift typical of immortalized cell lines, even though they are more difficult to establish and maintain [11]. More than 500 PDAC cell lines are available (https://www.cellosaurus.org/), of which 49 have been analyzed phenotypically and genotypically [12,13]. Unfortunately, 2D models lack the three-dimensional (3D) structural organization of primary tumors and consequently do not accurately mimic patient therapy responses or the interactions between tumor cells and their microenvironment. For example, 3D spheroids generated from human cancer cell lines are tunable and easy to maintain. At the same time, they lack the tissue architecture and clonal and genetic heterogeneity of PDAC.

1.2. Organoids and cocultures

Tumor organoids are *ex vivo*, three-dimensional models derived from fresh primary tumor tissue that reproduce the essential biological characteristics of the original tumor [14]. Patient-derived organoids generated by physical dissociation and enzymatic treatment of tissue can be established from resected primary and metastatic tumors, ascites or rapid autopsy specimens [15]. Furthermore, organoids can be expanded *in vitro* for several months and cryopreserved, like conventional cell lines, providing models for translational and personalized medicine. The complexity of organoid culture may be enhanced by co-culture with cells from the TME, such as CAFs and immune cells, recapitulating the essential biological characteristics of the primary tumor.

Hans Clevers' pioneering work with organoids showed that intestinal crypt-villus units can self-organize from a single $Lgr5^+$ stem cell, maintaining hierarchy and producing all differentiated cell types without a non-epithelial niche [16]. Furthermore, long-term culture with nicotinamide, an ALK inhibitor, and a p38 inhibitor enabled the development of organoids with $Lgr5^+$ progenitors that differentiate into both ductal and endocrine cells, demonstrating their bi-potentiality [17,18]. David Tuveson's group further contributed to developing PDAC organoid models from both normal and neoplastic tissues, effectively replicating tumorigenesis from early neoplasms to invasive carcinomas for studying disease progression [14]. Since then, optimized 3D culture conditions have been established to generate patient-derived tumor organoids from human pluripotent stem cells, modeling PDAC with mutation-specific phenotypes while preserving tumor heterogeneity and patient-specific physiological traits for precision medicine [19]. Furthermore, it has been shown that pluripotent stem cell lines injected into immunodeficient mice generated PanIN, which progressed to invasive PDAC [20]. Similarly, pancreatic organoids developed dysplasia and adenocarcinoma *in vivo* due to Kras(G12D) expression, p53 loss, or both [21].

1.3. Tissue slices and circulating tumor cells

Organotypic slice cultures (OTSC) are short-term *in vitro* cultures obtained from slicing tumor tissue that maintain the multicellular 3D architecture of the primary tumor, including the TME[22]. Lastly, circulating tumor cell (CTC)-derived adherent and spheroid cultures have also been described in PDAC [23], but this technology is not as advanced as other *in vitro* systems. The different systems will be discussed in the following sections, highlighting their advantages and disadvantages to study the features that contribute to poor outcomes in PDAC.

2. The pancreatic cancer microenvironment

The development of pancreatic cancer promotes a pro-inflammatory TME, which leads to a cycle of abnormal wound healing and the development of fibrosis [24]. As a consequence, PDAC tissue is unusually desmoplastic and immune-suppressed. To accurately mimic human disease, it is therefore crucial that experimental models reflect these features, and the sections below describe the two most characteristic of these.

2.1. Cancer-associated fibroblasts and extracellular matrix

During the formation of pancreatic cancer *stroma*, both stromal CAFs (the most abundant cellular constituent of the TME) and tumor cells engage in bidirectional support and signaling [25]. Hence, it is of paramount interest to evaluate and model the role of CAFs and their functional heterogeneity as well as plasticity in the TME using advanced *in vitro* or *ex vivo* models (Table 1).

For this purpose, hydrogel scaffolds are often used for co-culture models containing PDAC cells, the ECM and stromal cells such as CAFs [26]. Three methods can be distinguished: reconstituted submerged cultures, 3D microfluidic cultures, and air-liquid interface (ALI) cultures [27]. Most commonly used in reconstituted TME models, cancer cell organoids are cultured in growth factor- and inhibitor-tailored ECM domes, such as Matrigel [28].

Organoid models have been pivotal in studying CAFs heterogeneity in PDAC (Table 1). Ohlund et al. identified two distinct CAF subtypes in a mouse model of PDAC: myofibroblastic CAFs (myCAFs), located close to tumor cells, and inflammatory CAFs (iCAFs), enriched in regions distal to the tumor. MyCAFs are characterized by high α-SMA expression and are induced by direct tumor-cell contact and TGF-β signaling, while iCAFs are driven by IL-1-induced JAK/STAT activation and secrete pro-inflammatory factors such as IL-6 [29]. Elyada et al. extended this understanding by identifying antigen-presenting CAFs (apCAFs) *in vivo* using single-cell RNA sequencing. These apCAFs express MHC class II molecules and exhibit antigen-presenting capabilities, a feature validated in *ex vivo* organoid cultures [30]. While *in vivo* studies have been foundational in defining these subtypes, *ex vivo* and *in vitro* models, such as tumor organoid and CAF co-cultures, have been instrumental in elucidating the functional plasticity of CAFs. For instance, JAK inhibition in PDAC organoid models disturbs the balance toward myCAFs, increasing extracellular matrix (ECM) deposition and reducing tumor cell proliferation [31]. However, clinical trials targeting these pathways, such as ruxolitinib (a JAK1/JAK2 inhibitor), have not demonstrated improved survival in metastatic PDAC patients, highlighting the complexity of CAF biology [22]. In addition to prior studies, recent research has explored whether specific conditions can drive the emergence of distinct CAF subtypes. For example, hypoxic environments

Table 1
Cell types of the PDAC tumour microenvironment (TME) and the *in vitro* models used to study them.

Cell type	Subtype(s)	Features and role in PDAC	Section	Reference	Appropriate models
Cancer-associated fibroblasts (CAF)		The most abundant component of the TME that produces and remodels ECM	2.2 3.1 3.2 6.1 7	[180]	2D mono or co-cultures, co-culture in spheroid/organoid models, organ on chip, OTSCs
	myofibroblast-like (myCAFs)	In close proximity to the cancer cells. Express α-SMA^{high}/IL-6l^{ow} and represent a classical TGF-β activated subtype		[29]	co-culture in spheroid/organoid models
	inflammatory CAFs (iCAFs)	Distant from cancer cells, secrete inflammatory modulators like IL−6, after upregulating JAK/STAT and NFκB signalling. They are α-SMA^{low}		[29] [31]	co-culture in spheroid/organoid models
	metabolic CAFs (meCAFs)	meCAFs have high glycolytic activity. They associate with higher risk of metastasis and a poor prognosis but a better response to immunotherapy.		[181]	not determined yet
	antigen-presenting CAFs (apCAFs)	Appear to originate from mesothelial cells. Express MHC class II-related genes and can induce T-cell receptor ligation in CD4 + T cells in an antigen-dependent manner. Contribute to decreased antitumour immunity effects.		[30] [182]	Co-culture in spheroid/organoid models
Immune cells		These cells play crucial roles in tumour growth, metastasis, and response to therapy. Their composition can vary greatly between patients.	2.2 3.2 4.2 6.1 7.	[183]	Mono-culture, co-culture in spheroid/organoid models, OTSCs
	tumour infiltrating lymphocytes (TILs)	T effector cells are divided into CD4 + T cells and CD8 + cells. The latter are the most studied in PDAC and are related to better survival.		[184]	OTSCs
	Regulatory T-cells	Exert an immunosuppressive role, PDAC tumours are enriched for this cell type.		[184]	OTSCs
	dendritic cells (DCs)	DCs are antigen-presenting cells. Few can be found only in the peripheral zones of PDAC. Are associated with better survival.		[184] [185]	OTSCs
	B cells	CD20 + B cells are particularly present in PDAC tissues. They infiltrate PDAC in response to local chemokines and can have a pro or anti-tumorigenic effect.		[186]	OTSCs
	tumour-associated macrophages (TAMs)	Dichotomous categorization of macrophages (M1 and M2) does not accurately describe the plethora of macrophages present in PDAC. TAMs contribute to the immunosuppressive TME of PDAC.		[44] [187]	OTSCs
	tumour-associated neutrophils (TANs)	Neutrophils are one of the most abundant immune cells in PDAC TME and they favour the progression of pancreatic cancer by promoting the proliferation of cancer cells.		[188]	OTSCs
	myeloid-derived suppressor cells (MDSCs)	MDSCs block the anti-tumour activities of the effector CD4 + and CD8 + T cells.		[185]	OTSCs
	NK cells	Natural killer cells are part of the Innate lymphoid cells (ILCs), a heterogeneous group of cells derived from common bone marrow lymphoid progenitors. Few is known about NKs in PDAC.		[184]	OTSCs
	monocytes	Little is known on monocytes in PDAC. PDAC patients have more circulating monocytes in case of tumour spread to nerves and higher monocyte levels were linked to more aggressive PDAC and worse survival.		[189]	OTSCs
Vasculature	tumour-associated endothelial cells (TAECs)	TAECs expressing COL4A1 + promote angiogenesis	5. 6.2 6.3	[190]	Organ-on-chip, OTSCs
	pericytes	Ectopic α-smooth muscle actin (αSMA) expression.		[191]	Not determined yet
Neuronal cells	neurons	Secrete stimulatory factors to accelerate PDAC tumorigenesis but also provide metabolic support to PDAC cells.	6.4 6.1	[192] [193]	Co-culture with PDAC cells, OTSCs
	Schwann cells	They are the most prevalent cell type in peripheral nerves. They migrate toward tumour cells and are associated with a poor prognosis in PDAC. Detected around PanINs. Promote cancer cell metastasis by secreting cytokines, or by direct cancer cell contact.		[194]	OTSCs
Adipocytes		Adipocyte infiltration facilitates abundant cytokine secretion and is positively associated with anticancer immune signatures. Adipocytes can elicit increased CD8 + T-cell infiltration. High adipocyte infiltration predicts better outcomes of PDAC, lower tumour mutational burden (TMB).	3.2	[195]	Not yet determined
Cancer cells		Ductal cells from the exocrine pancreas.	2.1 2.2 2.3 6.2 6.3 3.2 4.2 7.	[196]	2D mono or co-culture, co-culture in spheroid/organoid models, organ on chip, OTSCs
	Cancer stem cells (CSCs)	Associated with relapse, metastatic seeding, drug resistance. Difficult to accurately identify using currently available markers.	7.	[150]	Suspension cultures, OTSCs

(*continued on next page*)

Table 1 (*continued*)

Cell type	Subtype(s)	Features and role in PDAC	Section	Reference	Appropriate models
Metastasis-associated cells			2.3 6.2 6.3		
	Circulating tumour cells (CTCs)	Drive metastases formation by disseminating through the bloodstream		[23]	2D cultures, spheroids
	Hepatocytes	First site of metastases formation in PDAC.		[197]	OTSCs, organotypic bioreactor models
	Peritoneum	The second site of metastases formation in PDAC. Associated with very poor outcomes.		[197] [134]	three-dimensional artificial human peritoneal tissues (AHPT)

have been shown to influence CAF states by enhancing the production of inflammatory cytokines in cancer cells. This creates a potential feed-forward mechanism involving autocrine cytokine signaling that can alter CAF phenotypes. Specifically, hypoxia has been linked to the induction of IL-1α, which promotes the differentiation of CAFs into the inflammatory subtype (iCAFs), characterized by their pro-inflammatory cytokine secretion [32,33].

In a more recent study a new "mini-tumor" model was proposed in which PDAC organoids and CAFs are pre-incubated in ultra-low attachment plates before seeding in Matrigel. This model showed that organoid-CAF "mini-tumors" secrete collagen type I, creating an extracellular matrix and inducing epithelial-mesenchymal transition [34,35]. This finding showed how tumor-stroma interactions contribute to disease progression. The model also showed that CAFs confer oxaliplatin resistance to PDAC organoids and demonstrated its potential for high-throughput drug screening [36].

The origin of CAFs in PDAC models differs and may derive from human-induced pancreatic stellate cells. One study utilized an ALI co-culture system of patient-derived PDAC cells with induced pancreatic stellate cell-derived cells to generate a fused PDAC organoid model. The model identified two phenotypes of PDAC, namely, quiescent organoids that were drug resistant due to the desmoplastic microenvironment and proliferative organoids that showed renewed cell growth following chemotherapy. This model provides a unique opportunity to understand the mechanisms behind cancer recurrence [37].

Other models of PDAC that accurately recapitulate key CAF characteristics can also be established *ex vivo*. These include tumor explants and organotypic slice cultures, which maintain cellular and extracellular components of the tumor microenvironment, and are essential to gain insights into tumor-TME interactions and how TME contributes to tumor progression and drug response. However, unlike long-lived organoid and cell line co-cultures, tumor explants and OTSCs are relatively short-lived. Therefore, depending on the tumor-TME crosstalk feature of interest, and the timescale at which these features apply, tumor explants offer a unique snapshot of the tumor dynamics in a native-like environment, whereas culture-based systems are perhaps less complete representations but have a far greater practical flexibility [38].

2.2. *The immune tumor microenvironment*

Although immune checkpoint blockade has been demonstrated to confer prolonged benefit in numerous solid tumors, its efficacy in PDAC clinical trials has not yet been demonstrated [39–41]. PDAC is regarded as an "immune-cold" tumor, exhibiting a paucity of immune cell infiltrate, limited antigen presentation, and an abundance of immunosuppressive signals. The TME, presents a barrier to therapeutic immune cell infiltration and activation in PDAC. Cellular elements include CAFs, suppressive immune cells, endothelial cells and adipocytes, while acellular components encompass the ECM, cytokines, hypoxia, metabolites, and matrix stiffness. Modeling response to immunotherapy in PDAC in an *in vitro* setting (cell-based, antibody-based, checkpoint inhibitor, cytokine) is challenging due to the need to represent the heterogeneity, structure, cell-to-cell and cell-matrix contact, cancer-immune cell interactions and the TME. PDAC is characterized by a considerable intra- and inter-heterogeneity in immune infiltrate. The density and location of the immune infiltrate have been identified as predictive factors for patient outcome, tumors with cancer cells surrounded by CAFs and macrophages with rare T cells have the poorest prognosis [42,43]. Single-cell RNA sequencing demonstrated the existence of discrete subtypes of immune cells within the PDAC TME. Subclustering of T-, and B cells, and macrophages revealed several subsets for each of these cell types [44]. Two effector CD8 + T cell clusters, naïve CD4 T cells, cells resembling central memory T cells, and T-effector cells (TEGs) were identified. Additionally, the traditional M1 vs. M2 classification of macrophages was found to be inadequate to reflect the diversity of macrophages present in the TME, as sub-clustering of single cell sequencing data revealed five distinct clusters [44]. Modeling such heterogeneity *in vitro* is a formidable challenge (Table 1).

In conventional 2D screening of cell-based immunotherapy, a mixture of immune cells with cancer cells is used to assess the efficacy of targeted cancer cell killing. Although this approach has the advantages of a low cost and high turnover, the cultures it produces are overly simplistic. The initial *in vitro* model for examining immune infiltration in the local microenvironment, which emulates the solid tumor, was developed in 1977 by Sutherland and colleagues [45]. This model employed mammary spheroids cultured with mixed leukocyte cultures, which demonstrated in situ destruction of tumor cells. It is now established that tumor cells grown in spheres exhibit decreased HLA-I and HLA-II surface expression, thereby mimicking the phenomenon of tumor immune evasion [46]. More advanced models include cancer cell-CAF co-cultures in Matrigel domes, where lymphocytes can be introduced as an overlay. Following a designated culture period, lymphocytes in the media can be removed for flow cytometry analysis, and the spheroids with infiltrated lymphocytes can be examined by fluorescent microscopy [15]. Incorporating CAFs is crucial, as FAP-positive CAFs have been demonstrated to facilitate immunosuppression in dendritic and cytotoxic T cells [47]. The spheroid in co-culture with T cells and NK cells was employed to examine the potential of CXCL12 inhibition with the clinical-stage l-RNA-aptamer NOX-A12 on immune infiltration in various cancer types, including PDAC [48]. Treatment enhanced the infiltration of both T and NK cells and had a synergistic effect on the activation of T cells with the PD-1 checkpoint in the spheroids. Patient-matched peripheral blood lymphocytes can be used to study lymphocyte infiltration. These can be co-cultured in Matrigel domes or embedded in collagen and loaded onto microfluidic devices/chips for assessment. The use of microfluidic Mcultures to model cytokine release in response to PD-1 inhibition has been demonstrated with both mouse- and patient-derived organoids [49]. This approach has also been shown to recapitulate both response and resistance to treatment. This is a significant finding, as the activation status of T cells (as indicated by the CD62L/L-selectin population) differs depending on the media formulation used for culture [15].

Ultimately, the most physiologically relevant *in vitro* culture system for investigating the TME is the OTSC, which preserves the intact microenvironment and native extracellular matrix. In the OTSC, immune, endothelial, and cancer cells are found within morphological structures, can be imaged in real time, and can capture the phenotypic and genetic heterogeneity for a limited duration. It has been established

that following an initial decline in overall cell viability, the percentage of CD45 + cells within the total viable cell population remained stable for up to a week, with macrophages representing the predominant immune cell type [50]. This is a significant finding, as CD163 + or CD204 + tumor-associated macrophages (TAMs) in PDAC tissue have been linked to significantly shorter survival [51]. The OTSC platform is suitable for screening immuno-modulators or combination treatments and for the evaluation of adoptive cellular immunotherapies [50,52]. Over the course of 6–9 days in culture, T cells, macrophages, and αSMA+ myofibroblasts in the OTSC exhibited a similar global protein profile to fresh PDAC tissue [52]. For the evaluation of adoptive cellular immunotherapies, fluorescently labelled single-cell suspension of autologous splenocytes (for mouse model-derived OTSC) or PBMCs (for patient derived-OTSC), can be added to the top of the slices and their migration throughout the slices can be followed during 4 days of culture [52]. Advances in technology have enabled the expansion of OTS analysis to encompass a multitude of functional readouts, including histological staining, high-content imaging following immunostaining, gene expression and sequencing, immune profiling with flow cytometry, and cytokine profiling with immunoassays [15,53–55]

The presence of macrophages in the OTSC is of importance, as TAMs are abundant in the PDAC microenvironment and the presence of $CD163^+$ or $CD204^+$ macrophages in PDAC tissue is associated with significantly shorter survival [51]. TAMs in PDAC are derived from both tissue-resident macrophages and inflammatory monocytes and have an immunosuppressive pro-fibrotic phenotype [56]. To study the role of macrophage-cancer cell interaction in a simpler setting, U937 monocyte lineage cell line can be differentiated to resemble monocytes, macrophages or dendritic cells [57]. Upon treatment with phorbol myristate acetate (PMA) they can differentiate into macrophage-like cells, and then upon interleukin treatment, into M2-like phenotype and used in co-cultures. Alternatively, bone marrow cells can be isolated from femurs and tibias of mice and differentiated with CSF1 to generate macrophages [58]. These have been used in triple culture of macrophages, cancer cells and CAFs to show that CAFs can induce macrophage proliferation in a contact-dependent manner. Although, one has to bear in mind that murine myeloid cell populations are not fully analogous to human myeloid cell populations, and myeloid cells in the peripheral circulation are often different from their tissue-resident counterparts [59].

3. Heterogeneity

Heterogeneity exists at all levels of the central dogma of biological information, from genetic diversity to the differential regulation of protein activity and metabolic wiring. It is now well accepted that treatment efficacy can be much improved if patients are selected for (or against) treatments based on the characteristics specific to their individual tumor. Also, understanding the differences between cell populations *within* a tumor is likely key to understanding why treatments do not show the anticipated efficacy.

3.1. Intertumor heterogeneity

Studies aiming to delineate genetic heterogeneity between PDAC cases into classes with distinct tumor biology and clinical characteristics have yielded subtypes based on the patterns and degree of DNA aberrations [60]. Organoids and other advanced *in vitro* cultures have limited use in studying genetic heterogeneity [61]. In addition, most research has centred on the analysis of mRNA [62], classifying PDAC into varying numbers of molecular subtypes [63–67]. One subtype feature has epithelial biology that reflects, to some extent, the pancreatic ducts, with a relatively favorable outcome. A more mesenchymal subtype is consistently associated with aggressive growth and poor outcome. Additional subtypes of yet unknown relevance have been reported, such as those resembling the exocrine features found in the healthy pancreas [63,64,66]. Single cell analysis has largely supported the existence of a two-tier heterogeneity, but also cell types that fall outside these categories [68,69]. Initially, molecular subtypes were found to be strongly prognostic, largely a consequence of the particularly aggressive growth of mesenchymal subtype tumors. However, subtypes were later found to predict response to standard-of-care systemic therapies (the mesenchymal/basal subtype is relatively resistant to FOLFIRINOX), greatly increasing their relevance for clinical decision making [70,71].

However, resected specimens are used for most subtyping studies and organoid development pipelines, leading to an overrepresentation of early-stage disease models, and an underrepresentation of aggressive mesenchymal subtypes associated with metastatic disease. Likewise, the use of tumor models to guide systemic therapies is most relevant in the metastatic setting where access to tissue to generate models may be limited. Furthermore, organoid cultures enrich for, or reprogram to, the relatively indolent epithelial subtypes and it is uncertain how well organoids mimic patient tumors at the mRNA level [68]. On the other hand, cell lines grown adherently in serum-containing media are thought to enrich for mesenchymal phenotypes. Therefore, *in vitro* models still come with inherent biases for molecular subtyping. For relatively short-term experiments, it is likely that *ex vivo* tissue slices most accurately reflect the tumor's subtype but challenges with subtype calling and experimental manipulations exist. Another option is organotypic coculture systems that circumvent these biases, but they are labor intensive, need established cell lines, and are not amenable to high throughput [72].

3.2. Intratumoral heterogeneity

PDAC also harbors a high intratumor heterogeneity. For instance, within one tumor, cell clusters displaying a broad spectrum of differentiation grades may be observed. Some form gland-like structures, while others assemble into compact nests of large atypical cells. Single-cell profiles or multiple biopsies of the same tumor have shown significant variations in the genetic composition of malignant cells within distinct areas of the same tumor, a phenomenon known as spatial intratumor heterogeneity [73]. Both intracellular and extracellular factors drive intratumor heterogeneity. Intrinsic mechanisms encompass cell-to-cell variability in genetic alterations and non-genetic influences such as epigenetic modifications, gene expression, and signal transduction. Extracellular factors that further contribute to heterogeneity originate from the tumor microenvironment. These various sources of heterogeneity can coexist and interact over time, contributing to the complexity of cancer.

Two hypotheses have been proposed on the source of genetic heterogeneity in PDAC and why it persists [74]: 1) Intratumor heterogeneity may occur through the positive selection of genetic variants that arise randomly from the preneoplastic period throughout tumor development;or 2) high levels of subclonal heterogeneity may persist in some patients from the very early stages of tumor development [65]. Recent studies show that there are subclones with different genetic changes in PDAC tumors, and that these clones undergo clonal evolution by competing within the tumor microenvironment [75,76]. In accordance, Grünwald et al. determined that organoid models obtained from 5 patients proliferated more when placed in a 'reactive' subtumor microenvironment (containing fibroblasts with plump morphology and enlarged nuclei, containing non-poor cellular components, but often containing a rich inflammatory infiltrate). Furthermore, tumor cells located in a reactive subtumor microenvironment had a more basal-like phenotype, squamous features, solid sheets, or cribriform glands while deserted subtumor microenvironment harbored well-polarized glands [77]. Similarly, Williams et al. found that when culturing 77 patient tumor samples in an environment different from their natural *in vivo* environment may lead to changes in cancer cell subtype fractions. However, spatial distribution must be considered when evaluating the suitability of model systems for patient tissue samples [78].

Single-cell and spatial transcriptomics have significantly advanced our understanding of intra-tumor cellular diversity; however, achieving a detailed characterization of the proteomic landscape at single-cell resolution remains a formidable challenge [79]. Single-cell proteomics in PDAC leverages technologies such as mass cytometry (CyTOF) and antibody-based multiplexed imaging to analyze protein expression in individual cells. This approach has highlighted protein-level differences in tumor and stromal cells, revealing new therapeutic targets and biomarkers [70]. Spatial proteomics has further advanced our understanding by combining proteomic analysis with spatial mapping, providing insights into how protein expression varies across different regions of the tumor. Mass spectrometry imaging (MSI) and multiplexed ion beam imaging (MIBI) are emerging tools that enable the localization of proteins within the pancreatic tumor microenvironment [79]. These technologies have unveiled spatial patterns of protein expression associated with drug resistance, immune evasion, and metabolic reprogramming [80].

As technologies advance, combining these approaches with multi-omics data integration and artificial intelligence promises to unravel the intricate mechanisms underlying pancreatic cancer and improve patient outcomes. Considering the above, as well as the challenges associated with models for intertumor heterogeneity mentioned in the previous section, it is likely that *ex vivo* models such as tissue slices, obtained from multiple sites of a tumor are the most appropriate system. Other model systems do not sufficiently cover spatial heterogeneity or non-tumor cell-derived cues.

4. Angiogenesis

Another PDAC hallmark is the hypovascular and hypoxic TME (Table 1). The increased presence of stromal components, including collagen, fibronectin, and hyaluronic acid, results in elevated stromal pressure, which impairs microvascular density and facilitates tumor progression [81,82]. A reduction in blood flow to PDAC tissues of approximately 60 % compared to normal pancreas hampers drug delivery and limits the effectiveness of chemotherapy [83]. However, the consequences of altered vascular density in most solid tumors, including PDAC, are still under debate. The hypovascular nature of PDAC is known to prevent the entry of cytotoxic immune cells and chemotherapeutics. Consistent with this point of view, another study suggested that increased vascularity of PDAC may be associated with improved patient outcome due to more effective delivery of cytotoxic immune cells. The analysis of PDACs with low and high CD31 expression, a marker of vascularity, indicated that the patients with high CD31 expression have better overall survival with increased CD4 + T cells, CD8 + T cells and naïve B cells [84].

Despite poor oxygenation resulting from collapsed intra-tumoral blood vessels, new blood vessels (angiogenesis) are formed in the periphery of PDAC. This phenomenon occurs because of the stimulation of hypoxia-induced factor-1α (HIF-1α), a well-described transcription factor that is upregulated in hypoxia in response to low oxygen levels. The increased expression and stability of HIF-1α promotes the expression of vascular endothelial growth factor (VEGF), which is one of the main drivers of angiogenesis [85]. The newly formed vessels are poorly perfused with a heterogeneous distribution, and their density is impaired due to stromal pressure. These pathological landmarks of PDAC are associated with early recurrence, metastasis, and poor survival after tumor resection [86]. Furthermore, increased vascularity may facilitate metastasis and tumor progression, indicating that vascular density in PDAC is a double-edged sword. Thus, further research is needed to determine its role in tumor progression, which requires models that accurately recapitulate tumor vasculature.

Many *in vitro* experimental models lack a vascular system, resulting in constraints in their size, complexity, maturation, and function due to oxygen and nutrient deprivation, as well as the accumulation of metabolic waste [87,88]. To address this limitation, research has been directed towards incorporating vascular systems into organoid models. In this section, we will examine the few published studies that attempted to recapitulate angiogenesis of PDAC.

In one study, the vascular niche was formed by co-culturing organoids with human umbilical vein endothelial cells (HUVECs). The paracrine and direct interactions between endothelial cells and cancer-initiating cells (CICs) facilitated the maintenance of CICs. Inhibition of Wnt and Notch signaling in endothelial cells resulted in the suppression of organoid formation and the maintenance of CICs, indicating that Wnt and Notch signaling play a role in the interaction between CICs and endothelial cells [89]. In another study, the interaction between tumor cells and blood vessels was facilitated when PDAC cells in a three-dimensional matrix were cultured near perfusable endothelial lumina. This resulted in the invasion of cancer cells into the blood vessels and the destruction of endothelial cells by tumor cells [90]. This organ–on-a-chip model functions as an *in vitro* platform that represents PDAC invasion of blood vessels, which may enhance our knowledge of the hypovascular nature of PDAC. In another study, an organoid-on-a-chip vasculature system was developed in which PDAC organoids were cultured in a tri-culture system composed of fibroblasts and endothelial cells to evaluate the effect of the stroma on drug delivery [91]. The findings of the study highlight that implementing a perfusable vascular system enhances the cultivation and expansion of organoids, facilitating the examination of fibroblast-mediated drug delivery into these structures.

5. High metastatic potential

Based on data from mouse models, metastatic dissemination in PDAC manifests early on, even before the primary tumor is detectable by imaging [92]. Even patients with resectable and potentially curable disease (tumors <2 cm) have a low 5-year survival rate of less than 18 %, implying that this may also occur in the human clinical scenario [93]. Proceeding from the study of clonal populations that give rise to distant metastases, it is predicted that five years are required for the acquisition of the metastatic capacity of the initial non-metastatic founder cell [94]. Therefore, there is a window of opportunity for early detection and treatment to prevent and/or limit metastatic dissemination. Metastatic spread is a complex process with distinct sequential steps. These include cancer cell detachment, migration, and local invasion through adjacent tissues, intravasation and survival in the circulation, extravasation, attachment and adaptation to the new microenvironment, establishment of micrometastases, and finally formation of macrometastases. Each of these steps independently, can be modeled *in vitro* to a certain extent (Table 1).

5.1. Modeling migration and invasion

Cell motility is critical for both escape from the primary tumor site and colonization of the distant sites [95]. Traditionally, cancer cell migration has been measured in 2D assays as collective migration, where groups of cells move while maintaining cell-to-cell contacts [96]. This experimental design is valid when a novel receptor-matrix interaction is explored, as it can be performed on coated matrices or in presence of CAF-conditioned media. Another commonly used migration assay is a two chamber or Transwell assay to assess chemotaxis and the cells' ability to change shape and contract during migration through a pore of a confined size. Scratch and Transwell assays have shown that PDAC patient-derived CAFs promote epithelial to mesenchymal transition (EMT) in tumor cells and increase their migration capacity in indirect co-culture [97]. Transwell assays can also be adapted to model invasion, by adding a matrix coating of the Transwell membrane, which requires cells to first degrade or squeeze through the matrix before migration through the pores. In 3D, cells can migrate even without any ECM adhesion [98]. Single cell invasion in PDAC is considerably rare and can be measured by cell speed and persistence tracking with live cell

microscopy [99]. Mesenchymal and amoeboid modes of movement through matrices are dictated by both cell intrinsic characteristics and the microenvironment through which it invades [96].

In PDAC, the cross-linked and highly dense ECM is one of the major barriers that cancer cells need to overcome to migrate out of the primary tumor. Collagen and hyaluronan are present at very high levels in both primary and metastatic lesions of PDAC, and patients with higher ECM deposition have a worse prognosis [100]. Increasing collagen concentration in gels or crosslinking can model the physiological range of substrate stiffness up to 2000 Pa [101]. To study the modes of migration and the effects of different matrix components, spheroid cultures of cancer cells can be embedded in ECM matrix and monitored over time. As CAFs play a significant role in PDAC metastasis through their cross-talk with cancer cells via multiple secreted factors, spheroid co-cultures with CAFs in collagen gels are used to evaluate invadopodia formation, migration, and phenotypic shifts of cancer cells [102,103]. Cancer cell spheroids embedded in collagen in the presence of CAFs in microfluidic chips have been used to demonstrate that PDAC cells show both individual and collective cell migration [104]. Furthermore, models with cancer spheroids in basement membrane gels in the presence of CAFs and supplemented with TGFβ have an *in vivo*-like tumor stiffness of 1500 Pa, even after 45 days of culture [105].

TAMs can also promote PDAC cell invasiveness through cytokine signaling, exosome delivery of miRs and induction of MMP production [106,107]. PDAC patient-derived TAMs secrete exosomes loaded with miR-202–5p and miR-142–5p and *in vitro* co-culture can promote the invasiveness and migratory potential of PDAC cells. TAM-conditioned medium also contains transforming growth factor-β (TGF-β) and IL6, PDAC cells have higher migration and invasion rates in transwell assays in presence of TAM-conditioned media [108–110]. Ideally, TAMs can be isolated from PDAC patients, but for *in vitro* modelling THP-1 monocytes (monocyte cell line isolated from peripheral blood from an acute monocytic leukemia patient) can be exposed to PDAC cell-conditioned media to derive tumor-driven-like macrophages that express CD68 (macrophage marker), CD163 and CD204 markers (M2 phenotype macrophages) [111].

To study matrix remodeling, quenched fluorescent peptides that release fluorescence upon MMP cleavage can be incorporated into gels and are used to indirectly quantify MMP activity, while invadopodia can be visualized with TKS5 staining [101,112]. Additional microenvironmental factors like hypoxia and nutrient availability are considered important influencers of the metastatic journey and can be introduced as parameters in modeling invasion in co-cultures. Apart from CAFs and TAMs, other non-malignant cell types are present in the PDAC TME including other types of immune cells, endothelial cells, pericytes and adipocytes but have not been systematically introduced in metastatic *in vitro* models yet.

5.2. Modeling extravasation and intravasation

Intravasation and extravasation require cancer cells to move through the endothelium into the bloodstream and from the bloodstream into the secondary metastatic site, respectively. The simplest model of cell transmigration through the endothelium is a modified Transwell assay where the upper chamber is seeded with an endothelial cell layer [113]. More recently, fabricated tumor-microvessel platforms have been developed, in which cancer cells are added to an artificial vessel embedded in ECM and monitored by live cell microscopy [114]. In microfluidic devices, vascular flow can be mimicked and the spatially-restricted chemokine stimulation can be introduced to model the metastatic site [115]. As stated in a previous section, vessels can be assembled from HUVEC or microvascular endothelial cells specific for the metastatic site organ [116]. However, tumor vessels have altered permeability and decreased αSMA-positive perivascular cell coverage, and it is important to include the TME factors that contribute to vessel remodeling to model these parameters [117]. TAMs can also contribute to cancer cell interaction with the vasculature by the production of pro-angiogenic factors and MMPs [118]. To model their effects on the tumor-vasculature interaction, 3D microfluidic device with channels that form a tumor-vascular interface have been developed and show that macrophages increase trans endothelial cancer cell migration [119].

Once the cancer cells leave the primary tumor and enter the bloodstream, they are referred to as CTCs (Table 1). In PDAC mouse models, CTCs have a mesenchymal phenotype and stem cell properties[92], whereas human CTCs isolated from whole blood show a hybrid EMT state [120]. EpCAM-based CTC isolation is ineffective for PDAC, and CTC isolation based on size have been more successful [121]. PDAC CTC isolation and detection has been used as a prognostic marker but could potentially be used in extravasation and metastatic seeding assays, as they better represent the PDAC metastatic tumor cell heterogeneity.

5.3. Modeling the metastatic seeding

PDAC preferentially metastasize to the liver [122] and patients with liver metastasis have worse survival compared to the other metastatic sites [123]. Cytokines, growth factors and extracellular vesicles secreted by the primary tumor enhance the vascular permeability, remodel the matrix and provide attachment sites for CTCs, thus helping in the establishment of the permissive niche in the liver [122,124]. In response to these signals, resident cells in the liver (e.g. metastasis-associated macrophages) secrete cytokines and exosomes that prime the premetastatic niche for seeding [125]. Maintenance of human hepatocytes *in vitro* is challenging and requires specific environments to preserve cell-cell contacts, cellular architecture, and polarity [126,127]. In the last decade, organotypic 3D liver models have started to emerge [128, 129], incorporating primary human hepatocytes, liver sinusoidal endothelial cells and Kupfer cells. These models are highly demanding in terms of cellular sources and maintenance and so far, have been mainly used to assess drug metabolism and toxicity. For *ex vivo* modeling of cancer cell hepatic seeding, organotypic liver bioreactors that incorporate the fluid flow and biological functionality of a hepatic parenchyma have also been developed [130]. These can incorporate human hepatocytes, human nonparenchymal liver cells and oxygen sensors, which are micropumps capable of generating varying profiles of nutrients and soluble factors that enable real-time sampling and monitoring of the microenvironment upon cancer cell seeding [131,132]. These models can sustain functioning hepatocytes for more than 15 days, effectively integrate cancer cell lines into the hepatic niche, and present a useful platform for elucidating adaptive behaviors of cancer cells that underline establishment of macrometastases. Unfortunately, apart from establishment and functional validation of the bioreactor models, they have not been widely implemented in *in vitro* research due to their complex maintenance.

The second most common site for PDAC metastasis after liver is the peritoneum, accounting for 50 % of PDAC patients at the time of death [133]. With advances in tissue engineering, three-dimensional artificial human peritoneal tissues (AHPT) harboring the blood or lymphatic vascular network by the cell-accumulation technique have been developed for visualization of metastatic seeding and invasion [134]. This model has been successfully used to observe different dynamics of tissue infiltration of common PDAC cell lines using light and electron microscopy [134].

5.4. Neural invasion

Neural invasion (NI) in PDAC is linked to pain and reduced survival and serves as a low-resistance conduit for metastasis [135,136]. Given these considerations, several methods to study PDAC cell–neuron interactions have been put forward. For instance, PDAC cells have been co-cultured with dorsal root ganglion (DRG) neurons to evaluate the impact on NI of neuron-derived chemokines, CXCL10 and CCL21 [137]. Although this study is important for identifying factors involved in NI, it

solely focused on indirect interaction between the two cell types, which restricts its broader impact. In addition to neurons, Schwann cells are recognized as the first contact point in NI. Various techniques have been employed to study the interaction between these and PDAC cells. For instance, Schwann cells have been embedded in Matrigel, with PDAC cells seeded on top. In another model, Schwann and cancer cells were co-cultured in 2D wells, with adjacent microchannels allowing 3D movement [138]. Schwann cells indirectly co-cultured with PDAC cells in both 2D and 3D environments revealed that Schwann cell-derived TGF-β promotes cancer cell motility [139]. Another study demonstrated that Schwann cells physically guide pancreatic cancer cells toward nerves, facilitating NI in a contact-dependent manner [140].

The use of complex models like organoid co-cultures or organ-on-a-chip systems to model nerve-cancer interaction remains limited. Demir et al. developed a protocol for co-culturing pancreatic cancer organoids with neural cells, using induced pluripotent stem cells SC-derived neural crest cells and DRG explants to investigate neurogenesis and NI processes [141]. In another study, mouse pancreatic cancer organoids were co-cultured with human brain organoids to mimic pathophysiology of NI. Analysis of the changes in structural crosstalk, interaction and growth pattern of organoids demonstrated that organoid co-culture systems recapitulated the NI as observed in mouse models [142].

Considering the complex microenvironment of PDAC, incorporating other components like immune cells and fibroblasts into co-culture systems is likely necessary. Likewise, to accurately model functioning neurons, specific culturing methods are required. For instance, Kawada et al. developed a nerve-organoid model in a specially designed microdevice, which seemed to form nerve-like structures that are electrically active [143]. Another study using a human nerve-on-chip platform with both neurons and Schwann cells showed that the peripheral nerves are myelinated and functioning [144]. In summary, while the number of studies mimicking functional nerve structures has been increasing [145, 146], their integration into models for PDAC is an unmet challenge.

6. Therapy resistance

PDAC is characterised by high intrinsic and acquired resistance to various therapeutic modalities, including chemotherapy and radiation therapy, which limits treatment efficacy and contributes to poor patient outcomes [147]. Many properties described in the previous sections contribute to therapy resistance and often cooperate to this end. Furthermore, it is now clear that therapy responses differ between subjects according to their specific genomic and transcriptomic signatures. Therefore, preclinical models must capture the appropriate combinations of these features to discover how resistance is achieved and identify ways to target or circumvent resistance.

The simplest model to study resistance are adherent (2D) cell lines. This model is relatively easy to handle and manipulate, and also offers the benefit of enriching tumor cellularity, the compartment in which most resistance mechanisms will be activated [10]. Thanks to cell lines, several chemoresistance mechanisms have been identified [148] and led to important observations. First, cross-resistance to different drugs was frequently observed, underscoring that PDAC resistance is a complex multistep process. Moreover, common pathways to treatments were identified, such as metabolic adaptation, activation of EMT properties, and the stemness profile's re-activation. This last aspect is crucial since CSCs are thought to contribute to the resistance and recurrence of nearly all cancer types, due to their slower proliferation, which makes them less sensitive to chemotherapies that act on highly proliferating cells [148, 149]. In addition, CSCs' pluripotency leads to new cancer cells, contributing to disease relapse and dissemination. CSCs can be cultured in suspension and tested for their ability to form tumor spheres [150, 151]. However, a key caveat for PDAC research is that there are currently no specific markers that exclusively identify PDAC CSCs. Researchers have combined cell surface markers and functional assays to enrich for these cells, but uncertainties regarding the actual presence and activity of CSCs in PDAC remain [152,153].

On the other hand, 3D (co)cultures and ex-vivo tissue slices are other options to identify and address resistance mechanisms accurately. Specifically, 3D models like spheroids and organoids better resemble *in vivo* conditions than adherent cell lines. Gene expression profiles of cells cultured in 3D are more similar to the expression levels found in patients than those from adherently grown cell lines [154]. Furthermore, spheroids and organoids show higher treatment resistance than 2D cell lines, which is in agreement with the poor responses to therapy observed in patients [155,156]. Moreover, 3D co-cultures incorporating different cell types are crucial when considering that therapy resistance in PDAC is largely also driven by the complex and dense TME [157]. Organoids co-cultured with patient-derived CAFs show higher proliferation and reduced chemotherapy sensitivity than monocultures. Moreover, coculturing of organoids induced EMT in cancer cells and pro-inflammatory phenotypes in CAFs [34]. These findings highlight once again the importance of the complex interplay between PDAC cells and the TME. Given these challenges, immunotherapy was also proposed as a PDAC treatment, including immune checkpoint inhibitors, cancer vaccines, oncolytic viruses, and adoptive cell therapy [158]. Unfortunately, these approaches have not yet shown convincing clinical results in PDAC and further investigation is needed. To this aim, organoid co-cultures with immune cells can be used to study immunotherapy approaches efficacy. Zhou and collaborators created PDAC organoids co-cultivated with T-cells and found that the administration of two epigenetic inhibitors combined with anti-PD-1-based therapy had a higher effect than anti-PD-1 therapy alone proving the potential of co-culture systems [159,160]. Similarly, the contributions of the immune system and other TME constituents can be studied using OTSCs [161]. However, as already mentioned, one major limitation of OTSs is their limited lifespan, typically around a week [162]. As a consequence, the possibility of studying the drug pharmacokinetics is precluded in this model. On the contrary, to study the drug mechanisms organ-on-chip devices or *in vivo* models can be used.

Lastly, a more recent aspect that should be considered when studying therapy resistance in PDAC is the increasingly recognized role of the microbiome. Microbiota, especially of the gut, have been identified as possible contributors to resistance in different tumors including PDAC [163,164]. In particular the gut microbiota can change the pharmacodynamics and pharmacokinetics of drugs. For example, it has been reported that indole-3-acetic acid (3-IAA) derived from the microbiota metabolism is correlated with the better response of PDAC patients to treatment with chemotherapy [165]. However, this area of research is still in its infancy, and these studies are at risk of reverse causation bias as they are often performed retrospectively [166]. To avoid this issue, *ex vivo* models and *in vitro* co-cultures could be combined with the intratumor microbiota or selected bacteria and complex admixtures to resemble the *in vivo* molecular mechanisms and assess the actions of the microbiome that contribute to therapy response.

To conclude, understanding and overcoming therapy resistance in PDAC necessitates a comprehensive approach that considers intrinsic tumor characteristics, the multifaceted TME, and emerging factors such as the microbiome. Continued advancements in preclinical models, especially 3D co-cultures, will be crucial for elucidating the underlying mechanisms of resistance and identifying novel therapeutic strategies.

7. Other considerations

When selecting an appropriate translational model, there is more to consider than the tumor biological aspects described above as there are practical considerations that come into play. Each model system requires specific infrastructure and dedicated personnel with specific expertise. The quality of the starting material is also a crucial factor, with large samples from freshly resected tumors being ideal. Furthermore, the composition of the culture media has a significant impact on organoid morphology, transcriptome, and the *in vitro* response to both

chemo– and targeted therapy [167]. In addition, one of the most demanded features of these models is time efficiency, specifically a rapidly established model that can provide an assessment of treatment sensitivity or resistance within the time constraints of the clinical scenario of the disease. The recent study by Kim et al. showed how fresh EUS-FNB samples can be used to establish PDO, with a high success rate of 83.2 % from over 100 biopsy samples, with sufficient culture available after 3 weeks to perform drug sensitivity assays [168]. This would allow the use of such models as *avatars*, bearing in mind the limitations of organoid monocultures.

Organoid formation and expansion rates in different cancer types are frequently less than 30 %, due to variations in the quality of the initial material and tumor cell content [169]. Modifying the culture medium may improve success rates, but it remains uncertain whether this improvement can be replicated across all cancer types. Batch-to-batch variability is an additional issue arising from non-standardized cancer tissue samples, imprecise medium formulations, and also diverse animal-derived matrices [170]. Matrigel, the key matrix for cancer organoid cultivation, is mouse-derived and demonstrates significant variability [170]. Additionally, it contains impurities that can affect organoid phenotype. Even growth-factor-reduced Matrigel exhibits inconsistent protein levels across batches. This discrepancy has driven the innovation of tailored culture mediums and matrices. Recently, synthetic materials such as HA, PEG, and gelatin are being created to maintain uniform quality when culturing organoids [171]. Designer matrices are modular synthetic hydrogel networks that can include a specific ECM protein (e.g. fibronectin, laminin) can be tuned to model stiffness of the microenvironment and also be used in this setting to better mimic PDAC stiffness and achieve consistent take rates of organoid cultures [172].

A lack of multi-cellular components and vasculature is still considered a significant defect of tumor organoid cultures. Primary tissue used as a starting material of organoid cultures still contains stromal cells such as immune and fibroblast cells, which are gradually lost during the long-term culture in Matrigel and stem cell-like culture medium [37]. In Table 1, we provide an overview of the cell types that exist in PDAC tissue and should be considered when designing experiments. We also provide a suggestion for optimal *in vitro* setup. Carefully chosen and optimized matrices as described above may offer a 3D environment suitable for incorporating various cell types like immune cells, endothelial cells, or CAFs.

Another gap to address is that organoids, organoid cocultures, or tissue slices are not “connected” to an organism. A key omission is typically the lack of blood flow with influx of signaling cues and cell types. Microchip technology offers a controlled setup for culturing different cell and tissue types, with the flow of media across the cells. Encapsulation methods like microcapsules and microfibers function to maintain organoid structure [173]. These platforms allow organoids to grow with surrounding blood vessels, tissue support, immune elements, and other components. Additionally, it can accurately regulate drug dosages and monitor cell health, death, and growth. Microfluidic devices incorporating epithelial cells can faithfully replicate the flow profiles and mechanical strains of the gastrointestinal tract [174]. Also, the progression or inhibition of a tumor depends significantly on its surrounding milieu, which is intricate and comprises diverse chemical and protein transport systems, blood vessel formation, and pressure discrepancies. Bioengineered microfluidic platforms and semi-permeable scaffolds have been developed to allow for the precise manipulation of chemical and physical parameters, aiding the creation of organoid tumor cultures that closely mimic *in vivo* tumor behavior and microenvironment interactions.

To boost comparability between experiments and foster reproducibility, a case can be made for the standardization of *in vitro* model systems and experimental procedures. This is likely best achievable in those models that are established using widely available constituents such as cell lines and immortalized fibroblasts. However, in models that require higher complexity and cell types that need to be freshly derived, for instance matched immune cells, this would likely be harder to achieve. These limitations would argue for the use of animal models but also here, despite many strains being inbred, environmental influences differ across facilities which may impact on for instance tumor take [175].

Another point to consider when designing and interrogating increasingly complex model systems is the advent of genome-wide screening [176]. Using silencing RNA, or genome editing methods, it is now possible to perturb all the genes in the genome and assess their contributions to for instance tumor growth, therapy resistance, and metastatic potential [177,178]. However, these experiments require highly tractable cell systems and are not conducive to complex admixtures of cells, and adherently cultured cell lines are the most appropriate model to use. A related technology is the barcoding of cellular populations to allow the interrogation of for instance therapy responses of hundreds of cell types in parallel [179]. Also here, multicellular systems are hard to use, and the field will likely apply relatively simple culture systems in the foreseeable future.

8. Summary

Considering all the above, the choice of *ex vivo* and *in vitro* models for PDAC depends on the scientific question at hand and more complex systems are not always required. In most cases, monocultures will insufficiently capture the intrinsic tumor biology that makes PDAC such as difficult tumor to treat in the clinic. The issue of tumor heterogeneity can be addressed by studying models from multiple patients, or multiple sampling sites or biopsies from a single tumor. Furthermore, it is important to consider whether a model will be used to assess responsiveness (i.e., to select for a particular treatment) or to determine resistance (to decide against a particular treatment), as models to select an effective therapy and optimize drug combinations require short-term cultures that involve less time investment and avoid the culture-induced changes observed in longer-term cultures. In contrast, models aimed at studying resistance mechanisms and understanding why certain treatments fail, require cultures that are stable in the long-term. Depending on the feature studied, outcomes of the model should be benchmarked to clinical scenario as far as possible, to ensure that the model accurately reflects the complexity and behavior of tumor in the clinic. This enhances the predictive validity of the model, making it more reliable and increases the translational potential of research findings to the patient.

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Data availability

No data was used for the research described in the article.

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