TRANSLATIONAL INSIGHTS INTO PANCREATIC DUCTAL ADENOCARCINOMA

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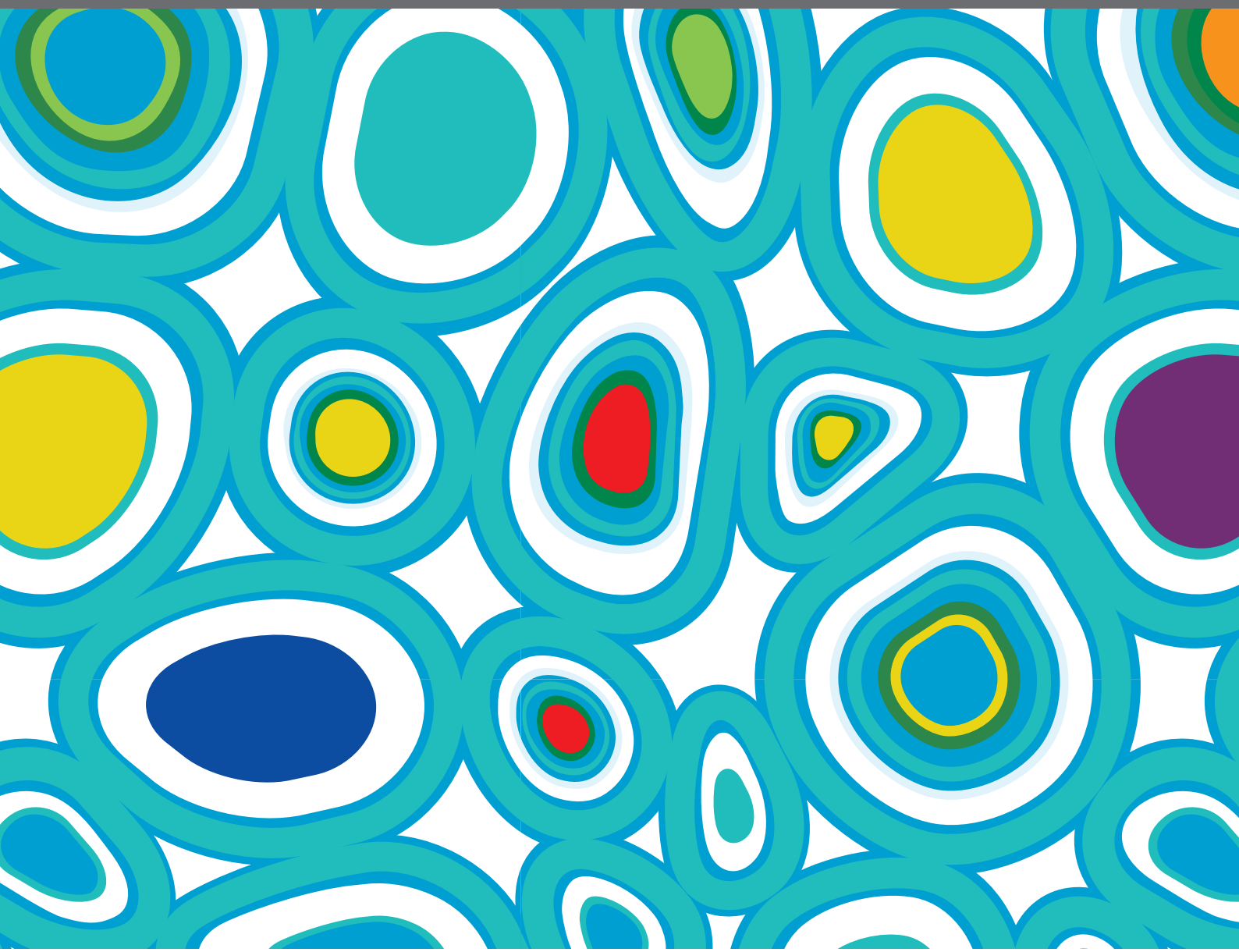

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TRANSLATIONAL INSIGHTS INTO PANCREATIC DUCTAL ADENOCARCINOMA

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EDITORIAL
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Editorial: Translational Insights Into Pancreatic Ductal Adenocarcinoma

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Keywords: PDAC—pancreatic ductal adenocarcinoma, organoids, tumor microenvironment, cell plasticity, cancer subtype classification, multi-omics analyses, tumor stroma, preclincal model

Editorial on the Research Topic

Translational Insights Into Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) carries the worst prognosis of all cancer types, and its incidence is increasing year on year. Currently available treatment options in both the resectable setting as well as inoperable disease are insufficiently effective. In this Frontiers Topic, members of the European Commission-funded PRECODE consortium and the wider pancreatic research community, provide their views on several key features of PDAC that contribute to its poor outcome. These include the abundant collective of non-tumor cells and material known as the stroma, the immune landscape unique to this disease, and intrinsic molecular subtypes that exhibit distinct neoplastic characteristics and plasticity in response to both stromal cues and therapy (**Figure 1**). A major goal of the PRECODE consortium was the establishment of a well characterized pancreatic organoid resource for the study of PDAC pathobiology and the development of much-needed novel therapies. The reviews in this topic support this goal and provide detailed commentaries on the application of organotypic pre-clinical models for the study of PDAC biology and the development of next generation treatments and decision-making tools. This Frontiers topic issue contains 14 reviews and one original research article.

Despite being driven by a relatively small number of recurrent driver mutations, PDAC is characterized by distinct *heterogeneity* at the gene expression level (**Figure 1**). It is now clear that distinct subgroups can be identified, and that these differ mostly in the degree to which tumor cells have acquired a motile, resistant phenotype that is called *mesenchymal*. Schreyer et al. and colleagues describe the currently available array of biomarkers and omics technologies, and the limitations that stand in the way of clinical implementation such as patient selection. Xu et al. further review the clinical relevance of molecular profiling and how to achieve effective clinical translation. In an original article Zhang et al. and colleagues introduce a 5-gene TP53 mutation-associated gene signature helpful to optimize prognostic stratification. Further adding to the contributions of heterogeneity and subtypes to poor therapy responses is the readiness with which PDAC cells transition between cell states (and at the tissue level; subtypes). This plasticity also applies to the origins of the disease: it is suspected that pancreatic ductal carcinomas find their origin in acinar cells that have transitioned to a ductal fate. The reviews by Malinova et al., van Roey et al., and Sankarasubramanian et al. provide an overview of the mechanisms currently known to govern lineage infidelity and phenotype switching, and how these may contribute to drug resistance. The signaling cascades, metabolic factors, transcription factor networks, and epigenetic gene regulation that contribute to plasticity, epithelial-mesenchymal transition (EMT) and disease progression are explained. Highly related to the molecular subtypes that may associate with response is the subgroup

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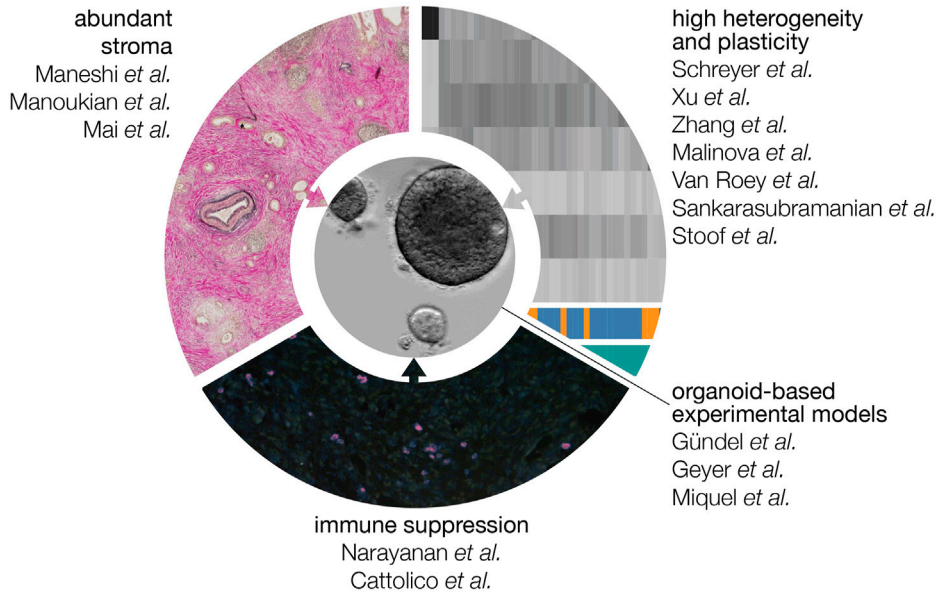

FIGURE 1 | Features that contribute to the poor prognosis of PDAC. The abundant stroma, high degree of heterogeneity at the level of gene expression, and immune suppressive microenvironment all contribute to a tumor type that quickly progresses to an incurable stage and is resistant to currently available regimens. In this Frontiers topic, the contributors explain how the organoid culturing system can be used and modified to address these tumor-promoting features experimentally.

of PDAC tumors that are defective for DNA damage repair. Stoof et al. describe how this deficiency can be leveraged to design and select for therapeutic strategies.

The sheer abundance of non-tumor cells and material known collectively as the *stroma* is known to impact tumor progression and therapy response but the exact outcomes and mechanisms remain elusive. The review by Maneshi et al. summarizes how extracellular matrix deposition and the resultant mechanical properties of the stroma impact on signaling pathways and subsequent tumor biology. The origins of the most abundant cell type in the stroma, the cancer-associated fibroblasts (CAFs) are a topic of debate. Likewise, a high degree of heterogeneity has been described to exist in this cell population that impacts on disease progression and therapy response. Manoukian et al. describes these different subsets of fibroblasts in PDAC, and how this relates to the suspected cellular origins of CAFs. Recently, another non-tumor cell entity, platelets, has been found to strongly interact with tumor cells beyond their well known roles in coagulation. For instance, they are a rich tumor biomarker matrix but also impact on tumor biology. Mai and Inkielewicz-Stepniak colleagues describe the contributions of platelets to pancreatic cancer and how these drive tumor progression.

In many tumor types, immune therapies such as checkpoint inhibitors have demonstrated promising efficacy in the clinic. In PDAC, this has not yet been the case. The abundant stroma explained above is suspected to contribute to this, as may additional features specific to PDAC. In the review by Narayanan et al. properties that allow PDAC tumors (for instance the above-mentioned low mutational burden) to evade immune surveillance are discussed in depth. Cattolico et al. further explain why the immune response is ineffective, despite detection of features in PDAC that should activate the immune system. The focus in this review is on the role of interferons in tumor-immune crosstalk, how PDAC cells bypass this signaling, and how to reactivate this signaling to improve therapeutic outcome.

Given the above considerations, the question arises how to design experiments that will give meaningful answers and allow the development and testing of therapeutic options that are effective despite the challenges specific to PDAC. Which model systems are required and appropriate, and how should they be modified to suit the specific research question? Most authors in this Frontiers topic propose that the organoid system is a solid basis from which to develop such models, and that plasticity can be faithfully captured in these cultures. In the case that contributions of the stroma are to be studied, relevant stromal cell types can be incorporated (**Figure 1**). This should be done in a fashion that does not perturb, for instance, their phenotype or orientation relative to the tumor cells as seen in patient tumors. A comprehensive summary about existing preclinical *in vitro* and *ex vivo* models is given by Gündel et al. and colleagues. Geyer and Queiroz explain how organ-on-chip technologies can further improve co-culture models for PDAC by more accurately modeling the physicochemical properties of PDAC. Miquel et al. make a case for the development of better models for metastatic PDAC, and how these could be developed from existing technologies.

In conclusion, this Frontiers topic issue summarizes the current challenges of PDAC treatment, pointing out that there is an urgent need to establish proper models to identify novel vulnerabilities of this devastating disease. Organotypic pre-clinical models of PDAC provide a tractable model system for understanding the dynamic interplay between neoplastic and stromal cell types. The co-culturing of genomically characterized pancreatic organoids with cell types that

are constituents of the PDAC tumor microenvironment promises new insights into the pathobiology of PDAC. These approaches will also drive the development of next generation treatment options that target key tumor-stroma interactions and/or modify neoplastic programs that effectively augment systemic chemotherapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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TP53 Mutational Status-Based Genomic Signature for Prognosis and Predicting Therapeutic Response in Pancreatic Cancer

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TP53 mutation is a critical driver mutation that affects the carcinogenesis and prognosis of patients with pancreatic cancer (PC). Currently, there is no driver mutation-derived signature based on TP53 mutational status for prognosis and predicting therapeutic response in PC. In the present study, we characterized the TP53 mutational phenotypes in multiple patient cohorts and developed a prognostic TP53-associated signature based on differentially expressed genes between PC samples with mutated TP53 and wild-type TP53. Comprehensive investigations were carried out in prognostic stratification, genetic variation, immune cell infiltration, and efficacy prediction of chemotherapy and targeted therapy. We found that TP53 mutation commonly occurred as a survival-related driver mutation in PC. In total, 1,154 differentially expressed genes were found between two distinct TP53 mutational phenotypes. A five-gene TP53-associated signature was constructed in The Cancer Genome Atlas (TCGA) cohort by least absolute shrinkage and selection operator (LASSO)–Cox analysis and proven to be a robust prognostic predictor, which performed well in three independent Gene Expression Omnibus (GEO) validating cohorts. Remarkably, patients in the low-risk group were characterized with decreased tumor mutation burden and activity of immunity, with favorable prognosis. Higher fractions of macrophages M0 and impaired CD8 + T cells were observed in patients in the high-risk group, suggesting immunosuppression with poor survival. Patients in the high-risk group also demonstrated enhanced response to specific chemotherapeutic agents, including gemcitabine and paclitaxel. Several targeted inhibitors, like histamine receptor inhibitor, were screened out as promising drugs for PC treatment. Collectively, the TP53-associated signature is a novel prognostic biomarker and predictive indicator of PC. The signature could contribute to optimizing prognostic stratification and guide effective PC treatments.

Keywords: TP53 mutation, pancreatic cancer, signature, prognosis, therapeutic responses

INTRODUCTION

Pancreatic cancer (PC) is an aggressive and lethal malignancy with a dismal 5 years survival rate of 4% and 47,050 cancer-related deaths in 2020 (Klint et al., 2010; Siegel et al., 2020). Because of the limited treatment options and deficiency of robust biomarkers for early stage screening, 80% of patients with PC were typically diagnosed in advanced stage and not candidates for surgical intervention (Sohn et al., 2000; Winter et al., 2006). Moreover, the survival of PC has not significantly improved even for those who received surgery at the early stage (Kasumova et al., 2018; Strobel et al., 2019). Currently, several targeted drugs have emerged as potentially effective treatments for PC; however, the limitation is that only a small subset of patients with specified characteristics may benefit from these targeted approaches (Kleeff et al., 2016; Osmani et al., 2018; Zhu et al., 2018). As a consequence, there is an urgent need to better stratify prognosis and develop more suitable therapeutic strategies for patients with PC.

The tumor suppressor gene TP53 is one of the frequent pan-cancer mutated genes, linked to unfavorable prognosis in multiple cancers and more than 500 million deaths (Kandoth et al., 2013). Functionally activated by a series of stress stimuli, wild-type TP53 protein exquisitely manages complex transcriptional processes involved in apoptosis and anti-proliferation (Kastenhuber and Lowe, 2017). Mutation of TP53 occupies one of the identified major driver mutations presented in the complex mutational landscape of PC (Jones et al., 2008; Makohon-Moore and Iacobuzio-Donahue, 2016). Occurring in about 70% of examples, TP53 mutation often leads to an oncogenic process and is associated with aggressive and metastatic phenotypes (Makohon-Moore and Iacobuzio-Donahue, 2016; Hashimoto et al., 2019). The tumor-suppressive effect of TP53 and the prevalence of TP53 mutation have encouraged the development of precise therapy targeting TP53 network in cancers. For example, MK-1775, SGT-53, Alisertib, and AMG900 are several promising anti-PC drugs that target TP53 and tested in ongoing clinical trials. Interestingly, recent studies depicted that TP53 mutational status is closely associated with various antitumor immune responses (Swidnicka-Siergiejko et al., 2017; Hashimoto et al., 2019). The regulatory effect on immune response of TP53 mutation has been proposed (Butin-Israeli et al., 2019; Blagih et al., 2020). P53 induction was associated with peptide processing and major histocompatibility complex (MHC)-I surface expression, thus it might prevent the cytotoxic T lymphocytes (CTLs) from killing tumor cells (Wang et al., 2013).

Previous studies have assessed the prognostic value of pancreatic driver mutations. However, to date, few robust and reliable driver mutation-related biomarkers were identified to predict prognosis and therapeutic response. Here, we present a comprehensive study to describe the mutational landscape of PC and the difference of TP53 mutational status and then constructed a TP53-associated prognostic signature in The Cancer Genome Atlas (TCGA) cohort. External validation was performed in the GSE28735, GSE62452, and GSE78229 cohorts to illustrate its prognostic efficacy. Furthermore, the associations of TP53 mutational signature with genetic mutation, tumor microenvironment, and multidimensional therapeutic application were investigated. This novel model could be used for screening, prognostic assessment, and treatment approaches in PC.

MATERIALS AND METHODS

Data Source and Processing

The public VarScan2 somatic mutations, corresponding transcriptional expressions, and full clinical annotation of PC patients were obtained from TCGA[1] and Gene Expression Omnibus (GEO)[2] databases. In total, 151 patients from TCGA-pancreatic adenocarcinoma (PAAD), 42 patients from GSE28735, 66 patients from GSE62452, and 49 patients from GSE78229 cohorts were collected for analysis. For the transcriptional profile in TCGA, transcripts per kilobase million (TPM) values or log2 transformations were performed in the gene expression data. In the GEO microarray data, batch effects were eliminated *via* the combat algorithm of "sva" package, and then data normalization was conducted by "limma" package (Ritchie et al., 2015). The downloaded data were utilized according to TCGA and GEO data access requirements. All mutation data, gene expression profile matrix, and clinical feature data of PC are publicly available.

Identification of Differentially Expressed Genes

To identify the differentially expressed genes (DEGs) based on different TP53 mutational statuses, we classified patients into two TP53 mutational phenotypes. Under the threshold of $|\log2\ \text{fold change}|$ ($\log^2\text{FC}$) ≥ 1 and false discovery rate (FDR) < 0.01, the "limma" R package was applied to determine DEGs between 82 PC samples with mutated TP53 and 69 PC samples with wild-type TP53 in TCGA cohort (Ritchie et al., 2015).

Construction and Validation of a TP53-Associated Prognostic Signature

A total of 151 PC samples with complete TP53 mutation data, gene expression profile, and survival data in TCGA cohort were subjected to analyses. Univariate Cox regression analysis was performed among DEGs *via* "survival" R package to figure out significantly prognostic DEGs associated with overall survival (OS). Next, the key prognostic DEGs were screened out, and the collinear problem was removed by the analysis of least absolute shrinkage and selection operator (LASSO). Multivariate Cox regression analysis was implemented to figure out the independent prognostic DEGs. We applied LASSO–penalized Cox regression analysis to further narrow the OS-related DEGs and construct a five-gene signature panel in TCGA cohort. The signature risk scores were calculated according to the sum of the multivariable regression coefficients multiplied by the expression level of each model gene. Defined by the cutoff equal to the

[1]https://portal.gdc.cancer.gov/repository

[2]https://www.ncbi.nlm.nih.gov/geo

median risk score, samples were classified into the high- and low-risk groups. Survival was measured utilizing the Kaplan–Meier method and log-rank test.

Estimation of Immune Cell Infiltration

To characterize the tumor-infiltrating immune cell fraction in PC, gene expression profile was normalized and written by standard annotation file, subsequently uploaded to the Cell type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) approach combined with the LM22 gene signature (Newman et al., 2015). Then, we quantitatively evaluated the abundance of 22 types of immune cells between high- and low-risk groups based on the signature. We access the marker gene set for infiltrated immune cell types offered by Bindea et al. (2013).

Tumor Immune Dysfunction and Exclusion

The Tumor Immune Dysfunction and Exclusion (TIDE)[3] is a data-driven Web platform that integrates large-scale omics data of over 33,000 cases from 188 cohorts, 998 tumor samples from 12 immune checkpoint blockade (ICB) clinical studies, and eight clustered regularly interspaced short palindromic repeats (CRISPR) screens. The TIDE could contribute to hypothesis generation and immunological biomarker optimization (Fu et al., 2020). In the present study, we used the TIDE to evaluate the impact of expression of the five genes on T cell dysfunction, immune-suppressive rejection score, and therapeutic response of ICB.

Prediction of Chemotherapeutic and Targeted-Therapeutic Response

Individual chemotherapeutic response was estimated according to the Genomics of Drug Sensitivity in Cancer (GDSC)[4], a public pharmacological Web portal accessible to predictive sensitivity of 138 common chemotherapeutic agents. We used the ridge regression to estimate the half-maximal inhibitory concentration (IC_{50}) and implement 10-fold cross-validation *via* "pRRophetic" R package. Then, the Connectivity Map (CMap) database was used to search for potential inhibitors or compounds that targeted TP53-associated signature ($p < 0.05$). Mode-of-action (MoA) analysis was performed to figure out the potential mechanism of those candidate drugs (Lamb et al., 2006).

Statistical Analysis

The statistical significance of mean value of variables between two groups was calculated by unpaired Student's *t*-tests. We adopted two-sided Fisher's exact tests to analyze contingency tables. As for the correlation between risk score and patients' outcome, the cutoff value of each subgroup was determined using the "survminer" R package. The survival curves were generated *via* Kaplan–Meier method, and the significance of survival differences was determined using the log-rank test. Univariate and multivariate Cox proportional hazard models were utilized to calculate the hazard ratios of variables and determine independent prognostic factors. LASSO analysis was carried out to get rid of the collinear problem and screen important prognostic genes. The predictive accuracy of the prognostic models was quantified through time-dependent and -independent receiver operating characteristic (ROC) curves. The waterfall function of "maftools" R package was used to describe the mutation landscape in patients with high and low risk in TCGA-PAAD cohort. Correlation coefficients between risk score and tumor-infiltrating immune cells were computed using Spearman and distance correlation analyses. "pRRophetic" R package was implemented for chemotherapeutic response prediction. All data processing was performed in R software 3.6.2. All statistical significance was set at $p < 0.05$.

RESULTS

Phenotypes Based on TP53 Mutational Status in Pancreatic Cancer

Data on somatic mutational variations in TCGA cohort were applied to elucidate the mutational landscape of PC and evaluate 20 of the most important gene mutations. In PC, TP53 mutation is one of the frequent somatic mutational types; among them, missense mutation is the most common aberration (**Figure 1A**). A significant association was observed between TP53 mutational status and prognosis in a deleterious direction (p = 6.723e-04, log-rank test), indicating that PC patients with TP53 mutation had worse prognosis than patients without TP53 mutation (**Figure 1B**). The chi-square test was performed to evaluate the correlation between the TP53 mutational phenotypes and clinicopathological factors. The results indicated that PC patients with TP53 mutation exhibited higher grade of PC than patients without TP53 mutation (p = 0.0015; **Figure 1C**). The single-sample Gene Set Variation Analysis (ssGSVA) method revealed enriched pathways between PC patients in various TP53 mutational subtypes. The direct comparison of the TP53 mutation group vs. the TP53 wild-type group revealed estrogen response and KRAS signaling as the top enriched pathways in PC (**Figure 1D**).

Identification of Differentially Expressed Genes and Construction of a TP53-Associated Signature

In view of the significant association between TP53 mutational status and prognosis of PC patients, we aimed to construct a robust prognostic signature based on the DEGs between PC samples with and without TP53 mutation. Differential transcriptional expression analysis was performed using the limma package and meeting the standard of $\log^2FC \geq 1$ and FDR < 0.01. In total, 245 upregulated genes and

[3]http://tide.dfci.harvard.edu

[4]https://www.cancerrxgene.org

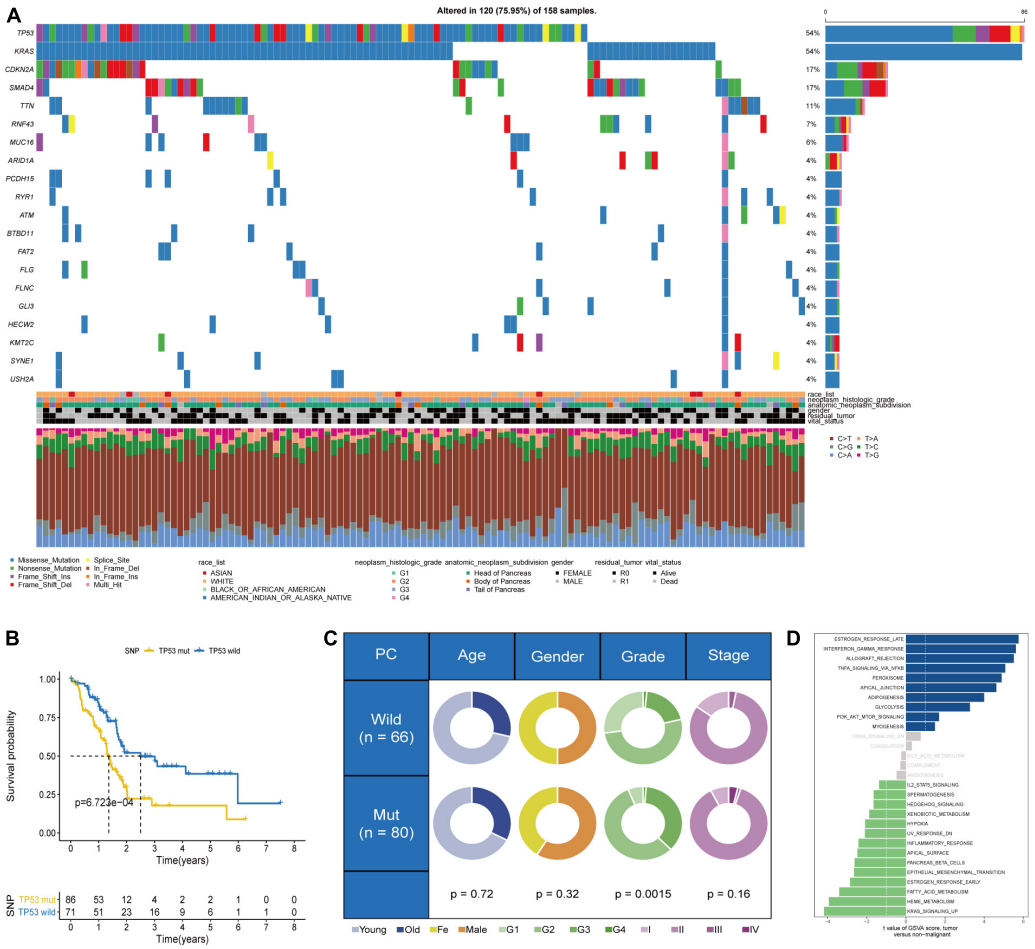

FIGURE 1 | Somatic mutational phenotypes based on the TP53 mutational status in The Cancer Genome Atlas (TCGA) dataset. **(A)** Genomic mutational landscape of pancreatic cancer (PC) in TCGA dataset. **(B)** Kaplan–Meier survival analysis showing that overall survival (OS) was significantly worse in PC patients with TP53 mutation than those without TP53 mutation. **(C)** Pie charts showing the chi-square test of clinicopathological factors in PC tumor samples. **(D)** Difference in the pathway activities scored by Gene Set Variation Analysis (GSVA) between PC patients with and without TP53 mutation.

909 downregulated genes were identified (**Figures 2A,B** and **Supplementary Table 1**). Univariate Cox regression analysis indicated that 492 genes were significantly correlated with prognosis of PC patients ($p < 0.05$). Then, those prognostic genes were subjected to the LASSO and multivariable Cox regression analysis (**Figures 2C,D**). Finally, a TP53-associated signature was constructed based on five genes. Risk score = Exp_{UCA1} * 0.314 − $Exp_{SLC26A11}$ * 0.395 + $Exp_{LINC01559}$ * 0.387 − Exp_{TRIM67} * 0.374 − Exp_{ARNT2} * 0.269 (**Figure 2E**). Within the signature, solute carrier family 26 member 11 (SLC26A11), tripartite motif-containing 67 (TRIM67), and aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) were downregulated in TP53-mutated PC and positively correlated with each other, while the expressions of urothelial carcinoma-associated 1 (UCA1) and long intergenic non-protein-coding RNA 1559 (LINC01559) were upregulated and positively correlated with each other ($p < 0.05$; **Figures 2F–K** and **Supplementary Table 2**). Then, we calculated individual risk score and categorized them into high- or low-risk groups according to the optimal cutoff point in TCGA cohort.

Evaluation and Validation of the Prognostic TP53-Associated Signature in the Cancer Genome Atlas and Gene Expression Omnibus Cohorts

To evaluate the prognostic ability and robustness of the aforementioned TP53-associated signature, its performance was assessed in TCGA and three independent GEO cohorts, including GSE28735, GSE62452, and GSE78229 cohorts. The individual risk score and survival status of patients in the cohorts were shown in **Figures 3A,D,G,J**. Survival analysis in TCGA cohort indicated that patients in the high-risk group were significantly associated with poor OS ($p < 0.0001$, log-rank test; **Figure 3B**). The area under the time-dependent ROC curve of the signature was 0.726 at 1 year, 0.788 at 3 years, and 0.871 at 5 years (**Figure 3C**). Moreover, the TP53-associated signature had well above AUC values compared with the TP53 mutation and clinicopathological factors (**Supplementary Figure 1A**). Consistent with the performance for OS prediction in TCGA cohort, we found

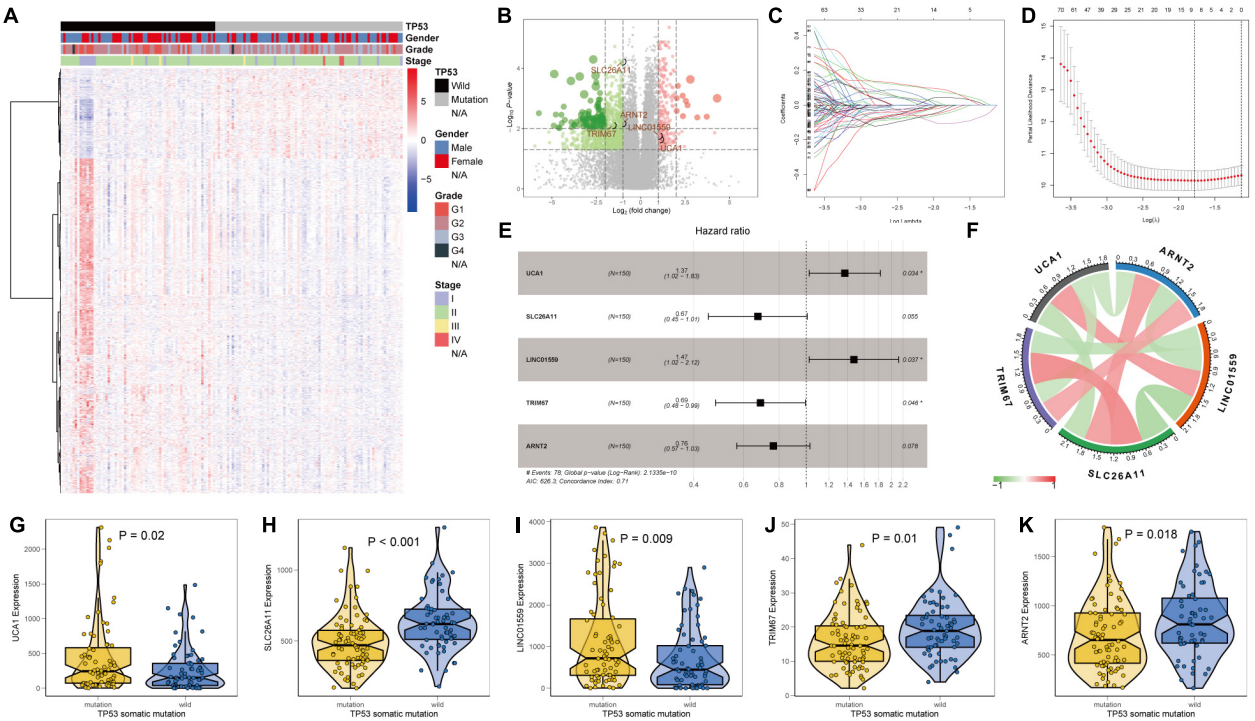

FIGURE 2 | Identification of differentially expressed genes (DEGs) and construction of a TP53-associated signature. **(A)** Heat map and **(B)** volcano plot of DEGs between pancreatic cancer (PC) samples with and without TP53 mutation. Five genes enrolled in the signature were emphasized. **(C)** Least absolute shrinkage and selection operator (LASSO) coefficient profiles of 495 genes. **(D)** Five prognostic genes obtained from LASSO regression with 10-fold cross-validation using minimum lambda value. **(E)** Forest plot, **(F)** mutual correlations, and **(G–K)** relative mRNA expressions of urothelial carcinoma-associated 1 (UCA1), solute carrier family 26 member 11 (SLC26A11), long intergenic non-protein coding RNA 1559 (LINC01559), tripartite motif-containing 67 (TRIM67), and aryl hydrocarbon receptor nuclear translocator 2 (ARNT2).

that the TP53-associated signature also worked well in external GEO cohorts, where patients in the high-risk group had unfavorable OS (GSE28735, p = 0.0125, **Figure 3E**; GSE62452, p = 0.0060, **Figure 3H**; GSE78229, p = 0.0059, **Figure 3K**). Moreover, the high accuracy of the signature remained stable in the independent cohorts (GSE28735, **Figure 3F**; GSE62452, **Figure 3I**; and GSE78229, **Figure 3L**). Conditional survival analysis described the probability of achieving 5 years survival in 307 patients from combined TCGA and GEO cohorts increased from 17 to 25, 43, 56, and 72% per additional year survived (i.e., 1, 2, 3, and 4 years, respectively; **Supplementary Figure 1B**).

Independent Prognostic Value of the TP53-Associated Signature and Its Correlation With Clinicopathological Characteristics

Univariate and multivariate regression analyses showed that the prognostic power of the TP53-associated signature for the OS of PC patients is independent of clinicopathological factors in TCGA cohort (**Figure 4A**). **Figure 4B** showed the comparison of clinicopathological factors between high- and low-risk patients. Analysis in the GSE28375, GSE62452, and GSE78229 cohorts also validated that the TP53-associatd signature is an independent prognostic factor. Besides, we performed subgroup survival analysis and risk stratification in patients with varied TP53 mutational statuses, ages, genders, grades, and stages of tumors. Importantly, the TP53-associated signature can also serve as a promising prognostic marker to predict OS in stratified subgroups of patients with PC in TCGA cohort, including TP53 mutation and TP53 wild-type subgroup (p = 0.0001 and p = 0.02851, respectively; **Figure 4C**), age > 60 and age < 60 (p < 0.0001 and p = 0.0079, respectively; **Figure 4D**), male and female gender (p = 0.0002 and p = 0.0032, respectively; **Figure 4E**), grades 1 + 2 and 3 + 4 (p < 0.0001 and p = 0.0446, respectively; **Figure 4F**), TNM stages I and II–IV (p = 0.0086 and p = 0.0156, respectively; **Figure 4G**). These results demonstrated that the TP53-associated signature is an independent prognostic biomarker.

Mutational Landscape Based on TP53-Associated Signature

As PC is a malignant disease characterized with highly somatic mutations, we next investigated the association between tumor mutation burden (TMB) and TP53-asociated signature. Patients in the high-risk group and

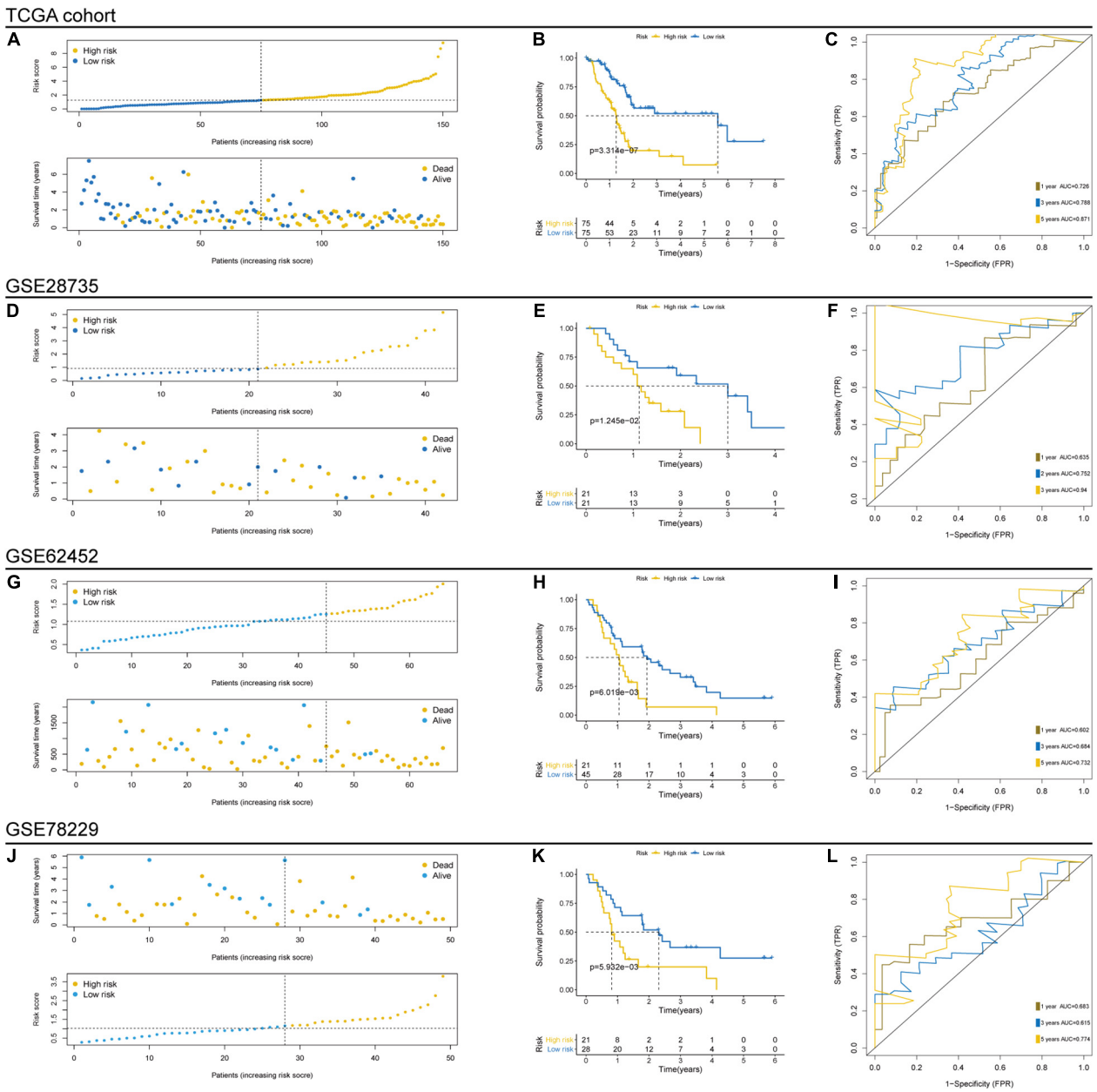

FIGURE 3 | Prognostic evaluation and independent validation of the TP53-associated signature in The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) cohorts. Risk score arrangement, survival status, Kaplan–Meier and time-dependent receiver operating characteristic (ROC) analyses in TCGA **(A–C)**, GSE28735 **(D–F)**, GSE62452 **(G–I)**, and GSE78229 **(J–L)** cohorts.

those with TP53 mutation displayed high TMB level ($p = 0.002$, **Figure 5A**; $p < 0.001$, **Figure 5B**). We found that the risk score and TMB are significantly correlated with OS (**Figure 5C**). Associations among the risk score, TMB, TP53 mutational status, survival status, and overall response were demonstrated in **Figure 5D**. Furthermore, the mutational landscape based on this mutational signature was depicted, and we found that KRAS mutation markedly increased in the high-risk group (**Figure 5E**). The above results demonstrated the stratified ability of the TP53-associated signature in predicting tumor malignancy.

Characterization of Immune Cell Infiltration in Distinct TP53 Mutation-Associated Risk Phenotypes

Recently, several studies emphasized that TP53 mutation status may trigger immune responses and be used as a predictor of immunotherapy in cancers (Dong et al.,

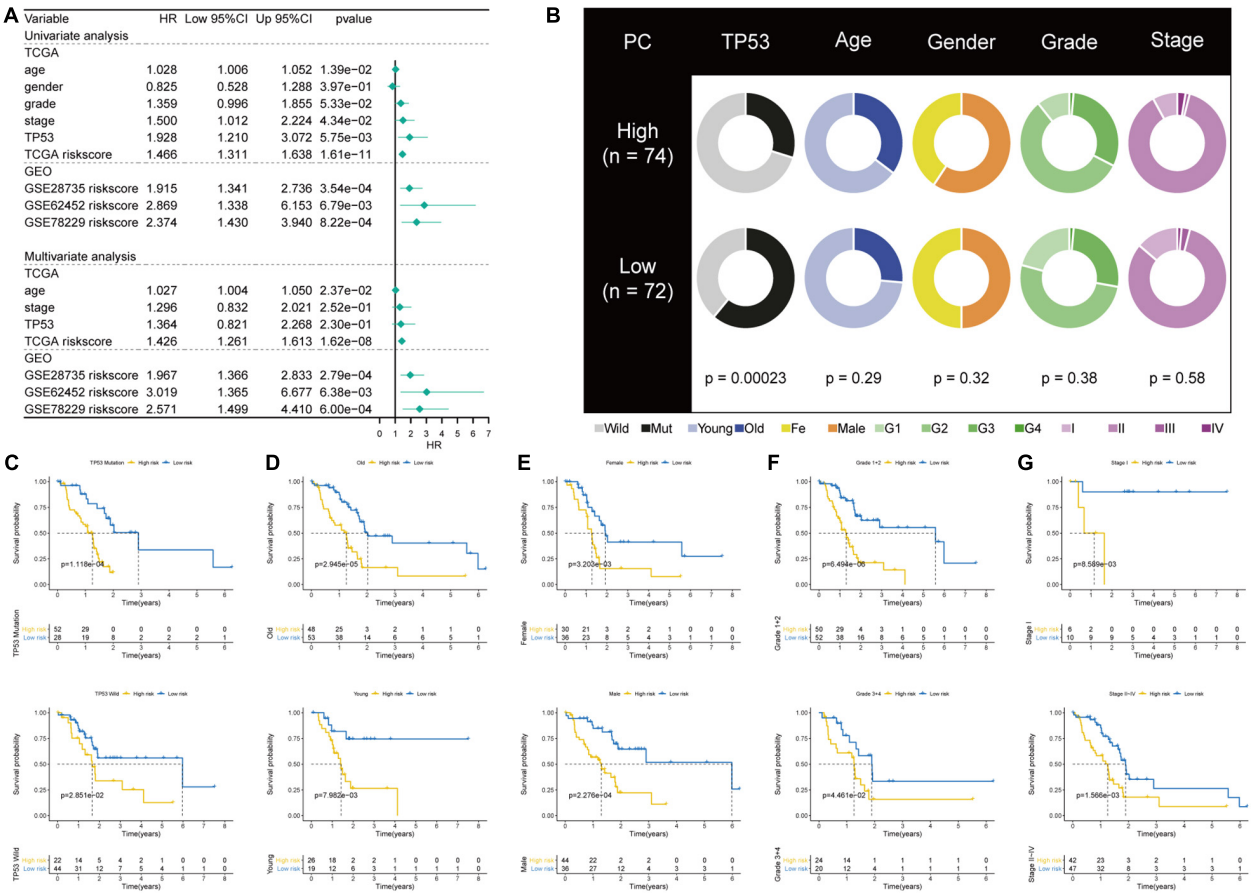

FIGURE 4 | Correlations with clinicopathological characteristics and prognostic independence of the TP53-associated signature. **(A)** Univariate and multivariate regression analyses of the TP53-associated signature and other clinicopathological factors. **(B)** Pie charts showing the chi-square test of clinicopathological factors in pancreatic cancer (PC) samples from The Cancer Genome Atlas (TCGA) cohort based on the TP53-associated signature. **(C–G)** Kaplan–Meier survival analysis of **(C)** TP53 mutation and wild-type subgroup, **(D)** age > 60 and age < 60 subgroup, **(E)** male and female subgroup, **(F)** grades 1 + 2 and 3 + 4 subgroup, **(G)** TNM stage I and II–IV subgroup.

2017; Skoulidis et al., 2018). Therefore, we explored the characterization of immune cell infiltration in PC *via* CIBERSORT. Our results showed the difference of tumor-infiltrating immune cells between patients in the high- and low-risk groups (**Figure 6A**). The variations identified in the immune landscape promoted us to gain insight into the intrinsic traits of individual characteristics. We further investigated the differential composition and association of tumor-infiltrating immune cells by CIBERSORT. Patients in the high-risk group had significantly higher levels of Macrophages M0 and resting natural killer (NK) cells but lower levels of naive B cells and $CD8^+$ T cells ($p < 0.05$; **Figure 6B**). TP53-associated signature was found to be positively correlated with Macrophages M0 and resting NK cells and negatively correlated with naive B cells and $CD8^+$ T cells ($p < 0.05$; **Figure 6C**). Differential and correlated analyses showed consistence in the immunologic characterization. Weak to moderate correlations existed between different types of tumor-infiltrating immune cells (**Figure 6D**). The immunosuppressive role of the five genes included in the signature was demonstrated by the TIDE database, which involved CRISPR screening, T cell dysfunction score, immune-suppressive rejection score, and therapeutic response of ICB (**Figure 6E** and **Supplementary Table 3**). Therefore, the heterogeneity of immune cell infiltration in PC may serve as a novel indicator and has potential clinical implication in immunotherapy.

Potential Predictive Biomarker for Chemotherapy and Targeted Therapy

Besides immunotherapy, chemotherapy and targeted therapy are currently two major adjuvant therapies in PC treatment (Bear et al., 2020; Christenson et al., 2020). Since chemotherapy is a classical and effective way in treating PC, we assessed the therapeutic responses of the two risk phenotypes to 138 chemotherapeutic agents. We put our predictive signature into the GDSC database for training. A significant difference in the estimated half inhibitor concentration (IC_{50}) between the two phenotypes was observed, where patients in the high-risk group demonstrated high sensitivity to 48 types of representative or

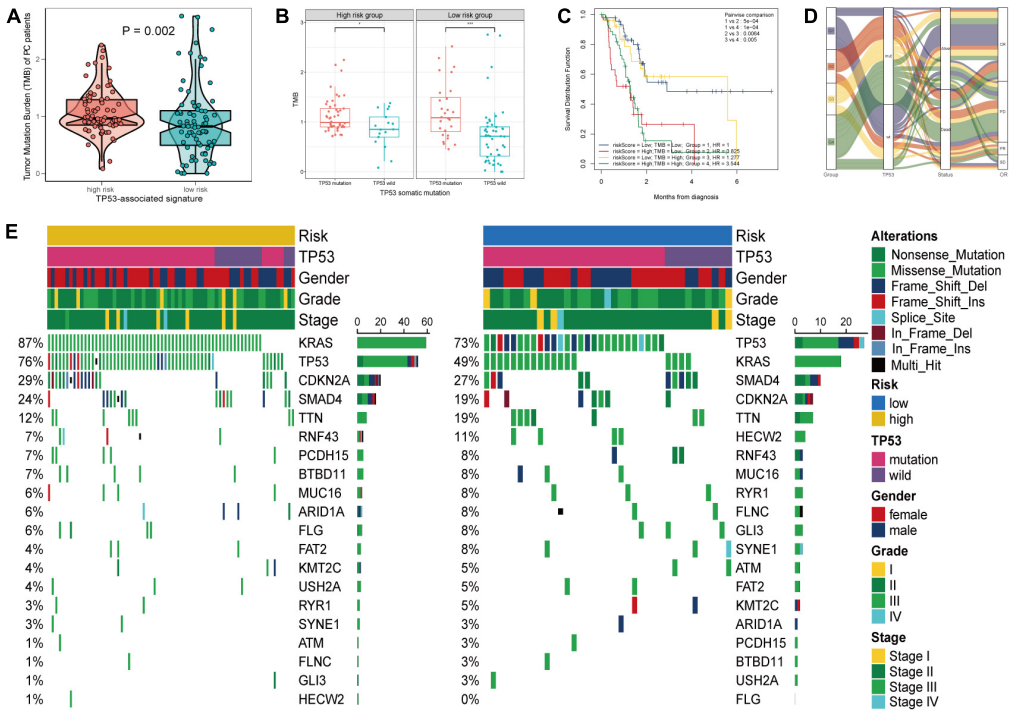

FIGURE 5 | Mutational landscape of the TP53-associated signature. **(A)** Significant difference of tumor mutation burden (TMB) between patients of high- and low-risk group. **(B)** Differential TMB between TP53 mutation and TP53 wild-type pancreatic cancer (PC) based on TP53-associated signature. **(C)** Survival of patients with PC based on the TMB and risk scores. **(D)** Sankey alluvium showing dynamic correlation of patients with PC. **(E)** Waterfall plot of somatic mutation displayed mutational landscape correlated with the signature.

promising chemotherapeutic drugs (**Supplementary Figure 2**), like gemcitabine ($p < 0.0001$; **Figure 7A**), paclitaxel ($p = 0.030$; **Figure 7B**), cisplatin ($p = 0.009$; **Figure 7C**), and pyrimethamine ($p = 0.018$; **Figure 7D**). Next, analysis on the CMap approach was conducted and identified five of eight available candidate compounds/inhibitors that targeted the TP53-associated signature, including doxylamine, econazole, fuldroxycortide, ondansetron, and W-13. Using the mode-of-action (MoA) analysis, the aforementioned potential drugs are unveiled enriched in calmodulin antagonist, glucocorticoid receptor agonist, histamine receptor antagonist, lanosterol demethylase inhibitor, serotonin receptor antagonist, and sterol demethylase inhibitor (**Figure 7E** and **Supplementary Table 4**). Taken together, the established TP53-associated signature might provide guidance for selecting sensitive chemotherapeutic agents and developing individualized targeted drugs for PC.

DISCUSSION

Mutated in a wide range of cancer types and in over 70% of PC (Waddell et al., 2015; Kastenhuber and Lowe, 2017), tumor suppressor TP53 played a pivotal role in cellular stress response and acted as a tumor suppressor gene in PC (Kruiswijk et al., 2015; Kleeff et al., 2016). The past few years have witnessed tremendous efforts toward the development of promising candidate biomarkers in PC, among them the prognostic significance of TP53 mutation had been proposed (Grochola et al., 2011; Ormanns et al., 2014). Although the oncogenic role of TP53 mutation has been well documented, currently, there is a lack of a prognostic and therapeutically predictive biomarker based on the TP53 mutational status in PC. In the present study, we investigated the landscape and phenotypes of TP53 mutation in PC. We found that TP53 mutation played a prognostic and oncogenic role in PC, contributing to pathways of tumor growth and progression. We profiled a differentially expressed gene set affected by TP53 mutational status and generated a TP53-associated signature that could identify patients with poor OS, enhanced immune infiltration, and remarkable therapeutic sensitivity. Our results revealed promising value of the TP53-associated signature in clinical prognostic assessment. It could also be used to determine drug therapeutic strategy for patients who are not suitable candidates for surgery. To our knowledge, this study was the first to describe a novel model for prognostic assessment and therapeutic response based on the mutational status of driver genes in PC.

In this study, LASSO–Cox analyses were employed to construct a TP53-associated signature, composed of UCA1, SLC26A11, LINC01559, ARNT2, and TRIM67 genes. These five genes possessed the potential for individual target and may perform better in combination. These genes were proposed to play roles in regulating immune functions. Induced by hypoxia-inducible factor (HIF)-1α in the presence of hypoxia, UCA1

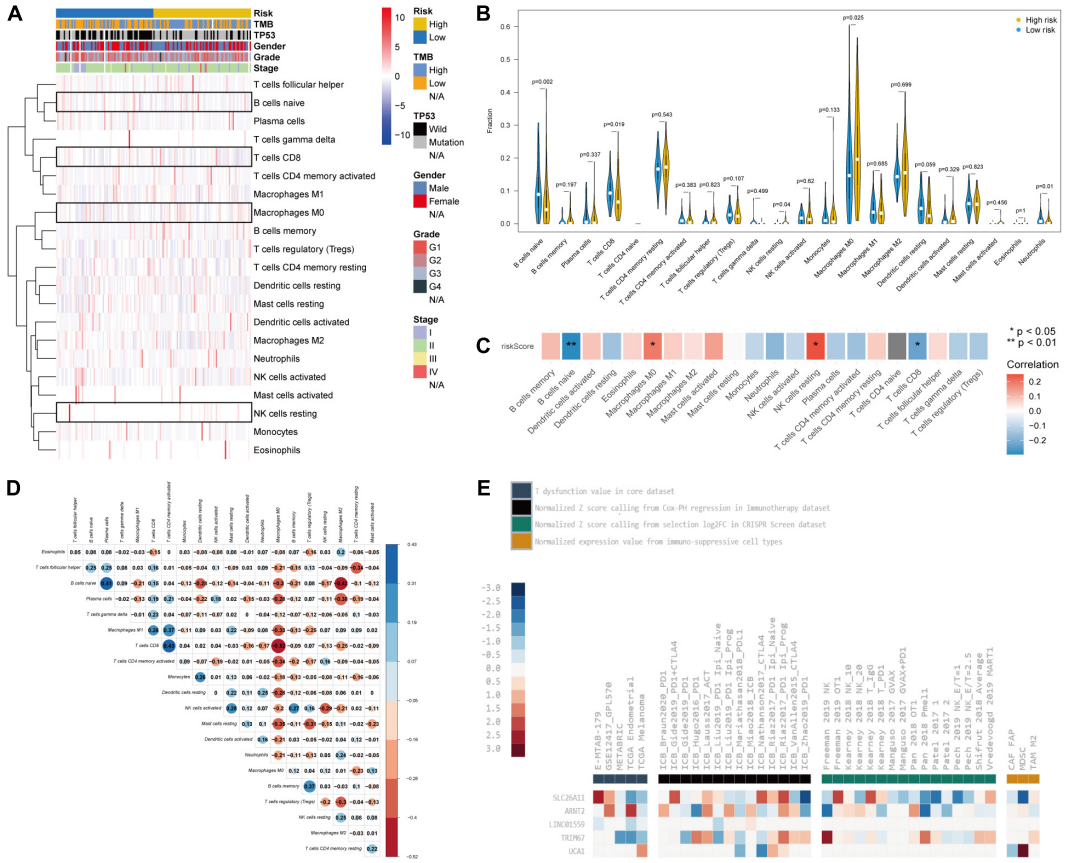

FIGURE 6 | The characteristics of immune cell infiltration and correlation with the TP53-associated signature. **(A)** Immune cell heatmap and clinicopathological characteristics of two phenotypes defined by TP53-associated signature. Four kinds of immune cells were highlighted with boxes. **(B)** Violin plots reflecting the differential composition of 24 types of tumor-infiltrating immune cells. **(C)** Correlation matrix visualizing the relationship between tumor-infiltrating immune cells and risk scores based on the TP53-associated signature. **(D)** Mutual correlations between 24 types of tumor-infiltrating immune cells. **(E)** The role of the five genes in T cell dysfunction, prognosis after immune checkpoint blockade (ICB) therapy, and immunosuppressive T cell rejection.

is dysregulated in tumors and plays a role in carcinogenesis (Chen et al., 2018). In PC, hypoxic exosome-mediated UCA1 could promote tumor angiogenesis and accelerate tumor growth *via* the miR-96-5P/AMOTL2/ERK1/2 axis and serve as a novel therapeutic target (Guo et al., 2020). Previous study reported that upregulated UCA1 promoted programmed death ligand 1 (PDL1) expression through the repression of miRNAs and contributed to immune escape in cancers (Wang C. J. et al., 2019). SLC26A11 is a chloride transporter facilitating acid secretion, with the localization of the vacuolar H + -ATPase in the A-intercalated cells of the kidney (Xu et al., 2011). TIDE analysis revealed that SLC26A11 is downregulated in the TP53-mutated PC and had remarkable association with immune-related processes, including interactions with cytotoxic T cells to affect patient prognoses, regulation of immunosuppressive cells that promoted T cell rejection, and ICB. Our results demonstrated the significance of SLC26A11 in immune-regulatory and oncogenic processes. In a previous study, a myriad of evidence has demonstrated the crucial roles of LINC01559 and ARNT2 in carcinogenesis and tumor progression. For example, LINC01559 could facilitate pancreatic tumor proliferation and migration through the regulation of Rubisco accumulation factor (RAF)1 overexpression and Yes-associated protein (YAP)-mediated pathway (Chen et al., 2020; Lou et al., 2020); located at the hub of transcription factor network, ARNT2 functions as a key component of oncogenic signature, contributing to cancer cell aggressiveness (Bogeas et al., 2018). However, whether immunological factors play critical roles in oncogenesis remain enigmatic, and our research revealed the immune-related oncogenic effects of LINC01559 and ARNT2 for the first time. Functioning as a transcriptional target bounded by p53 and crucial tumor suppressor, TRIM67 boosted apoptosis and p53-induced tumor growth suppression and improved chemotherapeutic responsiveness (Wang S. et al., 2019). Therefore, these five genes might play roles in cancers partly by affecting immune responses.

Further analyses suggested the accuracy, independence, and robustness of the TP53-associated signature in our study. We found that patients in the high-risk group had remarkably worse outcomes with the mean of AUC more than 0.75 in TCGA cohort and validated in the GSE28735, GSE62452, and GSE78229 cohorts. Moreover, this signature was proven to be an

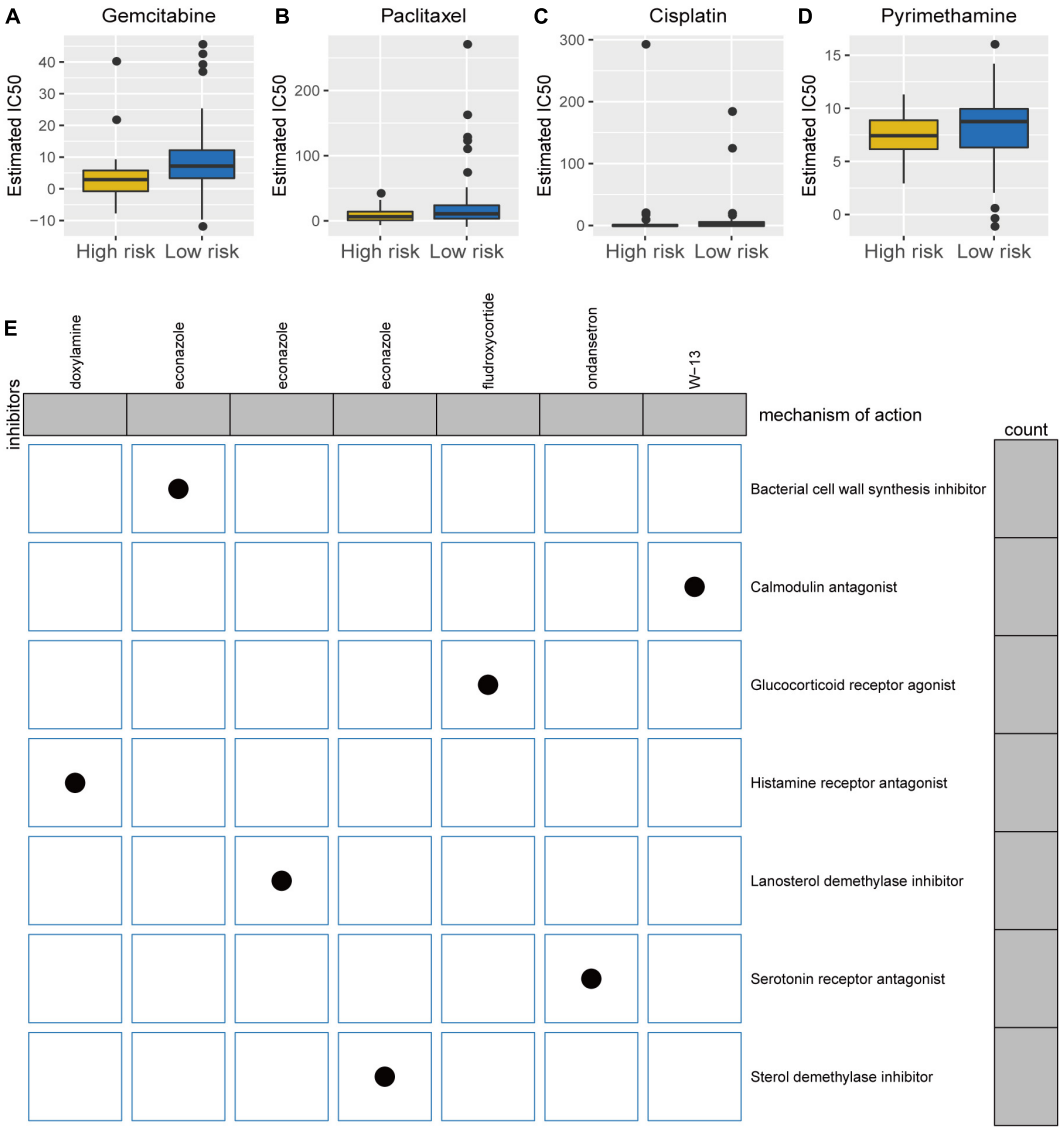

FIGURE 7 | Differential chemotherapeutic responses and targeted traits of patients of high- and low-risk pancreatic cancer (PC). **(A–D)** Estimated IC_{50} for gemcitabine **(A)**, paclitaxel **(B)**, cisplatin **(C)**, and pyrimethamine **(D)** between high- and low-risk patients with PC. **(E)** Intrinsic mechanisms of candidate inhibitors targeted the established signature: Connectivity Map analysis.

independent prognostic factor upon multivariable and stratified survival analyses of several clinical characteristics. Therefore, this TP53-associated signature has the potential to improve prognostic accuracy of traditional clinical factors and could serve as a promising tool for clinical use.

The tumor microenvironment mediated by epithelial–stromal cell interactions is emerging as a critical contributing factor of pancreatic cancer relapse and metastasis, impairing the effectiveness of chemotherapy and immunotherapy (Chronopoulos et al., 2016; Ren et al., 2018). Extensive studies on tumor microenvironment have suggested the pivotal role of immune cell infiltration in tumor dissemination, progression, metastasis, as well as immunotherapeutic response (Zeng et al., 2018; Zhang et al., 2020). Here, we investigated the characteristics of immune cell infiltration based on the TP53-associated signature and intrinsic traits related to the efficacy of cancer immunotherapy. High-risk PC patients tended to possess high proportions of macrophages M0 and resting NK cells and low proportions of naive B cells and $CD8^+$ T cells. Tumor-associated macrophages (TAMs) are capable of promoting tumor growth and progression during almost all stages of cancers *via* the secretion of immunosuppressive factors like interleukin-10 (IL-10) (De Palma and Lewis, 2013). Associated with unfavorable prognosis, TAMs are also attractive targets due to their effect on immunotherapy, chemotherapy, and monoclonal targeted therapy (Noy and Pollard, 2014). TAM-secreted cytokines are known to weaken the anticancer effect of tumor-infiltrating lymphocytes (TLSs).

$CD8^+$ T cell is one of such TLSs whose abundance is linked to favorable prognosis and immunotherapeutic response (Vassilakopoulou et al., 2016). Pioneering studies have suggested that recruitment and reactivation of $CD8^+$ T cell infiltration could be objectives of immunotherapies (Zhang et al., 2017). To meet this objective, intercellular interactions in tumor-infiltrating cells are more crucial than single-agent activity. For example, facilitating BAG3 blockade leads to higher infiltration of $CD8^+$ T cells in PC possibly due to decreased secretion of TAM-derived factor (Iorio et al., 2018). Trafficking into pancreatic tumor microenvironment, endogenous $CD8^+$ T cells reactivate recognition and destruction of neoplastic cells by the combination of programmed cell death protein 1 (PD-1) and C-X-C chemokine receptor type 4 (CXCR4) blockade as the basis for combination immunotherapy in PC (Seo et al., 2019). We suggested that the induction of immunosuppressive microenvironment likely underlies poor prognosis and treatment-refractory nature of the high-risk patients.

The optimal treatment strategy of locally advanced or metastatic PC remains challenged, given the absence of selecting population most likely to benefit from available standard chemotherapeutic regimens. Analysis on GDSC showed the difference of chemotherapeutic sensitivity between TP53-associated risk phenotypes. High-risk patients are more sensitive to the 48 chemo drugs, including gemcitabine and paclitaxel. Recently, pioneering investigation endorsed the activity and safety of nab-paclitaxel plus gemcitabine (AG) as the first-line chemotherapy in localized PC (Perri et al., 2020; Philip et al., 2020). In addition to AG, some other chemotherapeutic agents, like istiratumab (NCT02399137) (Kundranda et al., 2020), capecitabine and cisplatin (NCT01730222) (Reni et al., 2018), were shown to improve the treatment efficacy on metastatic PC. Besides, high-risk patients demonstrated high sensitivity in some novel agents in cancer treatment, such as ABT-263 (Navitoclax) and pyrimethamine, providing great insight into novel chemo drugs for PC treatment. Moreover, five potential inhibitors that target TP53-associated signature were screened out according to CMap database and MoA analysis. Previous studies have seldom reported the application of these drugs in the treatment of PC, with the exception of doxylamine [histamine receptor antagonist (HRA)]. A similar analysis has previously identified doxylamine as a potential drug that targeted lncRNA in non-homologous end joining pathway I in early stage pancreatic ductal adenocarcinoma based on genomic expression profile (Shang et al., 2020). Interactions between HRA and metformin may play a crucial role in inhibiting pancreatic carcinogenesis (Broadhurst and Hart, 2018). The present study suggested doxylamine and HRA as promising drugs for PC. However, the mechanism and effectiveness of specified agents for treatment of PC warrant further investigation and elucidation.

In summary, we constructed and validated a genomic signature based on TP53 mutational status in PC. The TP53-associated signature can be used for prognostic stratification and can reflect immune cell infiltration. It can also serve as a multifaceted therapeutic indicator.

DATA AVAILABILITY STATEMENT

All data used in this work can be acquired from the TCGA (https://portal.gdc.cancer.gov/repository), GEO (https://www.ncbi.nlm.nih.gov/geo), GDSC (https://www.cancerrxgene.org), and TIDE (http://tide.dfci.harvard.edu). The accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

FZ, MY, and YL designed this work. FZ, WZ, and KH integrated the data and conducted the analyses. FZ and HL wrote this manuscript. YL, MY, and KH edited and revised the manuscript. All authors approved this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.665265/full#supplementary-material

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REVIEW
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The Cellular Origins of Cancer-Associated Fibroblasts and Their Opposing Contributions to Pancreatic Cancer Growth

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Pancreatic tumors are known to harbor an abundant and highly desmoplastic stroma. Among the various cell types that reside within tumor stroma, cancer-associated fibroblasts (CAFs) have gained a lot of attention in the cancer field due to their contributions to carcinogenesis and tumor architecture. These cells are not a homogeneous population, but have been shown to have different origins, phenotypes, and contributions. In pancreatic tumors, CAFs generally emerge through the activation and/or recruitment of various cell types, most notably resident fibroblasts, pancreatic stellate cells (PSCs), and tumor-infiltrating mesenchymal stem cells (MSCs). In recent years, single cell transcriptomic studies allowed the identification of distinct CAF populations in pancreatic tumors. Nonetheless, the exact sources and functions of those different CAF phenotypes remain to be fully understood. Considering the importance of stromal cells in pancreatic cancer, many novel approaches have aimed at targeting the stroma but current stroma-targeting therapies have yielded subpar results, which may be attributed to heterogeneity in the fibroblast population. Thus, fully understanding the roles of different subsets of CAFs within the stroma, and the cellular dynamics at play that contribute to heterogeneity in CAF subsets may be essential for the design of novel therapies and improving clinical outcomes. Fortunately, recent advances in technologies such as microfluidics and bio-printing have made it possible to establish more advanced *ex vivo* models that will likely prove useful. In this review, we will present the different roles of stromal cells in pancreatic cancer, focusing on CAF origin as a source of heterogeneity, and the role this may play in therapy failure. We will discuss preclinical models that could be of benefit to the field and that may contribute to further clinical development.

Keywords: pancreatic ductal adenocarcinoma, stroma, heterogeneity, cancer-associated fibroblasts, cellular origins, resistance, radiation, pre-clinical models

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Abbreviations: Heterogeneity, The quality or state of elements being diverse or dissimilar; Phenotype, The total of all observable properties including morphological and functional aspects; Population, The total number of members in a particular area that are present at the same time; Subset, A group of unique elements/members that are contained within a population; Stellate cells, Typically quiescent fibroblast-like cells found in the liver or pancreas that are involved in tissue fibrosis; Fibroblasts, A connective-tissue cell of mesenchymal origin that secretes proteins, especially molecular collagen from which the extracellular fibrillar matrix of connective tissue forms; Cancer-associated fibroblasts, Constitutively activated fibroblasts that reside within a tumor; Mesenchymal stem cells, Multipotent cells isolated from various organs that are able to proliferate and self-renew, as well as to give rise to progeny of at least the osteogenic, chondrogenic, and adipogenic lineages.

INTRODUCTION

The tumor microenvironment (TME) comprises both cellular and non-cellular components (Bremnes et al., 2011). It consists of a rich admixture of cells that harbor distinct activities and contribute differently to tumor growth and progression (Kiaris et al., 2004). In terms of cellular members, the stroma is mainly composed of fibroblast populations and other mesenchymal stromal cells, both of which are involved in forming connective tissue and extracellular matrix components; however, other cell types such as endothelial cells, pericytes, adipocytes, and immune cells also populate the TME (Valkenburg et al., 2018). Although most cells in the stroma possess certain tumor-suppressing capabilities, these cells are thought to be eventually coerced by the cancer cells and instructed to promote cancer growth, invasion, and metastasis (Valkenburg et al., 2018). Nevertheless, the exact contributions of stromal constituents involved in extracellular matrix (ECM) deposition and remodeling to cancer progression and therapy response have still to be fully understood. One unanimously agreed upon fact is that cancer-associated fibroblasts (CAFs) are prominent components of tumor stroma that have a large impact on nearly all aspects of cancer cell biology (Tao et al., 2017; Monteran and Erez, 2019). The roles of CAFs are quite extensive and will be discussed in following parts of the manuscript. These include the ability to: shape the ECM; modulate the innate and adaptive immune microenvironments; recruit and regulate leukocyte migration and inflammation via cytokines, chemokines, and growth factors; provide metabolic support (amino acids, lipids, and tricarboxylic acid cycle intermediates); and contribute to paracrine activation of mitogenic and pro-survival cellular signaling via cell surface receptor-ligand interaction and secreted proteins or exosomes (Krisnawan et al., 2020).

Initially, CAFs were assumed to be a homogeneous population of stromal cells. However, various studies have revealed heterogeneity within the CAF pool and identified that these cells can possess both pro- and anti-tumorigenic properties (Öhlund et al., 2014). Indeed, the diverse roles of CAFs have become evident in various cancer types giving rise to the notion of different CAF phenotypes based on morphological, behavioral, and functional properties (Allinen et al., 2004; Tchou et al., 2012; Liu et al., 2019b). This led to several studies with the aim of identifying different CAF subsets, their roles in the tumor microenvironment (TME), as well as their significance with regard to the clinic (Sahai et al., 2020). At first, two subsets of CAFs (iCAFs and myCAFs) were identified as the dominant fibroblastic populations in pancreatic cancer stroma (Öhlund et al., 2017). Shortly after that, an additional subpopulation, namely antigen-presenting CAFs (apCAFs), was identified and found to have antigen-presentation capabilities (Elyada et al., 2019). Importantly, there are some indications that certain CAF subsets derive from specific cell types (Helms et al., 2021), which may be relevant for the failure of recent targeting strategies. It is for this reason that investigating the subtype and cellular origin of CAFs has become relevant. Thus, in this review we will focus on CAFs, with emphasis on the concept of CAF origin as a source of intra-stromal heterogeneity and as the main culprit behind therapeutic shortcomings.

THE CELLULAR ORIGINS OF CANCER-ASSOCIATED FIBROBLASTS

In context of pancreatic cancer, CAFs commonly derive from three major sources: resident fibroblasts (Nair et al., 2017), pancreatic stellate cells (PSCs) (Vonlaufen et al., 2008), and tumor-infiltrating mesenchymal stem cells (MSCs) (Miyazaki et al., 2020); however, other cell types may also be recruited to enrich the CAF pool and feed the desmoplastic reaction (summarized in **Figure 1**).

Resident Fibroblasts

Fibroblasts are mesenchymal cells with essential roles throughout embryonic development as well as adult organ function where they provide mechanical support and maintain tissue architecture (Di Carlo and Peduto, 2018). During normal physiological conditions, these cells typically remain in an inactivated state; however, they are involved in maintaining homeostasis and are activated to aid in wound repair for a short period, after which they revert to a quiescent-like state or are eliminated (McAnulty, 2007). Unfortunately, this is not always the case, as in a diseased state, these cells can be activated for an abnormally long period and lead to fibrosis, thereby impairing normal tissue function (Chan et al., 2019). A similar phenomenon occurs in cancer tissues, where fibroblasts (typically characterized by α-SMA expression) are perpetually activated and contribute to desmoplasia as cancer associated fibroblasts (McAnulty, 2007).

Pancreatic Stellate Cells (PSCs)

Hepatic stellate cells were originally identified by Kupffer (1876) and mislabeled as a type of endothelial cell. They were later properly identified in 1952 and characterized two decades later as the major storage site of retinoids, and vitamin A homeostasis (Kawada, 1997; Pinzani and Gandhi, 2015). Subsequently, pancreatic stellate cells (PSCs) were identified in the mouse pancreatic duct in 1982 as a cell type that is enriched in lipid droplets and that has the capacity to store vitamin A (Watari et al., 1982). They were then observed in healthy sections from human and rat pancreas and named pancreatic stellate cells (Ikejiri, 1990). When activated from their resting state, PSCs adopt a myofibroblast-like phenotype and secrete various ECM components, thereby feeding into the CAF pool and promoting pancreatic fibrosis (Omary et al., 2007). However, beyond that, most of our knowledge on these cells is assumed from their resemblance to hepatic stellate cells (HSCs). Of note, stellate cells express mesenchymal, endodermal, as well as neuroectodermal markers (Kordes et al., 2012), which has complicated identifying their exact source. However, cell lineage tracing studies have confirmed that HSCs derive from mesenchymal cells and have evolved from a mesodermal origin (Cassiman et al., 2006; Asahina et al., 2011). It is worth mentioning that no such studies have been performed for their pancreatic counterparts, up till now, and little is known beyond the fact that the bone marrow

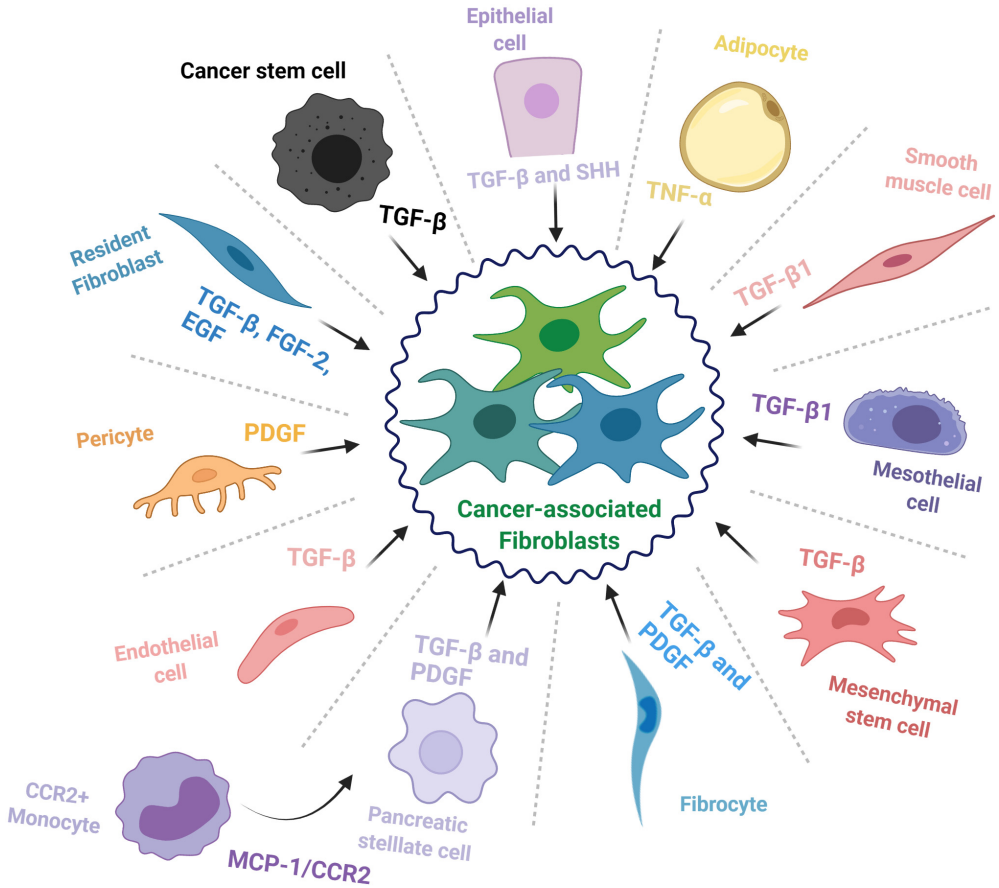

FIGURE 1 | The cellular origins of cancer-associated fibroblasts. The cell types that contribute to the cancer-associated fibroblast (CAF) population and some of the major factors and signaling pathways involved in the transition toward a CAF phenotype.

can be a source of PSCs (Sparmann et al., 2010). Thus, the lineage of PSCs still needs to be mapped in full to reach a complete understanding of what these cells really are, how they influence the pancreatic TME, and how they contribute to different CAF populations in PDAC.

Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells are multipotent adult progenitor cells that were initially discovered in the bone marrow but were later documented in multiple other tissues, including umbilical cord and fat tissue (Pittenger et al., 1999). Mesenchymal stem cells hold self-renewal properties and the ability to differentiate into multiple tissues/cell types including bone, cartilage, muscle and fat cells, as well as connective tissue (Ding et al., 2011). Recent studies have introduced the possible role of MSCs during inflammation, immune response, wound healing, and cancer progression (Chamberlain et al., 2007). Current knowledge suggests that MSCs are recruited into pancreatic tumors where they contribute to disease progression and facilitate cancer therapy resistance (Saito et al., 2018). As a matter of fact, MSCs have been proposed as potential delivery vehicles for anticancer agents in the clinic for many cancer types, including PDAC, due to their ability to home toward tumor sites (Spano et al., 2019). Naturally, it is for this specific property that MSCs are considered one of the main sources of CAFs; for instance, bone marrow-derived MSCs are recruited to PDAC tumors where they differentiate into CAFs or tumor-associated MSCs (TA-MSCs), which can act as yet another source of CAFs and further enrich the population (Liu et al., 2019a). However, it is not yet known how differently sourced MSCs contribute to CAF subsets in PDAC. A recent study showed that adipose tissue-derived MSCs could differentiate into two different CAF subpopulations: direct contact co-culture with a PDAC cell line could induce their differentiation toward either myCAF or iCAF phenotype, while an indirect co-culture induced differentiation into only iCAFs (Miyazaki et al., 2021). This sheds light on the role of MSCs in feeding the CAF population, but whether this is the case for MSCs from other sources as well needs to be studied.

Cells From Non-fibroblastic Lineage

Besides the abovementioned sources, CAFs have been documented to transdifferentiate from seemingly unrelated cell types such as epithelial cells (Iwano et al., 2002; Kalluri and Weinberg, 2009), endothelial cells (Zeisberg et al., 2007),

adipocytes (Bochet et al., 2013), pericytes (Hosaka et al., 2016), mesothelial cells (Rynne-Vidal et al., 2015; Koopmans and Rinkevich, 2018), and smooth muscle cells (McAnulty, 2007). Further, fibrocytes, a circulating mesenchymal cell population of monocytic origin, may contribute to the pool of CAFs in the TME (Reilkoff et al., 2011; Gunaydin et al., 2015). A recent study reported that CCR2+ monocytes can migrate to the pancreas following activation through MCP-1/CCR2 signaling and differentiate into PSCs (Ino et al., 2014). Another interesting source of CAFs is the cancer stem cell (CSC) population; indeed, in some cancer types, CSCs have been documented to adopt a myofibroblast-like phenotype, by undergoing EMT, and subsequently contribute to tumor growth (Petersen et al., 2003; Huang et al., 2015). This shows how deep the repertoire of CAF progenitors may be and underscores the need for comprehensive studies on the relation between CAF origin and function.

THE CONTRIBUTIONS OF SINGLE CELL SEQUENCING (scRNA-Seq) TECHNOLOGY TO CANCER-ASSOCIATED FIBROBLAST IDENTIFICATION

In a recent time-course scRNA-seq study, fibroblasts from pre-invasive human and mouse pancreatic lesions were analyzed to shed some light on gradual changes during pancreatic tumorigenesis and possibly the origin of such cells (Schlesinger et al., 2020). A mouse model with inducible expression of Kras-G12D was used to profile the changes in stromal and acinar cells during the progression from preinvasive lesions to PDAC. Two clusters of fibroblasts were initially observed (Igfbp5+ cells and Il6+ cells). Interestingly, however, during the late stages, Il6+ cells expressed high levels of cytokines (including Ccl2, Ccl7, and Cxcl2) and were reminiscent of inflammatory CAFs (iCAFs). Whereas myofibroblasts (Acta2-positive cells) expressing high levels of Des and Igfbp5 compared to the IL6+ faction were observed. Further, three additional subpopulations of fibroblasts expressing distinct sets of genes were apparent: (i) proliferating fibroblasts (most cells expressed Acta2); (ii) fibroblasts that were recently discovered as MHC-II positive and expressed additional related genes such as CD74 and CD8323; as well as (iii) 15 months' CAFs, which clustered uniquely, that expressed Acta2, Tgfb1, and Cx3cl1, and members of the Wnt signaling pathway, such as Wnt265.

Another single cell transcriptomic study also investigated the biology of primary PDAC tumors as well as metastatic lesions from human patients (Lin et al., 2020). A noteworthy observation was that cells from primary PDAC tumors clustered into seven major populations whereas those from metastatic lesions clustered into three, which directly revealed that the two settings are more dissimilar than alike and calls for more focused studies. Interestingly, unsupervised clustering of CAFs from the primary tumors led to three major clusters (dubbed as c0, c1, and c2), which, in contrast to tumor cells, did not cluster by patient. This finding indicated that CAFs from different patients were more similar in their gene expression profiles than their matching tumor cell populations. The team also set out to determine whether the CAF clusters identified in their analysis matched the three classical CAF subtypes. Only one cluster (c0) was enriched for previously described (myCAF) markers, whereas the remaining two clusters were not enriched in signature genes associated with either iCAFs or apCAFs. In fact, it was found that the signature genes that define cluster 1 were more enriched with those associated with quiescent CAFs; while cluster 2 displayed an expression signature that resembles that of smooth muscle cells, which drove the authors to postulate that these cells might be mural cells including pericytes and vascular smooth muscle cells from the blood vessels.

A recent scRNA-seq paper by Chen K. et al. (2021) described a novel subgroup of CAFs with complement-secreting capacity (csCAFs) in human PDAC tumors that, despite their resemblance to iCAFs, qualified as a unique subgroup. Subsequently, the group demonstrated the existence of these cells and their location by performing by RNA ISH (RNA *in situ* hybridization) and IF (immunofluorescence) on human PDAC sections with different clinical stages. The findings of this study suggested that csCAFs may play a tumor-suppressive role in PDAC microenvironment and that this population is gradually lost during tumor progression.

Another prominent study described a mass cytometry approach that allowed the analysis of mesenchymal stroma in both normal and tumor murine pancreatic tissues (Hutton et al., 2021). The findings of this study revealed extensive stromal heterogeneity across both tissues and led to the identification of coordinated relationships between mesenchymal and immune cell subsets in PDAC. Remarkably, it was found that the expression of CD105 could distinguish two stable and functionally distinct pancreatic fibroblast lineages in both human and murine settings. It was also evident that the CD105-positive fibroblast population is tumor-permissive, while CD105-negative fibroblasts are highly tumor suppressive in a manner entirely dependent on functional adaptive immunity.

Recent advances in single cell technology, including scRNA-Seq as described above, have partly overcome previous shortcomings in the identification of CAF subsets. Nonetheless, the information available in literature regarding CAF *origin* is still somewhat limited. This underscores the need for more comprehensive approaches that make good use of scRNA-Seq findings, perhaps together with lineage tracing technologies to better understand how tumor stroma is shaped and identify the contributions and roles of different CAF progenitor cells in PDAC tumors. A recent paper highlights the importance of such tracing studies for understanding PDAC; targeted ablation specifically of PSC-derived CAFs revealed non-redundant functions for this defined CAF population in shaping the PDAC microenvironment (Helms et al., 2021). This finding links stromal evolution from distinct cells of origin to transcriptional heterogeneity among PDAC CAFs and demonstrates unique functions for CAFs of a defined cellular origin, further supporting the notion of cellular origin being a driver of CAF heterogeneity and urging more studies.

TUMOR CELL-DERIVED SIGNALS INVOLVED IN FIBROBLAST-LIKE CELL RECRUITMENT AND ACTIVATION

Numerous studies have focused on the aspect of tumor-stroma interactions in PDAC. Crosstalk between pancreatic cancer cells and the surrounding stroma is complex, but many key elements in this process have now been identified (Valkenburg et al., 2018). The main mechanisms of stroma cell-tumor cell interaction include the exchange of extracellular molecules, such as extracellular vesicles, cytokines, and chemokines. Other means of communication, such as direct cell-cell interaction, have also been shown to influence the recruitment and activation of CAFs (Sperb et al., 2020). Finally, the roles of key intracellular signaling pathways such as JAK/STAT, mTOR, Sonic Hedgehog (SHH), and NF-kB are relatively well defined in the context of PDAC-stroma crosstalk (Rhim et al., 2014; Duluc et al., 2015; Wörmann et al., 2016; Garg et al., 2018).

It is well established that cancer cells secrete factors like chemokines to recruit inflammatory cells, MSCs, and PSCs toward tumor sites, as well as to instruct them to create a nurturing environment that encourages tumor progression (**Figure 2**; Roy et al., 2017; Geismann et al., 2019). One example study demonstrated that in a coculture setting with Panc-1 cells, normal skin fibroblasts were driven to secrete collagens (I and III), PDGF, as well as fibronectin, thereby contributing to a more desmoplastic environment (Mahadevan and Von Hoff, 2007). The authors went further to identify that transforming growth factor beta 1 (TGF-β1) and fibroblast growth factor (FGF)-2 were involved in this phenomenon and led to the proliferation of both cell types. Typically, many of the signaling processes in the stroma influence the behavior of the surrounding cancer cells as well. In fact, increased TGFβ signaling in the TME seems to form an autocrine-paracrine loop that serves to promote invasion and metastasis of tumor cells during later stages of many cancers, including PDAC (Bierie and Moses, 2006).

Various processes are involved in the transition of stromal cells toward a CAF phenotype and are typically dependent on stimuli such as local hypoxia, oxidative stress, and growth factor release. Indeed, certain factors including TGF-β, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor 2 (FGF2) are considered key regulators of fibroblast recruitment and activation (Karagiannis et al., 2012; Wu et al., 2021). In pancreatic tumors, PSCs can become myofibroblast-like and express α-SMA upon activation by growth factors (TGF-β and PDGF), inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, IL-10, etc.), as well as other factors such as EMMPRIM, ET-1, Angiotensin II, SHH, and further enrich the CAF pool (Heinemann et al., 2014; McCarroll et al., 2014). Furthermore, once activated, PSCs perpetuate their own activity via autocrine loops, which in turn promotes vicious stromal development and ECM deposition (Erkan et al., 2012).

The SHH pathway is an important signaling axis in PDAC as it is involved in pancreatic fibrogenesis (Jung et al., 2011). Initially, multiple lines of evidence indicated that blockade of this pathway with small-molecule inhibitors can inhibit the development of pancreatic tumors (Bai et al., 2016). This attracted a great deal of attention in the pancreatic cancer field and proposed novel therapeutic regimens. Nonetheless, these inhibitors have yielded rather contradictory findings. Several clinical trials (NCT01064622; NCT01088815) have investigated the efficacy of SHH inhibition in pancreatic cancer but overall no significant benefits were observed, despite promising pre-clinical findings (Olive et al., 2009; Özdemir et al., 2014). Since then, papers have emerged that argue against Hedgehog (Hh) pathway inhibition as a therapeutic approach in PDAC. Preclinical studies have shown that genetic and pharmacological inhibition of Hh pathway activity in fact accelerates PDAC progression (Lee et al., 2014). One study described the role of Shh signaling in driving the formation of a fibroblast-rich desmoplastic stroma in PDAC (Rhim et al., 2014). Indeed, the findings of this study show that not only did Shh-deficient tumors have reduced stromal content, but that they were, surprisingly, more aggressive and exhibited undifferentiated histology, increased vascularity, and heightened proliferation. This was an indication that Hedgehog-driven stroma may have a suppressive role in PDAC. This was then linked to the findings of other studies wherein depletion of CAFs and subsequent fibrosis could induce immunosuppression and accelerate pancreatic cancer progression (Özdemir et al., 2014). A recent paper shed further light as to why Shh inhibition may not be the most ideal approach for PDAC treatment (Steele et al., 2021). The findings of this study showed that Shh signaling was specifically activated in myCAFs and that inhibiting this pathway using the smoothened antagonist LDE225 inhibited Shh signaling, reduced myCAF numbers, and increased iCAF numbers in a PDAC mouse model, resulting in an immune suppressive microenvironment.

Epigenetic regulation of normal fibroblasts has been recently highlighted as a means of conversion into CAFs in different cancers (head and neck, lung, and breast). Mechanistically, exposure of the fibroblasts to pro-inflammatory leukemia inhibitory factor (LIF) triggers an epigenetic switch leading to constitutive activation of Janus kinase 1–signal transducer and activator of transcription 3 (JAK1–STAT3) signaling, which is sustained by the DNA methyltransferase DNMT1, and activates the fibroblasts into pro-invasive CAFs with increased acto-myosin contractility (Albrengues et al., 2015); this suggests a shift toward a myCAF-like phenotype but goes against previous findings that linked the iCAF and myCAF phenotypes with JAK/STAT and TGF-β signaling, respectively (Biffi et al., 2019; Chen and Song, 2019). Lactate-mediated epigenetic reprogramming has also been noted in human pancreatic cancers where it regulates the formation of CAFs (Bhagat et al., 2019); in fact, epigenetic reprogramming, in the form of widespread loss of DNA methylation and gain of cytosine hydroxymethylation at selective promoters, was observed in both MSC-derived and primary (patient-derived) pancreatic CAFs and linked the process to increased alpha-ketoglutarate production.

It is worth noting that pancreatic cancer cells can use extracellular vesicles (see also paragraphs on *exosomes* below) to educate members of the TME and contribute to carcinogenesis (Stefanius et al., 2019). One study assessed the ability of pancreatic cancer to recruit PSCs *in vitro* by performing Transwell assays and *in vivo* using a mouse

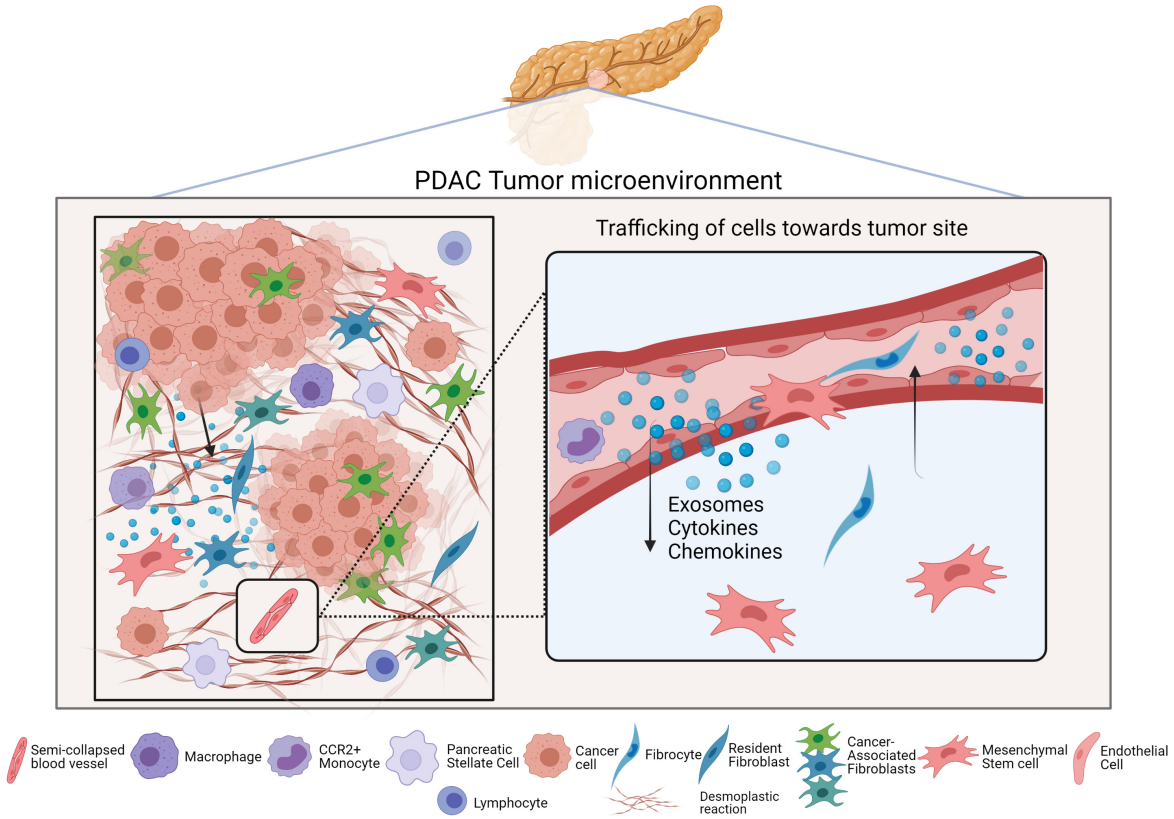

FIGURE 2 | Cell trafficking and the PDAC tumor microenvironment. The **left** panel of the figure is a simplified depiction of the PDAC tumor microenvironment and includes the major cell types that reside in it. The **right** panel is a magnified view of how different secreted factors serve to recruit CAF progenitors into PDAC tumors wherein they enrich the CAF population.

model (Zhang et al., 2019). The group showed that pancreatic cancer cell-derived exosomes containing Lin28B (Exo-Pan and Exo-Mia) promoted the recruitment of PSCs by activating the Lin28B/let-7/HMGA2/PDGFB signaling pathway, which highlights the importance of exosomal signaling in cancer.

Importantly, specific cancer cell genotypes can differently instruct CAFs. One team analyzed tumors harvested from two mouse models with different metastatic propensities and saw that p53 mutant PDAC cells can drive CAFs into establishing a prometastatic and chemoresistant microenvironment due to increased nuclear factor-κB (NF-κB) signaling and high levels of the NF-κB target gene tumor necrosis factor-α (TNF-α) (Vennin et al., 2019). Another finding from this study was that p53 mutant-educated CAFs could induce the invasion of p53 null cancer cells, which are normally considered poorly invasive, supporting the notion that CAFs may play a role in mobilizing aggressive cells within the tumor (Sperb et al., 2020). Similarly, another group identified a unique, highly rigid, matricellular-stromal (also described as mesenchymal-like) phenotype in PDAC (linked to integrins, YAP, and SOX2) that results in reduced epithelial TGF-β signaling and elevated tumor cell contractility as well as tumor progression and aggression (Laklai et al., 2016).

In conclusion, we highlighted how pancreatic cancer cells shape the TME by recruiting and educating CAFs from different sources/precursor cells and presented the main signaling pathways that are involved in these phenomena and how they dictate CAF subtype. We also went further and stressed the importance of cancer cell genotype in further increasing heterogeneity in the CAF pool and generating CAFs that encourage PDAC cell aggressiveness and support tumor progression.

CANCER-ASSOCIATED FIBROBLAST-DERIVED SIGNALS THAT SUPPORT PANCREATIC CANCER

Cancer-associated fibroblasts are a substantial source of signals that can promote tumor growth and impact on therapy responses (Chen et al., 2014; Ali et al., 2015; Richards et al., 2017). CAFs can foster tumor cell growth, angiogenesis, and invasion by secreting paracrine factors, such as pro-inflammatory cytokines, chemokines, prostaglandins (PGE), growth factors, as well as proteases, and by remodeling the extracellular matrix (ECM) (Chan et al., 2019). Moreover, the production of TGFβ, leukemia

inhibitory factor (LIF), growth arrest-specific protein 6 (GAS6), fibroblast growth factor 5 (FGF5), growth differentiation factor 15 (GDF15), and hepatocyte growth factor (HGF) promotes invasive and proliferative behavior in cancer cells (Sahai et al., 2020). VEGF-A and Tenascin-C are other relevant markers in context of stromal contribution to cancer progression; the expression of the former is a driver of angiogenesis and metastatic colonization, while that of the latter provides cancer cells protection from apoptosis (O'Connell et al., 2011). PSCs have also been documented to influence cancer cells through hypoxia-inducible factor 1α (HIF-1 α)-mediated signaling and thereby leading to the subsequent activation of various genes involved in cell survival, progression, invasion, and metastasis (Omary et al., 2007). One study identified a subpopulation of CAFs designated as cancer-associated mesenchymal stem cells (CA-MSCs) and demonstrated that they contribute to cancer invasion via granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion (Waghray et al., 2016). These findings were confirmed by another study which identified that the mechanism underlying GM-CSF-induced invasion is related to downregulation of E-cadherin and upregulation of TWIST1 (which are associated with resistant cancer cell phenotypes) and vimentin via the JAK2/STAT3 pathway (von Ahrens et al., 2017).

Another method by which fibroblasts interact with tumor cells is the exchange of metabolites. Many recent studies have focused on metabolites and their importance in tumor-stroma interaction. Here, we will briefly mention some examples of how stromal cells support pancreatic cancer growth by providing metabolites. For instance, fibroblasts are known for undergoing autophagy in response to stimulation by cancer cells and thereby releasing alanine, which subsequently contributes to pancreatic cancer cell energy production by feeding the tricarboxylic acid (TCA) cycle (Sahai et al., 2020). PSC-derived alanine can also promote PDAC cell growth in nutrient-limited conditions by acting as an alternative carbon source to glucose and glutamine and fueling the TCA cycle, supporting lipid and non-essential amino acid biosynthesis, as well as shunting glucose for serine/glycine biosynthesis (Xu et al., 2020).

Many recent studies have been aimed at studying the role of extracellular vesicles, such as exosomes, in tumor-stroma interaction (Doyle and Wang, 2019). Exosomes are small membrane-enclosed vesicles (<150 nm) of endosomal origin that contain numerous molecular components including proteins, mRNAs, and miRNAs (Ruivo et al., 2017). Exosome secretion has been proven to play a crucial role in long-distance communication in various tissue types (Dai et al., 2020). MSCs and fibroblasts seem to be a rich source of exosomes and have been recorded to interact with tumor cells and participate in tumorigenesis as well as tumor progression (Hu et al., 2015; Yin et al., 2019). For instance, miR-142-3p transfer via BM-MSC-derived exosomes, as well as those from fibroblasts, has been noted to increase the CSC population in colorectal cancer (Li and Li, 2018). CAFs also seem to protect pancreatic cancer cells from gemcitabine treatment via exosomal Snail and miR-146a that enhance the proliferative capacity of the cancer cells and induces EMT, which is linked with resistance and metastasis (Richards et al., 2017); interestingly, exosome secretion was upregulated as a result of gemcitabine treatment, which highlights the importance of CAFs in *acquired* pancreatic cancer resistance. Many efforts aimed to explore the secretome of CAFs and various secreted factors and active substances have been identified to be involved in tumor-stroma crosstalk and might serve useful to better understand CAF heterogeneity and establish effective treatment approaches.

THE TUMOR-SUPPRESSIVE POTENTIAL OF CANCER-ASSOCIATED FIBROBLASTS IN PDAC

The notion of a tumor-suppressive population of CAFs has gained a lot of attention. This led to the proposal of markers, such as α-smooth muscle actin (SMA), as candidates to identify tumor-restraining CAFs in PDAC. However, other studies had already revealed a correlation between the number of α-SMA+ CAFs and poor outcome in various types of human solid cancers, leading to an opposing hypothesis which states that α-SMA+ CAFs are in fact tumor-permissive and tumor-promoting (Miyai et al., 2020). Interestingly, a recent study provided insight into the nature of tumor-restraining CAFs and suggested that they may share molecular properties with PSCs and MSCs (Mizutani et al., 2019). Indeed, markers that are representative of PSCs and MSCs seem to be associated with favorable prognosis in PDAC; for instance, the number of CD271+ stellate cells is associated with good prognosis in pancreatic cancer (Fujiwara et al., 2012). CD36-expressing fibroblasts with a low expression of CD36 contribute to the deposition of collagens and fibronectin, to a higher degree than their highly CD36-expressing counterparts do, thereby contributing to a more desmoplastic environment. The expression of Meflin, a glycosylphosphatidylinositol-anchored protein as a marker of mesenchymal stromal/stem cells, in CAFs has also been correlated with favorable outcome in human PDAC (Maeda et al., 2016). Remarkably, Meflin has the capacity to suppress αSMA expression (myofibroblastic differentiation) in CAFs as well as ECM remodeling, a crucial process for cancer progression, which might give some insight into the functionality of tumor-suppressive CAFs and how they inhibit tumor growth. Of course, more comprehensive studies in genetic animal models are necessary to confirm these *in vitro* findings and whether targeted approaches could be safe and efficacious in the clinic.

There, thus, appears to be a link between CAFs expressing markers that are typically attributed to MSCs and/or PSCs and them being tumor-repressive. However, since MSCs and PSCs are major sources for CAFs, this suggests one of two scenarios; (i) that tumor-repressive CAFs are less activated and therefore more similar to MSCs/PSCs, or (ii) that there is heterogeneity among MSCs/PSCs that persists after they become CAFs. These notions seem less farfetched when you take into account the findings of Waterman et al. (2012) who showed that MSCs can have two phenotypes that present opposing effects on cancer growth and metastasis.

Exosomes are also involved in mediating the anti-cancer effects of stromal cells in pancreatic cancer. One study demonstrated that BMSC-derived exosomes could suppress

proliferation, invasion, and metastasis as well as promote apoptosis in pancreatic cancer cells by transferring miR-126-3p and, thereby, downregulating ADAM9 (Wu et al., 2019). Another study showed that low miR-1231 expression in peripheral blood-derived exosomes was significantly correlated with the TNM stage of pancreatic cancer, hinting toward a potentially inhibitory effect of exosomal miR-1231 on the occurrence and development of the disease (Shang et al., 2019).

To sum up, we know that certain aspects dictate whether CAFs exert tumor-suppressive or -promoting activities and that this needs to be fully understood in order to improve therapeutic regimens and/or adopt more targeted approaches. Taking the abovementioned information into account, there seems to be a link between the tumor-suppressive activity of CAFs and them expressing PSC/MSC markers and that tumor-suppressive CAFs seems to have reduced α-SMA expression and ECM remodeling abilities compared to their tumor-promoting counterparts. We propose that more advanced *ex vivo* or animal models should be utilized in order to corroborate these findings and make way to clinically relevant targeted approaches that target specific CAF subpopulations in PDAC. We will discuss this in a subsequent section that discusses available technologies and how they may be utilized for improving PDAC modeling.

THE ROLE OF THE STROMA IN CONFERRING RESISTANCE TO CHEMOTHERAPY

Cancer-associated fibroblasts, ECM components, as well as immune cells can all directly confer a resistant phenotype in tumor cells (Krisnawan et al., 2020), here, we will mainly discuss the role of CAFs in this regard. Many studies have identified these cells as promoters of resistance; however, the molecular mechanisms underpinning these phenomena remain unclear. One group demonstrated that CAF-secreted SDF-1 drives gemcitabine resistance in pancreatic cancer by forming a positive feedback loop that drives paracrine induction of SATB-1 in the pancreatic cancer cells (Wei et al., 2018). Another team identified the role of miR-21 expression in CAF activation, through PDCD4 upregulation, as well as resistance to gemcitabine using tumor samples from PDAC patients (Zhang L. et al., 2018). This led to some mechanistic insights as the authors revealed that high miR-21-expressing CAFs secreted elevated levels of MMP-3, MMP-9, PDGF, as well as CCL-7, thereby promoting the invasion of PDAC cell lines and mediating gemcitabine resistance in an *in vivo* setting. USP27X is another interesting candidate in context of stroma-mediated chemoresistance. In fact, USP27X is activated by TGFβ, a known inducer of EMT (Shen et al., 2017), and plays a major role in both TGFβ-induced EMT and fibroblast activation (Lambies et al., 2019), which suggests it contributes to a positive activation loop. The IL-1β-IRAK4 signaling pathway is yet another major player in PDAC cell response to chemotherapy (Zhang D. et al., 2018); CAFs robustly express IRAK4 and NF-κB and support PDAC cell chemoresistance. Interestingly, this axis may be a valuable therapeutic target to potentially circumvent chemoresistance in pancreatic cancer as targeting IRAK4 or IL-1β could render PDAC tumors less fibrotic and more sensitive to gemcitabine and, potentially, other chemotherapeutic agents (Zhang D. et al., 2018; Elahi-Gedwillo et al., 2019).

Another group showed that Periostin, which is exclusively expressed by PSCs, confers resistance to gemcitabine in pancreatic cancer cells (Dauer et al., 2017). PSCs have also been reported to promote the expression of HES1 in pancreatic cancer cells, thereby making them more resistant to chemotherapy. The SDF1/CXCR4 pathway is a major signaling axis that contributes to resistance in pancreatic cancer; SDF1 is secreted by CAFs and interacts with CXCR4, its receptor, which is present on the cancer cells, and induces gemcitabine chemoresistance in these cells by paracrine-induced activation of the intracellular FAK-AKT and ERK1/2 signaling pathways and a subsequent autocrine IL-6 signaling loop (Zhang et al., 2015). Interestingly, IL-6 was also found to induce the production of Survivin, an inhibitor of apoptosis, in cancer cells (Duluc et al., 2015); the authors also identified that mTOR/4E-BP1 signaling is activated in cancer cells as a response to CAF-secreted factors and plays a role in imparting chemoresistance. Another interesting finding was that CAFs serve as a source of CYR61 in co-culture models and also induce chemoresistance by downregulating the nucleoside transporters hENT1 and hCNT3, which are known to mediate cellular uptake of chemotherapeutic drugs such as gemcitabine (Hesler et al., 2016).

Pancreatic stellate cells also contribute to shaping a hypovascular and hypoxic microenvironment, which is a characteristic of pancreatic tumors (Margetis and Drekolias, 2015) and a major obstacle for the delivery of chemotherapeutics (Sheth et al., 2013). These features intensify chemoresistance and encourage fibrosis in a self-perpetuating hypoxia-fibrosis cycle (Margetis and Drekolias, 2015). This not only promotes EMT and genetic instability in cancer cells, but also impairs drug delivery (McCarroll et al., 2014); it is worth noting that stromal depletion prior to chemotherapy administration could enhance intratumoral drug perfusion, rendering tumors vulnerable to cytotoxicity and, thereby, inhibiting tumor growth and prolonging overall survival. However, removing the stroma, which acts as a tumor-containing fibrotic barrier, may have also unintentionally encouraged the metastatic evolution of aggressive clones (Özdemir et al., 2014), which might explain the disappointing results of a phase II clinical trial following the paper initially describing this concept (Olive et al., 2009). Interestingly, PSCs survive in patients treated with full-dose gemcitabine plus concurrent hypo-fractionated stereo-tactic radiosurgery, and display a more activated phenotype following this regimen (Cabrera et al., 2014). In addition to contributing to mechanical properties and hypoxia-induced chemoresistance, PSCs can directly impact cancer cell response to chemotherapy; PSC secretions have been shown to confer a chemoresistant phenotype in pancreatic cancer cells by suppressing H2O2-induced apoptosis (Vonlaufen et al., 2008) in addition to decreasing pancreatic cancer cell sensitivity to gemcitabine, 5-fluorouracil (5-FU), cisplatin, doxorubicin as well as radiation therapy (Miyamoto et al., 2004; Hwang et al., 2008).

Despite the current knowledge, however, there are not enough comprehensive studies on the roles of different CAF subsets with respect to pancreatic cancer cell sensitivity to chemotherapy, and whether certain subsets exist that strictly alleviate resistance. In fact, most of the available data on resistance cannot be correlated with CAF subtype due to the lack of expression data for relevant markers such as α-SMA or IL-6. We should also mention that most studies that are currently published tackle gemcitabine resistance but there are not enough studies on more recently implemented regimens such as 5-FU, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) (Chiorean and Coveler, 2015). This is something to acknowledge and warrants further research.

THE TUMOR STROMA AND RADIORESISTANCE

Radiation therapy has a prominent place in treating locally advanced pancreatic cancer (Hall and Goodman, 2019). Unfortunately, however, the molecular pathways that contribute to resistance to ionizing radiation (IR) in pancreatic cancer remain poorly understood (Brunner et al., 2005; Kimple et al., 2010). Much evidence has emerged to support the role of PSCs and CAFs as major contributors to radio-resistance (Krisnawan et al., 2020). These cells were shown to confer resistance by multiple modes of action. On the one hand, they could achieve this feat by direct contact with surrounding cancer cells mainly via β-Integrin-FAK signaling (Mantoni et al., 2011). On the other hand, they could contribute to a resistant cancer phenotype by secreting a plethora of factors (Linares et al., 2021).

A recent study described the role of stromal fibrosis in activating pro-survival and epithelial-to-mesenchymal transition (EMT) pathways in PDAC. The group identified two cell-surface proteins, a disintegrin and metalloprotease 10 (ADAM10) and ephrinB2, as drivers of fibrosis and tumor progression after radiation therapy (RT) and suggested that activation by ephrinB2 drives fibroblasts toward a myofibroblast differentiation, thereby driving cancer invasion (Mueller et al., 2021). Unfortunately, there are few studies that include data on fibroblast heterogeneity and/or phenotype with respect to resistance to RT and most studies focus more on the mechanisms underpinning RT resistance in general; as such, this section will be a more general viewpoint on the role of CAFs in mediating/alleviating radioresistance in PDAC. We will also use this opportunity to encourage more studies on different CAF populations and subsets and how they are affected by RT and by which underlying mechanisms.

As was introduced above, exosome transfer is a prominent mechanism for tumor-stroma signaling. This mode of communication is also implicated in conferring resistance to radiation (Zhang H. et al., 2018). For instance, paracrine anti-viral RIG-I and juxtacrine NOTCH (NOTCH3-JAG1) have both been identified as contributors to therapy-resistance, which they facilitate by inducing tumor-initiating cell expansion in a STAT1-dependent fashion (Boelens et al., 2014). Exosomal lipids have been shown to induce drug resistance in MiaPaCa-2 cells, via the C-X-C motif chemokine receptor 4 (CXCR4)/stromal cell derived factor (SDF)-1α signaling axis (Yan et al., 2018). Some studies have indicated that exosomes can increase intracellular ROS levels in pancreatic cancer cells, thereby making them more susceptible to DNA-damage and radiation-induced killing. Mechanistically, these effects were linked to miR-6823-5p, within exosomes originating from irradiated cells, which contributed to modulating superoxide dismutase 1 (SOD1) levels (Nakaoka et al., 2021).

Another study highlighted the important role of tumor stroma in hampering or even negating the beneficial effects of radiotherapy in PDAC treatment (Zhao et al., 2015). Specifically, the authors demonstrated the effectiveness of Cyclopamine, a SHH pathway inhibitor, and its promise as a radio-sensitizing and stromal disruptive agent in PDAC treatment. Interestingly, this seems contradictory to other studies wherein targeting SHH in PDAC was found to contribute to an immune-suppressive environment (Steele et al., 2021).

Cancer-associated fibroblasts and bone marrow cells have been noted to protect breast cancer cells by inducing interferon (IFN)-related DNA damage-resistance in a STAT1-dependent manner, leading to radio-resistance (Boelens et al., 2014). Although the effect of IFN-γ on resistance has not yet been explored in pancreatic cancer, its role in inhibiting the growth of, as well as tumor-associated macrophage trafficking in, pancreatic cancer has already been established (Detjen et al., 2001; Zhang et al., 2020); thus, the aforementioned concept is worth exploring in PDAC and might be a successful strategy to improve the efficacy of radiation-based combinatorial regimens.

Similarly, conditioned medium from PSCs led to a dose-dependent induction of pancreatic cancer cell proliferation, migration, invasion, and colony formation and caused resistance to gemcitabine and RT (Hwang et al., 2008). The mechanism underpinning these phenomena was found to be through MAPK/AKT pathway activation in the tumor cells. The authors also postulated that PSC-secreted factors such as interleukin-1β (IL-1β) and TGFβ were implicated in this process, the latter of which has been already correlated with gemcitabine resistance (Hesler et al., 2016). In addition, CAFs can promote irradiated cancer cell recovery and tumor relapse after RT by producing insulin-like growth factor-1/2 (IGF-1/2), C-X-C motif chemokine ligand 12 (CXCL12), and β-hydroxybutyrate (Wang et al., 2017). CXCL1 signaling is another contributor to radioresistance. Both cancer cells and CAFs express and secrete CXCL1 which then leads to ROS accumulation following RT via inhibition of the ROS-scavenging enzyme SOD1 (Alafate et al., 2020).

Stromal cell-mediated radioresistance can be also induced through direct contact-mediated signaling. In fact, PSCs promote radioprotection and stimulate the proliferation of pancreatic cancer cells through β1 integrin signaling, which is known to modulate genotoxic stress-induced cellular responses such as RT (Cordes, 2006). Notably, inhibiting β1 integrin could abolish PSC-mediated radioprotection in pancreatic cancer cells in both single-dose and fractionated RT settings (Mantoni et al., 2011). Further, other integrins have been revealed to play a role in mediating radiochemoresistance in pancreatic cancer; of which, β8 Integrin emerged as a crucial determinant and as a potential druggable target (Jin et al., 2019). Besides, the stroma may

also lead to a radioresistant phenotype in pancreatic cancer by activating Akt signaling (Toulany and Rodemann, 2013).

Considering the relevance of RAS signaling in PDAC, it would be interesting to better understand how this pathway facilitates treatment resistance in this disease and how this could be exploited in the clinic. Indeed, inhibiting Ras activation could be a potential strategy for tumor-specific radiosensitization in a large majority of pancreatic cancer patients (Cengel et al., 2007; Kimple et al., 2010; Chang et al., 2014). Another likely target to alleviate resistance to therapy is ADAM9, which is overexpressed in PDAC tumors (Grutzmann et al., 2004). Notably, silencing ADAM9 could promote both radio-sensitivity and chemosensitivity in cancer cells (Josson et al., 2011). Despite all this work, again limited information is available regarding the role of specific subsets of CAFs, or their precursors for that matter, in dictating treatment responses.

IMPROVING IMMUNOTHERAPY RESPONSE BY TARGETING CANCER-ASSOCIATED FIBROBLAST SUBSETS

Various studies have demonstrated the complex contributions of immune cells to pancreatic cancer, and how they communicate with cancer cells as well as other members of the TME (Gorchs and Kaipe, 2021; Hanley and Thomas, 2021). An important point worth considering is that these cells may be differentially shaped depending on CAF phenotype. Most studies in this regard focus on myCAFs or iCAFs, the latter of which are hallmarked by inflammatory features. These cells are capable of secreting high levels of IL-6, which suppresses NK cell activity and leads to PDAC metastasis (Huang et al., 2019). In fact, high IL-6 levels have been previously correlated with reduced response to therapy in general and recorded to impair some ketogenic responses, thereby leading to a systemic metabolic stress response that hinders anticancer immunotherapy in PDAC (Flint et al., 2016). Following these discoveries, blocking IL-6 signaling gained momentum in the field and yielded positive preliminary findings in animal models; a combination of PD-L1 blockade and IL-6 inhibition could effectively suppress tumor progression and enhance overall survival in murine models of PDAC, which gave rise to a clinical trial (NCT04191421) adopting a similar approach. Beside iCAFs, apCAFs and csCAFs might be key stromal elements that affect immunotherapeutic approaches in PDAC as both have immunological activities as depicted above. These cells have not been studied as comprehensively as other CAF subtypes, especially in the immunotherapy department; this warrants further understanding of CAF origins and subtypes and how they differentially shape the immune system and affect therapeutic regimens.

Myofibroblasts are perhaps the most studied stromal cells in PDAC and the contributions of fibroblasts falling under the myCAF phenotype have been investigated from different angles with respect to PDAC progression and therapy response/resistance. Inhibition of TGF-β, a promoter of the myCAF phenotype, has gained some attention in the clinic and has been successfully utilized in concert with gemcitabine to improve overall survival in unresectable PDAC patients (Melisi et al., 2018). Interestingly, inhibiting TGF-β receptor can reduce IL-6 production in CAFs, leading to decreased STAT3 activation in tumors and reversed immunosuppression in mouse models (Huang et al., 2019). Further, blocking both PD-L1 and TGF-β using a double-fusion protein (M7824) inhibited tumorigenesis in mouse models (Lan et al., 2018). This shows the potential of multimodal approaches that combine PD-1/PD-L1 blockade with TGF-β inhibitors and chemotherapeutic regimens. CXC chemokines and their receptors are also relevant with respect to immunotherapy in PDAC. Indeed, CAF-secreted CXCL12 has been documented to induce an immunosuppressive environment in PDAC tumors; blocking the effect of CXCL12 on PDAC cells could enhance antitumor immunity (Garg et al., 2018). Besides, inhibition of CXCR4, using AMD3100, in combination with PD-L1 blockade induced T-cell accumulation in a KPC mouse model leading to reduced cancer proliferation (Feig et al., 2013). The COMBAT trial, a phase IIa clinical trial, was recently conducted to evaluate the efficacy and safety of the CXRC4 antagonist BL-8040 with pembrolizumab and chemotherapy in metastatic PDAC (NCT02826486). The results showed that combined CXCR4 and PD-1 blockade expanded the benefit of chemotherapy in PDAC (Bockorny et al., 2020). Moreover, the CXCL3-CXCR2 axis could stimulate the transformation of CAFs to myCAFs, which secrete type III collagen and accelerate tumor metastasis (Sun et al., 2021). In fact, inhibition of CXCR2 and CCR2 could reverse tumor progression promoted by type I collagen deletion in myCAFs as was evident in a PDAC mouse model potentially by cytotoxic T cell trafficking (Chen Y. et al., 2021). It is worth noting that both CXCR2 and CCR2 promote infiltration of suppressive myeloid cells as well. The CCR2/CCL2 axis also plays a particularly important role in attracting monocytes, which, after interactions with tumor- and stromal-derived factors, differentiate into suppressive tumor-associated macrophages at the site (Nakaoka et al., 2021). A combined blockage of CCR2 and CXCR2 in a murine PDAC model prevented CCR2+ macrophages (Burfeind et al., 2020); this approach may also reduce the number of stellate cells and ultimately CAFs in the TME as CCR2 signaling is involved in monocyte recruitment to PDAC tumors (Ino et al., 2014).

As outlined earlier, SHH signaling is important to consider in PDAC also from an immunological perspective. Initially, it was shown that depletion of CAFs could induce immunosuppression in PDAC tumors with indications that the Hedgehog pathway may play a role in this phenomenon (Rhim et al., 2014; Özdemir et al., 2014). This is in accordance with previous literature that describes the role of SHH in shaping the immune environment (Bai et al., 2016). A recent paper provided more insight as to why Shh inhibition is complex in PDAC treatment (Steele et al., 2021). It was evident in this study that inhibiting Shh signaling using the Smoothened antagonist LDE225 decreases the myCAF/iCAF ratio in the tumor stroma, resulting in an immune suppressive microenvironment. The observed effects were linked to a decrease in the number of cytotoxic T cells and

an increase in that of regulatory T cells, as occurs in an immune-suppressive environment, and suggests that targeting SHH would be counterintuitive in PDAC patients.

Another study also explored the premise of altering the fibroblast composition as a therapeutic strategy for PDAC. It was identified that tumor-secreted IL1, predominantly through autocrine LIF, activates the JAK/STAT pathway in CAFs (Biffi et al., 2019). Subsequently, JAK/STAT signaling maintains an inflammatory CAF phenotype through a positive feedback loop involving STAT3-mediated upregulation of IL1R1. Interestingly, however, treating tumor-bearing KPC mice with the JAK inhibitor AZD1480 led to a significant decrease in cancer cell proliferation and tumor growth as well as a significant increase in collagen deposition; It was also apparent that AZD1480-treated tumors had increased levels of αSMA, suggesting that JAK inhibition may promote a shift from an iCAF phenotype toward a myCAF-like state (Biffi et al., 2019). This approach was deemed likely to improve therapeutic outcomes in PDAC patients as it simultaneously targets potential tumor-promoting components, such as iCAFs, along with components that impede drug delivery, such as myCAF-derived desmoplasia. Nonetheless, further research should be performed to establish safe and effective fibroblast altering strategies for the clinic.

Together, the abovementioned studies introduce some of the pathways by which CAFs are involved in response to immunotherapy and underline the importance of targeting select CAF subsets or certain pathways that underpin resistance to immunotherapy rather than using general stromal disruptive agents or targeting the entire CAF population.

THE INVOLVEMENT OF CANCER-ASSOCIATED FIBROBLASTS IN PANCREATIC CANCER METASTASIS

Metastatic dissemination is a process that is heavily reliant on stromal cues and tumor-stroma communication (Joyce and Pollard, 2009). CAFs can promote the invasiveness of cancer cells as well as angiogenesis by secreting a plethora of growth factors and extracellular matrix molecules. CAF-mediated ECM deregulation may lead to biomechanical and biochemical changes to the TME, thereby facilitating tumor cell invasion and metastasis (Cao et al., 2016; Glentis et al., 2017). Moreover, PDAC cells can educate CAFs and drive them into establishing a prometastatic microenvironment; these fibroblasts contribute to the formation of a niche that supports the survival and expansion of extravasated cancer cells (Brodt, 2016; Houg and Bijlsma, 2018). In one study, weakly metastatic cancer cells stimulated co-cultured MSCs, a source of CAFs, into secreting the chemokine CCL5, thereby promoting the invasion of the cancer cells and metastasis (Makinoshima and Dezawa, 2009). Nonetheless, it is not yet clear whether all CAFs contribute to the metastatic dissemination of PDAC cells. One group identified a population of matrix-remodeling CAFs expressing the Endo180 (*MRC2*) receptor that supported tumor growth and metastasis (Jungwirth et al., 2021).

The paired-related homeobox 1 (Prrx1) transcriptional factor is a key regulator of epithelial-to-mesenchymal transition (EMT) and metastatic colonization in PDAC. Prrx1 is also highly expressed in PDAC stroma and was reported to mediate CAF activation, leading to increased ECM deposition, improved tumor differentiation, fewer circulating tumor cells, and reduced metastasis (Feldmann et al., 2021). CAFs expressing Prrx1 could promote EMT and chemotherapeutic resistance in tumor cells through paracrine HGF signaling. It is also noteworthy that high Prrx1 expression was correlated with the squamous subtype, whereas low stromal Prrx1 expression was predominant in classical tumors, which may indicate differences in stromal content between the two subtypes. Other studies have shown that overexpression of ETV1, another transcription factor, drastically increases the incidence and volume of micro- and macro-metastases in mouse models through stromal expansion (Heeg et al., 2016).

Besides, characterizing stroma within metastatic lesions in an autochthonous model of PDAC indicated that myofibroblasts appear when metastases are as small as 6–7 cells and that cell populations within these lesions become more epithelial during growth (Aiello et al., 2016). Interestingly, fibroblasts at metastatic sites differ from CAFs within primary tumors and are often termed metastasis-associated fibroblasts (MAFs); MAFs make significant contributions to the establishment of pre-metastatic niches and, subsequently, metastatic lesions and encourage therapeutic resistance in metastatic tumors. These cells are capable of remodeling the extracellular matrix of metastatic tumors, modulating immune cells in the tumor microenvironment, promoting angiogenesis and enhancing malignant tumor phenotypes (Wang et al., 2021). MAFs in liver metastases of pancreatic cancer seem to promote angiogenesis and resistance to antiangiogenic drugs through secretion of CCL2 and CXCL8; preclinical studies suggest that targeting MAFs can alleviate the progression of metastatic cancer and mitigate therapeutic resistance (Pausch et al., 2020). Indeed, others have also demonstrated the crucial role the immune system plays in the process of PDAC metastasis. A study showed that the regulation of stroma within PDAC liver metastases is unique and dependent on immune interactions, which may precede cancer cell metastasis. They further demonstrated that metastasis-associated macrophages (MAMs) derived from bone marrow cells rather than native Kupffer cells; in contrast, metastasis-associated fibroblasts were found to be of local origin, presumably hepatic stellate cells, which raises some questions regarding the cellular origins of CAFs in distant metastases compared to primary tumors (Nielsen et al., 2016; Quaranta et al., 2018). Nielsen et al. (2016) also showed that chemical ablation of MAMs in mice after metastatic seeding had occurred was sufficient to decrease the accumulation of activated myofibroblasts as well as reduced the size of the area covered by metastatic cells; although, it did not significantly reduce the metastatic frequency. They went further to identify that macrophage-conditioned media could activate quiescent fibroblasts through granulin, which was, remarkably, only expressed in bone marrow-derived macrophages found in liver metastases and not in those found at the primary tumor site, raising some questions that warrant

further studies. The CAF-tumor-associated macrophage (TAM) axis is a major player in PDAC metastasis. In fact, CAFs produce high levels of IL-33 that induces an M1-to-M2 transition in TAMs, which then exhibit elevated levels of MMP9; in mouse and human fibroblast-rich pancreatic tumors, genetic deletion of IL-33 or MMP9 markedly blocked metastasis (Andersson et al., 2018; Yang et al., 2021).

One paper eloquently introduces the processes involved in the metastatic dissemination of pancreatic cancer cells (Shan et al., 2017). It was described that CAFs activated through paracrine Hedgehog signaling in turn induce the Snail transcription factor in PDAC cells, thereby leading to EMT in the cancer cells (as indicated by vimentin upregulation and E-cadherin downregulation) and enhancing their invasive capacity. It was also hypothesized that after circulating tumor cells home into a new environment, the paracrine action of normal non-activated fibroblasts downregulates this axis in the cancer cells, leading to the formation of new metastatic foci, which in turn activate CAFs and initiate a new cycle.

Together, the abovementioned studies highlight the importance of CAFs in pancreatic cancer metastasis and illustrate the need for more comprehensive studies on the cellular origins of CAFs or MAFs in PDAC metastases and how they phenotypically differ from their counterparts in primary sites.

THE EFFECTS OF CANCER THERAPIES ON CANCER-ASSOCIATED FIBROBLASTS

Another aspect that is important to consider is treatment-induced changes to the TME. During the process of being activated, and in response to therapeutic regimens, CAFs undergo changes that grant them resistant characteristics; this mainly occurs through a defective p53/p21 response pathway and high expression of the cancer marker Survivin (Hawsawi et al., 2008; Arandkar et al., 2018; Wang et al., 2019). Since the stroma presents the larger fraction in PDAC tumors, and taking into account stroma-driven resistance, it is not unlikely that this adds yet another obstacle for the delivery of therapeutic regimens.

Cancer-associated fibroblasts and mesenchymal stem cells have been consistently shown to be enriched in chemotherapy-treated human tumors, including PDAC, wherein they promote cancer growth and treatment resistance by secreting various paracrine factors (Chan et al., 2019). Not only that, but exposing these cells to cytotoxic agents seems to also alter them toward a senescence-like secretory phenotype that encourages stemness features and aggressiveness in the surrounding cancer cells (Lotti et al., 2013). Similarly, quiescent PSCs have also been recorded to undergo a phenotypic and functional transition toward an activated myofibroblast state in response to noxious agents such as alcohol (Charrier et al., 2014). As such, it would not be too farfetched to assume that this phenomenon also takes place when cytotoxic agents and radiation regimens are introduced. Considering the fact that a highly dense stromal compartment supports cancer cell resistance by providing a mechanical barrier that diminishes the potency of anticancer drugs (Miao et al., 2015), the contributions of stellate cell activation to PDAC progression should be fully understood.

Molecular analysis-based studies on neoadjuvant chemotherapy–treated human PDAC tumors and orthotopic tumor xenografts revealed that traditional, or maximum-tolerated dose, chemotherapy regimens induce persistent STAT-1 and NF-κB activity in CAFs, subsequently resulting in high expression and secretion of ELR motif–positive (ELR^+) chemokines. On the contrary, administering the same overall dose of a certain drug over a longer time frame, as a low-dose metronomic chemotherapy regimen, largely reduced therapy-induced stromal ELR^+ chemokine paracrine signaling and, thus, enhanced treatment response and improved mouse survival rates (Chan et al., 2016).

Chemotherapy-induced oxidative stress plays a controversial role in cancer and has raised some concerns regarding the effectiveness of combinatorial approaches (Shacter et al., 2000; Liu and Wang, 2015; Li et al., 2020). A recent study suggested that oxidative stress could be another contributor to PDAC desmoplasia in the sense that it can induce p38-mediated monocyte-to-myofibroblast transdifferentiation (MMT), thereby leading to stromal activation, modulating immunosuppression, as well as promoting tumor progression (Huang et al., 2020). This contributes to the uncertainty regarding the use of oxidative stress-inducing agents in cancer treatment and urges more comprehensive studies.

An important consequence that may take shape because of RT is chronic inflammation that drives fibrosis and leads to an increase in stromal members in the TME as well as more ECM components (Straub et al., 2015). CAFs tolerate relatively high doses (30 Gy) of radiation without apoptosis; however, doses higher than 10–12 Gy often result in a senescent CAF phenotype (Ragunathan et al., 2020). Premature senescence seems to be also induced in normal human fibroblasts as a result of chronic low dose rate (LDR) exposure (5 or 15 mGy/h) of gamma rays (Fujimori et al., 2005). In addition to inducing premature cellular senescence, exposing CAFs to RT results in the potent induction of multiple DNA damage response (DDR) foci as well as the inhibition of the proliferative, migrative, and invasive capacity of these fibroblasts (Goel et al., 2013; Li et al., 2018; Im et al., 2020). Senescent CAFs have been described to present a senescence-associated secretory phenotype that is characterized by the upregulation and secretion of various substances (e.g., CXCL12, TGF-β1, IGF-1, IGFBP2, and NO) (Li et al., 2016; Ansems and Span, 2020), some of which are pro-tumorigenic factors, such as IL-6, IL-8, and osteopontin, and are linked to stroma-mediated therapeutic resistance (Krisnawan et al., 2020). Various cytokines such as TGF-β1, TNF-α, IL-1, IL-4, and IL-13; chemokines such as MCP-1 and MIP-1β; as well as angiogenic and growth factors are involved in RT-induced fibrosis (Ansems and Span, 2020).

NEXT STEPS FOR PDAC MODELING

In the previous part of this review, we focused on the concept of CAF heterogeneity in PDAC and how CAF subsets may have functionally different roles in the tumors. We also discussed

how CAFs behave in response to tumor-secreted factors as well as therapeutic regimens and raised some concerns regarding the potential heterogeneous responses of different CAF subsets. Indeed, we would like to re-emphasize the importance of considering CAF heterogeneity, which may be facilitated by CAF cellular origins, when designing targeted therapeutic regimens for PDAC patients to avoid unwanted contraindications (Öhlund et al., 2017; Elyada et al., 2019). Of course, the dose and frequency of regimens likely affect therapy response and CAF diversity and senescence, and should be further investigated. Despite all this information, we are still limited in our understanding of tumor-stroma interactions and CAF-induced changes on tumor progression as well as chemo- and radio-resistance. We believe that considerations and improvements should be made to establish advanced *ex vitro* tools for effectively modeling PDAC and revamping therapeutic strategies. Therefore, in the next section of this paper, we will suggest pre-clinical models that might be useful for modeling PDAC as well as recapitulating CAF heterogeneity and the processes of communication that take place within these tumors.

THE CONTRIBUTION OF PRE-CLINICAL MODELS TO MODELING TUMOR-STROMA INTERACTIONS

For a long time, animal models and conventional two-dimensional (2D) cell culture systems have been used to study and understand human pathology. However, despite the fact that these models led to various scientific advances, their utility for modeling human physiology and pathology is limited for several reasons (Velasco et al., 2020). Hence, it is quite evident that there is a need for advanced *in vitro* or *ex vivo* tools to model desmoplastic diseases, especially those with complex stromal dynamics like PDAC.

To fill this gap, effort has been invested to establish more advanced technologies in order to generate 3D human tissue-like models; this led rise to the current systems we know as organoids and spheroids (Costa et al., 2016; Kim et al., 2020). Subsequently, many modifications have been made to improve and/or adapt these 3D models for different cancer types. In the context of pancreatic cancer, organoid-based models gained the most attention and have since become a standard for modeling the disease (Baker et al., 2016). Nonetheless, despite the success of organoids in recapitulating pancreatic cancer, the lack of stromal components in these models is still a major obstacle that limits their clinical worth. Indeed, most organoids lack the characteristic fibrotic stroma that is a major obstacle for drug delivery as well as a crucial abettor for cancer cells as was evident throughout this review. Further, inclusion of immune cells in *ex-vivo* culture systems is a critical step to establish a platform for the study of immunotherapy in pancreatic cancer (Tiriac et al., 2019). Fundamentally, a robust patient-matched co-culture system is the optimal approach for researchers and clinicians to model PDAC and assess various therapeutic strategies before proceeding to the clinical setting.

To that end, many groups sought out to establish co-cultures of organoids and stromal cells including resident fibroblasts, cancer associated fibroblasts, pancreatic stellate cells, and immune cells (Hwang et al., 2019). The first move toward this goal arose in the form of combining pancreatic organoids/spheroids with CAFs or PSCs which led to valuable advances in studying invasion, matrix remodeling, and drug response (Khawar et al., 2018; Che et al., 2020). Nonetheless, there was still a shortage of models that took into account the immune microenvironment and which allowed co-culturing a number of different cell types. Recently, more progress has been possible due to technological advances in biomimetic scaffolds, 3D bioprinting techniques, as well as microtechnology-based systems (Sun et al., 2019). Indeed, some microfabricated organoid models, which are generated through the use of techniques and methods implemented in the development of microelectromechanical systems, such as micropatterning and microfluidics have the potential to transform organoid production (Velasco et al., 2020). These new methods are of great utility to the cancer field as they allow us to culture several cell types together and take one step closer toward truly recapitulating the *in vivo* setting (Velasco et al., 2020). Recently, many microfluidic 3D platforms were described for culturing PDAC. One study provided evidence that PDAC cells can be cultured on the HepaChip, a novel microfluidic chamber, which maintains cell vitality, morphological appearance, and growth characteristics (Beer et al., 2017). The group argues that their microfluidic system allows for continuous perfusion, which is where previous models have failed. Moreover, more advanced strategies tried to combine organ-on-a-chip and organotypic technologies to better emulate the vascular aspect of PDAC tumors. One group presented an organotypic PDAC-on-a-chip culture, featuring a 3D matrix containing juxtaposed PDAC and perfusable endothelial lumens, which emulate vascular invasion and tumor–blood vessel interactions (Nguyen et al., 2019). An arguably even more interesting concept is maintaining primary-derived tissue in short culture for downstream analyses. A recent study described a promising pre-clinical model of PDAC that maintains the viability, 3D multicellular architecture and microenvironmental cues of unmanipulated patient derived tumors (Kokkinos et al., 2021). The group presented a simple and cost-effective model that bridges the gaps and provides unprecedented opportunity to closely study the biology of pancreatic cancer; of course, such models are quite valuable for identifying novel therapeutic targets involved in tumor-stroma crosstalk as well as testing new combinatorial treatment regimens. In parallel, other groups have focused on 3D bioprinting approaches to design miniaturized tumor models that are capable of self-organization (Langer et al., 2019); one spheroid-based array showed great promise for studying the formation of precursor PDAC lesions and cancer progression (Hakobyan et al., 2020).

Taken together, the abovementioned tools present good possibilities for modeling PDAC. Considering the dynamic and heterogeneous nature of PDAC tumors, it is essential to use advanced models that allow for co-culturing cancer cells with different members of the stroma in order to recreate

the *in vivo* environment and identify precious biomarkers for targeted therapies. It has become common knowledge in the field that CAFs have different cellular sources and that they can have different functions within a PDAC tumor depending on interactions with cancer cells and other factors. Indeed, there are indications that CAFs behave, or are influenced, differently depending on geographical factors and culture conditions such is the case in the work of Öhlund et al. (2017) and a more recent study on AD-MSC differentiation into iCAFs vs. myCAFs (Miyazaki et al., 2021). Therefore, to be able to effectively and accurately model pancreatic cancer, we are in need of models that can (1) maintain the morphological and growth characteristics of parent tumors as well as their mutational profiles; (2) the models should also take non-cancer compartments into account by including most of the stromal cell types that are present in the *in vivo* setting; and (3) be able to recapitulate the phenotypes and functionality of different CAF populations and subsets in PDAC tumors. Of course, this is essential to allow proper drug response predictions as the stroma is one of the main obstacles for drug delivery as well as an important contributor to resistance in pancreatic cancer.

LIMITATIONS AND CONCLUDING NOTES

It has become apparent throughout this paper how important CAFs are in PDAC tumors and how complex and seemingly opposing their contributions can be. It is for this reason that we would like to stress the urgency of more advanced studies that focus on the cellular origins of CAFs, how these precursors contribute to the CAF population, and how this drives stromal heterogeneity in PDAC. We are also in need of markers that can be used to accurately identify different CAF subsets and their potential function, possibly as potential biomarkers to select for (novel) targeted therapeutic approaches. It might, thus, be relevant to investigate and better understand the gradual variations in the stromal profile during PDAC progression. In order to make headway on this matter, we believe that more advanced *ex vivo* models need to be implemented in combination with lineage tracing and scRNA-seq technology. This combination might not be currently practical or feasible in light of technological limitations; however, this does not reduce from value of establishing and implementation such platforms in the near future. In conclusion, we believe that accurately modeling PDAC and unraveling the paradigm that is CAF heterogeneity are crucial for reaching clinically relevant findings and making strides toward personalized or targeted treatment approaches.

AUTHOR CONTRIBUTIONS

PM drafted the manuscript and figures. HL and MB provided supervision and revised the manuscript. All authors have read and accepted the final version of the manuscript.

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REVIEW
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DNA Damage Repair Deficiency in Pancreatic Ductal Adenocarcinoma: Preclinical Models and Clinical Perspectives

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers worldwide, and survival rates have barely improved in decades. In the era of precision medicine, treatment strategies tailored to disease mutations have revolutionized cancer therapy. Next generation sequencing has found that up to a third of all PDAC tumors contain deleterious mutations in DNA damage repair (DDR) genes, highlighting the importance of these genes in PDAC. The mechanisms by which DDR gene mutations promote tumorigenesis, therapeutic response, and subsequent resistance are still not fully understood. Therefore, an opportunity exists to elucidate these processes and to uncover relevant therapeutic drug combinations and strategies to target DDR deficiency in PDAC. However, a constraint to preclinical research is due to limitations in appropriate laboratory experimental models. Models that effectively recapitulate their original cancer tend to provide high levels of predictivity and effective translation of preclinical findings to the clinic. In this review, we outline the occurrence and role of DDR deficiency in PDAC and provide an overview of clinical trials that target these pathways and the preclinical models such as 2D cell lines, 3D organoids and mouse models [genetically engineered mouse model (GEMM), and patient-derived xenograft (PDX)] used in PDAC DDR deficiency research.

Keywords: DNA damage response (DDR), pancreatic duct adenocarcinoma (PDAC), preclinical model, cell line, organoid, genetically engineered mouse model (GEMM), xenograft, targeted therapy

INTRODUCTION

Pancreatic cancer is one of the most lethal cancers worldwide, accounting for 2.6% of all new cancer cases but causing 4.8% of all cancer deaths (Ferlay et al., 2019). Despite recent advances in personalized and targeted therapy, little progress has been made to improve overall survival (OS) and the 5-year survival rate is estimated at 9% (Siegel et al., 2020).

Currently, curative treatment is limited to low-stage, resectable disease but over 80% of patients present with advanced or metastatic disease (Bilimoria et al., 2007; Stathis and Moore, 2010; Ilic and Ilic, 2016). Current standard of care treatment for advanced pancreatic ductal adenocarcinoma (PDAC) consists of either combination treatment of nab-paclitaxel with

gemcitabine or 5-fluorouracil, leucovorin, irinotecan, oxaliplatin (FOLFIRINOX) (Mohammed et al., 2014; Mohammad, 2018; Adel, 2019). In 2013, the MPACT trial showed that nab-paclitaxel with gemcitabine improved OS by 1.8 months compared to gemcitabine alone (8.5 vs. 6.7 months, $p < 0.001$) (Von Hoff et al., 2013). The PRODIGE trial found that FOLFIRINOX improved OS by 4.3 months compared to gemcitabine alone (11.1 vs. 6.8 months, $p < 0.001$) (Conroy et al., 2011). However, FOLFIRINOX is associated with higher toxicity profiles and is therefore generally reserved for patients with a good performance status and given as a modified regimen. Systemic treatment options for PDAC include gemcitabine, gemcitabine with erlotinib, FOLFIRINOX, gemcitabine with nab-paclitaxel, nano-liposomal irinotecan with 5-FU, pembrolizumab (patients with microsatellite instability), larotrectinib/entrectinib (patients with NTRK-fusion), and olaparib (patients with g*BRCA* mutation).

Genomic analyses have revealed a complex mutational landscape that is predominated by mutations in *TP53*, *KRAS*, *SMAD4*, and *CDKN2A* (Bailey et al., 2016; Aguirre et al., 2018). Despite extensive research, targeted therapies for these mutations have not reached clinical practice (Qian et al., 2020). In addition, PDAC is characterized by genome instability (Alexandrov et al., 2013). Genome instability has been described as one of the enabling hallmarks of cancer by Hanahan and Weinberg (2011) and can be attributed to multiple sources, including increased sensitivity to mutagenic agents, defects in the genomic maintenance machinery, loss of telomeric DNA, and aberrant surveillance mechanisms. While these aberrations can partly be contributed to these four commonly mutated genes, additional pathway deficiencies are also involved. The DNA damage response (DDR) pathway plays a central role in genome maintenance and repair. In contrast to *TP53* and *KRAS*, DDR deficiency is targetable, with multiple drugs already available in the clinic for non-PDAC cancer types, such as breast and prostate cancer (Wengner et al., 2020).

This review briefly covers the role and definition of DDR deficiency in PDAC and provides an overview of clinical trials that investigate DDR targeting drugs. The main focus is on how cell lines, organoids, and mouse models are used to study DDR deficient pathways in PDAC.

MAIN

DNA Damage Repair Pathways in Pancreatic Ductal Adenocarcinoma

To combat the development of mutations and the effects these may have on the cell a complex network of DNA damage repair (DDR) pathways exists (Giglia-Mari et al., 2011). At the core of this network are the pathways for base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), interstrand crosslink repair (ICL repair), and double strand break repair [both homologous recombination (HR) and non-homologous end-joining (NHEJ)] (**Figure 1**). Each pathway can roughly be divided into three phases or steps: recognition of the damage, excision or processing of the damaged strand(s), and the actual repair.

Base excision repair removes non-bulky single-base lesions such as oxidation or deamination damage (Lee and Kang, 2019). The damaged base is recognized and removed by one of multiple specific DNA glycosylases (such as UNG, SMUG1, or NEIL1), depending on the type of lesion. Next, the newly created abasic site is excised and processed by APE1 to generate a 3′-hydroxyl site. This 3′-hydroxyl is then used by DNA polymerase to fill the gap using the opposing strand as template.

Nucleotide excision repair is the main pathway for the removal of bulky lesions but can also remove intrastrand crosslinks and cyclobutene pyrimidine dimers that are produced by UV radiation (Schärer, 2013; Lee and Kang, 2019). While two subpathways can be distinguished – global genome NER (GG-NER) for the whole genome and transcription-coupled NER (TC-NER) for the transcribing strand of active genes, the general repair process is similar to BER. GG-NER recognizes distortions of the DNA helix through DDB1, DDB2, and XPC, whereas TC-NER CSA and CSB recognize blockage of the RNA polymerase. TFIIH opens up the DNA to enable XPD to verify the lesion upon which several other XP endonucleases and RPA are recruited to excise the lesion. Finally, the resulting 22–32 nt long gap is filled and ligated to the original DNA strand by DNA polymerases and ligases.

The MMR pathway removes single nucleotide mismatches and small insertions or deletions created by DNA polymerase during DNA synthesis (Gupta and Heinen, 2019). The lesions are recognized by the heterodimer MSH2/MSH6. The dimer recruits another heterodimer, MLH1–PMS2, and together they recruit several other proteins including Exo1 to excise the damage. Finally, polymerase eta or delta fills the newly created gap.

Interstrand crosslinks (ICLs) are caused by bifunctional alkylating agents that form covalent bonds between the two DNA strands (Deans and West, 2011; Hashimoto et al., 2016). In quiescent cells the lesion is recognized and repaired by the NER pathway, but during the S phase several steps take place to activate the HR pathway. When a DNA replication fork encounters an ICL the fork stalls and, through a complex containing FANCM, the lesion is recognized and the Fanconi anemia complex and BTR complex are recruited. These complex create a double-strand break (DSB) which is subsequently recognized and repaired by the HR pathway.

Double-strand breaks are repaired through two main pathways: HR and NHEJ (Giglia-Mari et al., 2011; Yang et al., 2016; Ranjha et al., 2018). HR can take place during the S- and G2-phase of the cell cycle when it can use the homologous sequence of a sister chromatid to accurately repair the break. The DSB is recognized by the MRN complex (consisting of MRE11, RAD50, and NBN), the ends of the break are resected, and RPA binds to and forms a filament between the newly resected single-stranded DNA section. Next, BRCA2 recruits RAD51 to replace the RPA-filament and, assisted by several other proteins, homology search and strand invasion of the sister chromatid takes place. Using the sister chromatid as template, polymerase delta synthesizes the missing nucleotides of the broken strand and the ends are ligated. NHEJ, in contrast, can take place during every phase of the cell cycle and is quicker than HR but is also error-prone and commonly results in small deletions.

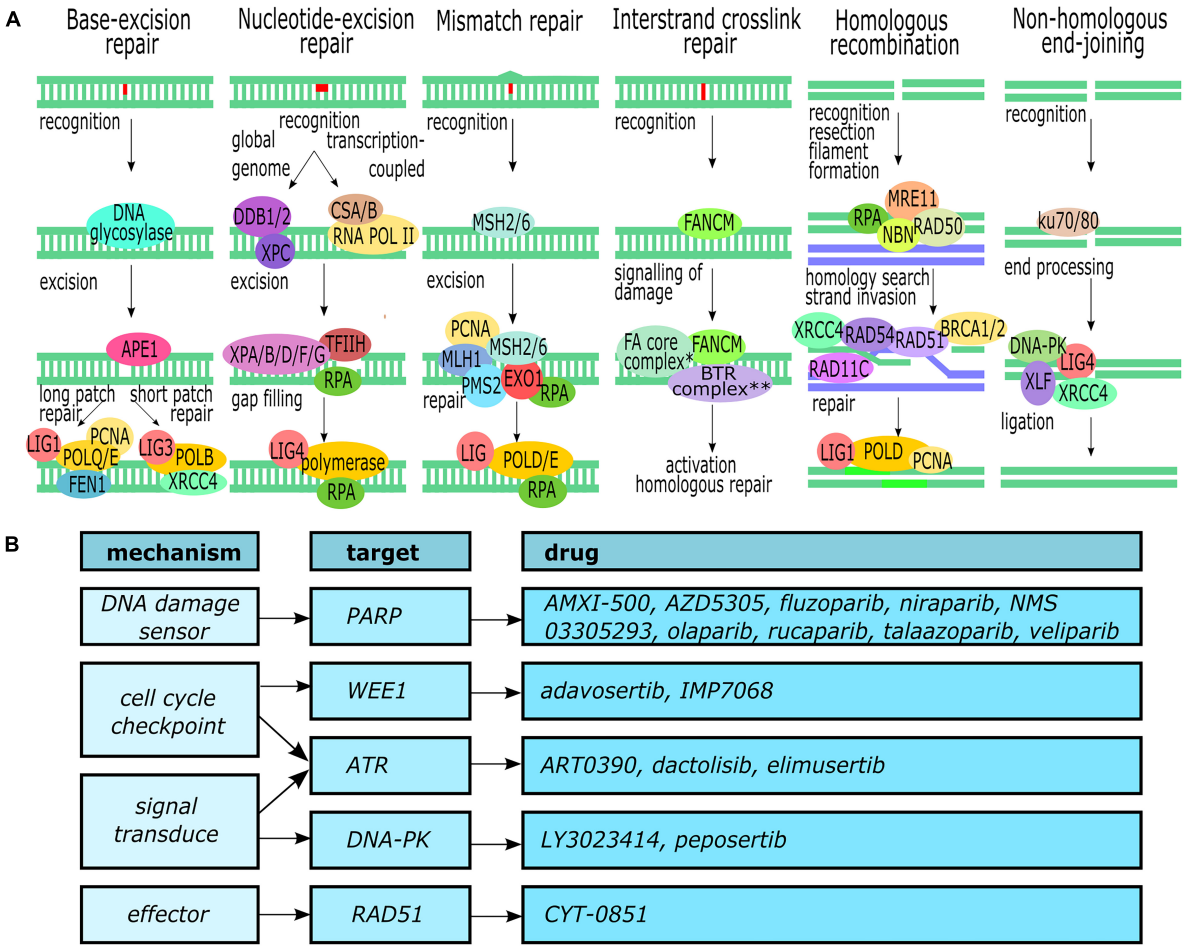

FIGURE 1 | DNA damage repair. **(A)** Overview of the major DNA repair pathways (Deans and West, 2011; Yang et al., 2016; Ranjha et al., 2018; Gupta and Heinen, 2019; Lee and Kang, 2019). *The Fanconi anemia core complex consists of *FANCA*, *FANCB*, *FANCC*, *FANCD*, *FANCE*, *FANCF*, *FANCG*, and *FAAP100*. **The BTR complex consists of *BLM*, *TOPOIII*, *RMI1*, and *RMI2*. **(B)** Molecular targets within the DDR pathways and available inhibitors. This figure was created in Inkscape.

The break is recognized by the ku70/80 heterodimer which subsequently recruits DNA-PKcs, XLF, XRCC4, and Lig4 to process and ligate the broken ends of the DNA strands. In recent years, important progress has been made in deciphering the molecular underpinnings of PDAC due to the unparalleled power of next generation sequencing (NGS) technologies. Constitutive mutations of PDAC have been described as selective DDR pathways in PDAC, however, the main problem encountered is the heterogeneity of somatic alterations among patients outside of the four most frequently mutated genes (KRAS, CDKN2A, TP53, and SMAD4) which poses a challenge to the identification of potential predictive and prognostic biomarkers.

Germline Mutations

Approximately 10% of all PDAC cases are considered familial; defined as a family with at least two first-degree relatives with PDAC (Turati et al., 2013). While several germline pathogenic alterations that increase an individual's lifetime risk of PDAC (e.g., hereditary pancreatitis and Lynch syndrome) have been characterized, the causative germline mutation of most familial cases remains unclear (Klein, 2012). The most commonly mutated genes in familial pancreatic cancer are *BRCA2*, *CDKN2A*, *BRCA1*, and *PALB2* (Perkhofer et al., 2020). Pathogenic germline alterations have also been identified in patients who do not meet criteria for familial PDAC, and may involve genes beyond those previously associated with hereditary pancreatic cancer. These pathogenic germline alterations are therapeutically considered actionable in 5–10% of patients, and clinical guidelines now support routinely offering germline genetic testing with a broad panel of known hereditary cancer predisposition genes to all PDAC patients.

Somatic Mutations

The presence of DDR gene mutations has been reported in 17–43% of all sporadic PDAC patients (Waddell et al., 2015; Aguirre et al., 2018). However, these papers focused on a limited selection of well-characterized DDR genes and potentially actionable DDR mutations may be more prevalent. We queried the GENIE cohort (The AACR Project GENIE Consortium, 2017) containing 3706 PDAC patients with somatic mutation profiling for the presence

of mutations in any of the genes of the six major DDR pathways (BER, NER, HR, NHEJ, ICL repair, and MMR) (Deans and West, 2011; Yang et al., 2016; Ranjha et al., 2018; Gupta and Heinen, 2019; Lee and Kang, 2019). A comprehensive list of 352 genes was collated based on the gene lists of the respective pathways in the Gene Ontology database. Mutations were reported in 117 (33%) of the genes, with 46 (13%) and 14 (4.0%) genes being mutated in more than 1 and 2% of the patients, respectively. The most commonly mutated genes were *TP53* (68.9%), *BRCA2* (4.4%), *ATM* (4%), and *PRKDC* (3.9%). An overview of these genes and associated pathways can be found in **Table 1**, **Figure 1**, and **Supplementary Table 1**.

The relatively high prevalence of DDR gene mutations opens up opportunities for targeted therapies based on the synthetic lethality principle: tumors with a DDR pathway deficiency are more dependent on alternative DNA repair pathways to repair double-stranded DNA breaks (Guo et al., 2011; Topatana et al., 2020). Synthetic lethality has been applied successfully in cancers harboring *BRCA1/2* mutations (homologous repair pathway) by treating them with *PARP* inhibitors (*PARP* is involved in the single-strand break repair pathway) (Lord and Ashworth, 2017). Unrepaired single-strand breaks will turn into DSBs during DNA replication which will accumulate to the point of cell death due to the HR deficiency.

DNA Damage Repair Pathways Genomic Profiling/Biomarkers

Multiple research groups have performed next-generation sequencing and expression profiling to classify molecular PDAC subtypes that can be used to tailor therapies and guide clinical decision making (Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016; Puleo et al., 2018). At its simplest, a distinction is made between classical and basal-like subtypes, though most classifications include more specific subtypes as well (**Figure 2**). Bailey et al. (2016) defined four PDAC subtypes (immunogenic, pancreatic progenitor, ADEX, and squamous) based on 10 discriminatory gene programs found by transcriptional network analysis. Over 50 DDR genes were included in the gene program "proliferation" which is associated with the squamous subtype. Functionally, the squamous subtype is associated with histological adenosquamous carcinoma and a poor survival. The classifications by Collisson et al. (2011), Moffitt et al. (2015), and Puleo et al. (2018) found no associations with DDR deficiency.

Instead of mutational signatures, Waddell et al. (2015) based their classification on structural variation. Using a dataset of 75 primary samples and 25 patient-derived cell lines (PDCLs) they defined four subtypes: stable, locally rearranged, scattered, and unstable. The unstable subtype co-segregated with inactivation of *BRCA1*, *BRCA2*, and *PALB2*, as well as a mutational signature of DDR deficiency. Mutations of *ATM* and other genes involved in DNA maintenance (e.g., *XRCC4/6* and *FANCM*) were also regularly found in these tumors.

Currently only gBRCA is used in the clinic as biomarker for sensitivity to PARP inhibition (PARPi) olaparib. Our query of somatic DDR mutations found that *BRCA2* is mutated in 4% of all PDAC patients indicating that a larger group of patients may benefit from targeted therapy. In addition, multiple clinical trials are recruiting patients for treatment with DDR inhibitors based on a larger selection of DDR mutations, including but not limited to *PALB2*, *CHEK2*, *ATM*, and *RAD51* (**Supplementary Table 2**). Their outcomes will show whether more DDR genes can be included as biomarkers for targeted therapy in the clinic.

Already, DDR deficiency has been associated with a significantly better patient survival compared to DDR proficiency independently of tumor subtype classification (Zimmermann et al., 2021). However, it should be noted that analysis of the interaction with precise treatment regimens is limited, in particular with regard to receipt of platinum based therapy, and this can be a considerable confounder. In addition, a small retrospective study in 36 patients treated with first-line FOLFIRINOX in a metastatic setting found that DDR deficiency, as based on a 14-gene panel, was significantly associated with improved survival (p = 0.04) (Sehdev et al., 2018). While the DDR deficient patient group also had a better median OS (14 vs. 5 months) this difference was not significant (p = 0.08). A similar retrospective study in 40 patients with metastatic PDAC treated with first-line platinum chemotherapy in combination with FOLFIRINOX was published a year later (Palacio et al., 2019). Based on a 35-gene panel, the patients with DDR deficiency had a significantly longer progression-free survival (PFS) (18.5 vs. 6.9 months, p = 0.003), with a trend toward superior median OS as well. Further research is needed to confirm these findings in a larger cohort and to investigate whether DDR deficiency is associated with response to FOLFIRINOX treatment or OS in general.

Preclinical Models

Despite the promising results for many targeted therapies in other solid tumors such as breast, lung, and colon, the use of targeted therapies in PDAC has had limited survival benefit in the clinic. Target discovery and successful development of targeted therapies is highly dependent on the relevance of the preclinical models used and therapies frequently fail at the transition to clinical trials. Multiple recent papers are available which review the preclinical models used in PDAC research (Moreira et al., 2018; Garcia et al., 2020; Swayden et al., 2020; Yu et al., 2021). This review will extend upon published literature by focusing on the application of these models to further target DDR pathways.

Cell Lines

Cell lines remain the most commonly used preclinical model for cancer research. Their widespread use has ensured that they are readily available and most commercial cell lines are well-characterized (Deer et al., 2010). The main advantages of cell lines are that they are cheap, require little maintenance, and are easy to manipulate. In addition, cell lines are considered to be more homogenous than other preclinical models, thus contributing to a better reproducibility which makes them well-suited for high-throughput drug screening. However, this also means that cell lines lack the complexity and heterogeneity typical of tumors. At the same time, clonality and adaptation to 2D culturing conditions as well as immortalization and repeated passaging can all contribute to genomic drift which can significantly affect

TABLE 1 | Prevalence of the top 25 most frequently found somatic DDR gene mutations and associated pathways in a cohort of 3706 PDAC patients.

Gene	Mutation frequency (%)	Affected pathways (respective GO term)					
		HR (GO:724)	NHEJ (GO:6303)	BER (GO:6284)	NER (GO:6289)	ICL repair (GO:36297)	MMR (GO:6298)
TP53	68.90				x		
BRCA2	4.40	x			x		
ATM	4.00		x				
PRKDC	3.90		x				
MCM4	3.50	x					
NIPBL	3.20	x					
POLQ	3.20	x	x	x			
RIF1	3.10	x	x				
WRN	2.40	x		x			
FAAP100	2.40					x	
FANCD2	2.40					x	
ERCC6	2.20	x	x	x	x		
EP300	2.10				x		
RECQL4	2.00	x					
HELQ	1.90	x					
CUL4A	1.80				x		
ARID2	1.80	x					
FANCM	1.80	x				x	
FANCA	1.80					x	
PAXIP1	1.70		x				
FAN1	1.60	x			x	x	
BRCA1	1.60	x	x				
MUS81	1.60	x				x	
SETD2	1.60	x					x
ATR	1.60					x	

HR, homologous recombination; NHEJ, non-homologous end-joining; BER, base-excision repair; NER, nucleotide-excision repair; ICL repair, interstrand crosslink repair; MMR, mismatch repair.

drug responses (Hughes et al., 2007; Monberg et al., 2021). Other disadvantages of 2D culture include loss of part of the normal 3D morphology, cell polarity, and cell–cell or cell–stroma interactions, especially the interaction with cancer-associated fibroblasts (CAFs) and immune cells (Feig et al., 2012). While some of these disadvantages can be resolved or diminished by adapting the culture methods (e.g., using early passage PDCLs instead of established cell lines, or co-culturing with fibroblasts) other disadvantages are inherent to the model system itself.

While the possible applications of cancer cell lines are diverse, ranging from biomarker discovery to functional studies, the use of cell lines to study the DDR pathways in PDAC has mainly been limited to drug sensitivity studies. Investigated drugs include PARP inhibitors (e.g., veliparib, olaparib, and rucaparib), WEE1 inhibitors, ATM inhibitors, ATR inhibitors, DNA protein kinase catalytic domain (PRKCD) inhibitors, and more.

DNA damage repair pathway deficiency has been shown to confer sensitivity to PARPi. Multiple studies found that the *BRCA2*-deficient cell line Capan-1 is significantly more sensitive to several PARP inhibitors and cisplatin, but not to gemcitabine, compared to the *BRCA2*-proficient cell lines MiaPaCa-2 and Panc-1 (Porcelli et al., 2013; Andrei et al., 2015; de Soto, 2020). In addition, restoration of *BRCA2* expression in Capan-1 cell lines was shown to reduce sensitivity to olaparib and HYDAMTIQ (Mini et al., 2017; Sullivan-Reed et al., 2018). Similarly, shRNA-mediated knockdown of *BRCA2* in Panc-1 cells impaired homology-directed repair and conferred sensitivity to BMN-673 (but not to veliparib) (Andrei et al., 2015). Furthermore, increased sensitivity to PARPi (olaparib, BMN-673, and rucaparib) and cisplatin has been found in DDR deficient PDCLs (Dreyer et al., 2021).

Acquired resistance is a problem in many cancer treatments. Likewise, long-term treatment of Capan-1 cells with low dose PARPi can induce resistance, including cross-resistance to other PARPi and cisplatin. Several mechanisms have been suggested for the development of resistance in Capan-1 *BRCA2*-deficient cell line, the simplest being the restoration of *BRCA2* expression. Sakai et al. (2008) found that 7 out of 14 Capan-1 clones that developed resistance to cisplatin treatment had additional mutations in the *BRCA2* gene which corrected the original frameshift mutation found in Capan-1. The truncated protein was also still present suggesting that these restorative mutations were preceded by gene duplication. However, the amplification of the truncated protein might in itself also contribute to resistance. Park et al. (2020) investigated PARPi resistant Capan-1 clones and did not detect reversion mutations, though several clones had additional copies of the mutant *BRCA2* allele as well as an increased *BRCA2* protein expression.

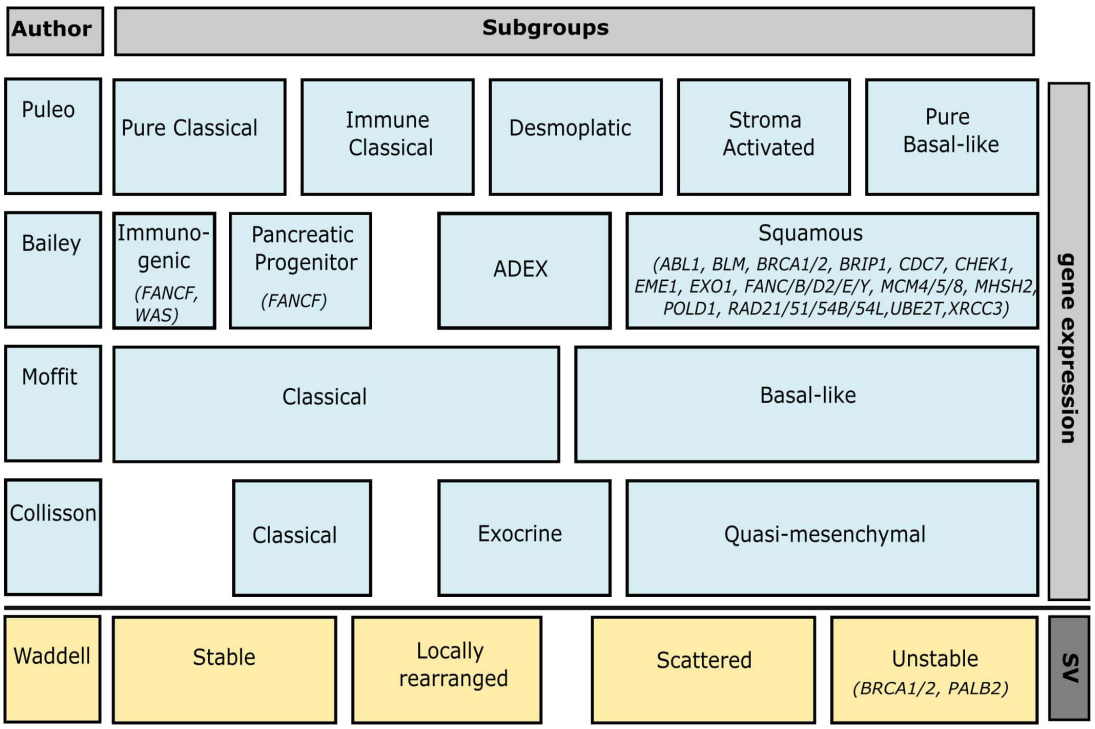

FIGURE 2 | Overview of genomic pancreatic subtypes and how they overlap. Associated DDR genes of which mutations have been found in PDAC patients (see **Supplementary Table 1**) are included under their respective subtypes. The bar on the right indicates whether the classification is based on gene expression or structural variation (SV). Associated DDR genes not mutated in patients include *CDC45*, *FEN1*, *GINS2/4*, *MAD2L2*, *MCM2/3/6/7*, *RMI2*, *RPA3*, *TIMELESS*, *HMGB2*, *POLA1*, *LIG1*, *DNA2*, *RDC2/3/4/5*, *PCNA*, *COPS5*, *BRIP1*, *HMGA2M*, *CETN2*, *UBC*, *TP73*, *PSMD14*, *POLR2D*, and *CDK7* for Bailey's squamous subtype, and USP7 for Bailey's Pancreatic Progenitor subtype. This figure was created in Inkscape.

Immunoprecipitation of *BRCA2* followed by mass spectrometry showed enrichment of *PALB2*, *RAD51*, *MLLT10*, and *DOT1L* in the resistant clones, but not in the parent cells, which may contribute to resistance. Alternatively, Chen et al. (2020) also found additional mutations in *BRCA2* in PARPi resistant Capan-1 clones, but these mutations resulted in truncated splice isoforms. In addition, they found overexpression of the anti-apoptotic proteins *COX-2* and *BIRC3*. Depletion of either *BRCA2*, *COX-2*, or *BIRC3* partially restored PARPi sensitivity. In contrast, combined depletion had no additive effect, suggesting that additional mechanisms contribute to PARPi resistance.

Sensitivity to the *WEE1* inhibitor AZD-1775 has been evaluated in multiple studies, but due to contradicting findings its role in DDR deficiency remains unclear. Two studies found that Capan-1 is markedly more sensitive to AZD-1775 than other (PDAC) cell lines, suggesting that *BRCA2* deficiency might play a role (Dréan et al., 2017; Parsels et al., 2018). However, while restoration of the *BRCA2* open reading frame due to secondary mutations induced by CRISPR-Cas9 reduced the sensitivity to PARP inhibitors olaparib and BMN-673, it did not affect the sensitivity to AZD-1775. On the other hand, Lal et al. (2016) investigated sensitivity to AZD-1775 in a panel of nine PDAC cell lines and reported a medium sensitivity for Capan-1. In addition, they found that knockdown of *BRCA2* by siRNA in MiaPaCa-2 and PL5 induced resistance to AZD-1775. These contradicting findings highlight the need for further investigation.

The application of ATR inhibitors in PDAC has been investigated in multiple *in vitro* studies in both human PDAC cell lines and mouse KPC and KPCB cell lines but so far drugs have shown limited potential and sensitivity to treatment does not correlate with the DDR status (Wallez et al., 2018; Elliott et al., 2019; Dreyer et al., 2021). However, multiple studies have found that ATRi (VE-821 and VE-822) sensitizes to gemcitabine and radiotherapy through impairment of the DNA repair (Fokas et al., 2012; Wallez et al., 2018). siRNA knockdown of another major signal transducer, ATM, in combination with ATRi in MiaPaCa-2 was able to prevent gemcitabine-induced activation of ATR completely (Wallez et al., 2018), suggesting that ATM-mutant tumors may be especially sensitive to this combination treatment. In addition, combination treatment with chloroquine, an autophagy inhibitor that is used in the treatment of malaria, significantly reduced proliferation in 24 or 17 out of 26 tested PDAC cell lines compared to VE-822 or chloroquine alone (Elliott et al., 2019). Azorsa et al. (2009) used an RNAi screen to identify which genes, when silenced, sensitized pancreatic cancer cells to gemcitabine. Silencing of *CHK1* was found to be most effective and was further validated with additional siRNAs and two small molecule inhibitors (SB218078 and PD407824) in MiaPaCa-2 and BxPC3 cell lines (Azorsa et al., 2009).

A disadvantage of DDR targeted therapy is that it is not inherently cytotoxic. By inhibiting multiple DNA repair genes the cancer cells will accrue DNA damage, but whether this results in cell death or senescence depends on additional factors, such as the proliferation rate and how well the cells tolerate replicative stress. Combination treatment with chemo- or radiotherapy can increase the anti-tumor effect by inducing additional DNA damage (Porcelli et al., 2013). Perkhofer et al. (2017) generated stable mouse cell lines from tumors with pancreas-specific loss of *Kras* (KC), and *Kras* and *Atm* (AKC). *Atm*-deficient AKC cells showed a significant increase in DNA damage markers 53BP1 and γH2AFX upon treatment with 5 Gy of ionizing radiation compared to KC cells ($p < 0.03$), indicating impaired DSB repair, and had decreased proliferation. No significant differences were observed in sensitivity to cisplatin, 5-FU, or gemcitabine. Treatment with olaparib or niraparib reduced viability in an *Atm*-dependent manner and was potentiated by combination with gemcitabine or radiation ($p < 0.01$).

The cell lines used for DDR pathway studies in PDAC are mainly limited to Capan-1 as model for DDR/*BRCA2*-deficiency, and MiaPaCa-2, BxPC-3, and Panc-1 for DDR-proficiency. Studies in additional cell line models are required to analyze the role of the DDR pathways in more depth. **Table 2** provides an overview of the mutations found in the 10 most frequently mutated DDR genes (excluding *TP53*) as per **Table 1** for all PDAC cell lines found in the Cancer Cell Line Encyclopedia. Twenty of the 46 cell lines had a mutation in one or more of the investigated genes, of which three (Capan-1, PL18, and SNU-324) had a mutation annotated as pathogenic or likely pathogenic in the ClinVar or COSMIC database.

Apart from Capan-1, SNU-324 is the only other available cell line with a suspected deleterious *BRCA2* mutation (Ku et al., 2002). SNU-324, established in 2001, is derived from a poorly differentiated primary pancreatic tumor of a 50-year-old male. The cells are mainly adherent, but a fraction of the cells grow in suspension and frequently form aggregates. SNU-324 does not contain mutations in *KRAS* or *TP53*, but is microsatellite instable (Ku et al., 2002). Despite its usefulness for *BRCA2*-deficiency studies no other publications are available which have used this cell line. Therefore there is a need for additional well-defined *BRCA2*-deficient PDAC cell lines.

Organoids

Patient-derived organoids are still a relatively new model for pancreatic cancer and there are few studies published that focused on the DDR of PDAC organoids. However, there is limited information on patient derived organoid (PDO) sensitivity to DDR-targeted drugs.

Driehuis et al. (2019) performed high-throughput drug screening of 76 drugs in 24 PDOs and found that, in general, PDOs have a similar response to agents that target the same biological process or molecular pathway. Drug response was found to be PDO-specific, thus reflecting patient heterogeneity. Of the 24 PDOs, 1 PDO had a *BRCA2* indel and was among the most sensitive PDOs for most of the tested drugs.

Tiriac et al. (2018) performed pharmacotyping on 66 PDOs for the drugs gemcitabine, paclitaxel, irinotecan, 5-FU, and oxaliplatin. They found that PDO response reflected interpatient variability. For nine patients, the PDO response could be compared to patient response. Eight out of nine patients exhibited an outcome consistent with their matched PDO. Additionally, they investigated the correlation between AUC distribution and genotype for a range of drugs, including olaparib. The three samples with the lowest AUC had *ATM* loss, *PALB2* loss, and *ATM* frameshift plus *BRCA2* loss, respectively. The researchers observed a trend between olaparib sensitivity and complete loss of *PALB2*, but these data must be interpreted cautiously as there were just four PDOs with complete *PALB2* loss. In addition, they state that single-copy losses of a range of genes involved in HR deficiency do not correspond with olaparib sensitivity. In line with these findings Dreyer et al. (2021) reported that they found no correlation between DDR status and the response to *ATR* or *WEE1* inhibition in the six PDOs they investigated.

Based on genomic, transcriptomic, and histologic data, organoids are representative models of PDAC (Tiriac et al., 2018; Driehuis et al., 2019; Gendoo et al., 2019). Yet, limited studies have been published highlighting their use in the study of DDR deficiency in PDAC. However, drug screening and correlation to patient response studies are promising and suggest that organoids are good models to determine drug sensitivity for targeted therapies, and might also be used to identify biomarkers for drug sensitivity.

Mouse Models

Patient-derived and cell line-derived xenograft models

Patient-derived xenografts (PDXs) are well established cancer models and have been reviewed extensively (Garcia et al., 2020; Shi et al., 2020). PDAC xenografts can be established from resection, biopsy material, and ascites. Copy number alterations and gene expression profiling are largely maintained between primary samples and PDX and genomic signatures can be fitted to the Collisson, Moffitt, and Bailey subtypes (Golan et al., 2017; Nicolle et al., 2017; Pham et al., 2021). Interestingly, even though the mouse hosts are immune-deprived, PDX tumor models can reproduce the immune-related phenotype that is found in certain human primary tumors (Nicolle et al., 2017).

However, the application of PDX toward studying DDR deficiency in PDAC is still used infrequently. Golan et al. (2018) developed six PDXs from metastatic lesions of germline *BRCA*-mutated patients to recapitulate the clinical scenario of *BRCA*-associated PDAC in xenografts. Patient samples were taken before treatment and during progression to represent treatment naïve and resistant patients. Four models had bi-allelic inactivation of *BRCA1/2* and demonstrated increased somatic mutational load compared to the two models that had retained one wild-type copy. Three PDX were treated with olaparib and cisplatin monotherapy, and PDX treatment response as well as HR deficiency profile were found to be associated with patient treatment response to platinum and PARPi.

In a similar study, Lohse et al. (2015) compared treatment sensitivity of four xenografts containing a germline mutation in *BRCA1/2* resulting in heterozygous or homozygous loss to three xenografts with wild-type *BRCA1/2*. Mice were treated

TABLE 2 | Mutation status of DDR genes in cell lines.

Cell line	Gene	Nucleotide change	Protein change	ClinVar	COSMIC FATHMM
BXPC3	*POLQ*		p.ILL1421fs	n/a	n/a
Capan-1	*BRCA2*	c.5946del	p.S1982fs	Pathogenic	n/a
	ATM	c.4755A>C	p.R1585S	n/a	n/a
CFPAC1	*PRKDC*	c.1945T>C	p.F649L	Uncertain significance	n/a
HPAC	*NIPBL*		p.T735I	n/a	n/a
HuP-T3	*BRCA2*	c.6131G>T	p.G2044V	Benign/likely benign	n/a
	NIPBL		p.1532_1532E>DK	n/a	n/a
KP2	*PRKDC*		p.G2261S	n/a	n/a
	FAAP100		p.K333R	n/a	n/a
MZ1PC	*PRKDC*		p.W1355C, p.W1355I, p.F1028V	n/a	n/a
Panc-02.03	*POLQ*		p.L1430fs	n/a	n/a
Panc-03.27	*ATM*	c.7052A>G	p.E2351G	Uncertain significance	n/a
Panc-04.03	*NIPBL*		p.S2389I	n/a	n/a
Panc-08.13	*ATM*		p.F1234S	n/a	n/a
PATU8988S/T	*ATM*		p.R919M	n/a	n/a
PK-45H	*POLQ*		p.G2225R	n/a	n/a
PK-59	*BRCA2*	c.6131G>T	p.G2044V	Benign/likely benign	n/a
PL18	*NIPBL*	c.3G>T	p.M1I	Pathogenic	n/a
			p.S1517*	n/a	n/a
PL4	*RIF1*	c.1331C>T	p.A444V	n/a	Neutral (0.12)
PSN1	*NIPBL*		p.K601fs	n/a	n/a
SNU-324	*BRCA2*	c.7480C>T	p.R2494*	Pathogenic	n/a
	ATM		p.Q2809fs	n/a	n/a
	MCM4	c.1579G>A	p.V527I	n/a	Pathogenic (0.96)
SW-1990	*NIPBL*		p.K1180*	n/a	n/a
TCCPAN2	*POLQ*		p.R6P	n/a	n/a

*Representation of the top 10 (excluding TP53) most frequently mutated DDR genes in PDAC cell lines. The genes WRN and FANCD2 were not found to be mutated in any PDAC cell line. When available the pathogenicity status/score is included in the table. The * indicates that the mutation results in an early terminated gene product. n/a, not available.*

for 4 weeks with gemcitabine or cisplatin. The *BRCA* mutant xenografts were significantly more sensitive to both gemcitabine and cisplatin compared to the *BRCA* wild-type xenografts ($p < 0.0001$). In another study, using a *BRCA2* mutant and a *BRCA2* wild-type xenograft, Lohse et al. (2016) found no significant difference in sensitivity to radiation treatment or olaparib. Additionally, olaparib did not sensitize to radiation but instead reduced the induction of DNA damage in the *BRCA* mutant xenografts which was attributed to an increased repair of DSBs by the NHEJ pathway and activation of DNA-PK in the *BRCA* mutant xenograft.

Waddell et al. (2015) compared gemcitabine and cisplatin treatment sensitivity of three PDX with an unstable genome and/or high *BRCA* mutational signature burden to four PDX without. None of the DDR-proficient xenografts responded to cisplatin, while two out of three DDR-deficient xenografts did. The DDR-deficient xenograft that did not respond had a *BRCA* mutational signature burden but no unstable genome or mutation in the *BRCA* pathway. Response to gemcitabine was varied in both groups.

Roger et al. (2020) compared the efficacy of multi-DDR interference as maintenance therapy to continuous FOLFIRINOX treatment in a cell line derived xenograft model. *Atm/Kras*-deficient PDAC mouse cell lines (AKC) were orthotopically transplanted in mice and treated with four cycles of FOLFIRINOX to mimic a clinical setting, followed by either a combination of olaparib, VE-822 (ATRi), and CC-115 (DNA-PKi); FOLFIRINOX; or vehicle until an ethical endpoint was reached. OS was significantly longer in the multi-DDR group compared to the FOLFIRINOX or placebo groups (28.5 vs. 24.5 vs. 18.0 days, $p < 0.02$). In addition, the FOLFIRINOX treatment was shown to select for more aggressive subclones, which could partly be erased by multi-DDR treatment. The combination of multiple targeted drugs allowed for lower dosing than used in monotherapy which reduced side effects to a similar level as for the FOLFIRINOX treatment.

Genetically engineered mouse models

The advance of genetic manipulation has allowed for the development of genetically engineered mouse models (GEMMs). Carefully chosen germline mutations induce tumor formation at an early age and at a relatively high penetrance. In contrast to xenografts, tumors in GEMM develop progressively and can therefore also be used to study precancerous lesions and low grade tumors (Gopinathan et al., 2015).

The most used PDAC models are the KC and KPC mice (Hingorani et al., 2003, 2005; Gopinathan et al., 2015; Lee et al., 2016; Ariston Gabriel et al., 2020). The KC mice is characterized by a germline mutation in *Kras* (*K-ras*$^{LSL.G12D/+}$) and the KPC mice has an additional mutation in *Tp53* (*p53*$^{LSL.R172H/+}$). The

presence of Pdx1-Cre removes the floxed transcriptional STOP cassette which silenced the mutant alleles in the pancreas. Both models develop PanINs and eventually also PDAC although the onset and penetrance of PDAC is later and lower in KC mice.

The KC and KPC mouse models have been instrumental in understanding tumor development in DDR-proficient PDAC but have also been used as a basis for DDR-deficient GEMM models. The following section will describe DDR-deficient GEMM models (*Brca2*-deficient and *Atm*-deficient) that have been published in literature (**Table 3**).

Brca2-deficient genetically engineered mouse model

Feldmann et al. (2011) established two *BRCA2*-mutated GEMM, abbreviated as CB (*Pdx1-Cre*; $Brca2^{flox/flox}$) and CBP (*Pdx1-Cre*; $Brca^{flox/flox}$; $LSL\text{-}Trp53^{R172H}$), and performed extensive histopathological characterization and survival analysis. Both models developed the full spectrum of PanIN lesions which replaced the pancreatic parenchyma and acinar tissue. The additional *Trp53* mutation in the CBP cohort enhanced the frequency of invasive neoplasia and resulted in earlier mortality (375 vs. 454 days, $p = 0.085$). Five mice from the CBP cohort developed metastatic lesions (after 15 months), two were categorized as moderately differentiated adenocarcinomas, two as a combination of adenocarcinoma and sarcomatoid carcinoma, and one as anaplastic carcinoma with giant cells. Sequencing of metastatic PanIN ($n = 2$) and PDAC ($n = 2$) in CB and CBP mice found no secondary *Kras* mutations in any of the mutation hotspots (codon 12, 13, and 61) indicating that *Kras* mutation is not prerequisite for tumorigenesis in the presence of *Brca2* mutation in mice.

These findings were contradicted by Skoulidis et al. (2010) who argued that *Brca2*-deficiency alone is not sufficient to induce carcinogenesis. They developed several mouse models with a combination of mutations in *Brca2*, *Trp53*, and *Kras* (Skoulidis et al., 2010). Of the resulting models, those with solely *Brca2*-deficiency ($CB^{Tr/\Delta 11}$: *Pdx1*-Cre; $Brca2^{Tr/\Delta 11}$) had a longer survival than those with combined *Brca2*-deficiency and *Trp53* loss ($PCB^{Tr/\Delta 11}$: $Trp53^{R270H}$, *Pdx1-Cre*; $Brca2^{Tr/\Delta 11}$ and $PCB^{Tr/WT}$: $Trp53^{R270H}$, *Pdx1-Cre*; $Brca2^{Tr/WT}$) which the researchers contribute to development of pancreatic insufficiency in a fraction of mice. Mice with triple mutation ($KPCB^{Tr/\Delta 11}$: KPC mouse with additional *Pdx1*-Cre; $Brca2^{Tr/\Delta 11}$) nearly all developed tumors and had the worst survival. However, homozygous *Brca2* inactivation did contribute to a significantly more aggressive disease with rapid clinical decline compared to wild-type or heterozygous loss in combination with *Kras* and *Trp53* mutation ($p < 0.002$). Tumors in KCB and KPCB mice displayed a range of histological features that can also be found in human pancreatic cancers, ranging from PDAC to sarcomatoid tumors and acinar-cell carcinoma.

In line with this Rowley et al. (2011) found that loss of *Brca2* alone is not enough to induce tumorigenesis, but that it can promote tumorigenesis in combination with *Trp53* inactivation. They developed *Brca2*-deficient ($CB2^{\Delta 11/\Delta 11}$: *Pdx1-Cre*; $Brca2^{\Delta 11/\Delta 11}$; $CB2^{wt/\Delta 11}$: *Pdx1-Cre*; $Brca2^{wt/\Delta 11}$) and *Trp53*-null ($CPB2^{\Delta 11/\Delta 11}$, $CPB2^{wt/\Delta 11}$, and $CPB2^{wt/wt}$) mice. The CB2 mice did not develop PDAC whereas the CPB2 mice did. Heterozygous and homozygous *Brca2* loss significantly reduced pancreatic cancer-free survival ($p < 0.0001$), with the strongest effect seen in the homozygous-loss mice. The tumors observed in these mice were similar to several human pancreatic cancer types: 40% were of ductal origin, 35% high grade undifferentiated carcinomas, 20% were mucinous tumors, and the remaining 15% were acinar carcinomas. $CPB2^{wt/wt}$ mice presented mainly with acinar and undifferentiated tumors. Seventy-two percent of the $CPB2^{\Delta 11/\Delta 11}$ mice were found to have PanIN lesions at the time of tumor resection or death, while this was less than 6% in $CPB2^{wt/\Delta 11}$ and $CPB2^{wt/wt}$ mice. Remarkably, additional $Kras^{G12D}$ mutation (CKB2 mice) decreased pancreatic cancer formation; Tumors were found in 66% of $CKB2^{wt/\Delta 11}$ and 61% of $CKB2^{wt/wt}$ mice, but in just 13% of $CKB2^{\Delta 11/\Delta 11}$ mice. The majority of these tumors (>90%) were PDACs.

These studies established that bi-allelic loss of *Brca2* in combination with *Tp53* deregulation can induce a spectrum of pancreatic lesions. Whether bi-allelic loss of *Brca2* alone can also induce pancreatic cancer remains unclear as Feldmann et al. (2011) did not investigate the mutation status of other genes besides *Kras* and *Tp53*.

Atm-deficient genetically engineered mouse model

Two studies have published a KC *Atm*-deficient mouse model. Russell et al. (2015) found that KC mice with floxed *Atm* (abbreviated as AKC) had developed more acinar-to-ductal metaplasia lesions and PanINs compared with KC mice at 10 weeks old. The higher tumorigenicity of KC *Atm*-deficient mice was confirmed by Drosos et al. (2017) who studied KC mice with either $Atm^{loxP/+}$ or $Atm^{loxP/loxP}$ (abbreviated as KCATMΔ+ and KCATMΔΔ). Post-mortem analysis identified pancreatic cancers in 94 and 62% of the KCATMΔ+ and KCATMΔΔ mice compared to 42% in KC mice. In addition, the *Atm*-deficient mice had a comparable and significantly reduced median OS in both studies ($p < 0.01$).

Both studies performed subtype analysis. Russell et al. (2015) performed gene expression profiling of 10-week-old KC and AKC mice and compared this to PDAC subtypes as described by Collisson et al. (2011). Hierarchical clustering revealed that AKC pancreatic tumors were closer associated with the quasi-mesenchymal human PDAC subtype than with KC pancreatic tumors. In contrast, Drosos et al. (2017) used *KC*, *KCATM*Δ+ and *KCATM*ΔΔ primary tumor cell lines for subtyping and concluded that their tumors were primarily of the pancreatic progenitor/classical phenotype based on the high expression of several progenitor marker genes (*Pdx1*, *Hnf1*β, and *Lgals4*) and a classical marker gene (*Gata6*). This discrepancy in subtyping may be explained by the sample material used. The subtyping as defined by Collisson et al. (2011) is based on FFPE material and therefore includes tumor cells, stroma, and normal tissue, by using cell lines the gene expression profile is altered compared to the primary tissue due to the lack of stroma which will likely affect the subtype definition, especially with respect to the quasi-mesenchymal subtype.

TABLE 3 | Overview DDR deficient pancreatic cancer GEMMs studies.

References	Model	Mutations	No. of mice	Phenotype
Hingorani et al. (2003, 2005)	KC	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*	33	100% developed PanIN which progressed to invasive and metastatic PDAC in a small minority.
	KPC	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*; *p53$^{LSL.R172H/+}$*	28	96% developed PDAC which metastasized in over half of the mice. Median survival of 22 weeks.
Feldmann et al. (2011)	CB	*Pdx1-Cre*; *Brca2$^{flox/flox}$*	25	15% developed invasive and metastatic PDAC, more mice developed PanIN. Median survival of 65 weeks.
	CBP	*Pdx1-Cre*; *Brca$^{flox/flox}$*; *LSL-Trp53^{R172H}*	33	100% developed invasive or metastatic PDAC. Median survival of 54 weeks.
Skoulidis et al. (2010)	CB$^{Tr/\Delta 11}$	*Pdx1*-Cre; *Brca2$^{Tr/\Delta 11}$*	24	No development of pancreatic cancer.
	PCB$^{Tr/\Delta 11}$	*Pdx1-Cre*; *Trp53^{R270H}*; *Brca2$^{Tr/\Delta 11}$*	22	No development of pancreatic cancer.
	PCB$^{Tr/WT}$	*Pdx1-Cre*; *Trp53^{R270H}*; *Brca2$^{Tr/WT}$*	25	No development of pancreatic cancer.
	KCB$^{wt/wt}$	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*	40	15% developed PDAC.
	KCB$^{Tr/wt}$	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*; *Brca2$^{Tr/wt}$*	40	30% developed PDAC.
	KCB$^{Tr/\Delta 11}$	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*; *Brca2$^{Tr/\Delta 11}$*	32	19% developed PDAC, though frequent development of pancreatic insufficiency.
	KPCB$^{wt/wt}$	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*; *p53$^{LSL.R172H/+}$*	30	80% developed PDAC. Median PDAC-free survival 24 weeks.
	KPCB$^{Tr/\Delta 11}$	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*; *p53$^{LSL.R172H/+}$*; *Brca2$^{Tr/\Delta 11}$*	30	87% developed PDAC. Median PDAC-free survival 12 weeks.
	KPCB$^{Tr/wt}$	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*; *p53$^{LSL.R172H/+}$*; *Brca2$^{Tr/wt}$*	30	97% developed PDAC. Median PDAC-free survival 20 weeks.
Rowley et al. (2011)	CB2$^{\Delta 11/\Delta 11}$	*Pdx1-Cre*; *Brca2$^{\Delta 11/\Delta 11}$*	12	No development of precursor lesions or PDAC.
	CB2$^{wt/\Delta 11}$	*Pdx1-Cre*; *Brca2$^{wt/\Delta 11}$*	21	No development of precursor lesions or PDAC.
	CPB2$^{\Delta 11/\Delta 11}$	*Pdx1-Cre*; *Trp53$^{F2-10/F2-10}$*; *Brca$^{\Delta 11/\Delta 11}$*	34	High frequency development of pancreatic cancer, >40% of ductal origin
	CPB2$^{wt/\Delta 11}$	*Pdx1-Cre*; *Trp53$^{F2-10/F2-10}$*; *Brca$^{wt/\Delta 11}$*	41	Development of pancreatic cancer, >40 of ductal origin.
	CPB2$^{wt/wt}$	*Pdx1-Cre*; *Trp53$^{F2-10/F2-10}$*	47	Development of pancreatic cancer, predominantly acinar, and undifferentiated.
Russell et al. (2015)	AKC$^{\pm}$	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*, *Atm$^{flox/+}$*	32	Development of PanIN. Median survival 36 weeks.
	AKC$^{-/-}$	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*, *Atm$^{flox/flox}$*	15	Development of PanIN. Median survival 45 weeks.
Drosos et al. (2017)	KC	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*, *Ptf1a$^{+/cre}$*	19	42% developed pancreatic cancer of which >80 of sarcomatoid histology, median survival 61 weeks.
	KCATMΔ+	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*, *Ptf1a$^{+/cre}$*, *Atm$^{loxP/+}$*	21	62% developed pancreatic cancer mainly poor and moderately differentiated, median survival 39 weeks.
	KCATMΔΔ	*Pdx1-Cre*; *K-ras$^{LSL.G12D/+}$*, *Ptf1a$^{+/cre}$*, *Atm$^{loxP/loxP}$*	18	94% developed pancreatic cancer with a mixture of moderate, poor, and undifferentiated tumors, median survival 39 weeks.

Clinical Trials Targeting DNA Damage Repair Deficiency Pathways

The past decade has seen a rising interest in the combination of cytotoxic chemotherapy with targeted approaches to exploit the synthetic lethality of this combinatorial approach. As of July 2021, there are 51 clinical trials registered on clinicaltrials.gov that investigate DDR targeted therapies alone or in combination with chemotherapy in PDAC (either in PDAC alone or as part of a larger cancer patient cohort). The majority of these trials (78%, n = 40) focus on *PARP* inhibitors, although *ATM/ATR*, *CHK1*, *DNA-PK*, and *WEE1* inhibitors are also being investigated (**Supplementary Table 2**).[1] Except for a single trial, all trials are either phase I or II, with limited numbers of patients and often single-arm treatment protocols which renders efficacy analysis more challenging.

PARP Inhibitors

The pivotal phase III trial leading to FDA approval for the use of PARPi in metastatic PDAC was conducted by Golan et al. (2019) and evaluated olaparib as maintenance therapy in metastatic PDAC patients with germline mutation of *BRCA* (NCT02184195). Patients were eligible if their tumor had not progressed on first-line platinum-based chemotherapy (e.g., cisplatin or oxaliplatin). Treatment with olaparib was compared to placebo and a significant increase in PFS was observed (7.4 vs. 3.8 months, p = 0.004), but at interim analysis (data maturity 46%) no significant difference was found in median OS (18.9 vs. 18.1 months, p = 0.68). The updated results of this study were presented in January 2021 (Golan et al., 2021). Disappointingly there was again no difference in median OS between the groups

[1]The status of the trials is not always up-to-date, several trials have been completed and published the results but are still marked as "active."

(OS 19.0 vs. 19.2 months, $p = 0.35$). Notably, however, PFS2, i.e., the time from randomization to second disease progression or death, was significantly longer in the olaparib-treated group (PFS2, 16.9 vs. 9.3 months, $p = 0.0061$).

A comparable phase I trial (NCT00515866) in PDAC patients with locally advanced or metastatic PDAC being treated in the first line setting included a comparison of olaparib combined with gemcitabine vs. gemcitabine alone in the expansion phase ($n = 22$) (Bendell et al., 2015). Patients were eligible for inclusion regardless of genetic/molecular status. No significant benefit was found regarding objective response rate (ORR), OS, or PFS for the combination treatment. While the researchers noted that nine patients had *BRCA* mutation status available, analysis of response by *BRCA* mutation status was not performed due to the small number of patients for whom this data was recorded.

O'Reilly et al.'s (2018) phase IB trial of the addition of another PARPi, veliparib, to first line chemotherapy (cisplatin, gemcitabine) demonstrated a striking ORR of 78% in patients with stage III/IV PDAC with *BRCA1/2* germline mutations and an equally impressive median OS of 23.3 months. The investigators then proceeded to a phase II trial of this combination in patients with PDAC and germline *BRCA* or *PALB2* mutations (O'Reilly et al., 2020). Response rate in the combination arm was 79 vs. 65.2% in the chemotherapy alone arm ($p = 0.02$). However, there was no statistically significant difference in PFS or OS between the groups (PFS 10.1 vs. 9.7 months, $p = 0.73$; OS 15.5 vs. 16.4 months, $p = 0.6$). The 2- and 3-year OS of 30.7 and 17.8%, respectively, in this study are the longest ever reported in a clinical trial in this cohort. Notably a phase II study of veliparib alone in the second line setting in patients with *BRCA* mutant PDAC reported no confirmed responses; 4 patients (4/16) had stable disease for 4 months (Lowery et al., 2018).

Two additional phase II trials have published results on the addition of veliparib to 5-FU based chemotherapy. The first study (NCT02890355) compared the combination of veliparib with modified FOLFIRI vs. FOLFIRI alone as second line treatment in metastatic pancreatic cancer patients (Chiorean et al., 2019). In total, 108 patients were included in the analysis. The addition of veliparib to FOLFIRI treatment was shown to increase toxicity. Moreover, veliparib did not improve either OS (5.1 vs. 5.9 months in combination vs. monotherapy arm, respectively, HR 1.3, $p = 0.21$) or PFS (2.1 vs. 2.9 months, HR 1.5, $p = 0.05$). Additionally, blood and tumor biopsies were collected at baseline to explore HR or DDR biomarkers. Nine percent of the tumors had HR deficiency (*BRCA1/2*, *ATM*, *PALB2*, *ATM*, or *CDK12* mutation), and an additional 20% had mutations in other DDR genes (*FANC*, *BLM*, *SLX4*, *CHEK2*, *POLD1*, *RIF1*, and *MSH2/6*). Correlative analysis of HR or DDR deficiency with treatment response is still ongoing.

Pishvaian et al. (2020) performed a phase I/II clinical trial (NCT01489865) to evaluate the safety and response of PDAC patients to combination treatment of veliparib with modified FOLFOX. For the phase I portion of the study patients were not selected based on genetic history; however, for the phase II part of the trial, patients were selected based on the presence of HR-DDR deficiency or family history suggesting breast or ovarian cancer syndrome, and a distinction was made between previously treated and untreated patients. The ORR was 20% in the phase I unselected cohort ($n = 23$) and 31% in the phase II cohort ($n = 33$) selected for HR-DDR deficiency. Further analysis of the phase II cohort showed that treatment-naïve patients had a better ORR and OS than previously treated patients (40 vs. 22%, and 13.0 vs. 4.5 months, respectively). In the treatment-naïve HR-DDR patients, the ORR was 57%. However, due to the lack of a placebo or control arm the magnitude of benefit attributable to the addition of veliparib is difficult to quantify. Previously reported OS of metastatic PDAC patients receiving FOLFOX as second-line treatment are in a similar range (3.3 and 6.3 months) (Yoo et al., 2009; Hecht et al., 2020).

Multiple trials for PARP inhibitors are currently recruiting or preparing for recruitment of patients with DDR deficiency (either for *BRCA* mutations specifically, or for a panel of DDR genes). This includes phase I and II trials for niraparib both as monotherapy and in combination with other drugs (NCT04673448, NCT04764084, NCT03601923, and NCT04493060), Olaparib (NCT04548752), rucaparib (NCT04171700), talazoparib (NCT04550494), and NMS-03305293 (NCT04182516).

CHK1 Inhibitor

Laquente et al. (2017) performed a phase I/II clinical trial for the *CHK1* inhibitor rabusertib (LY2603618) combined with gemcitabine vs. gemcitabine alone in patients with locally advanced or metastatic PDAC (NCT00839332). Although no significant differences were found in the number and severity of adverse events, no significant differences in OS, PFS, ORR, or duration of response were found either.

DNA-PK Inhibitor

A phase II trial on the safety and efficacy of the combination of the *DNA-PK* inhibitor LY3023414 with abemaciclib in previously treated metastatic PDAC patients compared to standard-of-care gemcitabine or capecitabine found that the combination treatment had a significantly worse PFS (1.81 vs. 3.25 months, $p = 0.012$) (NCT02981342).

Patient Selection

Preclinical models have shown that several targeted therapies are more effective in models that defects in complementary DNA repair pathways and this principle of synthetic lethality has been adapted by clinical trials. Multiple trials are currently running which select patients based on the presence *BRCA* mutations. While the application of targeted therapy in patients with of *BRCA* mutations has had success in other cancer types (most notably in breast and ovarian cancer), the fraction of patients with *BRCA* mutations is relatively small and patients with other DDR gene mutations may also benefit from these therapies. Several studies have included additional mutations in their selection criteria, but based on **Table 1** these panels could be extended upon. However, preclinical trials might be needed to warrant this.

Alternatively, DDR deficiency might serve as biomarker for response to non-targeted therapies. One study that is currently investigating this is the Precision Panc trial which recruits

PDAC patients for molecular profiling and allows patients to enroll in a PRIMUS trial (NCT0461417), one of which aims to test a DDR deficiency biomarker for response to FOLFOX-A treatment (NCT04176952).

DISCUSSION

The dismal survival rate of PDAC underscores the urgent need to develop new and more effective preventative and therapeutic strategies. So far, clinical trials for DDR targeting drugs have shown limited results beyond the approval of Olaparib in the maintenance setting for patients with germline *BRCA1/2* mutations. To improve treatment, representative models are needed, especially those that model DDR deficiency, to test drugs and to develop biomarkers that predict patient response. Clinical rationale exists to expand the use of DDR targeting agents, however, to date there are no validated predictors of treatment response with these agents for patients with DDR deficient tumors beyond *BRCA1/2*.

Therefore, in order to expand the impact of targeting DDR pathway genes, we performed a comprehensive review of published preclinical models which can potentially be used for DDR deficiency targeted drug screening. Using a 352 DDR gene panel we queried the GENIE database and found multiple frequently mutated DDR genes, some of which are not commonly used in DDR panels. This suggests that DDR deficiency might occur more frequently than previously thought but also provides additional options for biomarkers or targeted therapies.

Cell lines are by far the most frequently used model for PDAC. However, DDR deficiency studies are mainly limited to the *BRCA2*-mutant cell line Capan-1. Our query of the PDAC cell lines in the CCLE database for the top 10 most commonly mutated DDR genes (excluding *TP53*) found that 20 cell lines contain one or multiple DDR gene mutations. However, for the majority of these mutations the pathogenicity is unknown and functional characterization of the DDR status is warranted.

The application of organoids toward PDAC studies is still relatively new and at the time of writing no studies in PDAC organoids on DDR-deficiency specifically have been published. We therefore stress the need for characterization of DDR status in existing organoids based on NGS and functional profiling as well as the development and characterization of DDR deficiency in existing organoids through gene editing.

In contrast to cell lines and organoids, several DDR deficient mouse models have been published. So far the application of these models has mainly focused on the contribution of DDR deficiency on tumorigenesis and tumor progression, but there is opportunity for their application in drug sensitivity studies.

The process from biomarker/drug discovery to clinical practice is a long and often unsuccessful path, with 95% of the drugs failing at the translation from preclinical model to clinical trials (Seyhan, 2019). Preclinical models that closely recapitulate the primary tumor have a higher translational value and thus improve the chance of success.

Interest in DDR deficiency as a target for personalized therapy is rising, indicated by the high number of clinical trials currently open in this area. Of the 51 trials running, 29 phase I or II trials are currently recruiting or not yet recruiting. More evidence of the efficacy of these therapies in preclinical PDAC models are required to support their clinical rationale, as many studies have not achieved their desired endpoint. While limitations exist to preclinical *in vitro* and *in vivo* models (Nelson and Walsh, 2020), appropriate preclinical models can reflect histopathological subtypes, assist in early prioritization of promising therapies, be used in high-throughput screening to identify ineffective therapies earlier, and thus prevent the excessive time and money resources of a failed clinical trial.

AUTHOR CONTRIBUTIONS

NW and ML conceived the study and provided the significant guidance. NW drew up the outline of the review. JS wrote the manuscript and generated the illustrations. EH contributed with the section on clinical trials. SM contributed to the writing of early drafts. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.749490/full#supplementary-material

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REVIEW
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Pancreatic Ductal Adenocarcinoma: Preclinical *in vitro* and *ex vivo* Models

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most overlooked cancers despite its dismal median survival time of 6 months. The biggest challenges in improving patient survival are late diagnosis due to lack of diagnostic markers, and limited treatment options due to almost complete therapy resistance. The past decades of research identified the dense stroma and the complex interplay/crosstalk between the cancer- and the different stromal cells as the main culprits for the slow progress in improving patient outcome. For better *ex vivo* simulation of this complex tumor microenvironment the models used in PDAC research likewise need to become more diverse. Depending on the focus of the investigation, several *in vitro* and *in vivo* models for PDAC have been established in the past years. Particularly, 3D cell culture such as spheroids and organoids have become more frequently used. This review aims to examine current PDAC *in vitro* models, their inherent limitations, and their successful implementations in research.

Keywords: pancreatic ductal adenocarcinoma, 3D cell culture, spheroid, reporter assays, organoids

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INTRODUCTION

Over the past decades, researchers in cell biology recognized the limitations in clinical translation of both cell culture and animal models. Subsequently, effort was put into adjusting and accommodating to new demands. It is commonly accepted that there is no universally superior model but that instead the particular topic of research and the entailing experimental restrictions dictate model suitability (**Figure 1**). This review aims to survey common *in vitro* models used in pancreatic cancer research. As part of a review series, we will focus particularly on spheroid models, discussing examples of successful applications and limitations in PDAC research in this review.

Malignancies of the pancreas can originate from either the endocrine part or the duct system of the organ. The former are known as neuroendocrine tumors (NET) and have a much more favorable outcome than the latter. Pancreatic ductal adenocarcinoma (PDAC) as the most common tumor of the exocrine pancreas does often, but not exclusively, originate from the epithelial lining of the pancreatic duct. The classification of PDAC is done based on histological markers which

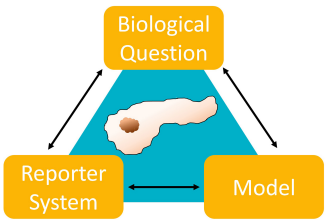


FIGURE 1 | Illustrating the codependences of model, reporter system and biological question. A relevant model needs to be suitable to emulate the biological environment dictated by the research question. Additionally, there needs to be a reporter system which will allow the observation of explicit parameters suitable to answer that question. Finally, the model and reporter system need to compatible.

overlap with those of healthy ductal epithelium. Of all the exocrine tumors, PDAC is by far the most common and accounts for the majority of deaths linked to pancreatic cancer. With a devastating median survival time of just 4.6 months (Carrato et al., 2015), PDAC was the 4th leading cause for cancer related deaths in Europe in 2020 (European Union, 2021) and is projected to become the 2nd leading cause of cancer related death by 2030 (Rahib et al., 2014).

Pancreatic ductal adenocarcinoma (PDAC) is characterized by dense and abundant desmoplastic stroma. The amount of cancer cells is often estimated at only 20% based on histological tissue inspection (Leppanen et al., 2019; Mayer et al., 2020). The most common cells in the tumor microenvironment (TME) are cancer associated fibroblasts (CAFs). They often stem from pancreatic stellate cells (PSCs) but can also arise from resident fibroblasts (Haber et al., 1999; Shek et al., 2002) and can be recruited from bone marrow derived stem cells (Marrache et al., 2008; Iwamoto et al., 2021). Upon injury to the tissue, caused by trauma or infection, these cells differentiate into myofibroblasts, which are rich in α-smooth muscle actin (α-SMA, ACTA2), and built up the major part of the extracellular matrix (ECM) (Haber et al., 1999). However, cancer cell lines also produce considerable amounts of matrix components (Löhr et al., 1994) and induce ECM formation in fibroblast by secretion of transforming growth factor β (TGF-β) (Abetamann et al., 1996; Löhr et al., 2001). Recent LC-MS/MS proteomics revealed that this property is not due to cell culture effects but verified the same findings in patient samples and a human-to-mouse orthotopic xenograft model (Tian et al., 2019). Furthermore, the type of secreted matrix components is not constant but changes as the cancer progresses. The main matrix components found in ECM are collagen types I and III, laminin, fibronectin, and hyaluronic acid (HA) (Fries et al., 1994; Löhr et al., 1994; Abetamann et al., 1996; Bachem et al., 2005; Naba et al., 2012). Like other glycosaminoglycans, HA is highly hygroscopic, causing a localized trapping of water which leads to increased interstitial fluid pressure and thus swelling of the tissue. This swelling exerts pressure, both onto the tumor itself and the surrounding healthy tissue, which is commonly summarized as solid stress (Nia et al., 2016; Voutouri et al., 2016). This compression triggers a number of mechano-sensitive responses, such as activation of latent TGF-β (Wipff et al., 2007), YAP (Zhang et al., 2014; Laklai et al., 2016), or fibronectin unfolding and interaction with collagen (Smith et al., 2007; Kubow et al., 2015). In a positive feedback loop TGF-β activates PSCs to differentiate into myofibroblasts which increase the contractile strain in the tissue (Biffi et al., 2019). Thus, solid stress causes a stiffening of the already dense matrix, exacerbating the restrictive nature of the TME in PDAC.

The physical changes of the TME have great ramifications for cancer therapy in PDAC. Commonly, blood vessels in solid tumors such as PDAC are leaky. Their formation is faulty due to the uncoordinated neo-angiogenesis found in tumors. This causes drugs of high molecular weight to exit the blood stream more easily in a tumor than in healthy tissue which leads to a drug enrichment in the target tissue (Maeda, 2012). This phenomenon is known as enhanced permeability and retention (EPR) effect and is widely exploited to reduce off-site effects in therapy (Maeda et al., 2000; Maeda, 2012). The EPR effect, however, is compromised in PDAC. Tumor blood vessels collapse due to their flawed construction and the pressure induced by the stroma and blood flow is more strongly directed out of the tumor rather than into it (Maeda, 2012). Unlike many other cancers, neo-angiogenesis is not common in PDAC either and the resulting lack of blood flow creates a highly hypoxic TME.

Hypoxia in tumors has long been linked to increased metastatic potential and, consequently, poor patient outcome in PDAC and other cancer types (Brizel et al., 1996; Höckel et al., 1996; Chang et al., 2011). Hypoxia represents evolutionary pressure for cancer cells. The ones which manage to adapt, are more resilient toward poor metabolic conditions as a result. Additionally, hypoxia itself sharply reduces the effectivity of radiotherapy as it relies on generating reactive oxygen species from elemental oxygen.

Unsurprisingly, these circumstances have made treatment difficult. Where applicable, surgery remains the best option for survival for now. However, improving *in vitro* models harbors two major advancements to improve patient outcome: the development of *in vitro* models describing the tumor microenvironment more accurately and thus furthering drug discovery; and by facilitating personalized medicine in the form of patient derived organoids (PDOs) or patient derived xenografts (PDXs). Combined, as *in vitro* models increasingly reflect the complexity of PDAC more accurately, they represent a better chance to identify ways to overcome resistance to conventional treatments.

2D CELL CULTURE

2D cell culture has been the standard of operating procedure for molecular life sciences for good reasons. It is easy to control and manipulate in experiments and hence provided good understanding for the fundamental processes in living cells. Compared to cell-free systems, e.g., in drug design screens, the introduction of cellular systems introduced parameters like membrane permeability, the impact of naturally occurring agonists and antagonists on the intended target and of course the cytotoxicity of the tested compounds (Blay et al., 2020). The

technical benefit of using 2D cell culture was indeed so great that commercial assays are often designed based on 2D cell culture, also given how widespread and easily accessible this model is.

This simplicity, however, is not sufficient when investigating increasingly complex systems such as cancer. Understanding the microenvironment of a tumor has been recognized as essential in eventually overcoming the challenges and heterogeneity of cancer. Pancreatic cancer in particular has a highly altered microenvironment (Kleeff et al., 2007), marked by excessive desmoplasia, hypoxia and poor nutrition. 2D cell culture fails to simulate this environment sufficiently on several accounts. Most strikingly, cells grow in monoculture unlike tissue, which consists of a multitude of different cell types. The surroundings of cells are also severely altered in 2D cell culture where there is none of the extracellular matrix preserved or commonly replicated. An inevitable drawback shared by any *in vitro* system is the selection for fast growing cells. In contrast, healthy tissue grows slowly and tightly regulated, even after injury. Additionally, while incubation chambers are supplied with carbon dioxide, they rely on normoxia when it comes to the oxygen content in the medium.

However, there are ways which enhance the biological relevance of this cell culture system. A more hypoxic environment can be achieved by using hypoxia chambers which are commercially available (Abdalla et al., 2019). With these, the cells can be cultivated under an altered atmosphere with partial gas pressures that resembles the ones found in tissue more closely (Geismann and Arlt, 2020). Another example is the use of trans-well plates which allows the cultivation of different cell types in one well. The medium containing signaling molecules and metabolites can diffuse through a membrane enabling crosstalk of solvent molecules. However, cell-cell-contact and its subsequent signaling is not possible.

Conclusively, PDAC research has recognized the complexity and significance of the TME with high levels of dense ECM. More detailed investigations into PDAC consequently require models which include these properties.

3D CELL CULTURE

When it comes to 3D cell culture, there are several levels of model complexity and consequently biological relevance. What they all have in common is that cells are not cultivated as a monolayer. By various means, which will be discussed in detail below, some aspects of the three-dimensionality of tissue are simulated or even preserved. Another commonality is that these models are not as established yet despite simulating *in vivo* conditions better compared to 2D cell culture models. For example, failure of potential drug candidates at early stages of drug development in more advanced models might prevent costly failures at later stages.

However, more advanced models also have drawbacks. More complex models are inherently less consistent as simpler models. Consequently, more complex models also generate less consistent samples. This reduced model fidelity carries the risk of masking significant results. It is hence an ongoing struggle to minimize this sample background heterogeneity by streamlining established protocols.

Spheroids

Spheroids are solid cell clusters generated from established 2D cell lines and do not require many additional changes in culture conditions compared to the 2D requirements. There are different types of spheroids and several ways to generate spheroids and based on the method used, the properties of the spheroids will differ (**Table 1**). Consequently, it is important to keep the methodology in mind as a source of heterogeneity when comparing different findings, especially when the conclusions drawn from the experiments contradict one another.

Common to all 3D culture is that the cells are forced not to adhere to the plastic surfaces of the culture vessels, but instead aggregate with other cells. To this end ultra-low attachment plastics as well as less costly methods using simple non-adherent plates in combination with crowding agents like methylcellulose or agarose coated plastics have been developed (Carlsson and Yuhas, 1984; Longati et al., 2013). Spheroids can be either grown just in liquid (media), embedded in or on matrix or by using a microfluidic platform.

The most common matrices used for spheroid generation are Matrigel and collagen hydrogels. These two natural matrices supply common ECM proteins, such as collagens and fibronectin, which allow cell-cell and cell-matrix interactions, e.g., by cadherins and integrins. A more specialized matrix is HA-based and for PDAC spheroids of particular interest as HA is

TABLE 1 | Comparison of different techniques used for spheroid formation and their attributes (Merck KGaA, 2021; The Cell Culture Dish, 2021).

Attributes	Matrix-embedded		Hanging drop	ULA-plate	ULA-plate with crowding agent	Bioreactor	Microfluidic system
	Animal derived matrices	Synthetic hydrogels					
Consistency of samples	+	++	+++	++	++	−	++
Cost efficiency	−	−	+	+++	+++	−	−
High throughput	+	+	++	++	++	+++	−
Long term culture	++	++	+	++	+	+++	+++
Sample retrieval	+	+	++	+++	+++	+++	−
Image analysis	+	+	+	++	++	−	+++

Suitability: +++ = High; ++ = Medium; + = Low; − = Very low. ULA = ultra low attachment.

a main component of the TME and the major driving force for the tumor interstitial fluid pressure (Scaife et al., 2008; Liu et al., 2018). Tissue stiffness was determined by atomic force microscope (AFM) to increase from 0.4 kPa in normal pancreas tissue to 1.2 kPa of a PDAC tumor (Rice et al., 2017). Collagen matrices have a compression force more akin to healthy tissue while modified HA matrices reach compression forces up to 1.5 kPa which compares well to the pressure measured in PDAC tumors (Chang and Lin, 2021). A major drawback of using natural compounds is their batch-to-batch variability. The substitution with synthetic polymers alleviates this problem and introduces more control and consistency to the model. The most widely used synthetic polymer is polyethylene glycol (PEG), a substance widely used in biomedical context due to its non-toxicity and non-immunogenicity. Polyethylene glycol can be modified to show properties similar to ECM proteins and enable cell aggregation (Liu and Vunjak-Novakovic, 2016).

While organoid culture, described below, relies heavily on these matrices, spheroid culture has alternative options by using microfluidic systems. The simplest way is preventing cell adhesion by using coatings, such as agarose (Carlsson and Yuhas, 1984; Ma et al., 2012). This approach is quite easy to accomplish and inexpensive but lacks spheroid uniformity, a feature important for consistent results. The same problem arises when using bioreactors, such as the spinner flask. While this method has a very high yield of spheroids and enables ongoing growth and culture, the produced spheroids come in any shape which limits their experimental use (Cui et al., 2017). As reproducibility and uniformity of size and shape is very important in research, the hanging drop method was favored over the previously mentioned methods (Timmins and Nielsen, 2007). Although more labor intensive than other spheroid generation methods and smaller overall size of spheroids, it provided more consistency and allowed medium throughput analyses (Cui et al., 2017). A recent advancement was combining aspects of microfluidics and matrix assisted growth by supplying the growth medium with the crowding agent methylcellulose (MC) in combination with non-adherent cell culture vessels (Longati et al., 2013). Like solid matrices, MC increases the viscosity of the medium enough to prevent cell sedimentation but unlike natural polymers it is not subject to batch-to-batch-variation. Spheroids are grown one per well, in scale to the well size, so it does not reach the high throughput levels of bioreactors. However, with liquid handling equipment commonly available at high throughput facilities spheroids were successfully grown even in 1536 well plates (Madoux et al., 2017). It grows consistently sized spheroids which are considerably larger than those produced by the hanging drop method, creating more distinct internal gradients.

Even the most basic form of spheroid culture which only uses a single cell type improves on mirroring the conditions of tissue compared to 2D cell culture (Friedrich et al., 2009; Longati et al., 2013; Wagner et al., 2013). By shielding the core of the spheroid from the medium, gradients of oxygen, nutrients, metabolic waste products and signaling molecules are generated. Often the core of the spheroid shows necrosis upon prolonged culture while the exterior layers still proliferate. As such spheroids might be viewed as avascular minitumors.

Dense stroma is a key hallmark of PDAC. Spheroids were shown to build up some matrix components (Longati et al., 2013), however, fall short in mimicking the combined physical properties of the tumor, like stiffness and compression, and thus cannot inherently model solid stress. External induction of solid stress can be simulated using a collagen-1-matrix for embedding.

Spheroids can be grown in coculture in several ways to investigate cell-cell-communication. The before-mentioned trans-well method can be used, e.g., a spheroid in solution and a 2D cell culture of CAFs, or a solid spheroid with immune cells in solution (Kuen et al., 2017). Even a combination of 3 types of cells was reported with heterospheroids of different pancreatic cancer cell lines and CAFs being exposed to monocytes. Histology allowed then to assess infiltration and drug response (Kuen et al., 2017).

Additionally, spheroids can also be generated from more than one cell type forming heterospheroids (Norberg et al., 2020). Subsequent analysis of crosstalk between the different cell types requires pre-labeling of these. For example, cell lines expressing fluorophores are often used to distinguish cell types within the spheroid or during cell sorting or image analysis (Öhlund et al., 2017). Cell sorting comes with the drawback of having to generate single cells from spheroids which entails substantial stress on the cells and sample loss. Other pre-labeling techniques like the use of isobaric tags have been carried out to investigate the proteomic shift between 2D and 3D culture in PDAC (Samonig et al., 2020) and to show how drug-induced Akt-inhibition increases the stemness of pancreatic cancer spheroids (Arasanz et al., 2020).

Alternative to pre-labeling, different cell types can also be vitrually "sorted" after cultivation by using cells from different species and exploiting the small differences in species specific DNA/RNA sequences (Conway et al., 2012; Liu et al., 2021). Our lab established one such model by generating heterospecies heterospheroids using human Panc1 cells and immortalized mouse pancreatic stellate cells (imPSCs) (Norberg et al., 2020; Liu et al., 2021). This allowed for the analysis of cell-cell crosstalk and could show that the coculture in heterospheroids substantially changed the gene expression pattern in key cancer pathways, PDAC type stratification as well as PSC/CAF phenotype (Liu et al., 2021). The RNA profile of Panc-1 in heterospheroids was more reflecting squamous like phenotype compared to Panc-1 in monospheroids. The CAF phenotype was shifting from myCAF to iCAF due to the presence of Panc-1 cells in the heterospheroids.

It could also be repeatedly shown that the spatial distribution of cells and the resulting gradients have substantial effects on chemosensitivity. Especially in the context of pancreatic cancer research with a large focus on drug discovery, these findings add weight to the progression toward three-dimensional cell culture as the base line for medical research which aims to identify novel treatment options.

As spheroids are grown from 2D cell culture sources, some of the limitations carry over while some characteristics are improved upon. Cell line identity can drift the longer a cell line is kept in culture. Especially cancer cells, which have lost many

DNA control/repair mechanisms, are more prone to accumulate mutations. In order to limit the influence of loss of cell line identity, cell lines should be tested regularly by short tandem repeat analysis and discontinued after 40-50 passages (Reid et al., 2013). Another limitation is that also spheroids cannot model the heterogeneity of PDAC. For one, established pancreatic cancer cell lines are limited in number (Moore et al., 2001) and do not reflect the genetic heterogeneity of mutations found in patients. Nor do co-cultured cell lines such as PSCs as recently shown (Lenggenhager et al., 2019). An additional problem lies in that not all cancer cell lines form spheroids and even less form heterospheroids with other cell types. Consequently, any findings carried out using only one model are not guaranteed to apply to all cell lines, all models and ultimately not all PDAC patients. PDAC, like many other cancers, is inherently heterogeneous and has several sub types. In this context, different 2D cell lines grown as spheroids can be viewed as models for different subpopulations of patients.

Other methods rely on single cell sedimentation into cell clusters (Lee et al., 2019). Uniform indentations in a silicon matrix allow for the sedimentation of 2D cells which then pack densely as a result of gravity. This is one of the novel approaches to develop 3D cell culture further, but it is too early to be assessed/discussed in a greater context for now.

Reporter Assays for Spheroid Cultures

Modern research in the field of cell biology is mostly dependent on reporters that rely on optical permeability at one point of the analysis with only few exceptions, e.g., radioactive tracers. We then interpret these optical readings in a biological/physiological context to draw conclusions. As such, models heavily rely on allowing light transmission.

Methods which treat spheroids as small tissue pieces were designed to deal with samples which cannot readily pass light. Consequently, practices like paraffin or OCT (optimal cutting temperature) compound embedding for immuno-histochemistry (Maftouh et al., 2014) following sectioning the sample are readily compatible with spheroid research and are being carried out routinely.

Commercially available biochemical assays have simplified sample analysis substantially by standardizing and simplifying protocols hence making experiments more reproducible between different research groups. However, most of these assays were designed for 2D cell culture which readily fulfills the important prerequisite of allowing light passage. Spheroids on the other hand exhibit a compact structure which allows for very little light transmission. Consequently, adapting reporter systems to be used for spheroid research is not trivial.

There are some classes of reporter assays which commonly adapt well to spheroid culture: those that involve lysis of the spheroid and those that test only a reporter in the culture medium. As mentioned previously, spheroid culture can be carried out without using a solid matrix but an altered medium instead. The combination of a liquid medium-based spheroid culture with colorimetric or fluorescent reporters which only samples the medium even allows a set up for high throughput screens.

More detailed analyses of the medium are being carried out as well. Metabolomics, i.e., detection of radioactively labeled substrates in combination with NMR have been successfully

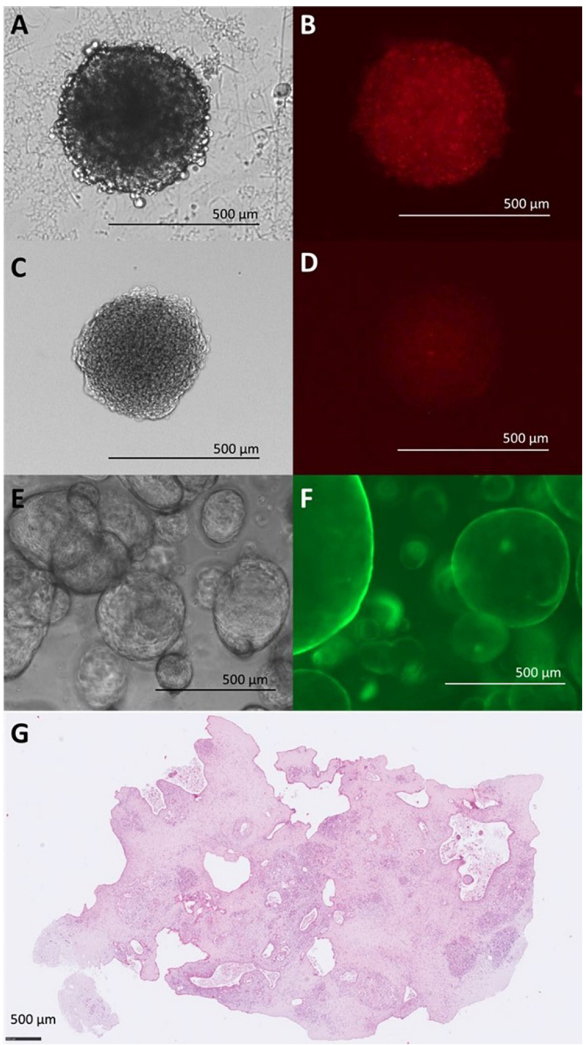


FIGURE 2 | Limitations of light microscopy for different *in vitro* models. **(A)** Bright field (BF) image of Panc-1 and imPSC heterospheroid at 4d. **(B)** Detection of mCherry fluorophore in Panc-1 and imPSC heterospheroid at 4d. Evenly distributed fluorescent signaling despite unevenly distributed cells in a spheroid. **(C)** BF image of Panc-1 and imPSC heterospheroid at 4d after clearing procedure. Clearing involved dehydration with ascending concentrations of ethanol causing some shrinking of the spheroid. **(D)** Detection of mCherry fluorescent protein in Panc-1 and imPSC heterospheroid at 4d after clearing. Fluorescent signal diminished due to clearing procedure but distribution of the signal aligns with cellular distribution with more concentrated signal in the center. **(E)** BF image of hollow organoids derived from a mouse tumor at 2d. **(F)** Detection of eGFP fluorophore in hollow organoids derived from a mouse tumor at 3d. The transparency of the organoid line allows unhindered observation. **(G)** Organotypic slice culture image after 72h in *ex vivo* culture. H&E staining. Images **(E,F)** were kindly provided by Daniel Öhlund, Umeå University, Sweden. Image **(G)** was kindly provided by Carlos Fernández Moro, Karolinska Institutet, Stockholm, Sweden.

applied to Panc-1 spheroids, or more precisely to the medium they were cultivated in Fan et al. (2018).

An example for a commercial adaptation to 3D samples is the cell viability kit CellTiterGlo 3D™ with enhanced lytic capabilities. As a result this assay is widely and successfully used (Norberg et al., 2020; Liu et al., 2021), and is the golden standard for cell viability in high throughput screens using spheroids. The APH assay is another cell viability assay optimized for 3D cell culture (Longati et al., 2013). It measures the activity of acid phosphatase in the cells. However, this assay requires a high pH step which impedes its implementation in high throughput settings due to its corrosive effects on metal parts of liquid handling equipment.

While these options offer a wide field of research, the most common reporter remains elusive: fluorescence. We previously mentioned fluorescence as a simple read out to distinguish different cell types in live cell imaging by making use of continuously expressed fluorescent proteins, but many available assays rely on activatable fluorescent probes as well. However, the larger the spheroid, the less applicable fluorescence as a reporter becomes. The sheer density of the "tissue" prevents light passage (**Figures 2A,B**). Confocal microscopy can be used to observe tissues too dense for brightfield imaging. However, the more elaborate image capture then limits the throughput capacity. Tissue clearing alleviates this problem altogether but also relies on embedding the tissue and precludes further culturing (**Figure 2C**). Spheroid protocols which provide highly homogenous spheroids can overcome this difficulty with larger sample number, so that different spheroids serve as single data points in timelines rather than one spheroid being continuously observed. A common problem with clearing protocols is the diminishing of pre-labeling fluorophores (**Figure 2D**). Fluorophores used on already cleared samples, e.g., during immunohistochemical staining, are not affected.

Single cell-analysis like flow cytometry relies on creating single cells from a spheroid which often entails substantial loss of cells. Additionally, there is uncertainty about the homogeneity of single cell origin. Central parts of the spheroid may not be fully separated into single cells and gated out of the analysis as a result. This would then bias the analysis toward cells on the outside of the spheroid which were less subjected to existing gradients. In the case different cell types are mixed in spheroids, the methods used to prepare single cell suspensions might preferentially harm one cell type introducing a different type of bias.

An ongoing trend in research is to add a spatial parameter to any given optical output and create chemical images of samples. One such method is matrix assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI). The spatially targeted MS-analysis allows for example to determine drug penetration in spheroids (Mittal et al., 2019).

To our knowledge spatial transcriptomics has not yet been introduced in PDAC spheroid research. This method, however, would be interesting to use in order to investigate the RNA profile along the gradients that build up in a spheroid. While single cell RNAseq (Liu et al., 2021) and transcriptomic profiling (Yang et al., 2018) have already provided much information about gene expression in pancreatic cancer spheroids, succeeding in adding the spatial aspect to this data would offer even more insight. High definition spatial transcriptomics (HDST) can be carried out with a resolution of 2 μm (Vickovic et al., 2019). Given the diameter of spheroids varying between 200 and 600 μm in diameter, based on cultivation method, spatial transcriptomics would provide a differentiated view on the impact of metabolic gradients on RNA expression.

Fourier-transform infrared (FTIR) spectroscopy imaging was successfully used for detecting necrosis in melanoma spheroids, a useful tool when determining toxicity of compounds in research (Srisongkram et al., 2020). The advantage of this method over others is the multivariate analysis that it is commonly linked with. It can quantify RNA, lipids and DNA and even subclasses of proteins present, as well as their folding status. As such it is a more comprehensive analysis of fewer samples. This method has not been carried out with PDAC spheroids yet but could provide interesting insights, for example when trying to determine which changes occur to the ECM during different treatments. This may reveal insights as to why certain drugs fail in 3D which seemed promising in 2D as FTIR imaging was also able to find substantial differences in biochemical composition between the two models (Srisongkram et al., 2020). Additionally, FTIR imaging can also be taught to identify certain compounds similarly to MALDI-MSI by incorporating specialized spectral libraries and so could also serve to keep track of drug delivery and metabolization.

However, what image-based analyses of spheroids have in common is that they are not adaptable to high throughput yet and so cannot replace 2D-cell-culture oriented assays. In the future, with implementation of machine learning, improvements in image acquisition and a more automated image analysis, image-based analysis has the potential to enhance and replace some of the methods for spheroid research described here. In the meantime, more work needs to be invested into establishing robust and efficient reporting systems as spheroids represent a significant improvement compared to 2D cell culture.

Particularly methods which are high-throughput ready need to be established on a broader base. The main parameter currently is cell viability but in order to distinguish PDAC-specific toxicity from general cell toxicity more information must be acquired in large drug screens, as for instance metabolic parameters such as pH, lactate or glutamate accumulation and consumption of glucose and glutamine.

Organoids

Since this review is part of a review series, we will not focus as much on organoids as this topic is being covered in several entries of the series.

In comparison, organoids are 3D structures that mimic properties and tissue organization of the organ the cells are derived from. An important aspect of organoids is the self-assembly into a micro tissue. As such their structure is more complex than that of spheroids and 2D cell culture while still being grown under laboratory conditions. While spheroids are grown from established 2D cell lines, organoids also known as PDOs are cultivated using primary cells. Consequently, they represent the heterogeneity of cancer much more accurately than spheroids or 2D cell lines. Organoids often preserve the polarity

and gene expression pattern of the original tumor during early passages (Baker et al., 2016). Organoids can grow as hollow spheres (**Figure 2E**) or small, more solid spheres which allow light passage and the use of fluorescence for analysis (**Figure 2F**). All parameters considered, organoids seem like a better suited candidate for *in vitro* drug testing.

However, the requirements for successful culture are considerably higher and more expensive than that of spheroids. Spheroids are easily propagated, quickly grown to the required amount and inexpensive to maintain. On the other hand, the self-organization of organoids into a tissue relies on signaling, both via solvent molecules, cell-matrix- and cell-cell-interactions. The most widely used matrix to facilitate this signaling is Matrigel®. It is animal-cell derived and provides a complex mix of matrix components that is able to mimic the natural matrix composition of the basement membrane. However, also due to its animal origin it is subject to significant batch-to-batch variations which negatively affect the reproducibility of organoid research. In addition, Matrigel is rather soft compared to the stiff collagen-I-rich stroma characteristic for PDAC (Chang and Lin, 2021). Attempts to move into a more homogenous matrix have not been successful yet but remain a target of intensive research. This obligatory embedding in matrix also poses a problem for sample accessibility. Whereas spheroids grown in liquid medium can be collected and split with ease, organoids need to be separated from the matrix. Precise organoid sample aliquots are not possible without breaking them down to single cell level, a condition some organoid lines have problems to recover from.

Given these difficulties, organoids have not yet firmly established their implementation into high throughput drug screening. However, with further improvements of the model, organoids will also play an increasingly important part in the 3Rs of humane animal research, to replace, refine and reduce animal experimentation in modern research as well as become a platform for drug screens of varying scale.

The most striking benefit of organoid research are the possibilities for clinical application. As previously mentioned, PDOs are generated from patient tumor tissue. Hence these organoid lines closely resemble the *status quo* of an individual patient. Potential therapies can then be tested *in vitro* for effectiveness before being administered to the patient, avoiding unnecessary side effects from ineffective treatments (Huang et al., 2015, 2020; Tiriac et al., 2018a). However, as before mentioned the median survival time for PDAC is around 6 months, meaning that a significant number of PDAC patients have a shorter life expectance than it takes to generate enough organoid material and perform the drug testing. To make a difference for patient therapy, organoid establishment, drug screen and data analysis must be carried out within a time frame short enough to warrant delaying immediate treatment. Current approaches cannot supply this information quickly enough yet. With better diagnostic markers being investigated in parallel by many research groups, this strained schedule could become more relaxed in the future. Until then, only the roughly 25 percent of patients amenable to surgery and thus with longer life expectancy can take advantage of this approach. Proposals to incorporate small scale PDO screens into clinical practice have been made as well (Frappart et al., 2020). In very recent years, protocols to derive organoids from fine needle aspiration biopsies are being established which could provide the necessary drug testing platform for patients not suitable for tumor resection (Tiriac et al., 2018b). However, acquiring enough cancer cells from the sampled tissue currently necessary for organoid establishment presents a major hurdle, especially when using fine needle biopsies in PDAC.

Organotypic Slice Culture

This model uses precision cut slices of tissue which is cultured submerged in medium adjusted to the tissue used (Moro, 2021). Only recently has this model been used in PDAC research (Jiang et al., 2017; Misra et al., 2019; Moro, 2021).

An outstanding benefit of this type of *in vitro* model is the maintaining of the TME and the spatial information of the tumor (**Figure 2G**) in combination with time resolved analysis. The activation and effects of $CD8^+$ T-cells following treatment was demonstrated using live-cell imaging in combination with confocal microscopy (Seo et al., 2019). Additionally to the tissue, the supernatant can be analyzed as well, e.g., to detect soluble signaling molecules (Jiang et al., 2017; Seo et al., 2019).

Organotypic slice culture can be viewed as an alternative to organoid culture in personalized medicine. Like organoids, it maintains the genetic tumor heterogeneity of the patient's tumor. Additionally, it retains the stromal environment, which gets inevitably lost during organoid generation. In detail, it could be shown that proliferation rate and grade of tumor differentiation could be maintained throughout culture duration of 4 days (Misra et al., 2019). Tissue slices also responded in a dose and time dependent manner to drug treatment which was confirmed by immunohistochemical measurements of cellularity and cleaved caspase-3 positive cells (Misra et al., 2019). A benefit compared to organoid culture is its immediate availability.

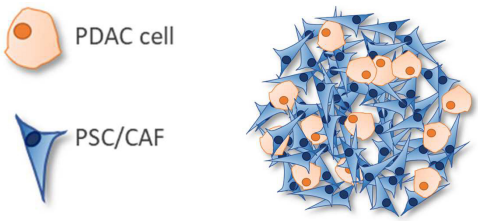
We discussed earlier the time sensitivity of PDAC treatment and how organoid culture takes too long under current conditions to be a tool in personalized medicine. Organotypic tissue slices, on the other hand, are available quickly and can be used to screen for specifically effective anti-cancer drugs (Ghaderi et al., 2020). Naturally, the size of the tumor limits the number of samples and the number of drugs which can be tested. Consequently, organotypic slice culture cannot serve as a model for drug discovery but remains an interesting tool for the advancement and clinical translation of personalized medicine in PDAC.

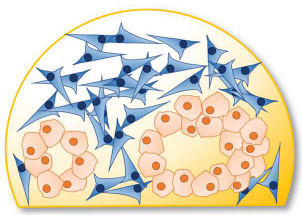
Conclusively, 3D cell culture is a diverse field of ongoing research with significant improvements compared to 2D cell culture in modeling a complex disease like PDAC (**Table 2**). However, more reporter systems which are tailored to these models are still required to make them an even more advantageous part of PDAC research.

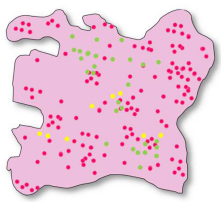
SPHEROIDS AS A STEPPINGSTONE

Spheroids are not exclusively used as a model for research. Instead, they are also used as a tool to refine animal models

TABLE 2 | Comparison of relevant parameters of spheroid, organoid and organotypic slice model.







	Spheroid model	Organoid model	Organotypic slice culture
Derived of	established 2D cell lines	primary tissue	Primary tissue
Complexity	homogenous sample generation with consistent growth progression cannot model tumor heterogeneity	varied organoid growth density and limited growth predictability retains the genetic expression pattern of the original tumor	cultivation of precision-cut tumor slices retains TME, its spatial distribution and tumor differentiation/grade
Co-culture system	• heterospheroid formation limited to few cell lines • trans-well co-culture with monospheroids • differentiated cell-type-specific analysis of crosstalk possible	• co-culture inside Matrigel dome • co-culture with suspended cells	• contains all cells of the patient's TME • additional non-adhesive cells can be added to medium to observe infiltration
Availability	compatible with regular cell laboratory facilities	requires additional storage, management and cultivation resources	compatible with regular cell laboratory facilities
Costs	similar to 2D culture, depends on protocol	additional costs caused by Matrigel and medium supplements	additional medium components necessary
Most applicable reporter systems	+ analyses of the medium + imaging after embedding + analyses of lysates use of radioactive tracers	+ image-based analysis with or without embedding + analysis of lysates + use of radioactive tracers	+ immunohistochemistry + live-cell imaging with confocal microscopy + medium analysis
Complications for reporter systems	limited light transmission of whole spheroids	limited accessibility due to matrix embedding and matrix interactions	tissue architecture prevents cell-type-specific biochemical analyses
High throughput application	analysis of medium and spheroid lysis	AI-assisted image analysis	Not applicable to high throughput due to very small sample size

and to advance other *in vitro* models. This last part of the review will focus on the implementation of spheroids in other preclinical models.

Xenografts

Xenografts are an *in vivo* model commonly used in translational research. Typically, suspended cells are injected orthotopically or subcutaneously. However, when injecting spheroids instead of 2D cells the resulting tumors grew more homogenously as well as more successfully. As such the implementation of spheroids reduced the number of animals subjected to cell injection as well as refining the *in vivo* model (Valta et al., 2016; Durymanov et al., 2019; Huang et al., 2020).

For PDAC there was one more substantial improvement. When transplanting spheroids consisting of Panc1 cancer cells and 3T3 fibroblasts, the resulting tumors contained more stroma than when suspended cells were injected, hence creating tumors which also more accurately resemble a patient's tumor (Durymanov et al., 2019).

Organ on Chip

Organ on chip systems use cells suspended in hydrogels in a small glass chamber. This model specializes on mimicking the influence of tissue-tissue interfaces or fluid-tissue interfaces. The latter is achieved by using microhydraulic systems which simulate blood circulation. The central drawback of this model is the inability to collect cells for sampling so any results need to be image-based (Tomás-Bort et al., 2020).

Models including spheroids grown from PDAC lines were established on several accounts. Established PDAC cell lines were embedded in collagen and offered ongoing nutritional perfusion (Beer et al., 2017). This model proved highly resistant to cisplatin and identified the matrix-interaction as a crucial factor in model establishing. Despite not forming spheroids, the cells responded more akin to those in spheroids rather than those in ECM-free 2D culture (Beer et al., 2017).

An organ on chip model was also designed to investigate solid stress found in PDAC. By increasing the interstitial fluid pressure to match that of a patient tumor an upregulation of the multidrug resistance protein family could be observed (Kramer et al., 2019).

A microfluidic system of Panc-1 and PSC cells was established to demonstrate the promotive effect of PSCs on cell motility (Lee et al., 2018). Additionally, an increased gemcitabine resistance facilitated by PSCs was demonstrated. The addition of medium circulation represents a platform to not only test for drug responses but also to test for dosage and treatment schedules.

A relatively new application is investigating multi-organ crosstalk (Wagner et al., 2013; Schimek et al., 2020). There, a liver spheroid was introduced to metabolize administered drugs to observe any possible adverse effect not only of the original drug but also its metabolized products.

While not yet attempted to our knowledge, the combination of liver metabolism to PDAC drug trials would represent a

considerable advancement. Not only due to the added metabolic degradation of the drugs but also to investigate liver toxicity. As metastases of PDAC are commonly observed in the liver, this organ is of additional interest in research seeking to improve PDAC treatment.

3D Bioprinting

Bioprinting uses cells typically suspended in hydrogels or other solidifying scaffolds to precisely determine the distribution of different cell types to one another. Unlike previously mentioned methods it thus seeks to recapitulate the morphology of organs or organ systems.

The use of scaffold also serves the purpose to give cells the correct cues for migration and differentiation which are naturally provided by the ECM.

Another novel approach was using scaffold embedded spheroids instead of scaffold embedded cells (Goulart et al., 2019). Compared to single cells, hepatic spheroids showed a more balanced metabolism and more importantly preserved the cell identity of the hepatocytes even in prolonged culture.

The bioprinting of neural spheroids was also recognized as more advantageous compared to single cell printing. Again a prolonged longevity of the culture could be observed, based on the enhanced self-renewal (Han and Hsu, 2017).

The importance of modeling the appropriate ECM for PDAC was also recognized in the area of bioprinting. However, only few studies have so far been carried out with PDAC cells. PDX derived cells were embedded along PSCs and human umbilical vein endothelial cells (HUVECs) (Langer et al., 2019) which created a dense and active stroma over time.

To summarize, spheroids seem to represent a way to stabilize the cellular identity. This conservation is especially important for fields of bioprinting which are slowly progressing into clinical application. Additionally, however, the benefit of *in vitro* models aiding in improving upon established models like the murine xenograft should not be overlooked.

CONCLUSION

To model complex and heterogenous pathologies like cancer, *in vitro* models must move beyond 2D cell culture as a failure-rich history in PDAC research has demonstrated. Likewise, the methods by which we build, interrogate and interpret these models must keep pace and develop further to meet the changing and increasingly complex questions of frontline research. Spheroids in particular exhibit great balance in recapitulating tissue conditions more authentically while also allowing controllable conditions which can be easily manipulated in experiments. Alongside spheroids, all 3D cell culture models will further expand our understanding how the TME can be modified in order to improve patient treatment. High throughput drug screenings and personalized medicine are merely the most prominent examples how 3D cell culture models could translate into relevant preclinical applications; the clinical effectiveness and truth of which will only be revealed by time.

AUTHOR CONTRIBUTIONS

All authors contributed to collecting literature, and to writing and revising the review.

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REVIEW
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Pre-clinical Models of Metastasis in Pancreatic Cancer

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Pancreatic ductal adenocarcinoma (PDAC) is a hostile solid malignancy coupled with an extremely high mortality rate. Metastatic disease is already found in most patients at the time of diagnosis, resulting in a 5-year survival rate below 5%. Improved comprehension of the mechanisms leading to metastasis is pivotal for the development of new targeted therapies. A key field to be improved are modeling strategies applied in assessing cancer progression, since traditional platforms fail in recapitulating the complexity of PDAC. Consequently, there is a compelling demand for new preclinical models that mirror tumor progression incorporating the pressure of the immune system, tumor microenvironment, as well as molecular aspects of PDAC. We suggest the incorporation of 3D organoids derived from genetically engineered mouse models or patients as promising new tools capable to transform PDAC pre-clinical modeling and access new frontiers in personalized medicine.

Keywords: metastasis, pancreatic cancer, organoids, metastasis models, PDAC – pancreatic ductal adenocarcinoma, GEMMs

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) has the worst 5-year relative survival rate in comparison to all other solid tumors and has been prognosed to become the second leading cause of cancer-related mortality in the United States by 2030 after lung cancer (Chu et al., 2017; McGuigan et al., 2018). More than 90% of pancreatic cancers are exocrine tumors, being the most frequent type, PDAC. Other tumors like neuroendocrine tumors (PNET) are often indolent and treatable (Antonello et al., 2009). The poor outcome is correlated to late diagnosis, a result of non-specific symptoms, poor specificity of tumor markers, and non-accessible sites for routine palpation. Further, the PDAC is associated with a high capacity of metastatic dissemination to adjacent organs already in small tumor sizes. Common sites of dissemination are the liver, with metastases present in 76–80% of patients, peritoneum (48%) and the lungs (45%; Yachida et al., 2012). Even though surgical resection of the primary tumor is the only treatment with curative intention, 85–90% of patients are not eligible due to the systemic nature of the disease and a lack of early diagnosis. Even in the less than 20% operable cases, where the primary tumor has been completely removed (R0) and no manifestation of metastasis at resection, 75% of the patients will die of metastatic relapse in then 5 years after being operated (Chu et al., 2017; McGuigan et al., 2018).

Genetics

Pancreatic ductal adenocarcinoma is a complex genetic disease, mainly determined by oncogenic activation of Kirsten rat sarcoma virus (KRAS) and mutations in tumor suppressor genes such Cyclin Dependent Kinase Inhibitor 2A (CDKN2A), Transformation Related Protein 53 (TP53), Lysine Demethylase 6A (KDM6A), Breast Cancer Gene (BRCA1/2), and SMAD Family Member 4 (SMAD4; Yachida and Iacobuzio-Donahue, 2009). The signature mutations of PDAC were identified in precursor lesions namely pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasia (MCNs), and intraductal papillary mucinous neoplasm (IPMNs; Hruban et al., 2001). Activation of oncogenic Kras in pancreatic epithelial cells triggers initiation of PDAC in mouse models and when combined with Trp53, Cdkn2a, or Smad4 mutations PDAC progression is accelerated, recapitulating many characteristics of the human disease (Izeradjene et al., 2007; see section "Genetically engineered mouse models").

Subtypes

Using bulk tumor samples, separate studies identified at least two subtypes of PDAC (Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016; Chan-Seng-Yue et al., 2020), differentiated by markers of epithelial differentiation state, being the less differentiated subtype ("basal-like," "squamous," or "quasi-mesenchymal") the one correlating with worse prognosis compared to the better differentiated subtypes ("classical" or "progenitor"; Dreyer et al., 2021a).

In primary patient-derived cell lines and bulky tumors of the various PDAC cohorts, a replication stress signature linked with the squamous subtype was identified. This is linked with functional impairments in replication of DNA and might also be utilized as biomarkers and give alternative therapeutics choices to standard care platinum chemotherapy for patients with DNA replication abnormalities (Dreyer et al., 2021b). The squamous subtype has also been defined by a distinct metabolic phenotype due to loss of genes that specify endodermal lineage, Hepatocyte Nuclear Factor 4 Alpha (HNF4A), and GATA Binding Protein 6 (GATA6). This subtype is therefore more sensitive to Glycogen synthase kinase 3 beta (GSK3β) inhibition except for a subgroup with distinct chromatin accessibility which acquires rapid drug resistance (Brunton et al., 2020).

Employing laser capture microdissection and RNA sequencing on PDAC epithelia and adjacent stroma defined two stromal subtypes differing in the immune-associated and extracellular matrix-associated processes. This study showed that across the same tumors, epithelial and stromal subtypes were partially linked [Extracellular Matrix (ECM) rich stroma was associated with Basal-like epithelium and Immune-rich stroma was found more often in association with Classical epithelia], showing potential dependence in the evolution of the tissue compartments in PDAC (Maurer et al., 2019).

Another study, based on the methylation patterns of the tumor genomes, defined two different origins of adenocarcinomas. One type of tumor is formed directly from ductal cells lining the ductal system of the pancreas, whereas the other develops from glandular cells and is less aggressive (Espinet et al., 2021).

Despite the current classification consensus, Juiz et al. showed that Basal-like and Classical cells coexist in PDAC as described by single-cell analysis on pancreatic cancer organoids derived from biopsies indicating that both subtypes can coexist in the same patient (Juiz et al., 2020).

Chemotherapy

Even though clinical decision-making based on histopathological criteria is widely established in several cancer types, subtypes of PDAC currently do not guide treatment decisions (Collisson et al., 2019). The only treatment with curative intent is surgery, which can be preceded by a neoadjuvant treatment and followed by adjuvant therapies such as gemcitabine monotherapy. However, recurrence rates in operated patients are still high and long-term survival is limited (Bijlsma, 2021).

The current standard of care for metastatic PDAC includes highly toxic chemotherapeutic cocktails with limited specificities. Gemcitabine has become a widely used drug for advanced and metastatic PDAC since it was reported (Burris et al., 1997), despite its low influence on patient survival. There are two gold-standard combination regimens for metastatic PDAC: 5-fluorouracil/leucovorin with irinotecan and oxaliplatin (FOLFIRINOX; Conroy et al., 2011), and gemcitabine with nab-paclitaxel since 2011 (Peixoto et al., 2017). A detailed review of PDAC chemotherapy can be found elsewhere: (Zeng et al., 2019; Singh and O'Reilly, 2020). According to developments and advances in other cancer types, it is expected that improvements in PDAC treatment are likely to come from the combination of classical cytotoxics with novel targeted agents against PDAC. Important matters in hand related to new therapeutic approaches include immunotherapy, DNA damage repair strategies, targeting the stroma, as well as cancer-cell metabolism (Dreyer et al., 2021b).

Current targeted therapies in PDAC undergoing Clinical Trials are divided into three approaches (**Table 1**). Firstly, inhibition of dysregulated oncogenes such as KRAS, c-MYC, Neurotrophic tyrosine receptor kinase, Neuregulin 1, and related molecules. Since these options have not led to an improvement of patient survival, alternative strategies are being developed to target these oncogenes, namely modification of mutant residues by small molecules, simultaneously inhibiting multiple molecules or pathways, and RNA interference. Secondly, reactivate tumor suppressors or modulate related molecules such as TP53, CDKN2A, SMAD4, KDM6A, and BRCA1/2. In addition to genetic event-guided treatment, immunotherapies such as antibody-drug conjugates, chimeric antigen receptor T cells (CAR-T), and immune checkpoint inhibitors also indicate the potential to target tumors precisely. Nonetheless, targeted therapies have been largely unsuccessful in PDAC. Currently, the only targeted therapeutic agent approved for PDAC is Erlotinib, which only slightly prolongs survival in metastatic disease (Moore et al., 2007; Sinn et al., 2017) but showed negative results in the adjuvant setting (Bijlsma et al., 2017). A possible reason for the unsuccessful outcomes of targeted agents in PDAC is incorrect patient selection. Interestingly, when tumor samples

TABLE 1 | Potential therapeutic targets in PDAC undergoing Clinical Trials.

Gene alterations (Targets)	Mutation rate (% af all tumors)	Potential target	Therapeutic mechanism	Promising agents	Combination partner	Study phase	Reference Clinical Trial
KRAS	90%	EGFR	KRAS inhibition	Nimotuzumab	Gemcitabine	Phase II	OSAG101-PCS07, NCT00561990
				Afatinib	Capecitabine	Phase I	NCT02451553
				Erlotinib	Gemcitabine	Phase III	CONKO-005, DRKS00000247
			Inhibits the intracellular phosphorylation of tyrosine kinase associated EGFR	Erlotinib	Selumetinib	Phase II	NCT01222689
		KRAS G12D/G12V	Small interfering RNA	siG12D LODER	Gemcitabine + nab-Paclitaxel Folfirinox	Phase II	NCT01676259
		KRAS G12C	Small-molecule inhibitor	MRTX849 (Adagrasib)	Afatinib Pembrolizumab Cetuximab	Phase I-II	NCT03785249, NCT04330664
				AMG 510 (sotorasib)	anti PD-1/L1	Phase I-II	NCT03600883
		PI3K-PLK1	Disrupts RAF and PI3K family binding to RAS	ON 01910.Na (Rigosertib)	Gemcitabine	Phase III	NCT01360853
			Allosteric AKT inhibitor	MK-2206	Selumetinib	Phase II	NCT01658943
CDKN2A	60%	CDK4-6	Cell cycle blockade via pRb	Ribociclib	Trametinib	Phase I-II	NCT02703571
				LY2835219 (Abemaciclib)	LY3023414 Gemcitabine Capecitabine	Phase II	NCT02981342
SMAD4	50%	TGFβ	Inhibits signaling through TGFβ-I receptor	Galunisertib	Gemcitabine	Phase I-II	NCT01373164
BRCA1/2	5%	PARP	PARP inhibition	Olaparib		Phase III	POLO trial: NCT02184195
MSIH/dMMR	1%	PD1	PD-1/PD-L1Immune checkpoint inhibition	Pembrolizumab	BL-8040 Onivyde/5-FU/LV	Phase II	KEYNOTE-158, NCT02628067 COMBAT, NCT02826486
NRG1	0.5%	ERBB3	Targets the HER2:HER3 heterodimer	MCLA128 (zenocutuzumab)		Phase I-II	NCT02912949
NTRK	0.3%	TRK	TRK inhibition	Entrectinib Larotrectinib		Phase I-II trials	NCT02122913 NCT02097810 NCT02568267
			NTRK mutations inhibition	Selitrectinib Repotrectinib		Phase I/II trials	NCT03215511 NCT03093116
c-MET	0.3%	MET	ALK/ROS inhibitor	Crizotinib		Phase I/II trials	NCT04693468
CXCR4	0.3%	CXCR4	Chemokine receptor inhibitor	BL-8040 (Motixafortide)	Pembrolizumab Onivyde/5-FU/LV	Phase II	COMBAT, NCT02826486

All the Clinical Trials described include patients with locally advanced or metastatic disease except the CONKO-005 trial which is focused on R0-resected pancreatic cancer.

from several of the CONKO-005 trial participants were re-analyzed, a subgroup of patients with a combination of SMAD4 loss and low Mitogen-Activated Protein Kinase 9 (MAPK9) expression benefited from the addition of Erlotinib (Hoyer et al., 2021). Therefore, not only novel targeted therapies are needed but also integration with genomic profiling along with a full understanding of the tumor microenvironment and immunology (Qian et al., 2020). It is expected that in the future, comprehensive tumor analysis should become an essential part of diagnostic routines and guide treatment choice.

Since most PDAC patients succumb due to metastatic cancer, this accentuates the crucial need to develop novel therapies that target, not only the primary tumor, but also the vulnerabilities of metastatic cells (Singh and O'Reilly, 2020). The last results of the COMBAT/KEYNOTE-202 Trial showed that Triple combination of motixafortide, pembrolizumab and chemotherapy are safe, well tolerated and showed signs of efficacy in a population with poor prognosis and aggressive PDAC (Bockorny et al., 2021). Promising new anticancer compounds are tested pre-clinically into *in vitro* and *in vivo* models. However, 90% of those fail when moving to clinical trials (Lai et al., 2019), showing the need for more consistent and representative models for drug testing, recapitulating better genetics, immunology, physiology, and metabolism from the human disease. Recent studies have highlighted the use of patient-derived organoids (PDOs) as a personalized model suitable for High throughput screening (HTS) which might help overcome some of the current model limitations (Tiriac et al., 2019).

Stroma

Pancreatic ductal adenocarcinoma is characterized by dense desmoplasia, which can compose up to 80% of the whole tumor volume and low tumor cellularity, while metastases in the liver have less stroma and more tumor cellularity than primary tumors resulting in less overall survival (Rucki, 2014).

A complex network of inflammatory cells, fibroblasts, ECM and vasculature maintain tissue homeostasis in the stroma of normal epithelial tissues. In Contrast, around pancreatic cancer tissues, neoplastic cells corrupt the stroma to form a tumor-promoting environment which, at the same turn, promotes cancer cell proliferation and migration, and provides a reservoir for growth factors and cytokines.

The three dominant entities in the PDAC stroma are the vasculature, ECM, and cancer-associated fibroblasts (CAFs). Molecular subtypes of pancreatic CAFs have been described (Öhlund et al., 2017), most remarkably myofibroblastic CAFs and inflammatory CAFs, which have been speculated to participate in active crosstalk with cancer cells and pro-tumor and antitumor properties, respectively. Tumor progression is linked with disruption of the basement membrane integrity and desmoplastic reaction with enhanced production of type I collagen (Öhlund et al., 2009; Shields et al., 2012). Moffitt et al. (2015) by digitally separating tumor, normal and stromal gene expression, defined "normal" and "activated" stromal subtypes, which are independently prognostic of PDAC. Using a Hedgehog inhibitor, the decrement of stroma was beneficial in mouse models due to the blockade of stromal growth factors and elimination of the barrier for therapeutic delivery (Olive et al., 2009). Despite those observations, several strategies to target the ECM have been pursued in the last years but have so far failed to show an increase in patient survival (Hosein et al., 2020; Tomás-Bort et al., 2020). Experimentally manipulating stromal matrix content led to lower tissue stiffness, and increased tumor growth, resulting in decreased overall survival. Similarly, a multitude of anti-angiogenesis agents have been unsuccessful in late-stage clinical trials of PDAC probably due to hypovascularity (Hosein et al., 2020).

Since desmoplasia promotes hypovascularity and immunosuppression it results in a hypoxic environment due to limited oxygen diffusion through the tumor (Tuveson and Neoptolemos, 2012). In-depth research has shown that hypoxia modulates tumor biology promoting malignancy through hypoxia inducible factors (HIFs), which should be considered for targeted therapy (Shah et al., 2020). Hypoxia signaling also affects stromal cells, enhancing activation of macrophages, CAFs, stem cells, and secretion of specific ECM factors to produce widespread stroma of PDAC (Keith et al., 2012).

Metastasis

Metastasis is characterized by a sequential process initiated by the invasion of carcinoma cells into the basement membrane into the neighboring stroma, followed by invasion and survival into circulation (blood, lymph), extravasation into the parenchyma of distant tissues, and lastly by the reestablishment of foci of neoplastic cells at remote sites, even after a period of dormancy (Massagué and Obenauf, 2016; Massagué et al., 2017; **Figure 1**). Accordingly, a central step in the metastatic process is the gain of migratory and invasive phenotype. This demands pancreatic cancer cells to switch many of their epithelial characteristics for mesenchymal traits through a cellular program named epithelial to mesenchymal transition (EMT). The opposite process, mesenchymal to epithelial transition (MET), happens when colonies are re-established at the secondary organ (Beuran et al., 2015).

The decisive promoters of PDAC metastasis are not yet sufficiently understood, notably since the genetic composition of most metastases closely resembles the one of the complementary primary tumors (Campbell et al., 2010; Yachida et al., 2010; Makohon-Moore et al., 2017). The most studied drivers of metastasis in PDAC are: TP53 (Morton et al., 2010), SMAD4 (Ahmed et al., 2017), aberrant Wnt signaling (Yu et al., 2012), and aberrant ECM gene expression (Harris et al., 2017). Also, reduced expression in Liver Kinase B1 (LKB1) is correlated with liver metastasis, vascular invasion and thus, worse overall survival (Yang et al., 2015, 1). It has been suggested that Transforming growth factor beta (TGF-β) promotes invasion and migration via the initiation of EMT (Padua and Massagué, 2009; Aiello et al., 2017). Molecular perturbations coupled with this transition combine the loss of epithelial markers such as cytokeratins, E-cadherin, occludin, and claudin, with the gain of mesenchymal markers namely N-cadherin, fibronectin and vimentin (Maier et al., 2010), loss of cell-cell contacts and polarity leading to the gain of a mesenchymal migratory behavior (Thiery, 2002). Further, cells that have transitioned to the mesenchymal state

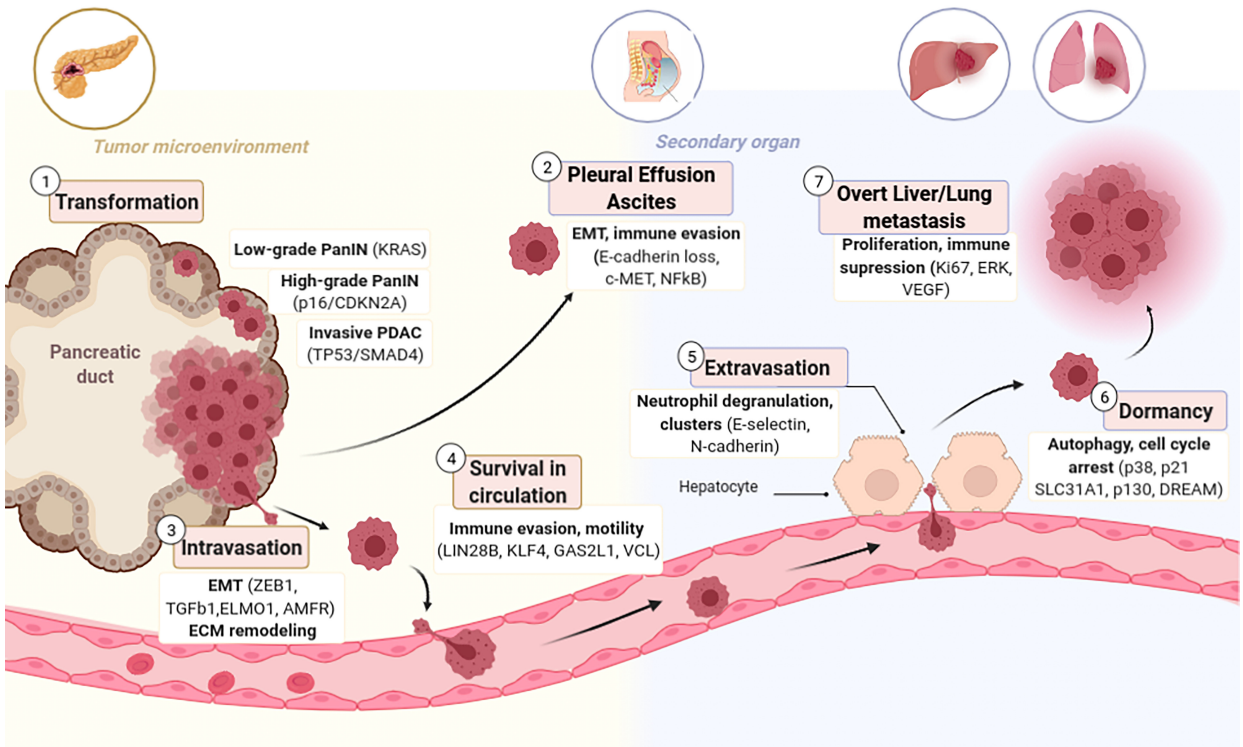

FIGURE 1 | Stages of Metastatic Progression with candidate genes responsible per each stage. (1) Normal epithelial pancreatic ductal cells acquire an aggressive phenotype through serial mutations that transform them firstly to PanIN and lately to PDAC. (2) Transformed cells are capable of detaching and colonizing the Peritoneum forming Ascites or Pleural Effusion. (3) PDAC cells have enhanced motility due to EMT that allows them to invade blood or lymphatic vessels. (4) CTCs in circulation are abundant, but only few survive this pressure. (5) Several CTCs have tropism for the pre-metastatic niche (PMN) and are able to extravasate to a secondary organ where they might remain dormant (6) for several years and eventually relapse and form overt metastasis (7).

embrace a spindle-like shape instead of a columnar one and have elevated invasiveness, migratory capacity, enhanced resistance to apoptosis and increased production of ECM factors (Rhim et al., 2012). It has been described that the PDAC EMT program is defined by an intermediate cell state "partial EMT" consisting of the maintenance of an epithelial program only at the protein level (Jolly, 2015). Partial EMT cells can migrate individually or as clusters while complete EMT cells mainly migrate in isolation (Duda et al., 2010; Grigore et al., 2016; Saitoh, 2018). The various mechanisms of dissemination (single cancer cells or clusters) seem to affect the metastatic capacity of cancer cells, since single cells do not have such a high metastatic capacity as tumor clusters (Friedl et al., 2012; Cheung and Ewald, 2016). Tumor clusters can be heterogeneous and integrated of stromal cells, such as CAFs, co-migrating with cancer cells to remote sites (Wang et al., 2016). Overall, in PDAC, the EMT program has been proved to enhance tumor-initiating potential (Rasheed et al., 2010) and drug resistance (Zhou et al., 2017).

When epithelial cells undergo EMT and enter circulation become circulating tumor cells (CTCs; **Figure 1**). CTCs isolated from patient blood express a cell motility gene signature consisting of upregulation of EMT and motility inducing genes such as Vinculin, Engulfment and cell motility protein 1, Autocrine Motility Factor Receptor, TFGß1 or p38 (Sergeant et al., 2012). CTCs necessarily contain the precursors of distant metastatic foci; thus, they may be characterized to identify drivers of dissemination, correlate gene set metastatic signatures, and develop targeted therapies to the "seeds" of metastasis (Franses et al., 2020). Using primarily CTCs collected from Genetically engineered mouse models (GEMMs), Growth Arrest Specific 2 Like 1 has been identified as a potential biomarker of CTCs in PDAC (Zhu et al., 2020). Contrary to the previous acceptance that PanINs are not able to undergo EMT, it has been reported that also low-grade PanINs, harboring only activating KRAS mutations, show indication of cells that have exfoliated and become CTCs expressing CD24 and CD44 (Rhim et al., 2012). Inflammation enhances the amount of circulating pancreatic preneoplastic cells, supporting the association between inflammation and PDAC (Mazur and Siveke, 2012). Consistently, treatment with anti-inflammatory agents reduces the amount of circulating PanIN cells and diminishes the number of PanINs in tissue (Tuveson and Neoptolemos, 2012).

The anatomical position of the primary tumor is a crucial determinant in the formation of peritoneal metastasis (Yachida and Iacobuzio-Donahue, 2009; Baretti et al., 2019). In some cases, the exfoliated cells directly attach to and invade organs and tissues in the peritoneal cavity (Jayne, 2007; Yachida and Iacobuzio-Donahue, 2009; Avula et al., 2020). It has been reported that

intraperitoneal metastases can also take place via blood vessels or lymphatic absorption through the hematogenous route (Jayne, 2007; Ge et al., 2017). Peritoneal spread of disease is found in around a third of patients with PDAC, which may lead to ascites accumulation in up to 20–30% cases or pleural effusion in around 15% of patients (Golan et al., 2017). Symptomatic retention of fluid with viable cells usually occurs late in the course of the disease, at the clinically treatment resistant phase (Baretti et al., 2019). A detailed analysis of molecular pathways leading to Peritoneal metastasis is reviewed in: (Avula et al., 2020). Briefly, E-cadherin loss, especially in a KRAS mutated background, and HGF/c-Met [hepatocyte growth factor (HGF)/mesenchymal–epithelial transition factor (c-MET)] pathway lead to EMT and cell detachment from the pancreas (Furuyama et al., 2000; Takiguchi et al., 2017; Sato et al., 2020). It is still a challenge to effectively treat peritoneal metastasis, thus further efforts in revealing its mechanism should be addressed in the future. Although there is extensive literature describing ovarian and gastric cancer cell immune evasion through the transition of the peritoneal cavity, this issue has not been exhaustively studied in PDAC.

Several studies prove that the hostile milieu of the liver is particularly preconditioned early to favor the engraftment and growth of disseminated tumor cells (DTCs), so-called pre-metastatic niche (PMN) formation. The formation of PMNs is directed by an intricate series of mutual interactions among the TME and tumor cells, along with the exploitation of recruited and resident cells in secondary target organs. Extracellular vesicles and soluble factors are secreted by the primary tumor or premalignant lesions, even before the initiation of PDAC dissemination. They help to form a supportive niche in the liver by providing vascular docking sites for CTCs enhancing vascular permeability, remodeling the ECM and gathering immunosuppressive inflammatory cells (Houg and Bijlsma, 2018). Hepatic metastases show unique characteristics, such as increased proliferation (Ki67), M2 macrophage infiltration, 3p21.1 loss, downregulation of EMT, and metabolic rewiring (Yang et al., 2021). Reichert et al. (2018) demonstrate, using multiple mouse models, that liver metastases highly depend on P120CTN-mediated stabilization of membranous E-cadherin, while the lung seems permissive to colonization by cells that are not MET-capable. Interestingly, PDAC patients with recurrent lung metastases show significantly better overall survival compared with patients with metastasis at other sites (Yamashita et al., 2015).

Next-generation genome sequencing of untreated pancreatic primary tumors and the corresponding patient metastasis showed that cells triggering distant metastasis are genetically indistinguishable with the different metastatic locus bearing the same driver gene mutations (Makohon-Moore et al., 2017). This implies that post-transcriptional or transcriptional modifications are pivotal to support the intricated series of biological bottlenecks that must be surpassed for PDAC to metastasize (Fidler, 2003; Lambert et al., 2017). DTCs display clonal diversification according to the location of the metastatic foci. Lineage tracing studies revealed that metastases in the lung and liver tend to be monoclonal, while those in the peritoneum and diaphragm exhibit polyclonality due to a different via of dissemination (Kleeff et al., 2016; Knaack et al., 2018). These observations suggest that heterotypic interactions between tumor subclones as well as site-specific selective pressures are both central to influencing metastatic initiation and progression.

Since CTCs do not express β2-integrins, they form clusters with blood cells using them as a linker to attach the capillaries and extravasate to distant organs (Charles Jacob et al., 2021). Extravasation may be controlled by E-selectins, N-cadherin, or galectin-3 from endothelial cells (Yadavalli et al., 2017). Once CTCs extravasate to the secondary organ, they remain dormant with high resistance to current therapies (Sosa et al., 2014). TGF-β and BMPs stromal signals have been identified as promoters of tumor dormancy by enforcing quiescence and inhibiting self-renewal of DTCs (Gao et al., 2012). The perivascular niche has also been reported to induce cancer cell dormancy (Ghajar et al., 2013). In contrast, contexts rich in fibronectin or type 1 collagen inhibit dormancy (Massagué and Obenauf, 2016). A lack of stromal growth factors and an abundance of growth-inhibitory signals can favor metastatic dormancy in experimental models. DTCs are also kept in a dormant state due to constant pressure from the immune system. However, the acquisition of further mutations, inflammation, microenvironmental alterations, as well as immune and stromal signals can promote arouse of dormant cells inducing local relapse or metastases still after curative therapy (Aiello et al., 2017; Lenk et al., 2018; Park and Nam, 2020). Dormany explains why most patients that were resected with no margin experienced a relapse and died of metastatic disease. Thus, it is a future goal to fully understand the pathways leading to metastasis outgrowth and improve current adjuvant combinations to not only eliminate the primary tumor but also to eradicate the dormant foci to prevent relapse.

The formation of metastatic foci occurs with a transition from mesenchymal to epithelial phenotype, leading to enhanced proliferation and metastatic tumor deposit (Makohon-Moore et al., 2017). The aim of adjuvant chemotherapy is the prevention of metastatic relapse. Nonetheless, the current pharmacological armory employed attacks proliferating tumor cells instead of targeting metastasis. Avoiding metastasis in high-risk patients would be ideal rather than treating them. However, the few approved drugs targeting the stroma of metastasis (bisphosphonates, Zometa, anti-RANKL antibody, and denosumab) have not yielded an improvement in the adjuvant setting (Smith et al., 2015; Perrone et al., 2019). Hence, the current standard of care does not instruct any agent to prevent metastasis (Massagué et al., 2017). There are several undergoing clinical trials of targeted agents designed specifically with patients suffering from locally advanced disease or metastasis (**Table 1**) hoping to improve patients' overall survival.

Conclusion

The early dissemination capacity of PDAC, even at the PanIN stage, explains why most patients have already found significant metastasis to the liver, lungs, peritoneum or lymph nodes at the time of diagnosis, and subsequent median survival is less than 1 year (Kleeff et al., 2016; American Cancer Society, 2021). Even though we hold an improved understanding of PDAC biology

and progression, the translation for patient benefit has been slow. On this subject, a possible contributing factor may be the lack of solid trustworthy models of human PDAC for preclinical testing and research.

IN VITRO PRECLINICAL MODELS

2D Cell Lines

Compared to other models, 2D cell culture is the simplest, fastest and most economic form to study metastasis and invasion. There are 49 PDAC cell lines described in Cancer Cell Line Encyclopedia with specific mutations and from different tumor sites (**Table 2**). Many PDAC cell lines from patients and murine tumors accompanied by different mutations of KRAS, p53, p16, and SMAD4 are widely used in PDAC metastasis research (Sun et al., 2001).

Several migration and invasion assays can be performed in cell lines to study metastasis. A fast method to assess migration in 2D cell culture is the wound healing assay, where a scratch is performed in the middle of confluent monolayer cells, and the measurement of cell migration is quantified via microscopy. Using this method several groups were able to identify genes that promote motility namely Yes1 associated transcriptional regulator (YAP1), H19 imprinted maternally expressed transcript (H19) or C-X-C motif chemokine ligand 12 (CXCL12; Cecati et al., 2021; Gao et al., 2021; Luo et al., 2021). However, this method is not able for non-adherent cells and the scratch can cause cell damage. Alternatively, invasion can also be studied using inserts in which cells are seeded on top and invasive cells are able to pass through such as the Transwell or Boyden chamber. To reproduce ECM degradation, it is possible to add a layer of ECM in the inserts (Kramer et al., 2013). Several studies analyzed the invasive capacities of PDAC cell lines through several substrates. Quite unanimously, Capan-1 and Mia Paca-2 have the higher invasion rates followed by PANC-1 and BxPC-3 with slight variations between groups due to subtle differences in methodology (Deer et al., 2010). It is also possible to study the morphology, directionality and velocity of migrating cells using optical mobility assays such as the TAXIScan (Yamauchi et al., 2017). Using this method, it has been shown that only a few cells are able to invade the ECM but they up-regulate the expression of invasion-promoting pathways such as PI3K-AKT (Fujita et al., 2014).

Another benefit of 2D cultures is the capacity to co-culture cancer cells with stromal cells, and model the signals from the TME. For example, co-culturing cancer cells with Pancreatic stellate cells (PSCs) showed that cancer cells had enhanced EMT markers and migration (Kikuta et al., 2010). Recently, the development of microfluidic assays has allowed investigating the biophysical parameters in PDAC metastasis modeling the chemical gradients, flow/shear stress and the complex interactions between several cell types (Kramer et al., 2019). Using PDAC cell lines and combining the previously mentioned assays with CRISPR-Cas9 technique has revolutionized the field of metastasis research. Currently, there are available genome-wide or custom made sgRNA CRISPR libraries that have helped identify genes promoting migration, chemoresistance or radioresistance such as Histone Deacetylase 1, ATP binding cassette subfamily G member 2, Endoplasmic reticulum-associated protein degradation (Du et al., 2021; Ramaker et al., 2021).

Lack of germline DNA and missing clinical annotation are general problems when working with established cell lines. Since 2D cell lines are separated from tissue and cultured on a flat cell culture surface, they undergo several *in vitro* selection steps. They will divide abnormally, become flat, and lose their differentiated phenotype (Kapałczyńska et al., 2016). Thus, some cell types are not well-represented and the tumor heterogeneity is reduced. Recently, single-cell sequencing technologies showed that there is a high degree of heterogeneity in commonly used PDAC cell lines, induced by culturing identical PDAC cell lines in different laboratories. They also question the use of immortalized, non-transformed pancreatic lines as control lines in the experiments (Monberg et al., 2021). Despite their limitations, cell lines have been pivotal tools for screening in pre-clinical settings the genes promoting migration and survival in PDAC as well as chemo- or radioresistance.

Spheroids

Despite being time and cost-effective, 2D cell cultures do not represent the architecture and structural complexity of human tissues. 3D culture of normal cells and their neoplastic counterparts was introduced in the 1970s (Emerman and Pitelka, 1977). Since the development of the hanging drop technique, spheroids have been utilized to study morphogenesis together with the composition and architecture of malignant tissues (Kelm et al., 2003) using several techniques summarized in **Table 3**. In semisolid matrices resembling the basement membrane, cell-cell contacts and cell-matrix interactions allow epithelial cells to develop polarized structures. Abounding ECM components namely collagen, fibronectin and laminin are necessary to support these cultures. Interestingly, studies that have compared transcriptomes between 2D and 3D cultures have shown that cells are highly influenced by cell-matrix interactions. Several PDAC cell lines are able to grow in spheroids but it is unclear their ability to reflect the properties of the human tumor since they undergo deep transcriptomic transformations in transitioning from 2D to 3D (Monberg et al., 2021).

Pancreatic stellate cells are the main source of stromal fibrosis, interacting closely with cancer cells to produce a supportive environment that drives local and remote neoplastic development. By co-culturing PDAC spheroids with ECM components, it is possible to model PDAC-stroma interaction. Some groups have shown that PSC co-cultured spheroids reflect PDAC chemotherapeutic responses (Lee et al., 2018; Wong et al., 2019; Liu et al., 2021). For instance, PSC/PDAC spheroids showed enhanced resistance to gemcitabine in comparison to PDAC-only spheroids, while c-MET inhibitors crizotinib, tivantinib, and PHA-665752 were similarly effective in both models (Firuzi et al., 2019). Recently, other groups also proved the increased chemosensitivity from heterospecies spheroids to gemcitabine, paclitaxel and SN38. Interestingly, this group also showed that

TABLE 2 | Most common Human derived pancreatic cancer cell lines and metastatic activity in orthotopic transplantation model.

Cell line	Mutation				Sample collection site	Metastasis *in vivo* (orthotopic)	References	Histology	COSMIC ID
	KRAS	TP53	SMAD4	CDKN2A					
PANC-1	G12D	R273H	WT	Hom. Del.	Pancreas	Liver, lungs	Loukopoulos et al., 2004; Zhang et al., 2020	Ductal carcinoma	1366282
AsPC-1	G12D	C135Afs*35	R100T	L78Hfs*41	Ascites	Lung, Liver, Lymph nodes	Loukopoulos et al., 2004	Ductal carcinoma	910702
BxPC-3	WT	Y220C	Hom. Del.	Hom. Del.	Pancreas	Lung, Liver, Lymph nodes	Loukopoulos et al., 2004; Razidlo et al., 2015	Ductal carcinoma	906693
CAPAN-1	G12V	A159V	S343*	Hom. Del.	Liver	Lung Liver Lymph nodes	Loukopoulos et al., 2004	Ductal carcinoma	753624
CAPAN-2	G12V/-	T125	WT	T18A19dup	Pancreas	Liver	Loukopoulos et al., 2004	Ductal carcinoma	910915
CFPAC-1	G12V/-	C242R	Hom. Del.	–; promoter methylation	Liver	Lung, Liver, Lymph nodes	Loukopoulos et al., 2004	Ductal carcinoma	906821
HPAC	G12D	G187R	D52Rfs*2	p.E120*	Pancreas	Lung, liver, peritoneum	Kuo et al., 2021	Carcinoma	1298136
HPAF-II	G12D/-	P151S	WT	R29A34del	Ascites	Lung, Liver, Lymph nodes	Fujisawa et al., 2009; Massey et al., 2019	Ductal carcinoma	724869
Hs766T	Q61H	WT	Hom. Del.	WT	Lymph node	Lymph, Liver, peritoneum, ascites	Fujisawa et al., 2009	Carcinoma	1298141
MIA-PaCa-2	G12C	R248W	WT	Hom. Del.	Pancreas	Lung, Liver, Lymph nodes, Peritoneum	Higuchi et al., 2017	Ductal carcinoma	724870
MZ1-PC	G12V	R209Kfs*6	–	R80*	Pleural Effusion	–	–	Ductal carcinoma	753595
PANC-02–03	G12D/-	R248Q	R135*	Y44*	Pancreas	–	–	Carcinoma	1298475
PANC-03–27	G12V/-	c.375 + 5G > T	–	Hom.Del.	Pancreas	–	–	Ductal carcinoma	925346
PANC-04–03	G12D/-	G245S	–	Y44*	Pancreas	–	–	Carcinoma	1298476
PANC-08–13	G12D	–	C123Mfs*2	–	Pancreas	–	–	Ductal carcinoma	925347
PANC-10–05	G12D/-	I255N/-	–	–	Pancreas	–	–	Ductal carcinoma	925348
PA-TU-8902	G12V/-	C176S	–	–	Pancreas	–	–	Carcinoma	1298526
PA-TU-8988T	G12V	R282W	Hom.Del.	–	Liver	Liver, kidney (SC)	Miao et al., 2020	Carcinoma	1240201
PL18	WT	E171del, R267W	–	–	Pancreas	–	–	Pancreatic adenocarcinoma	1240208
PL4	G12D	G266V	–	–	Pancreas	–	–	Carcinoma	1298533
PSN1	G12R	K132Q	Hom.Del.	Hom.Del.	Pancreas	Liver	Eyres et al., 2021	Ductal carcinoma	910546
SU8686	G12D	G245S, p.G360V	–	Hom.Del.	Liver	Primary	Liu et al., 2020	Carcinoma	1240218
SUIT-2	G12D	R273H	–	E69*	Liver	Liver, Lung, Kidney, Peritoneum	Higuchi et al., 2017	Carcinoma	1240219
SW1990	G12D	P191del	–	Hom. Del.	Spleen	–	–	Ductal carcinoma	910907
YAPC	G12V/-	H179R	R515Dfs*2	–	Ascites	–	–	Ductal carcinoma	909904

*Hom.Del. homozygous deletion, * deletion, WT Wild Type, and – unknown.*

TABLE 3 | Three-dimensional spheroid models for PDAC research and their applications in metastasis.

	Technique	Application	References
PDAC Spheroids model	Modified Hanging drop	PDAC-stroma interaction analysis and HT automated drug screening assays	Ware et al., 2016
	PANC-1 co-culture with mPSCs	Test chemosensitivity to gemcitabine, paclitaxel, and SN38	Liu et al., 2021
	Co-culture (type I collagen)	Stroma-mediated cell motility and drug resistance	Lee et al., 2018
	Co-culture (CS-HA coated plates)	Cellular interaction, migration, and drug resistance	Wong et al., 2019
	Co-culturing with microtissues	A testing platform for anticancer drugs in Tissue-on-chip technology	Brancato et al., 2017
	User-defined tumor compartment embedded in 3D matrix	A High-throughput testing platform for anticancer drugs screening	Puls et al., 2018

upon co-culture mPSC induces a shift from classical to a basal-like phenotype to PANC-1 spheroids showing the importance of TME in patient prognostic and metastasis development (Liu et al., 2021). CAFs are activated to myofibroblast and tumor-dependent lymphocyte infiltration is observed on co-culture reproducing the desmoplastic reaction of PDAC and providing a valuable tool for anticancer drug testing (Brancato et al., 2017; Tsai et al., 2018).

Immune cells interfere in treatment response and tumor progression (Qiu and Su, 2013). 3D approaches admit the inclusion of human immune cells in contrast to patient-derived xenografts (PDXs) which are established in immunodeficient mice. When T cells were added to monocyte and fibroblasts co-cultured with PDAC spheroids overexpressing Doublecortin-like kinase 1 -isoform 2, M2 monocytes were polarized via cytokine release which then inhibits CD4+ and CD8 + T cell activation and proliferation (Kuen et al., 2017; Chandrakesan et al., 2020). Since knockdown of DCLK-isoform2 resulted in enhanced CD8 + T cell activation and decreased pancreatic cancer cell viability—this study with spheroids suggested DCLK-isoform2 as a novel therapeutic target in PDAC (Chandrakesan et al., 2020). Importantly, another triple co-culture platform has been developed combining PANC-1, endothelial cells (HUVEC) and fibroblasts (MRC-5) which also mimicked the resistance to treatments observed *in vivo* to doxorubicin and gemcitabine hence proving the key role of a complex tumor microenvironment (Lazzari et al., 2018).

With additional therapies for stroma targeting and 3D patient models that replicate a patient's specific TMEs, it is an exciting time for PDAC research. Several Clinical Trials target the tumor microenvironment of PDAC (Tomás-Bort et al., 2020). 3D cell cultures, and specially PSC/PDAC spheroids, are important tools for screening of cancer and stroma targeting drugs permitting a validation step preceding animal testing and reduce the number of animals required (Ishiguro et al., 2018). 3D modeling of cell culture may aid in drug discovery and biological treatment. While current 3D spheroid invasion models more precisely replicate tumor invasion compared to conventional 2D models, they have limitations such as low reproducibility and the difficulty to interact with high-throughput (HT) systems.

To overcome this limitation, Puls et al. developed a 3D tumor-tissue invasion model for HT phenotypic drug screening. In short, PDAC cell lines are embedded in an Oligomer in suitable plates for HTS where it is possible to monitor invasion into the surrounding tissue. When CAFs were added this highly enhanced PDAC invasion, as is expected to occur *in vivo*. Additionally, they showed that gemcitabine inhibited proliferation while not fully eradicating the tumor or blocking invasion. These results line up with those from PDAC xenograft models which show gemcitabine substantially arrests tumor growth and proliferation but does not induce apoptosis or reduction of remote metastases and invasion related markers (Puls et al., 2018). Although 3D spheroids have proven useful in cancer cell research, it is acknowledged that a passive environment does not adequately represent the cellular development of these cancer cells. The tumor cells grew throughout time without being suppressed by the drugs; however, it is not clear how much of the development was hindered or accelerated as a result of static media supply (Holub et al., 2020).

Organoids

Inferring results from model systems to humans has been a major barrier in the drug discovery process. In the last decade, a surrogate *in vitro* 3D model for human and mice tissues, named organoids, has been refined. Unlike spheroids, organoids are not derived from cell lines but from primary cells. Moreover, organoids allow studies of tissue function since tissue-like structures are preserved (Marsee et al., 2021). Stem cells are isolated from mouse or human adult tissues and embedded in 3D matrices where they self-organize into epithelial structures (Tuveson and Clevers, 2019; Kim et al., 2020). They also maintain intra-tumor heterogeneity, cell polarity and interact with the ECM, resembling the molecular features of the original tumor. Not long ago, organoid cultures of pancreatic epithelium have allowed the culture of normal and neoplastic pancreatic epithelial cells for both humans and mice (Huch et al., 2013; Hindley et al., 2016; Boj et al., 2018).

To establish organoid cultures, it is necessary to mimic the homoeostatic surrounding of the normal tissue stem cells. For this purpose, cells are encapsulated in Matrigel, which contains the crucial components of the basement membrane, and complemented with the minimal essentials for sustainable growth of pancreatic epithelial cells left out mesenchyme. Since the majority of PDAC samples have high penetrance of KRAS activation (Yachida and Iacobuzio-Donahue, 2009), it is possible to apply selective pressure conditions withdrawing EGF or adding EGFR inhibitors to obtain a pure neoplastic culture.

Since organoids are genuine epithelial populations, they bypass the stromal suppression that primary tumors retain, allowing comparisons to normal ductal pancreatic cells (Boj et al., 2018). They can be established in several weeks even from small fine needle aspiration biopsies acquired from patients with advanced PDAC, enabling therapeutic testing and tumor response during treatment or disease progression. Employing a large cohort of PDOs, Tiriac et al. (2018) have developed a platform for testing single and targeted agents. They display, in retrospective case studies, that organoid response to therapeutic testing correlates with patient sensitivity to chemotherapy. By correlating the transcriptome and drug sensitivity profile of each organoid in the cohort, they defined transcriptomic signatures of chemosensitivity with prognostic clinical outcomes in treated cohorts of PDAC patients. Other authors found the same concordance when testing similar platforms (Huang et al., 2015; Romero-Calvo et al., 2019; Dreyer et al., 2021b). In PDOs obtained over multiple years in a metastatic PDAC patient, it was possible to show increased organoid resistance to chemotherapy in accord with treatment refractory cases (Tiriac et al., 2018). Organoid work has also shown that Beta-1,4-galactosyltransferase 1 (B4GALT1) promotes PDAC progression and chemoresistance via stabilization of CDK11^{p110} (Chen et al., 2021, 110). In the biomarker field, organoids showed that higher EV release is coupled to a high cell proliferation rate, promoted by Wnt pathway activation (Sándor et al., 2021).

Since their implementation, organoids have been able to demonstrate good genomic parallelization with the primary PDAC tumors (Tiriac et al., 2018; Gendoo et al., 2019; Romero-Calvo et al., 2019). Also, PDAC subtypes have been identified in independent cohorts of PDOs implying that the transcriptional programs are preserved. Seino et al. (2018) defined functional subtypes of PDAC and demonstrated an inverse correlation between and strict requirement for WNT-signaling and GATA6 expression (linked with classical subtype), thus implying that GATA6 acts as a key regulator of niche-dependency. This emphasizes the call for precision methods to select patients when considering Wnt pathway therapeutic approaches, for example with the porcupine clinical trials. In addition, organoids are genetically manageable for viral infection and transfection, allowing targeted evaluations of particular genes or genetic screening (Michels et al., 2020).

Co-cultures of organoids with PDAC stromal cells helped understand fibroblast heterogeneity and suggested new approaches for treating PDAC by blocking the fibroblasts that support the tumor and promoting tumor restraining fibroblasts (Öhlund et al., 2017; Tsai et al., 2018; Biffi et al., 2019). These co-culture conditions have also shown that CAFs modify the EMT phenotype and drive gemcitabine resistance induced by HGF derived from CAFs. Furthermore, high stromal expression of Paired related homeobox 1 (Prrx1), a transcription factor critical for activating CAFs, is displayed in the squamous subtype (Feldmann et al., 2021). Using organoids and mice, Walter et al. showed that MEK inhibition suppresses TGFβ-induced EMT and migration *in vitro* and eventually results in a greater decrease in CTCs *in vivo* (Walter et al., 2019). Further studies in PDAC metastasis have been achieved by creating organoid derived xenografts (ODX; section "*Organoid derived xenografts*").

The recent findings prove that organoids should be a focal point of future studies of PDAC. Overall, organoids recapitulate the human disease much closer than spheroids or cell lines. They allow tissue function studies and co-culture. Since it is possible to culture the normal and neoplastic compartment, organoids are well suited for therapeutic testing and have intermediate scalability. However, organoids are still a complex model that requires a lot of technical training and represent a high cost to establish and maintain (Marsee et al., 2021). Despite showing correlation with patient transcriptomics subtypes and chemoresistance signatures, it has been shown that there are transcriptomic switches during *ex vivo* passage that may restrict their predictive abilities (Monberg et al., 2021), thus correct passage monitoring is required. To bring to the clinic fast organoid testing of PDAC patients, further work is required in accelerating organoid establishment and testing techniques of valuable compounds.

Recently, a consortium named PRECODE (European Commission, 2021) was established where several laboratories collaborate working on Pancreatic Cancer Research in Organoids in different fields helping to push forward the understanding of this disease and get closer to the development of an effective treatment for PDAC.

IN VIVO PRECLINICAL MODELS

In vivo models are key to study alternative and innovative treatment approaches. Despite the great advantages of *in vitro* research, namely cost-efficiency and simplicity, these models are lacking a microenvironment, immune system and don't represent tumor heterogeneity. Thus, *in vivo* models have been widely used to overcome these limitations for metastasis research and allow the understanding of the complex crosstalks involved in metastasis and defining its stages.

Genetically Engineered Mouse Models

Transgenic models, using tissue or cell-type specific promoters, allow the ectopic and temporal expression of target genes in the mouse genome. Different pancreatic cancer lineage specific promoters have been employed in GEMMs like pancreatic and duodenal homeobox 1 (Pdx1), neurog3 (Ngn3), elastase (Ela), among others.

While several chemical and genetic approaches to generate PDAC in mice date back to the 1980s (Longnecker, 1984), it was the establishment of the Kras$^{LSL.G12D}$ mice (Johnson et al., 2001) in 2001 that permitted tissue-specific expression of oncogenic Kras under physiological control from the endogenous mouse locus. This model developed Lung cancer but not PDAC. After this, several GEMMs faithfully recapitulating the genetic, molecular, histological, and clinical hallmarks of human PDAC have been established (**Table 4**). A full review of GEMMs for PDAC is available in: (Westphalen and Olive, 2012).

While transgenic mice are fast to develop and permit the expression of human genes (Qiu and Su, 2013), the expression

TABLE 4 | Genetically engineered mouse models of pancreatic cancer summary of the most common GEMMs of PDAC driven by KrasG12D.

Name	Mutation	Phenotype	References
KC model ($Kras^{LSL.G12D/+}$; PdxCre)	Oncogenic Kras	Pre-invasive PanIN to PDAC	Hingorani et al., 2003
$Kras^{LSL.G12D/+}$; $Cdkn2a^{lox/lox}$; PdxCre	Oncogenic Kras, homozygous or heterozygous deletion of Cdkn2a	Rapid metastatic PDAC	Aguirre, 2003
KPC model ($Kras^{LSL.G12D/+}$; $p53^{R172H/+}$; PdxCre)	Oncogenic Kras, heterozygous deletion of Trp53	Pre-invasive PanIN to metastatic PDAC	Hingorani et al., 2005
($Kras^{LSL.G12D/+}$; Ink4a/$Arf^{lox/+}$; PdxCre)	Oncogenic Kras, heterozygous deletion of Ink4a/Arf	Rapid metastatic PDAC	Bardeesy et al., 2006b
($Kras^{LSL.G12D/+}$; $Tgf\beta r2^{flox}$; PdxCre)	Oncogenic Kras, homozygous deletion of Tgfbr2	PDAC with liver metastasis	Ijichi et al., 2006
KD model ($Kras^{LSL.G12D/+}$;$Dpc4^{flox/+}$;p48Cre/+)	Oncogenic Kras, heterozygous deletion of Smad4/Dpc4	MCNs to metastatic PC	Izeradjene et al., 2007
KPCZ model (KPC; $Zeb1^{fl/fl}$)	Oncogenic Kras, heterozygous deletion of Trp53, and homozygous deletion of Zeb1	Decreased local invasion and reduced metastatic competence	Krebs et al., 2017

occurs under foreign promoters. To circumvent these limitations, conditional models are used expressing the desired mutations within the endogenous locus by interbreeding mice carrying the mutant allele downstream of a "Lox-STOP-Lox" (LSL) cassette with Cre driver mice (Magnuson and Osipovich, 2013). In 2003 (Hingorani et al., 2003) by crossing PdxCre or p48Cre mice to $Kras^{LSL.G12D}$ mice the expression of $Kras^{G12D}$ was specifically targeted to the pancreas. In the $Kras^{LSL.G12D/+}$; PdxCre model (KC model), mice are born with normal pancreas but develop PanIN at 8 weeks and slowly increase in grade, with a subset of those developing PDAC. The KC model proved that Kras mutations are enough to initiate PDAC formation in mice while targeted conditional mutations in Cdkn2A, Smad4, or p53 did not lead to PanIN or tumor development with PdxCre expression. However, this long latency, background tumors and sporadic progression to metastasis hampered the utility of the KC model for preclinical applications.

By combining oncogene activation and tumor suppressor inactivation, it has been successful to generate metastatic PDAC models resembling human disease. Aguirre (2003) showed that homozygous deletion of Cdkn2a in the context of Kras mutation in the pancreas ($Kras^{LSL.G12D/+}$; $Cdkn2a^{lox/lox}$; and PdxCre) led to the rapid development of metastatic PDAC. Similarly, the loss of the Ink4a/ARF locus in Kras mutant mice promotes NF-kB, Notch signaling and metastasis (Bardeesy et al., 2006a). Conditional expression of $p53^{R172H}$ also accelerated $Kras^{G12D}$ pancreatic tumorigenesis. Although $Kras^{LSL.G12D/+}$; $p53^{R172H/+}$; and PdxCre mice (KPC mice) are born with histologically normal pancreas, they rapidly develop PanIN lesions, and die of PDAC in 5.5 months with ∼80% metastasis (Hingorani et al., 2005). This model showed indices of widespread genomic alterations, a characteristic that was previously missing in most GEMMs. Since KPC mice mirror the dynamics of the human TME, they are useful to study tumor-stroma interactions as well as disease progression and testing immunotherapies. Other models addressed the deletion of Smad4 in Kras mutant pancreatic cells but the histology of those tumors was more similar to MCNs or IPMNs (Bardeesy et al., 2006b; Izeradjene et al., 2007). Interestingly, homozygous deletion of transforming growth factor beta receptor 2 (Tgfbr2) combined with $Kras^{G12D}$ formed PDAC with metastasis with special tropism to the liver, suggesting that activated Ras signaling and hampered TGF-β signaling cooperate to advance PDAC progression (Ijichi et al., 2006).

Classical Cre-loxP GEMMs depend on a single Cre-mediated step of recombination to activate oncogenic Kras expression not allowing sequential multistep tumorigenesis and tumor heterogeneity, which are important hallmarks of PDAC. With a dual-recombinase system (Schönhuber et al., 2014), Krebs et al. (2017) generated the KPC; $Zeb1^{fl/fl}$ mice (termed KPCZ) model and discovered that the EMT-TF Zinc Finger E-Box Binding Homeobox 1 (Zeb1) is a crucial factor for driving metastasis.

Genetically engineered mouse models have enlightened the biology of PDAC, elucidated potential therapeutic and diagnostic targets, and accentuated the importance of the tumor stroma for pancreatic cancer immune evasion, maintenance, and drug resistance. Nonetheless, it is an expensive and labor-intensive model to generate and maintain. In addition, gene mutations are brought into the germline of mice, while they occur somatically and gradually in human tumors. Nonetheless, these limitations may be overcome by the use of CRISPR-Cas9 in mouse models. Recently, CRISPR-Cas9 technology has allowed more precise genome editing (Doudna and Charpentier, 2014; Platt et al., 2014). Using this method, Ideno et al. developed the Ptf1-Cre; LSL-Cas9 mouse model, which recapitulates human PDAC features such as PanIN or IPMN with potential advancement to PDAC (Ideno et al., 2019). Ischenko et al. (2021) used CRISPR-Cas9 to inactivate Kras in mice and demonstrated that in advanced tumors, Kras tumor growth dependence is diminished and is shown in the suppression of antitumor immunity.

All these models prove the crucial role of KRAS in the biology of pancreatic cancer; even though efforts to target KRAS directly have not been successful to date. Thus, Ras effector pathways namely Phosphatidylinositol 3-kinase (PI3K)- Protein Kinase B (AKT) and Raf- Mitogen-activated protein kinase kinase (MEK)- Extracellular signal-regulated kinase (ERK) have been investigated as potential surrogates (Mann et al., 2016). Following

this line, by crossing to KC mice and analyzing transposon insertions in the resulting tumors, it has been described a large number of candidate genes that may promote tumor progression in $Kras^{G12D}$ initiated pancreatic tumors namely TGF-β and p16/CDKN2A. Genes implicated in chromatin remodeling were identified, including Ubiquitin Specific Peptidase 9 X-Linked (Usp9X), which plays an important role in the pathogenesis of PDAC (Pérez-Mancera et al., 2012; Mann et al., 2012). Several groups followed Usp9X and showed its association with worse prognosis in PDAC (Liu et al., 2017b; Pal et al., 2018).

The grade of aneuploidy in human tumors leads to a great variety of intertumoral gene modifications, with a completely different appearance as in mice. Overall, these species-related differences hamper the capacity of GEMMs to predict the true therapeutic response of PDAC patients in clinical trials. To overcome these limitations, transplantation models might be used.

Transplantation Models

Transplantation models consist of the engraftment of mouse or human cells/spheroids/organoids/tissues into recipient mice. This provides the benefit of tractability and a relatively lower and more predictable tumor latency than transgenic models. The transplantation can be orthotopic (in the pancreas), or heterotopic (subcutaneous, intraperitoneal, intravenous, intrasplenic, or intra-cardiac) according to where the cells are implanted. Cells engrafted via orthotopic transplantation may spread from the primary tumor to remote organ sites, hence permitting the modeling of the entire metastatic cascade; whereas when injected heterotopically into circulation it is possible to reproduce the steps of dissemination, extravasation, and colonization (Gómez-Cuadrado et al., 2017). Different sites of vascular injection define the site of colonization. For example, injection of cancer cells in the tail vein leads to the development of lung metastases since the cells are rapidly trapped in the microvasculature of the lung. Intrasplenic injection leads to the formation of micrometastasis in the liver. On the other hand, intracardiac injections allow systemic dissemination and are used to model brain or bone metastasis (Khanna, 2004). Moreover, transplantation models can be xenogeneic (xenograft) or syngeneic (allograft).

Allograft transplantation models are established by the transplantation of mice derived neoplastic cells and tumors into mice. They permit the study of metastatic dissemination with an intact immune system, and hence more closely recapitulate the TME. Allografts from isolated cancer cells or tumor pieces derived from GEMMs were characterized by a faster and more consistent development of primary tumors and up to 90% metastasis in the liver compared to GEMMs (Li et al., 2019). The abundance of metastasis in this model is probably a result of focal disease formation, closely mimicking the random mutations in KRAS present in human disease (Tseng et al., 2010).

In contrast to allograft models, xenografts require the implantation of human tumors or cancer cells into immunocompromised mice. Pancreatic cancer cell lines or spheroids are a frequent source for transplant. Nonetheless, as phenotypic and molecular properties may switch in culture, xenograft models of cancer cell lines do not always anticipate clinical responses (Garcia et al., 2020) and thus 3D models are a better alternative.

Cell Line Derived Xenografts and Spheroid-Based Xenografts

One of the solutions to address the many shortcomings of 2D cell lines is to establish cell line derived xenografts (CDX). PDAC cell lines are implanted into mouse models to research and test the efficacy of anti-cancer therapies *in vivo* and metastasis formation. Several studies produced allograft models with C57BL/6, such as TB 32047 (Lu et al., 2020), KPC cell line (Torres et al., 2013), or Pan02 (Jiang et al., 2014). In contrast to allograft models, human PDAC CDX are established by transplanting PDAC cell lines into immunocompromised mice. Resuspended cells in Matrigel for injection to establish an orthotopic mouse model of PDAC is a common method. The orthotopic injection of SUIT-2 cells into the pancreas can induce a process similar to the spread of human PDAC (Higuchi et al., 2017). 3–14 days after inoculation, Higuchi et al. observed intraperitoneal dissemination, extrapancreatic invasion, and further hematogenous organ metastases of SUIT-2 cells (**Table 1**).

The lung and liver are the most common sites of metastatic PDAC at diagnosis. Most CDX models are generated by subcutaneously injecting PDAC cells into immunodeficient mice. However, subcutaneous xenograft tumors rarely metastasize and thus orthotopic models are a better alternative (**Table 1**). PANC-1 and KP3, AsPC-1 and KP2 develop liver metastases while only AsPC-1 showed signs of lung metastases (Zhang and Du, 2019). Interestingly, multinucleated cells and spindle cells have been observed in liver and lung metastases playing an important part in metastasis formation. There is also a lung metastasis model by injecting PDAC cells via the tail vein (Kong et al., 2020). Metastatic tumors can be observed in the lungs and other organs after about a month.

Pancreatic ductal adenocarcinoma orthotopic metastasis mouse models are successfully established by injection 2D cells into pancreas. There have also been some studies that xenografted 3D spheroids from PDAC cells (Durymanov et al., 2019; Azmi et al., 2020). 3D spherical culture, as opposed to classic monolayer cell culture, more nearly replicate *in vivo* conditions inside a microenvironment, which can improve the defects of 2D culture (Liu et al., 2021). Furthermore, in contrast to their cell-based counterparts, spheroid-based xenografts (SDX) show increased expression of pro-fibrotic and pro-survival PDAC hallmarkers (Durymanov et al., 2019). Orthotopic implantation can progress pancreatic tumor to liver and lung metastasis tumor, which is similar to humans. But some PDAC cell lines are difficult to metastasize. Tail vein and splenic injection can easily perform metastasis, but it doesn't produce a primary tumor. Secondly, immunodeficient mice successfully avoided rejection during xenotransplantation, but it also limits the study of metastasis progress since adaptation to the immune system plays an important role in the selection of metastatic mutations (Gonzalez et al., 2018). Another drawback of the CDX-SDX models is that they may not achieve the medical requirements

of individualization and precision. Cell lines cannot accurately reflect the complexity of tumor heterogeneity and can only represent patients with certain types of cancer (Xu et al., 2018).

Organoid Derived Xenografts

Orthotopic transplantation of human pancreatic tumor organoids into immunocompromised mice mimics the full spectrum PDAC progression, forming PanIN-like stages and advancing to invasive and metastatic PDAC (Boj et al., 2018). The only difference is that PanIN-like structures are not intraductal epithelial structures within the pancreas of the mouse. Conventional CDXs only repopulate the host environment but do not form any PanIN-like structure. In addition, ODXs closely recapitulate the dense collagen deposition present in human PDAC tissues and tumors from GEMMs, another feature missing in CDXs (Kim et al., 2009; Olive et al., 2009; Raimondi et al., 2020).

It is still unknown how engrafted PDAC organoids form PanIN-like structures. As organoid cultures can better retain tumor cell heterogeneity, it is possible that recovering several stem cells better mimics the different stages of disease progression upon implantation. In addition, it could be the organoid culture conditions that better reflect the cellular plasticity and epigenetic changes upon transplantation. The interaction of the matrix with pancreatic cancer cells in organoid cultures may help to switch into a PanIN-like biological stage (Hwang et al., 2016). Differentially from other xenograft models, ODXs offer a unique chance to study PDAC progression *in vivo* and early biomarkers. Undergoing research will evaluate the benefits of the ODXs for therapeutic and diagnostic development in comparison to classical PDAC models.

Patient-Derived Xenografts

Patient-Derived Xenografts have been established as a rising tool to recapitulate tumor heterogeneity, genetics, and cancer microenvironment of PDAC. PDXs are used to identify new biomarkers, enhance therapeutic outcomes and also as tools for personalized treatments of PDAC patients.

Patient-derived xenografts from patient tumor tissue represent a more favorable alternative to CDX, SDX, or ODX since there is no *in vitro* selection. Patient tumor pieces are implanted into immunocompromised mice orthotopically or subcutaneously for propagation *in vivo*, followed by passage of tumor fragments in subsequent generations (Tentler et al., 2012). PDAC PDXs develop between one and 4 months after transplantation, with an engraftment rate between 20 and 80% (Garcia et al., 2020). They have been shown to conserve metastatic potential and histology of the original tumor (Hidalgo et al., 2014) and closely mirror drug responses in human patients (Voskoglou-Nomikos et al., 2003). This may represent the fact that PDXs are not composed of cancer cell populations separated from human tumors and adapted to culture conditions. Since PDX models are established from tumor fragments, the tumor neoplastic cell architecture is retained (Loukopoulos et al., 2004; Rubio-Viqueira and Hidalgo, 2009). Although the initial human stroma is gradually replaced with cells of the murine host, these models can recapitulate the complexity of the TME in PDAC (Duda et al., 2004). Also, successively passaged PDXs normally show consistent biological properties and homogeneous histological and molecular phenotypes (Aparicio et al., 2015). Since it is necessary to use immunocompromised mice when generating xenografts, this represents a major drawback to study metastasis, since the adaptive immune system plays a crucial role in the selection of metastatic variants (Gonzalez et al., 2018).

Chick Chorioallantoic Membrane Xenografts

Chick chorioallantoic membrane (CAM) is a deeply vascularized extraembryonic membrane formed after embryonic day 5 rich in type IV collagen and laminin, which are similar to the human basement membrane. Chick embryos are not immune-competent until day 18 (Ribatti, 2016). More and more studies have confirmed that the CAM model can efficiently sustain tumor cell proliferation, making it a simple and rapid model for studying initial tumor development. It can reproduce all stages of tumor formation in a shorter amount of time as tumors can be detected after only 4 days of cancer cell injection (Komatsu et al., 2019). It has been shown that firefly luciferase-labeled primary PDAC cells can be engrafted onto the CAM with >80% success. A comparison of tumors harvested from the CAM with original human tissues by immunohistochemical staining showed similar positive staining for the PDAC markers cytokeratin, Cytokeratin 19, Cytokeratin 7, mucin-1, and Alcian blue. Importantly, the percentages of positive/negative cells within each model are very consistent (Rovithi et al., 2017). In addition to using fluorescently labeled cells, cell invasion can be analyzed by Alu PCR to estimate the presence of metastatic human cancer cells in the organs of the chick embryo. Li et al. extracted genomic DNA from chick CAM and liver, respectively, and Alu sequences in human cells were specifically detected by Alu PCR revealing that 31% of CAM and 0% of liver tissue were Alu positive in embryos with untreated Aspc-1 cells. After dexamethasone treatment, the invasion rate of Aspc-1 tumor cells to CAM and liver increased metastasis to 85% and 60%, respectively (Mira et al., 2002; Liu et al., 2017a).

In summary, CAM-assay is a flexible, cost-efficient, reproducible and rapid approach that can evaluate the metastatic capacity and aggressiveness of different PDAC cells within a short time *in vivo*. With the help of physiological and histological characteristics of PDAC, it is easy to assess the key features of tumor metastasis such as angiogenesis, intravasation and spontaneous metastasis. Therefore, CAM assay is an attractive metastasis model. Immune deficiency of chick embryos is up to 18 days, so the short observation periods (3–9 days) become an important limitation of CAM-assay. In addition, it cannot examine cancer-immune cell interactions. Another limitation to this system is that chick embryos are extensively vascularized organisms characterized by fast morphological changes (Lokman et al., 2020). CAM-assay for tumor research is also limited in its monitoring capabilities of tumor size. Because the tumor is encased by a radiopaque eggshell and has a modest structural size, it can

only be monitored from above, posing a challenge to existing imaging modalities. Even though repetitive ultrasonography can monitor tumor growth and vascularization, it also relies on the experience of the experimenter in ultrasound (Eckrich et al., 2020).

ZebraFish Model

Recently, the value of the zebrafish model has been appreciated. *Teleost zebrafish* (Danio rerio) shows large levels of physiologic and genetic analogies to mammals, closely resembling the clinical setting and allowing the natural history of the tumor to be monitored (Wu et al., 2017). Fishes are routinely maintained at 28°C, but the most favorable temperature for tumor cell proliferation is 37°C (Cabezas-Sainz et al., 2018). When engrafted zebrafish are raised at a compromise temperature (≤34°C), cancer cells do not proliferate at the same rate as when cultured in mice or humans. The Protein Kinase, DNA-Activated, Catalytic Subunit (prkdc) and Interleukin 2 receptor (il2rga) deficient zebrafish model can be raised at 37°C and can engraft a wide range of human malignancies (Yan et al., 2019). CRE/LOX technology and GAL4/UAS systems were combined to create the first kras-initiated PDAC model in zebrafish that highly recapitulates human PanIN development (Oh and Park, 2019). A xenograft model was established in zebrafish by transplanting human pancreatic cancer cells into the perivitelline cavity of 48 h post-fertilization zebrafish embryos (Guo et al., 2015). Subsequently, they observed that cells with kras mutations displayed significant proliferative and migratory behaviors invading the zebrafish vasculature system. Then xenotransplanted larvae were exposed to an inhibitor that targets the KRAS signaling pathway named U0126. There were fewer metastases in the bodies of the larvae in the following U0126 treatment group while the mock-treatment group displayed recurrent metastasis.

Zebrafish is a useful and economical *in vivo* animal model for speedy analysis of invasion and metastatic behavior. Zebrafish embryos are transparent, so it is easy to follow the invasion, circulation of tumor cells in blood vessels, migration and micro metastasis formation in real-time (Marques et al., 2009). The entire genome of zebrafish has been determined completely, and the genetic background is clear. Therefore, it can be used for large-scale genetic background screening (Gut et al., 2017). Zebrafish are highly reproductive, with a pair of zebrafish typically producing around 200 embryos (Hoo et al., 2016), and there is little difference between individuals, so they can be used for mass drug screening, such as anti-metastasis drugs (Nakayama et al., 2021). However, the most obvious shortcoming of zebrafish is that it is not a mammal. It is significantly different from humans directly and cannot fully simulate the type of human disease. In addition, antibodies against zebrafish protein are still lacking on the market compared to mice or humans (Hason and Bartùnik, 2019). In the process of zebrafish xenograft, the temperature of embryo incubation, the different sites for implantation of tumor cells, the interaction between cells and host in embryo, and the changes of tumor microenvironment all affect the experimental results of cell proliferation, invasion and metastasis (Cabezas-Sáinz et al., 2020). Therefore, the technology of xenograft still needs to be improved in different aspects.

CRISPR/Cas9 for Metastasis Research of Pancreatic Ductal Adenocarcinoma

CRISPR/Cas9 research technology has been developing in recent years and has become a versatile tool for making changes to the genome of many organisms. Here describes some research on CRISPR technology in PDAC metastasis. It has been reported that Mucin 16 (MUC16) contributes to the metastasis of PDAC through FAK mediated Akt and ERK/MAPK pathway. MUC16 knock-out cells generated by CRISPR/Cas9 also exhibit reduced mesenchymal expression and enhanced epithelial expression in PDAC cells and inhibit cell metastasis (Muniyan et al., 2016). Vorvis et al. merged genetic and microRNA profiling analysis with CRISPR/Cas9 technology and identified that transcription factor Forkhead box A2 (FOXA2) is implicated in PDAC pathogenesis. Furthermore, suppression of FOXA2 levels by CRISPR/Cas9 *in vitro* resulted in the activation of the Plasminogen activator, urokinase receptor gene known to be implicated in invasive malignancy. These results were consistent when FOXA2 expression was blocked by siRNA (Vorvis et al., 2016). Core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 was disrupted in human PDAC cells (T3M4 and CD18/HPAF) by CRISPR/Cas9 leading to enhanced O-glycosylation truncation on MUC16, which increases the formation of aggressive PDACs and metastasis in KPC mice. Stock, al. generated two cortactin knockout lines (PANC-1 and BxPC-3) using CRISPR/Cas9 technology to study the functional role of cortactin in PDAC (Chugh et al., 2018). In PDAC metastases, they detected more expression of cortactin and Tyr421-phosphorylation than the original tumor. Cortactin activation and the migratory ability of the PDAC cells both decreased significantly after treatment with an inhibitor of the Src family kinase. CRISPR/Cas9 technology is also applied to PDAC modeling and therapeutic research using a variety of animals. An individual mouse strain expressing Cas9 in the adult pancreas under a p48 promoter has been established to generate PDAC GEMMs of complicated genotypes with high efficiency (Ideno et al., 2019). By the use of an adeno-associated virus to transfer multiplexed RNA guidelines (sgRNAs) to an adult pancreas of p48-Cre; LSL-Cas9 mice, they produce a mutated Kras G12D allele using homology directed repair in combination with CRISPR-induced disruption of cooperative alleles [Trp53, AT-rich interaction domain 1A (Arid1A) and Lkb1].

Overall, CRISPR-Cas9 engineering provides new opportunities to model PDAC development. It allows the production of syngeneic and humanized mice which can help to eliminate transplant rejection by the host immune system without needing immunocompromised animals (Krempley and Yu, 2017). As a result, these models are particularly useful for researching immunotherapies, allowing to investigate several unanswered problems. For instance, it would be

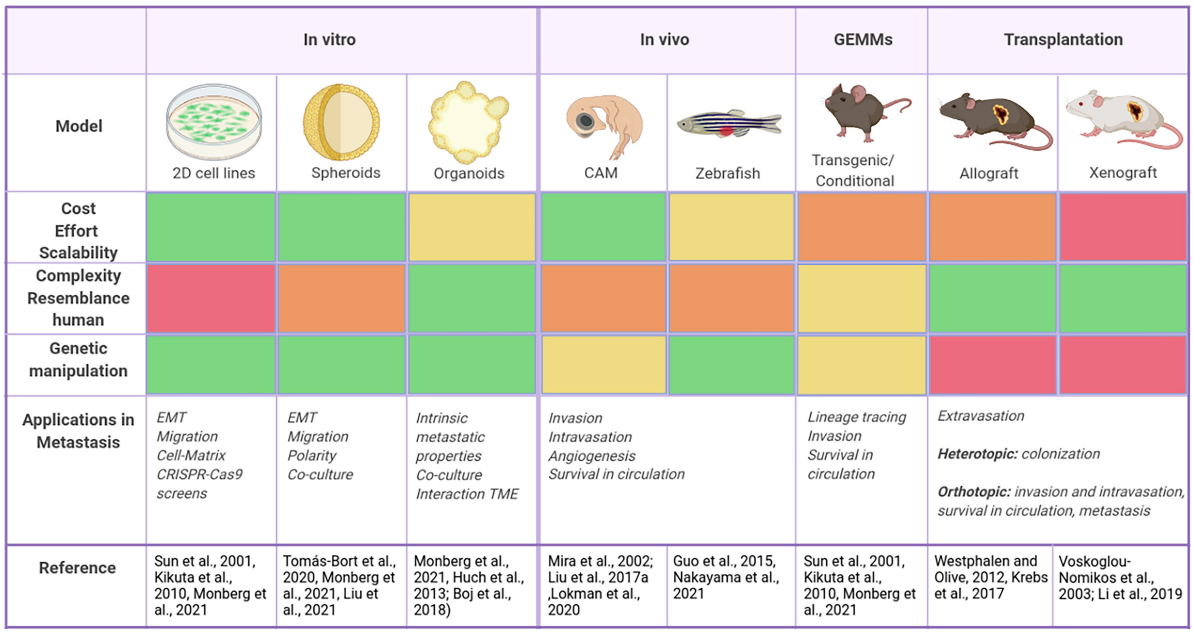

FIGURE 2 | Overview of the current models to study metastasis in PDAC. The color scale indicates whether a model is more suitable (green) or less (red) for each purpose.

compelling to see if tumors with distinct histopathological features reported in the model have substantial variations in target allele frequency and/or if further mutations have accumulated and how this model's total mutational and neoantigen load correlates to other GEMMs and human PDAC (Jørgensen and Hogg, 2016).

DISCUSSION

Several preclinical models for PDAC are accessible for basic and translational studies, which permit the description of the global genetic features of this disease (summarized in **Figure 2**).

Pancreatic ductal adenocarcinoma is characterized by an early and fast metastatic process partially related to the location of the pancreas leading to peritoneal, liver or lung metastasis. Nonetheless, a deep molecular insight into the metastatic process of PDAC is still missing since very few studies have studied the mechanisms behind PDAC metastatic organotropism. This is pivotal since the location of the metastases determines the clinical outcome of the patient. Each PDAC preclinical model described above (2D cell lines, spheroids or organoids, GEMMs, and PDXs models) has pros and cons, and the model of choice will vary according to experimental goals.

The correct combination of currently available models is necessary for the development of more trustworthy therapeutic strategies against PDAC. A good strategy is a combination of the methods in each step of the experimental process. Namely, 2D cell lines, PDCL or spheroids are good tools for HTS, studying tumorigenesis and progression. Organoids offer similar benefits as cell lines, adding a step closer to personalized medicine. *In vivo* models are useful to model the TME and immune response, key players in PDAC transformation and progression. Murine models are ideal platforms for understanding PDAC progression, pathophysiology and testing therapeutic modalities. Once candidates are selected, more relevant models like ODXs or PDXs models are suitable for functional validation.

AUTHOR CONTRIBUTIONS

MM wrote the abstract, introduction, some models sections, discussion, and elaborated the figures of the manuscript. SZ wrote some models sections, elaborated some tables, and revised the manuscript. CP revised the manuscript, provided critical feedback, and helped shape the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Context Matters—Why We Need to Change From a One Size Fits all Approach to Made-to-Measure Therapies for Individual Patients With Pancreatic Cancer

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Pancreatic cancer is one of the deadliest cancers and remains a major unsolved health problem. While pancreatic ductal adenocarcinoma (PDAC) is associated with driver mutations in only four major genes (*KRAS, TP53, SMAD4,* and *CDKN2A*), every tumor differs in its molecular landscape, histology, and prognosis. It is crucial to understand and consider these differences to be able to tailor treatment regimens specific to the vulnerabilities of the individual tumor to enhance patient outcome. This review focuses on the heterogeneity of pancreatic tumor cells and how in addition to genetic alterations, the subsequent dysregulation of multiple signaling cascades at various levels, epigenetic and metabolic factors contribute to the oncogenesis of PDAC and compensate for each other in driving cancer progression if one is tackled by a therapeutic approach. This implicates that besides the need for new combinatorial therapies for PDAC, a personalized approach for treating this highly complex cancer is required. A strategy that combines both a target-based and phenotypic approach to identify an effective treatment, like Reverse Clinical Engineering® using patient-derived organoids, is discussed as a promising way forward in the field of personalized medicine to tackle this deadly disease.

Keywords: pancreatic ductal adenocarcinoma (PDAC), tumor heterogeneity, KRAS, 3D cell culture models, personalized medicine, patient-derived tumor organoids, combined targeted and phenotypic approach, reverse clinical engineering

INTRODUCTION

Treating pancreatic cancer is a major clinical challenge. It is aggressive, often diagnosed late in its course and treatment options are not only limited but also with a low success rate—despite strong efforts in basic and clinical research to better understand and tackle this deadly disease.

The most frequent histological type of pancreatic cancer is the pancreatic ductal adenocarcinoma (PDAC) arising from epithelial ductal cells of the pancreas (Warshaw and Castillo, 1992; Li et al., 2004). PDAC is among the cancers with the worst prognosis with a 5-year survival rate of less than 9% (Siegel et al., 2021) and is predicted to be the second leading cause of cancer death by 2030 (Rahib et al., 2014).

The only curative treatment for PDAC so far is surgery, but most of the patients are diagnosed at late stages and already metastasized. 85% of PDACs are unresectable (Seufferlein et al., 2014; Orth et al., 2019) and currently the most common treatment for these patients is chemotherapy that includes combinations with gemcitabine and 5-fluorouracil (5-FU). Combination therapy of gemcitabine and nab-paclitaxel improved overall survival to gemcitabine therapy alone by 1.8 months (8.5 vs 6.7 months median overall survival) with the 2-years survival rate increasing to 9% on gemcitabine plus nab-paclitaxel compared to 4% on gemcitabine therapy alone (Von Hoff et al., 2013; Saito et al., 2017).

Improved therapy results were also shown for FOLFIRINOX, which is a combination of 5-FU, irinotecan, oxaliplatin, and folinic acid, but also exhibits increased side effects and affects the quality of life (Conroy et al., 2011). For patients treated with FOLFIRINOX, the overall survival increased by 4.3 months compared to gemcitabine (11.1 vs 6.8 months) (Conroy et al., 2011), and the response rate to gemcitabine or FOLFIRINOX therapy was only 10 and 31% respectively (Conroy et al., 2011; Bian et al., 2017).

While these two regimes were considered as a success story in the therapeutic arena of PDAC, the overall survival is still very low with only small improvements, illustrating that an effective treatment of PDAC is still missing. Further, chemoresistance of the tumor is prevalent and is one of the main reasons for the very low survival rate of this aggressive cancer (Juiz et al., 2019).

Targeted therapies aiming specifically at genomic aberrant pathways, often using specific molecular profiles of individual cancer to stratify patients, significantly enhanced cancer treatment—but not yet for PDAC. For example, colorectal cancer patients with wild type proto-oncogene *KRAS* or *BRAF* benefit often from treatment with monoclonal antibodies targeting the epidermal growth factor receptor (EGFR) (Amado et al., 2008; Karapetis et al., 2008; Di Nicolantonio et al., 2008). In PDAC, combination therapy of the anti-EGFR antibody erlotinib and gemcitabine has been approved as a first line therapy for metastatic disease, independent of *KRAS* mutational status, and showed clinical benefit compared to gemcitabine alone (Moore et al., 2007; Wang et al., 2015). However, also here the median survival time increased only to 6.24 months compared to 5.91 months for gemcitabine treatment alone in the initial trial (Moore et al., 2007) and *KRAS* mutational status was shown as not predictive for the treatment response to erlotinib in PDAC (Boeck et al., 2013).

In PDAC, some targeted therapies improved treatment but unfortunately only for a small proportion of patients with specific aberrations. PDAC patients with high microsatellite instability or DNA mismatch repair-deficient tumors (0.8% of PDAC cases) were shown to respond to immune checkpoint blockade inhibitors, such as anti PD-1/PD-L1, with durable responses (Diaz et al., 2017; Hu et al., 2018; Eso et al., 2020). Therapies targeting either DNA damage repair (i.e., PARP inhibitors) in germline *BRCA* mutated metastatic PDAC (about 7% of patients) (Vincent et al., 2011a; Golan et al., 2019) and therapies targeting specific oncogenes such as mutant BRAF (about 4% of patients) or kinase fusion genes in *KRAS* wild type tumors (about 4% of patients) (Luchini et al., 2020) were also shown to benefit patients in a clinically relevant fashion.

So why is there so little progress in PDAC treatment? The highly desmoplastic tumor stroma (Öhlund et al., 2017; Hosein et al., 2020) making it difficult for a drug to reach the tumor is considered as one reason for treatment failure in PDAC (not reviewed here). Another is the effective immune-evasion mechanisms employed by PDAC (Saka et al., 2020) (not reviewed here). But on the other hand, it is the individual differences at the molecular and cellular level of each tumor, its heterogeneity, as well as the interplay between various pathways and dysregulations, the context that confers different susceptibility to drugs. These tumor-cell intrinsic features are the topic of this review.

Much is known about the genetic landscape and mutations driving PDAC development and progression. PDAC is associated with mutations in only four major genes: the proto-oncogene *KRAS* as the main disease driver, followed by tumor suppressor genes tumor protein 53 (*TP53*), SMAD family member 4 (*SMAD4*) and cyclin dependent kinase inhibitor 2A (*CDKN2A*) (Hingorani et al., 2005). The current model for PDAC development is that genomic alterations occur in a stepwise manner with mutations in *KRAS* and *CDKN2A* preceding in early Pan-IN lesions, which are precursor lesions for invasive carcinoma, often followed by mutations in *TP53* and *SMAD4* which contribute to the tumor's invasiveness (Aguirre et al., 2003; Hingorani et al., 2005; Iacobuzio-Donahue et al., 2012; Ryan et al., 2014).

However, despite overwhelming research on PDAC biology and genetics, we were not yet able to harness this knowledge to develop effective targeted therapies for pancreatic cancer. The main lingering question that remains is how to translate the knowledge of disease biology and its heterogeneity into targeted therapies for individual patients. It is much needed to unearth the tumors' resistance mechanisms and identify chemo-sensitivity signatures in PDAC, which will increase the chances of identifying clinically effective targeted therapies.

This review delineates the difficulties in targeting highly heterogenous, mutant KRAS-driven PDAC cells due to the presence of dysregulations at multiple level. Dysregulations at genome level is just one aspect driving this disease but there are also dysregulations at the metabolic space, epigenetic alterations and additional pathway deregulations that enhance tumor progression. In addition, several compensatory mechanisms are utilized by PDAC cells for their survival when the highly dysregulated KRAS signaling pathway is targeted. Therefore, identifying and targeting vulnerabilities not only in the genetic landscape but also at the epigenetic and metabolic level is necessary for effective treatment. Further, it is required to approach PDAC therapy in an individualized manner to take the heterogeneity and the context of the multiple dysregulations into account. This review further highlights that patient-derived organoids are an excellent tool to functionally profile individual tumors and that Reverse Clinical Engineering®, an approach that combines target-based and phenotypic screening strategies as a single system in a potentially high-throughput manner holds an important component of the future of personalized oncology.

DISTINCT SUBTYPES REVEAL PDAC HETEROGENEITY

Molecular taxonomy of PDAC has been described in several gene expression studies in different, independent cohorts. Collisson and colleagues identified three subtypes (quasi mesenchymal, classical, and exocrine-like) in 2011, based on analyses of global gene expression from human and mice PDAC lines along with micro-dissected human PDAC tissue and found that these subtypes progress and respond to treatment differently (Collisson et al., 2011). A 2015 study by Moffitt et al. on bulk resected primary, non-treated PDAC tumors and metastases stratified PDAC into tumor-specific and stromal-specific subtypes using a sophisticated computational approach (Moffitt et al., 2015). Distinct molecular mechanisms associated with distinct tumor subtypes were identified by the Australian Pancreatic Cancer Genome Initiative in 2016 using integrated genomic, epigenomic, and transcriptomic analysis. Here, four subtypes were identified based on whole genome, deep exome, and RNA expression profiles: squamous, pancreatic progenitor, immunogenic and aberrantly differentiated exocrine- and endocrine-like (Bailey et al., 2016). Differential expression of transcription factors and their downstream effectors among these subtypes revealed the heterogeneity in PDAC pathophysiology.

Distinct classifications from different cohorts (Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016; Puleo et al., 2018) overlap (Raphael et al., 2017) and altogether sum up to two major tumor-specific subtypes based on high purity tumor samples: classical/pancreatic progenitor and squamous/basal-like. Both subtypes are associated with specific genetic programs and prognosis. The classical subtype has a higher level of differentiation and better prognosis compared to the basal-like/squamous subtype (Bailey et al., 2016).

A study from 2020 further improved the purification of PDAC tumor cells for genomic analysis using laser capture microdissection and classified the two major PDAC subtypes into further subclasses based on the degree of squamous signatures and clinical staging to classical-like A, classical-like B, basal-like A, basal-like B, and hybrid (Chan-Seng-Yue et al., 2020). Basal-like A phenotype was shown to be highly prevalent in metastatic advanced disease, enriched with squamous signatures and associated with increased genomic instability such as genome doubling and high *KRAS* imbalance (i.e., imbalance between wildtype and mutant *KRAS* alleles favoring the mutant *KRAS* allele) (Chan-Seng-Yue et al., 2020). However, if a relationship exists between high imbalance in *KRAS*, genomic doubling and increased expression of squamous signature is still unclear. Despite that, it suggests that increased mutant *KRAS* dosage may lead to increased KRAS signaling and promote metastasis.

Additional studies at single cell resolution revealed that most PDAC tumors harbor both classical and basal-like tumor cells, thereby showing that the two subtypes co-exist in a single tumor (Juiz et al., 2020). This demonstration of high intra-tumor heterogeneity even at the subtype level further highlights the need for personalized oncology. Treatment targeting for example basal-like tumor cells will not be sufficient if both subtypes are present in most tumors, demonstrating the complexity of targeting tumor cell subtypes.

In summary, distinct subtypes identified in massive sequencing studies revealed pronounced heterogeneity in PDAC with also less prevalent mutations, genome doubling, copy number changes, and chromosome alterations contributing to tumorigenesis besides mutations in the four major PDAC drivers (*KRAS, CDKN2A, SMAD4*, and *TP53*). This heterogeneity is the main reason for the current treatments not being more effective in PDAC, as the differences that prevail at molecular and cellular level of each tumor confer different susceptibility to treatment.

This demonstrates that while we need targeted therapies that specifically target the dysregulated molecular mechanisms driving tumor progression, there will never be a "one size fits all" solution in PDAC but a need for individualized treatment, for truly personal oncology.

COMBINATION THERAPIES FOR UNDRUGGABLE KRAS

90% of PDAC possess mutations in the proto-oncogene *KRAS* (Almoguera et al., 1988; Raphael et al., 2017) and *KRAS* mutations which were shown to initiate the disease (Hingorani et al., 2005). In addition to *KRAS* mutation, *KRAS* amplification also contribute for disease progression in cancers including PDAC (Silverman et al., 1990; Liu et al., 1998; Heidenblad et al., 2002; O'Hagan et al., 2002; Aguirre et al., 2003; Yamada et al., 2008). Given the strong *KRAS* oncogene addiction of PDAC (Eser et al., 2014; Zeitouni et al., 2016), it appears as the obvious aim for targeted PDAC therapy.

KRAS is a small GTP binding protein, localized at the lipid rich cell membrane and cycling between GDP-bound inactive and GTP-bound active states. Activated KRAS triggers a signaling cascade by activating further downstream kinase effectors such as mitogen-activated protein 3 kinases (MAP3K) RAF, which then activate MAP2K kinases like MEK, activating MAP kinases (MAPK) like ERK, activating finally transcription factors that regulate gene expression changes involved in cell cycle regulation, tissue repair, angiogenesis, and differentiation (Ullrich and Schlessinger, 1990; Adjei, 2001; Lemmon and Schlessinger, 2010).

Researchers in the academic and pharmaceutical arena are trying for over four decades to discover a drug that effectively targets mutated RAS. A crucial amino acid is the glycine at position 12 which upon mutation is often replaced with another amino acid. The consequence is a prevention of GTP hydrolysis, constantly favoring the active GTP-bound state activating the downstream signaling cascade responsible for cell proliferation and survival (Scheffzek et al., 1997). Inhibitors have been developed that target mutant $KRAS^{G12C}$ protein which were shown to be effective for lung cancer patients (Ostrem et al., 2013; Lito et al., 2016; Janes et al., 2018). However, in PDAC this mutation is only found in around 2% of cases, with $KRAS^{G12D}$ being the most common driver mutation (Cox et al., 2014). But despite tiring efforts, no clinically effective inhibitor

for $KRAS^{G12D}$ has been available for PDAC patients yet (Cox et al., 2014).

Efforts to develop effective KRAS inhibitors failed on one hand due to difficulties in targeting KRAS directly. Its high intrinsic affinity for GTP prevents the binding of competitive GTP inhibitors (Karnoub and Weinberg, 2008; Stephen et al., 2014). Another reason for the failure of KRAS inhibitors is that PDAC cells gain resistance based on other compensatory pathways (Karnoub and Weinberg, 2008; Stephen et al., 2014).

So indirect approaches to target KRAS appear to be required and one option would be targeting its downstream effector signaling. Several studies have provided compelling reason to consider the RAS-RAF-MEK-ERK pathway to target mutant KRAS-driven PDAC (Chen et al., 2016; Foster et al., 2016; Hayes et al., 2016; Riquelme et al., 2016; Raphael et al., 2017).

In a mutant KRAS-driven model of PDAC it was shown that mutant *KRAS* can be phenocopied by replacement with an activated mutant $BRAF^{V600E}$ allele (Collisson et al., 2012). In the rare cases of PDAC with wild type *KRAS*, a high percentage have a $BRAF^{V600E}$ mutation (Witkiewicz et al., 2015; Raphael et al., 2017; Seghers et al., 2020). However, BRAF selective inhibitors are effective only in BRAF mutant tumor models and have paradoxically activated ERK signaling in *KRAS* mutant or *RAS/RAF* wild type tumor models (Hatzivassiliou et al., 2010; Poulikakos et al., 2010). This supports the notion that these inhibitors might have opposing roles and function either as an inhibitor or activator of the same signaling pathway depending on the cellular context.

Inhibition of ERK as monotherapy is limited by normal cell toxicity and cancer cells often acquire resistance by ERK reactivation through loss of an ERK-dependent negative feedback mechanism (Ozkan-Dagliyan et al., 2020; Klomp et al., 2021). MEK inhibitors also caused feedback reactivation of ERK and have shown limited to no activity in *KRAS* mutant cancers (Samatar and Poulikakos, 2014).

Combination therapy inhibiting distinct nodes of the same pathway concurrently was suggested as a strategy to treat *KRAS* mutant PDAC (Ozkan-Dagliyan et al., 2020). Lower doses of RAF-ERK inhibitory combinations were shown to exhibit a synergistic suppression of activated ERK and caused cell cycle arrest and apoptosis (Ozkan-Dagliyan et al., 2020). Similarly, studies have also shown that combined RAF-MEK inhibition would overcome ERK reactivation in *KRAS* mutant or wild type cancer lines (Lamba et al., 2014; Yen et al., 2018), implicating this as a potential strategy for PDAC. Supporting studies from BRAF mutant melanoma have shown that combined BRAF-MEK inhibition is effective, exhibiting synergistic growth suppression and delaying onset of resistance, and has been clinically approved for this cancer type (Flaherty et al., 2012; Larkin et al., 2014; Long et al., 2014; Dummer et al., 2018). There is an ongoing clinical trial evaluating a pan-RAF inhibitor in combination with ERK or MEK inhibitors in *KRAS*-mutant non-small cell lung cancer and *NRAS*-mutant melanoma which will hopefully provide insights about RAF-ERK vs RAF-MEK inhibitory combinations (NCT02974725) (Ozkan-Dagliyan et al., 2020).

In summary, while mutant KRAS itself appears to be undruggable, identifying combination therapies that overcome pathway reactivation by targeting distinct compensatory mechanisms cancer cells use for their survival when KRAS signaling is suppressed is crucial for the development of effective combinatorial strategies that would result in synergistic tumor regression effects.

PI3K PATHWAY DRIVES PDAC PROGRESSION AND COMPENSATES WHEN KRAS SIGNALING IS SUPPRESSED

Another signaling pathway activated by KRAS and implicated in PDAC is the PI3K/AKT pathway (Edling et al., 2010). Phosphoinositide 3 kinase (PI3K) describes a family of heterodimeric enzymes composed of a regulatory and a catalytic subunit. The catalytic subunits are classified into p110α, p110β, p110γ, and p110δ encoded by the genes *PIK3CA*, *PIK3CB*, *PIK3CG*, and *PIK3D* respectively (Hawkins et al., 2006). Upon activation, PI3K convert the cell membrane component phosphotidylinositol (4, 5) bisphosphate (PIP2) to phosphatidylinositol (3, 4, and 5) trisphosphate (PIP3) (Cantrell, 2001). PIP3 acts as an activating anchor for 3-phosphoinositide-dependent protein kinase 1 (PDK1) which in turn activates protein kinase B (PKB), also called AKT (Alessi et al., 1997; Currie et al., 1999). AKT activates further downstream signaling components such as the mechanistic target of rapamycin kinase (mTOR) (Cantrell, 2001; Janku, 2017). PI3K pathway activation plays a major role in cell cycle regulation, survival, and differentiation (Alessi et al., 1997; LoPiccolo et al., 2008). The tumor suppressor protein phosphatase tensin homolog (PTEN) converts PIP3 back to PIP2, acting as a negative regulator of the PI3K pathway and preventing cellular over proliferation (Maehama and Dixon, 1998).

PI3K was shown to be activated by oncogenic KRAS in both human and mouse models of PDAC (Jimeno et al., 2008; Kennedy et al., 2011; Ying et al., 2011). PDAC tumors have further been shown to have gain of function mutations in the oncogene *PIK3CA* or loss of function mutations in the tumor suppressor gene *PTEN* (Ying et al., 2011; Foo et al., 2013; Waddell et al., 2015). Oncogenic *PIK3CA* expression phenocopied KRAS driven PDAC progression (Payne et al., 2015). *PIK3CA* was also shown to regulate tumor immunogenicity and a genetic ablation of *PIK3CA* rendered mutant KRAS/TP53 driven pancreatic tumors more immunogenic by increasing the expression of major histocompatibility complex class 1 (MHC 1) and CD80, both of which are needed for T-cell stimulation (Sivaram et al., 2019). Almost 60–70% of PDAC is also associated with increased AKT activity, for example through *AKT2* oncogene amplification (Cheng et al., 1996; Ruggeri et al., 1998; Schlieman et al., 2003).

Oncogenic KRAS was also shown to drive PDAC progression through PDK1 (Eser et al., 2013). Inactivation of *PDK1* in epithelial cells of the pancreas significantly reduced tumor formation driven by $KRAS^{G12D}$ (Eser et al., 2013). However, inactivation of *PDK1* using a recombinant strategy in epithelial cells of the lung have shown no decrease in progression of non-small cell

lung cancer (NSCLC) (Eser et al., 2013). Further, in mutant KRAS driven NSCLC inhibition of PI3K-mTOR did not reduce tumor growth substantially (Engelman et al., 2008) but inhibition of cRAF, another effector of KRAS, decreased cancer progression (Blasco et al., 2011; Karreth et al., 2011). This demonstrates that each tissue has its own specific molecular events and signaling requirements for tumor progression. Hence, it is important to investigate the differences of the activated effector pathways driven by oncogenic KRAS in a tissue and context specific manner (Eser et al., 2013).

Distinct mutations are also associated with different biological potency of driving cancer progression, as well as conferring distinct therapeutic vulnerabilities. Commonly found PDAC mutations $KRAS^{G12D}$ and $KRAS^{G12V}$ were both shown to elevate macropinocytosis, a nutrient-scavenging metabolic activity critical for PDAC growth, through the key effector PI3Kα (p110α) (Hobbs et al., 2020). However, a rarer $KRAS^{G12R}$ mutation, which is still found in about 20% of PDAC cases, does not interact with PI3Kα due to structural deformity, but the mutant cells still elevate macropinocytosis through a compensatory mechanism that activates the PI3K pathway through upregulation of another PI3K isoform, namely PI3Kγ (p110γ) (Hobbs et al., 2020). Hence, inhibitors selective to p110γ PI3K, but not p110α were effective in blocking macropinocytosis in $KRAS^{G12R}$ driven PDAC cells, whereas for $KRAS^{G12D}$ driven PDAC cells, both, a p110α or p110γ selective inhibitor, were effective. In addition, the drug sensitivity pattern to other targeted therapies differed between $KRAS^{G12R}$ and $KRAS^{G12D}$ mutant PDAC cells (Hobbs et al., 2020).

This study further illustrated that the distinct PI3K isoforms might have different roles in supporting cancer progression. Since these isoforms exist in both tumor and supporting stromal cells (Graupera et al., 2008; Thorpe et al., 2015; Conway et al., 2019), isoform specific targeting could enhance tumor regression and prevent off-target side effects in healthy tissues (Thorpe et al., 2015; Yap et al., 2015). In this line, combination therapies targeting EGFR and PI3Kα (p110α) in PDAC with high EGFR and AKT activity have shown promising efficacy (Wong et al., 2014).

Another study showed that p110α PI3K is required for KRAS-induced transformation of acinar to ductal metaplasia (ADM) via regulation of RAC1 (a known regulator of tumorigenesis) (Wu et al., 2014). Ablation of p110α but not p110β PI3K resulted in protection from tumorigenesis in a $KRAS^{G12D}$ driven pancreatic tumor mouse model. However, ablation or long-term inhibition of p110α PI3K lead to an activation of downstream AKT, probably due to compensatory activity of other PI3K isoforms, suggesting that both isoform specific targeting and combinatorial therapies are important (Wu et al., 2014).

PDAC cells were shown to use the PI3K pathway as a compensatory mechanism for their survival. When *KRAS* was ablated, PI3K was shown to activate MAPK signaling and an unbiased chemical screen identified *KRAS* ablated cells sensitive to PI3K inhibition (Muzumdar et al., 2017). KRAS inhibition was shown to activate AKT through the mTORC2 complex (Brown et al., 2020). Combinatorial inhibition of $KRAS^{G12C}$ and mTORC1/2 or MEK and mTROC1/2 however suppressed tumor growth in PDAC *in vitro* models and *in vivo* experiments (Brown et al., 2020), identifying another potential targeted combination to overcome resistance mechanisms.

In another study, a subset of mutant KRAS dependent PDAC cells acquired *de novo* resistance upon treatment with an ERK inhibitor through activation of the PI3K pathway, thereby overcoming ERK inhibition (Hayes et al., 2016). Modest anti-tumor activity was observed when MEK and PI3K were concurrently inhibited in mouse models of PDAC (Alagesan et al., 2015; Junttila et al., 2015). However, normal tissue toxicity is a limiting factor for combinatory inhibition of MEK and PI3K (Shimizu et al., 2012; Tolcher et al., 2015).

Multi-drug resistance pathways were shown to be activated by PI3K signaling in different cancers such as lung, breast, and chronic myeloid leukemia (Chen et al., 2018; Soltani et al., 2019; Wu et al., 2019). Chemoresistance through accelerated cell cycle processes was also credited to the activation of NF-κB, a downstream effector of the PI3K-AKT pathway, in several cancers including PDAC (Sui et al., 2014; Hu et al., 2015; Zhu et al., 2015; Eberle, 2019; Liu et al., 2020). Targeting the pathway component mTOR in cancer associated fibroblasts was demonstrated to reduce chemoresistance in PDAC (Duluc et al., 2015).

In summary, PI3K is another important signaling pathway involved in PDAC oncogenesis through various deregulations and a key player for the development of adaptive resistance to KRAS signaling inhibition, illustrating the need for combinatorial targeted treatment approaches beyond the KRAS-MAPK pathway.

METABOLIC ALTERATIONS DRIVE PDAC PROGRESSION

Several studies showed that PDAC cells tend to reprogram their metabolic activity to allow them to survive in the hypoxic tumor microenvironment (Commisso et al., 2013; Guillaumond et al., 2013; Son et al., 2013; Chini et al., 2014).

Increased glycolysis is a major hallmark acquired by cancer cells for their uncontrolled growth (Vander Heiden et al., 2009; Lu et al., 2012; Ying et al., 2012; Cai et al., 2013) and PDAC cells were shown to have an increased glycolysis conferred through the enzyme NADP(H) oxidase (NOX) (Lu et al., 2012). In a mutant KRAS driven mouse model of PDAC, the increase in glycolytic activity was driven by KRAS through the ERK-MAPK pathway and through loss of the oncogene *MYC* (Ying et al., 2012). Bryant et al., extended this observation to human PDAC in 2019 and showed that the glycolytic flux decreased upon both, KRAS suppression and ERK inhibition (Bryant et al., 2019).

Broad metabolic profiling stratified PDAC into different metabolic subtypes, lipogenic, and glycolytic, based on distinct metabolic reprogramming events and it was shown that each subtype has unique drug sensitivity profiles for specific classes of metabolic inhibitors (Daemen et al., 2015). The study also demonstrated that the lipogenic and glycolytic metabolic subtypes correlate with an epithelial and mesenchymal phenotype of PDAC respectively (Daemen et al., 2015).

The two major PDAC subtypes, classical and squamous, were shown to be driven by distinct metabolic phenotypes (Brunton et al., 2020). The worst prognostic squamous subtype of PDAC was shown to be highly catabolic, enriched with glycolytic transcripts and associated with increased glycolytic flux with high lactate secretion and decreased oxygen consumption (Bailey et al., 2016; Brunton et al., 2020). In homozygote $KRAS^{G12D/G12D}$ mutated lung cancer cells glucose metabolism was increased relative to $KRAS^{G12D/WT}$ heterozygous cells (Kerr et al., 2016). However, DNA sequencing analysis of PDAC established that $KRAS^{G12D}$ hetero- and homozygote cells were present across both PDAC subtypes (Brunton et al., 2020), therefore, another genetic or epigenetic event might have acted as a switch driving these metabolic changes in PDAC. It was shown that epigenetic loss of the genes *HNF4A* (hepatocyte nuclear factor 4 alpha) and *GATA6* (GATA binding protein 6), drives the metabolic reprogramming and switches the PDAC cells from classical to squamous subtype with increased glycolytic flux (Brunton et al., 2020).

Other metabolic pathways, namely macropinocytosis, an endocytic mechanism that PDAC cells utilize to accumulate essential amino acids (Commisso et al., 2013; Andreasson et al., 2020) and cholesterol metabolism (Chen et al., 2015; Guillaumond et al., 2015) also play significant roles in PDAC progression. Epidemiological and other studies have shown that statins, i.e., drugs reducing serum concentration of cholesterol, can be used to reduce the tumor load and improve the survival of PDAC patients (Walker et al., 2015; Wu et al., 2015; Huang et al., 2016). The signaling pathways that drive this dysregulation in metabolism and the order of direct and indirect signaling events is yet to be deciphered.

Another metabolic alteration associated with KRAS suppression or ERK inhibition in PDAC is impaired mitochondrial function (Viale et al., 2014; Kashatus et al., 2015; Bryant et al., 2019). A study performed in mouse PDAC cells showed that the PDAC cells resistant to oncogene ablation of $KRAS^{G12D}$ relied on increased mitochondrial respiration for survival (Viale et al., 2014). Mitochondrial fission, which includes fragmentation of mitochondrial matrix, was shown to be associated with KRAS induced transformation. Inhibition of the ERK-MAPK pathway resulted in increased mitochondrial fusion through blocking of mitochondrial fission, also reflecting increased oxygen consumption (Kashatus et al., 2015; Serasinghe et al., 2015). However, another study did not observe an increase in oxygen consumption with acute KRAS suppression or ERK inhibition in both human and mouse PDAC cell lines, instead the mitochondrial activity persisted at a lower level (Bryant et al., 2019). Potentially, ERK inhibition also suppressed the genes involved in mitochondrial biogenesis (Bryant et al., 2019).

Elevated autophagy was identified as a compensatory metabolic pathway that PDAC cells use when the glycolytic pathway and mitochondrial function were ablated through inhibition of KRAS signaling (Guo et al., 2011; Yang et al., 2011, 2014; Bryant et al., 2019; Kinsey et al., 2019). When KRAS was ablated by either siRNA, chemical inhibitors, or in a doxycycline-inducible model, all resulted in an elevated autophagic flux (Bryant et al., 2019). This metabolic alteration of increased autophagic flux was shown to be mediated mainly by the RAS-RAF-MEK-ERK pathway and the combination of ERK/MEK and autophagic inhibitors such as hydroxychloroquine resulted in a synergistic anti-tumor activity (Bryant et al., 2019; Kinsey et al., 2019). Several clinical trial studies have been initiated for this combined MEK/ERK and autophagy inhibition in RAS-mutant cancers including PDAC (NCT03825289, NCT04132505, NCT04214418, NCT04386057, and NCT04145297; https://clinicaltrials.gov).

In summary, metabolic alterations also drive PDAC progression and act as compensatory mechanisms under treatment. Therefore, metabolic dysregulation should also be considered in treatment strategies and there are indeed clinical studies underway that target autophagy in addition to KRAS signaling.

EPIGENETIC ALTERATIONS DRIVE PDAC PROGRESSION

Cancer cells attain malignant transformation via genomic instability, which serves as one of the main hallmarks for disease progression (Yao and Wei, 2014). Genomic instability can occur at both, the genetic and epigenetic level (Putiri and Robertson, 2011) and it increases as the tumor progresses. Epigenetics refers to the control of gene expression without changing the DNA sequence, e.g., via chemical modifications like methylation of the DNA, that can be passed on in cell divisions (Jones and Baylin, 2007; Akhavan-Niaki and Samadani, 2013; Ciernikova et al., 2020; Alonso-Curbelo et al., 2021). Epigenetic changes like gene silencing by promoter hypermethylation or gene overexpression by promoter hypomethylation were shown to impact cancer progression (Jones and Baylin, 2007).

Epigenetic drugs were shown to reduce tumorigenicity in pre-clinical models and some are already used in clinical trials (Hessmann et al., 2017; Ciernikova et al., 2020; Morel et al., 2020). However, so far, they are associated with limited efficacy and not successful as monotherapy.

Epigenetic changes should also be considered for biomarker discovery. CpG island (i.e., genomic regions with many CpG dinucleotide repeats) methylation that leads to loss of gene expression is widely studied in cancer and known to be an efficient biomarker since the 1990's (Jones and Baylin, 2002, 2007). Studies have also demonstrated that it is possible to detect DNA methylation markers in liquids secreted by the pancreas ("pancreatic juice") of PDAC patients which can be used for PDAC diagnosis (Matsubayashi et al., 2006; Kisiel et al., 2015).

Several studies have shown that aberrant hyper- and hypomethylation of specific genes contribute to PDAC development and progression (Ueki et al., 2000, 2001; Sato et al., 2003a, 2005). Genome wide studies have identified 1,658 loci which were differentially methylated in PDAC when compared to normal pancreas (Vincent et al., 2011b). Another study using a microarray platform to profile DNA methylation in a genome wide manner showed that hundreds of promoters and CpG island were aberrantly methylated in

PDAC cells (Omura et al., 2008). Utilizing the DNA-hypomethylating agent 5-Aza-dC and comparing the gene expression profiles before and after treatment in PDAC cell lines, resulted in the identification of several genes that showed abnormal methylation patterns at both CpG rich and CpG poor islands. This abnormal methylation was also detected in a selection of those genes in cancer tissue and pancreatic juice samples from PDAC patients (Sato et al., 2003b).

Genes with epigenetic dysregulation in PDAC include the cell cycle regulator *CDKN2A* and tumor suppressor *E-cadherin*, which were shown to be silenced by promoter DNA hypermethylation (Fukushima et al., 2002; Vincent et al., 2011b). Genes involved in the process of epithelial-mesenchymal transition (EMT), an important step in cancer progression, such as *TWIST1* and *BMP3* were found to be over-expressed with promoter hypomethylation (Tew et al., 2020).

An epigenetic mechanism behind an observed relationship between KRAS dependency and EMT in PDAC cell lines had also been suggested in an earlier study. The study found that KRAS dependent PDAC cells had a better differentiated epithelial phenotype with higher expression of the epithelial marker E-cadherin and upon EMT, KRAS dependency was reduced but there was no association with the mutational status of tumor suppressor and oncogenes other than KRAS, potentially suggesting an epigenetic mechanism behind the loss and gain of KRAS dependency (Singh et al., 2009).

KRAS also plays a role in gene expression regulation at the epigenetic level (Gazin et al., 2007; Serra et al., 2014). RAS was shown to mediate the epigenetic silencing of genes such as *FAS*, coding for the Fas cell surface death receptor which is needed for apoptosis in KRAS transformed mouse NIH3T3 and KRAS transformed human HEC1A cell lines (Gazin et al., 2007). A study performed in cell lines from lung cancer models showed that changes in DNA methylation were associated with mutant $KRAS^{G12V}$ overexpression influencing the expression of genes encoding for factors mainly involved in the biological processes of differentiation and development (Tew et al., 2020). In the same study, KRAS mutant and dependent pancreatic cancer lines also exhibited over 8,000 differential methylations of CpGs upon *KRAS* knockdown and differentially methylated promoters also showed an enrichment of genes involved in differentiation and development (Tew et al., 2020). Interestingly, while this study demonstrated an effect of KRAS on epigenetic changes, they also found that the observed DNA methylation changes were mostly random and strongly influenced by cell type, contributing to high heterogeneity between cell lines (Tew et al., 2020). Another noteworthy finding was that the ERK pathway, which is the main effector signaling pathway for uncontrolled proliferation in mutant KRAS driven PDAC (Hayes et al., 2016), was not responsible for the associated methylation changes over the observed short time frame (Tew et al., 2020), leaving the responsible signaling pathway to be deciphered.

The differences between the PDAC subtypes such as classical and squamous involve changes in the epigenetic landscape (Bailey et al., 2016; Lomberk et al., 2018; Somerville et al., 2018). The worst prognostic basal/squamous subtype of PDAC was shown to be highly hypermethylated (Bailey et al., 2016; Miyabayashi et al., 2020). This subtype was also associated with mutations in genes of several epigenetic regulators such as *KDM6A*, *KMT2C,* and *KMT2D* (Collisson et al., 2011; Bailey et al., 2016; Andricovich et al., 2018). Loss of gene expression that drive endodermal lineage specification, namely *HNF4A* and *GATA6,* through epigenetic silencing with promoter hypermethylation were shown to drive the cancer to a more squamous-like subtype (Brunton et al., 2020). Several studies have also shown that the cancer progresses to an invasive basal/squamous subtype through epigenetic modifications at the level of super enhancers mediated by the transcription factor TP63 (Andricovich et al., 2018; Hamdan and Johnsen, 2018; Somerville et al., 2018). However, more studies are needed to determine whether there is a consistent change in methylation patterns in the subtypes of PDAC and whether oncogenic KRAS controls the epigenomic changes that are crucial for cancer phenotype.

Epigenetic regulation has been shown to impact drug response. One example is the promoter methylation of the gene *MGMT* (O-6-methylguanine-DNA methyltransferase), coding for a DNA repair enzyme, which was shown to increase sensitivity to drugs such as carmustine and temozolomide in gliomas (Esteller et al., 2000; Hegi et al., 2005). In a multi-omic analysis on PDAC xenografts, the cholesterol transporter NPC1L1 (NPC1 like intracellular cholesterol transporter 1) was identified as a potential therapeutic target and found to be highly epigenetically deregulated. High levels of NPC1L1 were observed in PDAC tumors of the classical subtype and low levels in the basal/squamous subtype. Interestingly, the NPC1L1 inhibitor ezetimibe was more effective on basal subtype PDAC cells than classical subtype, as the inhibitor also functions as a cholesterol competitor, thereby also supporting the implication for metabolic approaches in PDAC treatment (Nicolle et al., 2017).

A potential resource to identify additional epigenetic deregulated targets are databases. Pancreatic Cancer Methylation Database (PCMdb) (http://crdd.osdd.net/raghava/pcmdb/) is a database that was developed to support the identification of DNA methylation-based biomarkers in pancreatic cancer. It provides data on the DNA methylation status of 4,342 genes from PDAC cell lines and tissues (Nagpal et al., 2014). It further integrated drug resistance data from another database, the Cancer Drug Resistance Database (CancerDR) (http://crdd.osdd.net/raghava/cancerdr/) which provides information on 148 anti-cancer drugs and their pharmacological profiling across distinct cancer cell lines (Kumar et al., 2013). These different database tools will facilitate the concept of personalized medicine and highlight the importance of utilizing DNA methylation of genes as an effective predictor of response to chemotherapeutic cancer drugs (Nagpal et al., 2014).

In summary, epigenetic regulation has implications in the phenotype, drug response and clinical outcome of PDAC and epigenetic alterations can be considered as another major driving

factor in PDAC oncogenesis. Therefore, targeting epigenetic changes in mutant KRAS dependent PDAC could be a new therapeutic intervention.

WNT PATHWAY IS ANOTHER FACTOR IN PDAC CHEMORESISTANCE

The Wnt signaling pathway is crucial for stem cell maintenance, tissue repair, embryonic development and differentiation and plays a vital role in pancreatic organ development (Clevers et al., 2014). For pancreatic specification during embryonic development, Wnt pathway inhibition is needed, while activation of the Wnt pathway is required for the growth and maintenance of the organ and differentiation of pancreatic progenitor cells into exocrine and endocrine lineages (Murtaugh and Kopinke, 2008a; Murtaugh, 2008).

The Wnt pathway can be divided into either canonical or non-canonical pathways (Miao et al., 2013). In the canonical Wnt signaling pathway, the central molecule is β-catenin (Miao et al., 2013). Upon binding of a Wnt-protein ligand to Frizzled receptors in association with the co-receptor lipoprotein receptor–related protein (LRP)-5/6, several downstream signaling cascades occur resulting in the accumulation of β-catenin and its translocation to the nucleus where it binds with the transcription factor TCF and activates the transcription of Wnt pathway target genes (Nakamura et al., 2003; Sano et al., 2016).

Mutations of β-catenin are uncommon in PDAC (Bailey et al., 2016). However, *in vitro,* and *in vivo* studies have shown that the canonical Wnt signaling pathway influences PDAC tumorigenesis, and the majority of PDACs are characterized by an upregulated Wnt/β-catenin transcriptional signature (Zeng et al., 2006; Magliano et al., 2007).

The Wnt signaling pathway has been described to promote resistance to apoptosis and maintenance of cancer stem cells (CSCs), resulting in the pathogenesis of PDAC (Modi et al., 2016) and is also upregulated in the worst prognostic squamous subtype of PDAC (Fang et al., 2017).

It was shown that higher expression of canonical Wnt ligands such as *WNT2*, *WNT5A*, and *WNT7A* are highly implicated in pancreatic carcinogenesis (Jiang et al., 2014; Bo et al., 2016; Wu et al., 2018; Makena et al., 2019). Also, other Wnt pathway associated genes, such as Wnt antagonists *DKK1* (Dickkopf-1) and *HMGA2,* a member of the non-histone chromosomal high mobility group (HMG), played an important role in PDAC oncogenesis (Tang et al., 2018). In addition, activation of the non-canonical Wnt signaling pathway through GATA-binding factor 6 (GATA6), cyclin-dependent kinase 8 (CDK8) and R-spondin lead to a progression of PDAC (Zhong et al., 2011; Xu et al., 2015; Chartier et al., 2016). GATA6 promoted Wnt activation and PDAC growth through transcriptional downregulation of the secreted Wnt inhibitor DKK1 (Zhong et al., 2011).

Targeting the Wnt/β-catenin pathway is an actively prosecuted strategy in the treatment of PDAC (Krishnamurthy and Kurzrock, 2018). Several novel inhibitors of the Wnt/β-catenin pathway have been developed as well as monoclonal antibodies against Wnt ligands to block their oncogenic activity (Krishnamurthy and Kurzrock, 2018). The monoclonal antibody vantictumab (OMP-18R5), for instance, showed growth inhibition in breast, pancreatic, colon, and lung cancer xenograft models (Smith et al., 2013). Vantictumab is effective against PDAC in transgenic and xenograft models alone or synergistically with chemotherapy, including gemcitabine or nab-paclitaxel (Gurney et al., 2012).

Wnt antagonists have been applied successfully to sensitize also for other drug treatments, such a taxane, in PDAC models (Fischer et al., 2017; Makena et al., 2019). Treatment with taxanes, a class of chemotherapeutics that inhibits mitotic spindle degradation, as monotherapy had no significant effect on tumor cells with high Wnt signaling activity, leading to tumor cell accumulation (Fischer et al., 2017; Makena et al., 2019). However, sequential administration of Wnt antagonists such as vantictumab and ipafricept followed by taxane treatment prevented this selection for Wnt-active taxane-resistant tumor cells and demonstrated superior antitumor activity in PDAC models (Fischer et al., 2017). This combination strategy was also incorporated into PDAC phase I clinical trials (NCT02005315 and NCT02050178).

It was also shown that KRAS signaling increases the interaction of β-catenin with cAMP response element binding protein (CREB)-binding protein (CBP), interestingly creating a potential point for therapeutic intervention (Manegold et al., 2018). In this study, the specific small molecule CBP/β-catenin antagonist ICG-001 was used to investigate its effect on human PDAC cells in both an orthotopic mouse model and a human patient-derived xenograft (PDX) model of PDAC. ICG-001 sensitized PDAC cells and PDX tumors to gemcitabine treatment which significantly decreased the tumor volume (Manegold et al., 2018). ICG-001 has demonstrated anti-tumor effects in several tumor types (Grigson et al., 2015). Another inhibitor, PRI-724, was also shown to block the interaction between β-catenin and CBP (Lenz and Kahn, 2014). In PDAC cell lines, PRI-724 promoted differentiation of chemotherapy-resistant cancer stem cells and decreased the metastatic potential (Lenz and Kahn, 2014). A phase I clinical trial demonstrated that PRI-724 can be safely administered in combination with gemcitabine in PDAC patients (Ko et al., 2016).

The E3 ligase RNF43 (Ring Finger Protein 43) inhibits Wnt signaling by ubiquitinating Frizzled receptors for degradation (Tu et al., 2019). Mutations in *RNF43* occur in approximately 5–7% of PDAC (Aguilera and Dawson, 2021) and may serve as a useful biomarker for patient selection during clinical development of Wnt inhibitors, as it was shown that RNF43 mutant PDAC cell lines and xenograft models were sensitive to the porcupine inhibitor LGK974 (Jiang et al., 2013). Porcupine is an acyltransferase required for the secretion and activity of Wnt ligands (Proffitt and Virshup, 2012) and its inhibitor LGK974 is currently tested in a phase I clinical trial in several cancers including PDAC (NCT01351103).

The microRNA MiR-29a was reported to induce gemcitabine chemoresistance via the canonical Wnt signaling pathway (Nagano et al., 2013). The inhibition of Wnt signaling could

reverse this chemoresistance to gemcitabine in PDAC (Nagano et al., 2013). MiR-33a, on the other hand, was reported to increase gemcitabine sensitivity in human PDAC cells by downregulating the nuclear translocation of β-catenin (Liang et al., 2015). The tyrosine kinase inhibitor masitinib also increased the sensitivity of pancreatic cell lines to gemcitabine by downregulation of the Wnt/β-catenin pathway (Jia and Xie, 2015). Wnt/β-catenin signaling is also associated with 5-FU resistance in PDAC cells as demonstrated in a 2018 study by Cao et al., which showed that the inhibition of glypican-4 (GPDAC4), a member of the glypican family and regulator of Wnt/β-catenin signaling, increased sensitivity to 5-FU in PDAC cells (Cao et al., 2018).

In summary, the Wnt signaling is another pathway involved in PDAC tumorigenesis and development of chemoresistance and targeting this pathway in combinatorial treatment approaches is an actively pursued strategy.

DUALITY ROLE OF TGF-β PATHWAY IN PDAC

The transforming growth factor β (TGF-β) pathway plays an important, context-dependent role as both a tumor suppressor and a promoter of PDAC and is altered in 47% of PDAC cases (Bailey et al., 2016; Dardare et al., 2020). SMAD4 is an essential signal transducer of the canonical TGF-β pathway (Dardare et al., 2020) and is inactivated in approximately 60% of PDAC cases (Hahn et al., 1996). Thus, precision targeting of the TGF-β/SMAD4 pathway could be critical in the treatment of PDAC (Ahmed et al., 2019).

An alteration in *SMAD4* is generally associated with worse overall survival in both primary and metastatic PDAC demonstrating the importance of the canonical TGF-β pathway (Singh et al., 2011; Yamada et al., 2015; Shugang et al., 2016; Zhao et al., 2018). The squamous molecular subtype also presents activation of TGF-β signaling pathway (Bailey et al., 2016).

In normal pancreatic cells and in stages I and II of PDAC, TGF-β signaling acts as a tumor suppressor inhibiting cell proliferation (Glazer et al., 2014; Dardare et al., 2020). On the other hand, TGF-β signaling has been shown to have tumorigenic activity in many late-stage malignancies, including PDAC, due to severe dysregulations, suggesting an explanation for the apparent TGF-β paradox (Principe et al., 2014; Melzer et al., 2017).

Upregulated and overexpressed TGF-β has been shown to induce stromal proliferation in PDAC tumor microenvironment, promote EMT leading to metastases and consequently is a potential target for cancer therapy (Pickup et al., 2013; David et al., 2016; Hussain et al., 2018). TGF-β targeted therapy is established for various human cancers and data from several preclinical and clinical studies indicates that TGF-β blockade could be effective in the treatment of PDAC as well (Ahmed et al., 2019).

There are three possible approaches to target TGF-β signaling: inhibition at the translational level, inhibition at the ligand-receptor level, and inhibition of receptor-mediated signaling (Massagué, 2008). The goal of each of these targeted therapy approaches is to inhibit the tumor-promoting function and maintain the tumor-suppressive function of TGF-β (Haque and Morris, 2017). Another approach is to target TGF-β induced EMT that plays a critical role in PDAC progression and metastatic disease development (Alvarez et al., 2019).

The use of anti-TGF-β-based therapies in phase I and II clinical trials in metastatic PDAC highlights the importance of understanding the role of TGF-β in PDAC progression (Alvarez et al., 2019). A phase I/II clinical study has shown a survival benefit in PDAC and melanoma using AP-12009, an antisense oligonucleotide that acts directly against the mRNA of TGF-β2 (Nemunaitis et al., 2006; Oettle et al., 2011). Monoclonal antibodies targeting the ligand-receptor binding and preventing subsequent signaling of the TGF-β pathway (Gomez-Puerto et al., 2019) are under clinical investigation such as lerdelimumab (CAT-152, Trabio™ for TGF-β2) and metelimumab (CAT-192 for TGF-β1) (Ahmed et al., 2019).

Immunological pathways have proven to be successful targets in the treatment of other cancers, but not in PDAC (Hilmi et al., 2018). It was previously shown that TGF-β exerts an immunosuppressive function in the tumor immune microenvironment by antagonizing interleukin 15-mediated proliferation of natural killer (NK) cells (Wilson et al., 2011). This immunosuppressive function of NK and T cells through the SMAD-dependent canonical TGF-β pathway is another key role for TGF-β in promoting tumor immune evasion (Thomas and Massagué, 2005; Trotta et al., 2008). TGF-β signaling inhibition was shown to reverse this immune evasion function by restoring immune activity against tumor cells (Wilson et al., 2011).

Checkpoint inhibitors are another immune-targeting approach in treating cancer (Darvin et al., 2018). Monotherapy with checkpoint inhibitors failed to elicit efficacy in PDAC patients (Henriksen et al., 2019). However, there is growing evidence that combining checkpoint inhibitors with TGF-β signaling inhibition may prolong survival in several cancers (Bai et al., 2019).

The SMAD-independent or non-canonical TGF-β pathways include several branches that lead to activation of the Rho-like GTPase signaling pathway, PI3K/AKT pathway and/or MAP kinase pathway (Zhang, 2009). The MAP kinase pathway component ERK upregulates the cyclin-dependent kinase inhibitor 1A (CDKN1A), also called p21, thereby facilitating TGF-β-mediated cell cycle arrest (Tang et al., 2002; Torii et al., 2006). An ERK-induced cell cycle arrest through TGF-β/SMAD4 mediated *CDKN1A* upregulation was also observed in benign pancreatic cell lines (Principe et al., 2017). While ERK can contribute to tumor-suppressive TGF-β signaling in normal pancreatic epithelial cells, TGF-β-induced activation of ERK can be very damaging in the disease state (Principe et al., 2017). For this reason, the crossover between TGF-β and ERK signaling pathways deserves further attention, particularly regarding the functional switch from tumor-suppressive to tumor-promoting TGF-β signaling.

Therapies targeting TGF-β signaling have been investigated in the preclinical and clinical setting and have shown efficacy in PDAC (Shen et al., 2017). However, the paradoxical effects of TGF-β in human cancers are poorly understood, and although

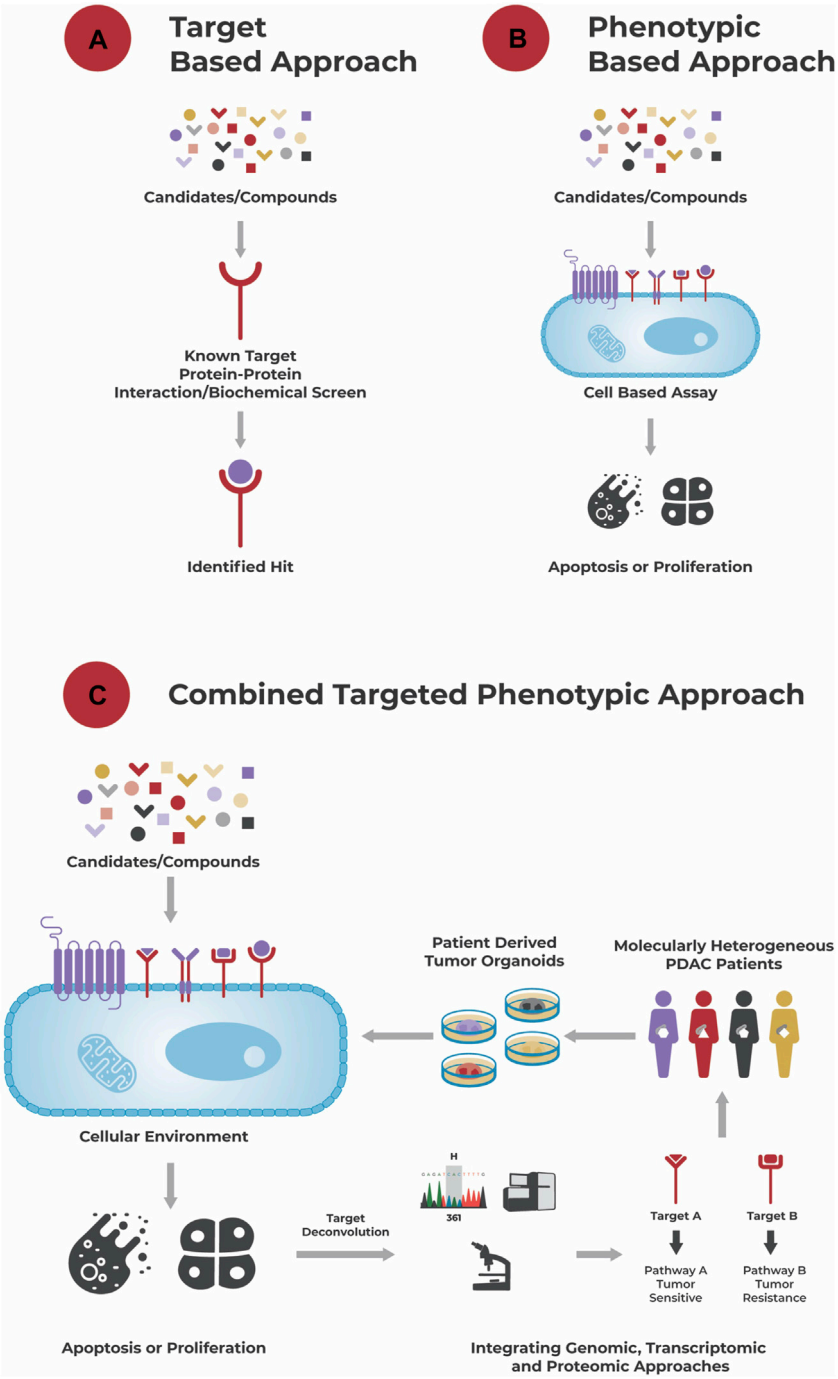

FIGURE 1 | Different approaches to drug discovery. **(A)**: A target-based approach aims to identify a hit to a known target using a biochemical screen. **(B)**: A phenotypic-based approach identifies hits based on their effect in a cell-based assay, e.g., their ability to induce apoptosis or growth arrest, without necessarily knowing the compound's biochemical target and its role in disease biology. **(C)**: A combined targeted phenotypic approach leverages both, a target-based and phenotypic approach. The effect of compounds is tested in a cellular environment and hits selected based on their effect on the cells' phenotype. A powerful model system here are patient-derived organoids that recapitulate the genetic and molecular alterations of a patient's tumor, and which are for example used in the approach of Reversed Clinical Engineering®. Then, using target deconvolution strategies like the integration of genomic, transcriptomic, and proteomic data, mechanisms (induced by the "hits") conferring to drug sensitivity and/or resistance and suitable compounds for treatment can be identified for each individual patient tumor.

therapies targeting the TGF-β pathway have merit, it is important to ensure that they target only the tumor-promoting effect.

COMBINED TARGETED PHENOTYPIC APPROACH UTILIZING PATIENT DERIVED ORGANOIDS—A PROMISING STRATEGY FOR PDAC TREATMENT DEVELOPMENT

So how can we translate our knowledge about the manifold dysregulations and compensatory mechanisms driving PDAC into more effective treatment options for patients?

To identify effective treatments for cancer, knowledge about commonly found aberrations and dysregulations is invaluable. This "target-based approach" (**Figure 1A**) aims to identify compounds that specifically act on a previously defined target in the context of such known, often genetic mutation-based dysregulations to attack the tumor. Unfortunately, in PDAC targeting major genomic dysregulations such as those involved in KRAS or PI3K signaling did not result in effective therapies until now (Van Cutsem et al., 2004; Javle et al., 2010; Milroy and Ottmann, 2014).

An alternative "phenotypic based approach" (**Figure 1B**) aims to identify compounds that elicit a growth inhibitory or apoptotic effect on tumor cells in a cell-based assay without necessarily requiring knowledge about the tumor's dysregulations and/or compounds' mechanism of action. This functional approach takes the tumor's complexity, interplay between different dysregulations, compensatory mechanisms and tumor heterogeneity into account and allows identification of compounds acting through unprecedented drug mechanisms (Moffat et al., 2017; Swinney and Lee, 2020).

One step further goes the "combined targeted phenotypic approach" (**Figure 1C**). After testing different single agents and combinatorial compounds in a phenotypic assay, allowing the rapid identification of responders and non-responders at a cellular level, integration of e.g., genomic, transcriptomic and/or proteomic data to those responders and non-responders allows identification of underlying drug sensitivity and/or resistance mechanisms (targets). Besides identification of effective compounds, potentially in a personalized manner, this approach can also be used for identification of biomarkers related to sensitivity or resistance towards a specific compound.

An essential prerequisite for the combined targeted phenotypic based approach is a suitable *in vitro* cancer model. Here patient-derived organoids are of specific interest and allow to identify the therapeutic responses of individual patient tumors including PDAC (Tiriac et al., 2018; Driehuis et al., 2019).

Patient-derived organoids are 3D cell culture models derived from small pieces of tissue, for example from a patient's primary tumor or a metastasis (Silvestri et al., 2017; Schumacher et al., 2019; Pfohl et al., 2021). Culture conditions allow cancer stem-like cells to self-organize and form miniature versions of a patient's tumor, i.e., organoids, that recapitulate the genetic, histologic, and molecular alterations of the tumor (Boj et al., 2015; Schütte et al., 2017; Driehuis et al., 2019), including its intra-tumor heterogeneity and functional phenotype (Schumacher et al., 2019; Pfohl et al., 2021). Further, patient-derived organoids were shown to be a suitable tool to predict treatment response in cancer patients (Vlachogiannis et al., 2018; Wensink et al., 2021) and can be used for high-throughput drug screens (Boehnke et al., 2016) and genetic manipulation (Boj et al., 2015).

Several studies have described the successful establishment of tumor organoid cultures from PDAC patients (Boj et al., 2015; Tiriac et al., 2018; Driehuis et al., 2019; Tuveson and Clevers, 2019). Organoid cultures were shown to recapitulate the hybrid nature of PDAC exhibiting distinct subtypes in a single tumor (Chan-Seng-Yue et al., 2020; Hayashi et al., 2020; Juiz et al., 2020).

Miyabayashi et al. performed an experiment in mice in 2020 where they carefully injected *in vitro* generated organoids directly into the pancreatic duct, the site where the pre-invasive pancreatic neoplasms originate and progress, and followed the patterns of intraepithelial neoplasms to gain cellular and molecular insights into the mechanisms promoting progression of PDAC (Miyabayashi et al., 2020). The organoids implanted in the duct, referred to as intraductally grafted organoid (IGO), gave rise to two different classes of neoplasm with distinct phenotypes, fast growing and slow growing, recapitulating the histologic heterogeneity of the tissue from which the organoids were derived. Fast progressor organoid-derived neoplasm recapitulated the basal-like or squamous subtype, showing invasiveness, migration from the duct, hyperactivation of the RAS pathway and activating cancer associated fibroblasts (CAFs). While the slow growing organoid-derived neoplasm recapitulated the classical or progenitor subtype and were more contained within the ducts (Miyabayashi et al., 2020). The study further showed that *KRAS* copy number was increased and the KRAS pathway was hyperactivated in the fast-growing organoids, whereas slow growing organoids showed increased expression of *GATA6*, a marker of the classical subtype, and low copy number for *KRAS* (Miyabayashi et al., 2020).

PDAC derived organoids were successfully used to assess drug sensitivity (Huang et al., 2015; Tiriac et al., 2018; Driehuis et al., 2019). Importantly, when treated with standard-of-care chemotherapeutics, the treatment response of PDAC-derived organoids was shown to correspond to that of the patients the models were originating from (Tiriac et al., 2018). This further supports the notion that patient-derived organoids have the potential to be used as personalized model of PDAC, predict therapy responses, thereby enabling prospective treatment selection and identification of new therapeutic strategies and can also be exploited for genomic and functional studies (Tiriac et al., 2018; Driehuis et al., 2019).

To allow utilization of the potential of patient-derived organoids as preclinical models but also to address the need for personalized oncology approaches, large collections of patient-derived tumor organoids and matching healthy (normal) organoids, i.e., organoid biobanks from various tumor entities including PDAC, were generated (van de Wetering et al., 2015; Drost and Clevers, 2018; Sachs et al., 2018; Calandrini et al., 2020; Botti et al., 2021). These

biobanks contain genetically diverse cultures and are suitable to represent disease heterogeneity. Together with the integration of genomic and drug screening data, these organoid biobanks facilitate drug development by predicting the drug response and toxicity profiles for individual patients (van de Wetering et al., 2015), thereby including personalized medicine in the treatment discovery process. Furthermore, biobanks allow future assessment of newly developed drugs or combinations on the same tumor models and direct comparison with current treatment effects.

However, there are also limitations such as the need for optimization of culture methods for individualized normal and tumor cultures (Kondo et al., 2011). Organoid models can only be established under specific culture conditions, and these dependencies often reflect differences in the tumor's mutational background (Fujii et al., 2016). However, in most cases the genetic background is not yet determined when establishing organoids from fresh patient tissues. This could lead to an underrepresentation of some alterations inhibitory to organoid derivation in the resulting biobanks. Poorly and moderately differentiated PDAC derived organoids could not be established in a biobank effort, further supporting the fact that distinct tumor subtypes require distinct culture conditions (Huang et al., 2015; Fujii et al., 2016).

Small scale drug screens on organoid biobanks already yielded promising results (Gao et al., 2014; van de Wetering et al., 2015; Sachs et al., 2018)) and further efforts to generate large, standardized, globally accessible banks of organoid models for the research community will continue to facilitate drug development and enhance personalized medicine.

Reverse Clinical Engineering® is an approach that combines a phenotypic based assay with a target deconvolution strategy in a single system utilizing patient-derived organoids (**Figure 1C**). It is a technique that establishes patient-derived organoids from individual patient tumors, exposes them to a wide range of therapeutic regimen and integrates the treatment response with genomic, transcriptomic, and proteomic data. This approach does not only allow identification of functionally effective compounds for the individual tumor, meaning patient, but also deciphers the tumor's molecular characteristics driving its therapy sensitivity or resistance. Reverse Clinical Engineering® can be performed on an individual patient-derived organoid model, identifying the Achilles heel of this specific tumor, or on a collection of patient-derived organoids from a biobank of e.g., a specific cancer type (PDAC or other) to identify for example a new treatment strategy and/or biomarker for this tumor type. Either way, this approach of functionally profiling tumors in a potentially high through-put manner is of high potential for real personalized oncology (Pfohl et al., 2021) and a promising strategy to finally identify effective treatment options for PDAC patients.

Patient-derived organoids have the potential to connect compound screening and clinical trials. However, establishing distinct culture and assay conditions for each distinct tumor entity and constant supply of patient material for large screens remain a major challenge in using this combined targeted phenotypic approach with patient-derived organoids (Boehnke et al., 2016). Efficient establishment of organoid cultures for different entities, drug assays including initial seeding material and density, treatment regimen, assay reproducibility, evaluation of drug response (e.g., IC50, area-under-the curve, Z-score IC50), drug validation, and assay scalability remain technical constraints and all need to be optimized (Boehnke et al., 2016; Phan et al., 2019; Bergdorf et al., 2020) In addition, potential off-target toxicity cannot be assessed by organoid monocultures (Pfohl et al., 2021). Nevertheless, patient derived organoids are a powerful tool which can be further expanded to take the tumor microenvironment into account, known to impact drug response, through suitable co-culture systems with e.g., cancer associated fibroblasts or immune cells (Tsai et al., 2018).

DISCUSSION

PDAC is a highly heterogeneous disease with a complex combination of genetic, epigenetic, metabolic, and microenvironment dysregulations. Each patient exhibits distinct molecular alterations, different gene expression profiles and specific therapeutic responses.

Although genomic studies support the notion that PDAC is associated with only 4 driver mutations (*KRAS*, *CDKN2A*, *TP53*, and *SMAD4*) and perceived to be uniformly aggressive, a high level of clinical heterogeneity exists. High level of intrinsic cell plasticity, a random nature of genomic instability, constellations of genomic aberrations rather than a single event, dynamic epigenetic modulations and involvement and contribution of non-genetic features such as tumor-microenvironment with high desmoplastic stroma and low vascularity together contribute for the emergence of distinct phenotypic states in PDAC, making it highly heterogenous and exhibiting different treatment responses, often with pronounced drug resistance (Adams et al., 2019; Chan-Seng-Yue et al., 2020; Miyabayashi et al., 2020). Further, studies have shown that for most PDAC cases there is no association between genetic mutations and therapeutic responses (Witkiewicz et al., 2016; Knudsen and Witkiewicz, 2017), supporting the notion that multiple mechanisms of dysregulation need to be taken into account.

The molecular und clinical heterogeneity of PDAC with distinct subtypes having different biologic and prognostic relevance, the alterations at various levels and multiple layers of dysregulation highlight the need for precision oncology to treat this complex cancer (**Figure 2**).

The pronounced heterogeneity of PDAC illustrates clearly that context matters and needs to be considered to develop effective treatment strategies. While 90% of PDAC contain a *KRAS* mutation (Almoguera et al., 1988; Raphael et al., 2017), strategies to target (just) this aberration did not yet lead to an improved PDAC treatment. As highlighted throughout this review, additional layers of dysregulation are present beyond genomic aberrations and other signaling pathways are deregulated in addition to KRAS, although often conferring

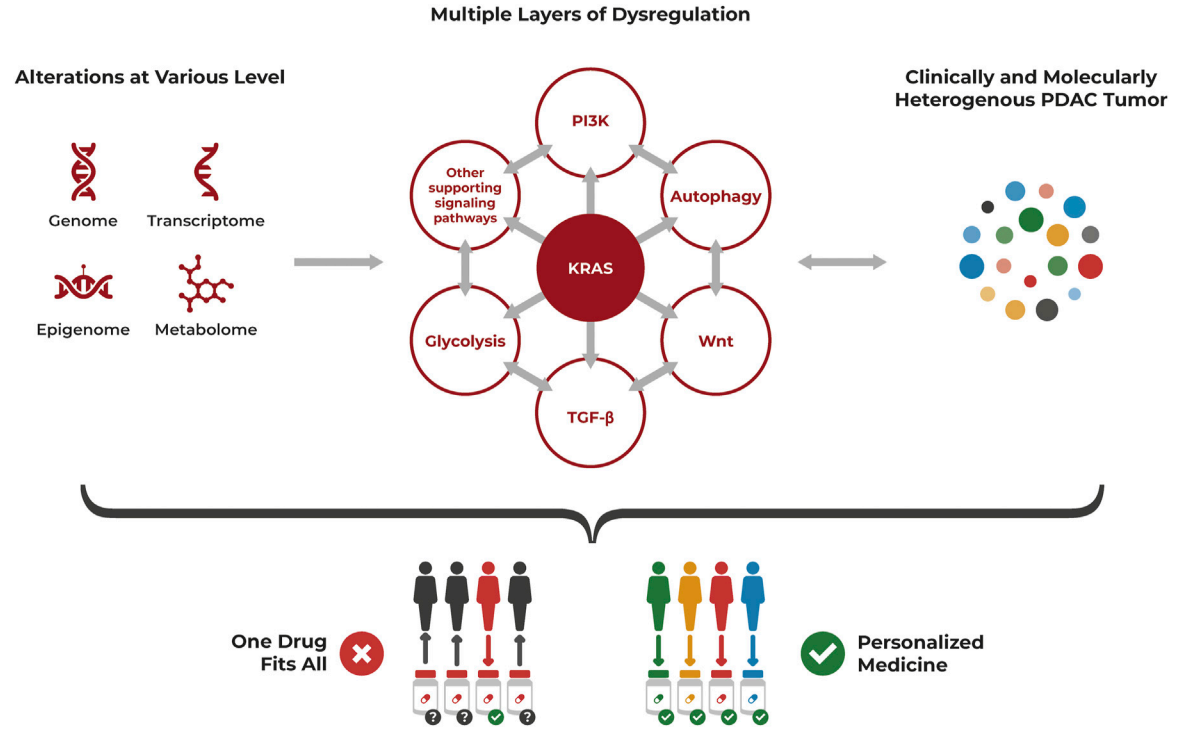

FIGURE 2 | PDAC heterogeneity requires a personalized approach for treatment. PDAC is a highly heterogenous cancer at the cellular level, often driven by *KRAS* mutations but with multiple layers of dysregulation affecting each other, that also need to be considered. Alterations occur at various levels including genomic, transcriptomic, epigenomic and metabolomic aberrations. The resulting clinically and molecularly heterogenous tumor is unlikely to be successfully treated with a "one drug fits all" approach but requires a personalized approach specifically targeting its individual dysregulations.

with it. These dysregulations are not only cancer drivers by themselves but can also act in a compensatory way if one aberration is targeted by therapy.

Despite several inhibitors for distinct effector pathways developed and tested in pre-clinical models and clinical trials, adaptive resistance is still a major hurdle. When KRAS or MEK or mTORC1/2 are inhibited individually in PDAC cells, tumor cell plasticity and rapid adaptation to stress activates compensatory pathways and favors the survival of tumor cells (Brown et al., 2020). Hence, identifying co-vulnerabilities in these tumors often driven by mutant KRAS and developing combinatorial and concurrent inhibitory strategies will prevent the ability of PDAC to survive through compensatory growth promoting pathways. However, limitations in combinatorial approaches include the selection of appropriate doses of individual therapies for optimal efficacy, considering for the presence of off-target, normal cell toxicity and different molecular subtypes of PDAC exhibiting different sensitivity profiles. In addition, the mechanisms of how cancer cells get adapted to several layers of inhibition is a complex phenomenon which is yet to be understood.

Each PDAC tumor is unique. Even if alterations occurred on the same level or by the same mechanism, the exact changes within the cancer cells are still heterogenous (Tew et al., 2020). Further, the combinations of dysregulations and alterations are heterogenous. One of the main reasons for multi-drug resistance is the genetic and molecular heterogeneity of cancer cells (Gottesman et al., 2002). The impact of genetic alterations depends on oncogenic contexts and several studies showed that dysregulations should be analyzed in a tissue- or cancer entity specific manner (Eser et al., 2013; Foggetti et al., 2021). Therefore, to identify much needed, effective treatment strategies for PDAC, its heterogeneity needs to be considered. This translates into the need for personalized oncology with better patient stratification and treating individual patients separately by functionally profiling the individual tumors.

As a potential solution, this review highlighted the combined targeted phenotypic approach as a promising strategy to identify effective compounds using a cell-based assay, allowing to take this tumor heterogeneity and the context of various unique alterations into account—given suitable cellular models are used. A powerful tool for this approach are patient-derived organoids as these are suitable models for tumor heterogeneity and personalized oncology, but also for high-throughput drug screens (Pfohl et al., 2021).

In summary, the tumor driving mechanisms and associated molecular alterations are different for each tumor and due to this heterogeneity, it is basically impossible to develop a drug or treatment regimen that will be effective for all PDAC patients. Overcoming chemoresistance and identifying chemo sensitive signatures using pharmacogenomic profiling is emerging as a new way forward and reason for hope. Although target-based

screening strategies that screened vast compounds for a single target have identified many "best in class" compounds, most of the "first in class" compounds in earlier decades were identified using phenotypic screening i.e., screening vast compounds on cells without a known target (Moffat et al., 2017). The sweet spot for the future lies in combining these target-based and phenotypic approaches in a single system.

Reverse Clinical Engineering®, a combined targeted phenotypic approach using patient-derived organoids, enables to identify effective drugs or drug combinations for an individual tumor within a cellular context, and deconvoluting the treatment's mechanism of action by utilizing technological advances in genomics, transcriptomic and/or proteomics. The application of patient-derived organoids allows to incorporate tumor heterogeneity—a factor not yet sufficiently considered in PDAC therapy.

This approach of functionally profiling individual tumors in a potentially high throughput fashion utilizing automated liquid robotic system offers several advantages and has potential applications also in the rare disease space (Puca et al., 2018; Phan et al., 2019). It offers the ability to model also rare tumors and poses an opportunity to identify drugs in a disease and mechanism agnostic manner (Phan et al., 2019).

In conclusion, PDAC is a complex and heterogenous cancer with currently insufficient treatment options. Drug screening using patient-derived organoids of PDAC can factor in tumor heterogeneity and the context of the multi-layered dysregulations to identify new treatment strategies and transform PDAC therapy to personalized oncology.

AUTHOR CONTRIBUTIONS

Conceptualization: SS, LW; resources: CRAR; writing—original draft preparation: SS, UP, and LW; writing—review and editing: LW, CR, and CRAR; visualization: SS, LW; supervision: LW, CRAR. All authors have read and agreed to the published version of the manuscript.

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Clinical Impact of Molecular Subtyping of Pancreatic Cancer

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Pancreatic ductal adenocarcinoma is a highly lethal malignancy, which has now become the seventh most common cause of cancer death in the world, with the highest mortality rates in Europe and North America. In the past 30 years, there has been some progress in 5-year survival (rates increasing from 2.5 to 10%), but this is still extremely poor compared to all other common cancer types. Targeted therapies for advanced pancreatic cancer based on actionable mutations have been disappointing, with only 3–5% showing even a short clinical benefit. There is, however, a molecular diversity beyond mutations in genes responsible for producing classical canonical signaling pathways. Pancreatic cancer is almost unique in promoting an excess production of other components of the stroma, resulting in a complex tumor microenvironment that contributes to tumor development, progression, and response to treatment. Various transcriptional subtypes have also been described. Most notably, there is a strong alignment between the Classical/Pancreatic progenitor and Quasi-mesenchymal/Basal-like/Squamous subtype signatures of Moffit, Collinson, Bailey, Puleo, and Chan-Seng-Yue, which have potential clinical impact. Sequencing of epithelial cell populations enriched by laser capture microscopy combined with single-cell RNA sequencing has revealed the potential genomic evolution of pancreatic cancer as being a consequence of a gene expression continuum from mixed Basal-like and Classical cell populations within the same tumor, linked to allelic imbalances in mutant KRAS, with metastatic tumors being more copy number-unstable compared to primary tumors. The Basal-like subtype appears more chemoresistant with reduced survival compared to the Classical subtype. Chemotherapy and/or chemoradiation will also enrich the Basal-like subtype. Squamous/Basal-like programs facilitate immune infiltration compared with the Classical-like programs. The immune infiltrates associated with Basal and Classical type cells are distinct, potentially opening the door to differential strategies. Single-cell and spatial transcriptomics will now allow single cell profiling of tumor and resident

immune cell populations that may further advance subtyping. Multiple clinical trials have been launched based on transcriptomic response signatures and molecular subtyping including COMPASS, Precision Promise, ESPAC6/7, PREDICT-PACA, and PASS1. We review several approaches to explore the clinical relevance of molecular profiling to provide optimal bench-to-beside translation with clinical impact.

Keywords: molecular subtypes, transcriptomes, structural variants, precision medicine, next generation sequencing, clinical trials, ESPAC

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), a distinct form of pancreatic cancer, remains a major oncological challenge (Kleeff et al., 2016). Globally the 5-year pancreatic cancer prevalence in 2020 was 4.87 per 10^5 per year (International Agency for Research on Cancer, 2021). The number of cases of pancreatic cancer worldwide in 2020 was 495,773 (world rank for all cancers = 13), with 466,003 deaths (world rank for all cancers = 7); incidence rates per 10^5 per year were 5.7 for men and 4.1 for women, and mortality rates of 4.9 and 4.5, respectively (International Agency for Research on Cancer, 2021). In Europe, there were 140,116 new cases with 132,134 deaths (International Agency for Research on Cancer, 2021). In North America, there were 62,643 new cases and 53,277 deaths, the fourth highest cancer mortality in both men and women (International Agency for Research on Cancer, 2021; Siegel et al., 2021). For Western Europe, the incidence rates per 10^5 per year were 9.9 for men and 7.4 for women, with mortality rates of 8.6 and 7.8, respectively (world rank first for pancreatic cancer) (International Agency for Research on Cancer, 2021). In 2017, in Germany, there were 18,687 new cases (with a rising incidence rate) and 18,005 deaths, but with a slight improvement in the 5-year survival rate from 8% in 2007–2008 to 9% in 2015–2016 (Zentrum für Krebsregisterdaten, 2021). In the United States, the 5-year survival rate for all stages has further improved to 10% (Siegel et al., 2021).

STANDARD THERAPIES FOR PANCREATIC CANCER

Most patients present with metastatic disease, with only 10–20% being diagnosed with localized pancreatic cancer that can be surgically removed, while the remaining 20–30% have non-metastatic locally advanced disease that cannot be removed by standard surgical techniques (Kleeff et al., 2016). Systemic chemotherapy is the only conventional approach for improving survival in patients with advanced disease with the best achievable median survival rates being 8–12 months for metastatic disease and 12–15 months for locally advanced pancreatic cancer (Burris et al., 1997; Cunningham et al., 2009; Conroy et al., 2011; Von Hoff et al., 2013; Gill et al., 2016; Wang-Gillam et al., 2016; Springfeld et al., 2019). Although chemoradiotherapy is often used for locally advanced disease especially in the United States, there is increased toxicity without improvement in overall survival (Sultana et al., 2007; Chauffert et al., 2008; Hammel et al., 2016). In patients with locally resectable tumors but without metastatic disease, advances in surgical techniques and the use of adjuvant systematic chemotherapy have increased 5-year survival rates from 8% with resection alone to 30–50% in conjunction with adjuvant chemotherapy most notably using gemcitabine and capecitabine or modified folinic acid, 5-fluorouracil (5-FU), irinotecan, and oxaliplatin (mFOLFIRINOX) combinations (Neoptolemos et al., 2004; Oettle et al., 2007; Neoptolemos et al., 2010; Kleeff et al., 2016; Uesaka et al., 2016; Neoptolemos et al., 2017; Conroy et al., 2018; Strobel et al., 2019). Patients with borderline resectable disease may benefit from neoadjuvant chemotherapy regimens comprising gemcitabine with capecitabine as well as mFOLFIRINOX, while regimens with chemoradiotherapy are inferior to chemotherapy alone (Ghaneh et al., 2020; Katz et al., 2021). Neoadjuvant therapy may also increase resectabilty with improved survival in patients with otherwise unresectable local disease due to major vessel encasement using comprising mFOLFIRINOX or gemcitabine-based regimens with either capecitabine or nab-paclitaxel (Hackert et al., 2016; Diener et al., 2021; Kunzmann et al., 2021). An argument has been made to extend the use of neoadjuvant chemotherapy to patients with resectable disease, but this appears to be inferior for overall survival compared to upfront surgery and adjuvant treatment (Sohal et al., 2021).

The evolution of molecular targeted therapies, aimed at advancing tumor control and cell killing of pancreatic cancer, has so far met with only very limited progress (Davis et al., 2019; Golan et al., 2019; Mosele et al., 2020; O'Kane et al., 2020; Pishvaian et al., 2020; Cobain et al., 2021). While systemic chemotherapy is the mainstay of treatment when added to surgery, its impact is limited by a wide variation in responsiveness that is related to intrinsic and acquired mechanisms of sensitivity and resistance by both the cancer cells themselves and the stromal environment (Greenhalf et al., 2014; Martinez-Balibrea et al., 2015; Noll et al., 2016; Amrutkar and Gladhaug, 2017; Geller et al., 2017; Martinelli et al., 2017; Schlitter et al., 2017; Neoptolemos et al., 2018; Tiriac et al., 2018; Dominguez et al., 2020; Kalimuthu et al., 2020; Sahai et al., 2020). Going one step further, the integration of molecular subtypes derived from global genomic and transcriptomic analyses into clinical trials is enabling translational insights into how we might better refine existing and evolving therapy modalities to improve pancreatic cancer treatment. Pancreatic cancer is almost unique in promoting an excess production of other components of the admixture of general tissue (stroma), resulting in a complex tumor microenvironment that contributes to

tumor development, progression, and response to treatment (Kleeff et al., 2016).

SINGLE GENE ALTERATIONS IN PANCREATIC CANCER

Mutations in genes responsible for producing classical canonical signaling pathways including driver oncogenes and dysfunction of tumor suppressor genes include KRAS, TP53, CDKN2A, and SMAD4 in most cases, and ARID1A, KDM6A, MLL3, TGFBR2, RBM10, BCORL1, and ROBO2 in 5–10% of tumors (Jones et al., 2008; Waddell et al., 2015; Bailey et al., 2016; Chan-Seng-Yue et al., 2020; Escobar-Hoyos et al., 2020). Genetic alterations occur in each of a core set of 12 cellular signaling pathways in 67–100% of the tumors, with representative genes listed below (Jones et al., 2008).

- Apoptosis: CASP10, VCP, CAD, HIP1
- DNA damage control: ERCC4, ERCC6, EP300, RANBP2, TP53, BRCA1/2, PALB2, ATM, ATR, MLH1, MSH2, MSH6, RPA1, STK11, FANCA, FANCC
- Regulation of G1/S phase transition: CDKN2A, FBXW7, CHD1, APC2
- Hedgehog signaling: TBX5, SOX3, LRP2, GLI1, GLI3, BMPR2, CREBBP
- Homophilic cell adhesion: CDH1, FAT, PCDH15, PCDHB16, PCDHGA1
- Integrin signaling: ITGA4, LAMA1, LAMA4, LAMA5, FN1, ILK
- c-Jun N-terminal kinase signaling: MAP4K3, TNF, ATF2, NFATC3
- KRAS signaling: KRAS, MAP2K4, RASGRP3
- Regulation of invasion: ADAM11, DPP6, MEP1A, PCSK6, APG4A
- Small GTPase–dependent signaling: AGHGEF7, ARHGEF9, CDC42BPA
- TGF-β signaling: TGFBR2, BMPR2, SMAD4, SMAD3
- Wnt/Notch signaling: MYC, PPP2R3A, WNT9A, MAP2, TSC2, GATA6.

Single genetic alterations occur in <5% of tumors, notably BRCA1/2 mutations, BRAF gene fusions/mutations, ERBB2 amplifications/mutations, RNF43, TGFBR2, MAP2K4, MLL3, PIK3CA, RBM10, SMARCA4, PBRM1, SLIT2, KDM6A, GATA6, BRAF, ATM, and mismatch repair (MMR) gene mutations (Waddell et al., 2015). MMR genes (MLH1, MSH2, MSH6, and PMS2) normally recognize mistakes in insertion, deletion, or mismatched incorporation of nucleotides arising from errors by DNA polymerases and then replacing them with the correct nucleotides. As well as gene mutations, loss of MMR protein function may arise through promoter methylation especially in the case of MLH1. The consequence is an accumulation of errors in DNA microsatellites (short repetitive sequences in DNA) causing high microsatellite instability (MSI-H) (**Table 1**; Waddell et al., 2015; Mosele et al., 2020).

Clinical applicability of genetic biomarkers has been classified by the European Society for Medical Oncology (ESMO) Translational Research and Precision Medicine Working Group into the ESMO Scale of Clinical Actionability for molecular Targets (ESCAT) (Mateo et al., 2018). There are four main levels defined as follows: I = the match of an alteration and a drug has been validated in clinical trials, and should drive treatment decision in daily practice; II = a drug that matches the alteration has been associated with responses in phase I/II or in retrospective analyses of randomized trials; III = alterations that are validated in another cancer, but not in the disease-to-treat; IV = hypothetically targetable alterations based on preclinical data (Mateo et al., 2018; Mosele et al., 2020). So far, the clinical utility of targeting drugs to specific molecular alterations is rather limited (Hong et al., 2020; Mosele et al., 2020):

TABLE 1 | List of actionable single gene alterations to in advanced pancreatic ductal adenocarcinoma in accordance with ESMO Scale for Clinical Actionability of Molecular Targets (ESCAT) levels I–III (Mateo et al., 2018; Mosele et al., 2020).

Gene	Alteration	Prevalence	ESCAT Level
BRCA1/2	Germline mutations	1–4%	I
BRCA1/2	Somatic mutations	3%	III
MSH1, PMS2, MLH1, and MSH6	MSI-H	1–3%	I
NTRK	Fusions	<1%	I
$KRAS^{G12C}$	Mutation	1–2%	II
PIK3CA	Hotspot mutations	3%	III
$BRAF^{V600E}$	Mutations	3%	III
MDM2	Amplifications	2%	III
ERBB2	Amplifications/ mutations	1–2%	III
NRG1	Fusions	1%	III
ALK	Fusions	<1%	III
RET	Fusions	<1%	III
ROS1	Fusions	<1%	III

mFOLFIRINOX –preferred for known germline BRCA1/2 or PALB2 mutations.

Olaparib – a PARP inhibitor as maintenance therapy in patients who have a germline BRCA1 or BRCA2 mutation and with metastatic pancreatic cancer that had not progressed during first-line platinum-based chemotherapy, resulting in improved progression-free survival.

Entrectinib – an inhibitor of tropomyosin receptor kinases (TRKs) of tumors with NTRK or ROS-1 gene fusions.

Laroctrenib – an inhibitor of tropomyosin receptor kinases (TRKs) of tumors with NTRK gene fusions.

Afatinib – an EGFR tyrosine kinase inhibitor in KRAS wild-type tumors with NRG1 gene fusions.

Sotorasib – a small molecule that targets the KRAS p.G12C mutation that is present in 1–2% of PDAC patients (Hong et al., 2020).

Also, erlotinib, a multiple tyrosine kinase inhibitor (including EGFR) used with gemcitabine, produces an improved survival in metastatic pancreatic cancer, but this benefit is only marginal with increased toxicity.

ACTIONABLE GENOMIC SUBTYPES

Structural variations amongst the 25,000 defined human genomes include deletions, amplifications, duplications, and translocations (International Cancer Genome Consortium et al., 2010; Waddell et al., 2015). The Waddell signature based on whole-genome sequencing and copy number variation identified four subtypes based on patterns of chromosomal structural variation with potential clinical utility (**Table 2**; Waddell et al., 2015).

> **Stable (20%)**, with <50 structural variations per genome, with widespread aneuploidy.
> **Locally rearranged (30%)**, with >200 structural variants clustered on 1–2 chromosomes. Of these, about a 35% had focal amplifications in KRAS, SOX9 and GATA6, as well as ERBB2, MET, CDK6, PIK3CA, and PIK3R3 but were only present in 1–2% of patients. The remaining local rearrangements involved complex genomic events such as breakage–fusion–bridge or chromothripsis (thousands of clustered chromosomal rearrangements occurring in a single event in localized and confined genomic regions in one or two chromosomes).
> **Scattered (36%)**, with non-random chromosomal damage in 50–200 structural variants per genome.
> **Unstable (14%)**, with >200 structural variants distributed across the genome indicating defects in DNA maintenance (BRCA1/2, and PALB2 gene defects) and a mutational DNA damage repair (DDR) deficiency, with potential sensitivity to DNA-damaging agents. The unstable structural variation subtype is responsive to platinum therapy and BRCA1/2 germline carriers also sensitive to both platinum and PARP inhibitors.

It is estimated that 24% of all pancreatic cancers may be sensitive to platinum therapy based on an unstable genomic structural variation subtype, and/or somatic and germline mutations in BRCA genes, and/or a BRCA-type mutational signature (Waddell et al., 2015). MSI-H occurs in 1–3% of pancreatic cancers, which is commonly associated with mutations in the MSH2 and MLH1 MMR genes, and can be detected by immunohistochemistry (MSH1, PMS2, MLH1, and MSH6 expression) or sequencing (single gene mutations and MMR mutational signature) (Waddell et al., 2015; Connor et al., 2017).

MSI-H tumors express a large number of neoantigens, potentially rendering them more susceptible to immunotherapy in comparison to those tumors with relatively few mutations. DNA replication stress producing single-stranded DNA will induce DDR of which the DNA damage checkpoint kinase ATR [Ataxia-Telangiectasia Mutated (ATM) and Rad3-related protein kinase] is a critical component. Genes encoding subunits of SWI/SNF (BAF) chromatin remodeling complexes, each composed of approximately 15 protein subunits, include ARID1A, ARID1B, ARID2, PBRM1, SMARCA4, and SMARCB1. ARID1A deficiency will impair cells to recruit topoisomerase 2A to chromatin causing cell cycle defects. The consequence is increased reliance on ATR checkpoint activity and thereby increased sensitivity to ATR inhibitor therapy (Williamson et al., 2016).

TRANSCRIPTOMIC SUBTYPES

Various transcriptional pancreatic cancer subtypes have also been described, most notably the Moffit, Collinson, Bailey, Puleo, and Chan-Seng-Yue signatures amongst others, which have potential clinical impact (**Table 2**; Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016; Cancer Genome Atlas Research Network, 2017; Puleo et al., 2018; Wartenberg et al., 2018; Maurer et al., 2019; Chan-Seng-Yue et al., 2020; Dijk et al., 2020). Each study has used a different approach to deal with the low cellularity and stromal contribution, leading to some debate regards the value of those subtypes. Two dominant transcriptional subtypes have emerged: a Classical subtype that tends to be more responsive to chemotherapy and a very aggressive poorly differentiated Squamous/Basal-like subtype.

Collisson et al. (2011) used micro-dissected tumor samples from resected primary PDAC from two different clinical series to define three specific gene expression subtypes.

> **Exocrine-like:** characterized by relatively high expression of tumor cell derived digestive enzyme genes.
> **Classical:** demonstrating high expression of adhesion-associated and epithelial genes, and epithelial cell terminal differentiation genes, notably GATA6; KRAS mRNA levels elevated relative to the other subtypes; Classical subtype cell lines are more sensitive to erlotinib.
> **Quasi-mesenchymal:** has high expression of mesenchyme associated genes; a relatively high proportion of high-grade tumors and poor patient outcomes; low GATA6 expression; QM-PDA subtype cell lines are relatively more sensitive to gemcitabine than those with the Classical subtype.

Moffitt et al. (2015) used a diverse collection of pancreatic gene expression microarray data, including normal pancreata samples as well as primary and metastatic cancer samples, to identify two tumor-specific subtypes as well as additional stromal Normal and Activated subtypes which were independently prognostic. To develop their two tumor-specific subtypes, Moffitt et al. excluded transcripts thought to be specifically enriched in either the normal pancreas or the tumor microenvironment. The two tumor-specific subtypes were referred to as Classical and Basal-Like (Moffitt et al., 2015).

> **Classical:** characterized by overlapping signature with the genes described in the Collisson classification including GATA6, and overall a better prognosis.
> **Basal-like:** associated with a worse prognosis than the Classical subtype but may have a better response to adjuvant therapy.

Bailey et al. (2016) described four subtypes using samples with >40% cellularity from resectable primary pancreatic cancer, based on differential transcription factor expression and downstream targets responsible for lineage specification and differentiation during development and regeneration.

TABLE 2 | Main molecular subtypes of pancreatic cancer.

Study name	Year	Sample source and number	Methodology	Tumor cellularity	Source of the DNA/RNA	Subtyping method	Molecular subtypes	Promising molecular biomarkers	Clinical relevance
Waddell et al., 2015	2015	Primary resected tumors (*n* = 100)	WGS		Cryo bulk tissue	Structural rearrangements	Stable, locally rearranged, scattered, unstable	BRCA mutation (frequent in unstable tumors)	Unstable genome and/or BRCA mutation tumors responded well to platinum-based therapy.
Collisson et al., 2011	2011	Primary resected PDAC (*n* = 27); PDXs (*n* = 19); Mouse cell lines (*n* = 15)	Gene expression microarray	High	Cryo samples underwent microdissection	Tumor transcriptional profiles	Classical, QM-PDA, exocrine-like	GATA6 (high in classical and low in QM-PDA)	QM-PDA subtype was correlated with poor outcome and was sensitive to gemcitabine; classical subtype was correlated with good outcome and was sensitive to erlotinib.
Moffitt et al., 2015	2015	Primary (*n* = 145) and metastatic (*n* = 61) PDAC; Cell lines (*n* = 17); Pancreas (*n* = 46) and distant adjacent normal samples (*n* = 88); PDXs (*n* = 37); CAF (*n* = 6)	Whole-genome DNA microarrays, RNA-seq, virtual microdissection		Cryo bulk tissue, cell lines, PDXs, CAFs	Tumor and stroma transcriptional profiles	Tumor: classical, basal-like Stroma: normal, activated	SMAD4 and GATA6 (high in classical and low in basal-like)	Classical tumors with normal stroma had best outcome and basal-like tumors with activated stroma had worst outcome; basal-like tumors responded to adjuvant therapy better than classical tumors.
Bailey et al., 2016	2016	Primary resected tumors (*n* = 456); Patient-derived cell lines (*n* = 41); Mouse cell lines	WGS, deep-exome sequencing, RNA-seq	>40% for WGS, 12–40% for deep-exome sequencing	Cryo bulk tissue	Tumor transcriptional profiles	Squamous, pancreatic progenitor, immunogenic, ADEX	TP53 and KDM6A mutations (frequent in squamous tumors); FOXA2/3, PDX1 and MNX1 (high in progenitor tumors)	Squamous tumors had worst outcome.
Raphael and Cancer Genome Atlas Research Network, 2017	2017	Primary resected PDAC (*n* = 150)	WES, custom targeted gene panel sequencing, RNA-seq		Cryo bulk tissue	Tumor transcriptional profiles	Validation of former classifications (Moffitt's classification was independent of tumor purity, while Collisson's and Bailey's classifications were correlated with tumor purity)	Low EMT and apoptosis pathway activity, high TSC-mTOR and RTK activity in better survival groups	
Wartenberg et al., 2018	2018	Primary resected PDAC (*n* = 110)	ngTMA		FFPE samples	Tumor transcriptional profiles	Immune escape, immune rich and immune exhausted		Immune escape tumors had poor outcome, and immune rich tumors had better outcome
Puleo et al., 2018	2018	Primary resected PDAC (*n* = 381)	RNA microarray, immunohistochemistry, DNA panel sequencing		FFPE samples	Tumor transcriptional profiles	Pure classical, immune classical, desmoplastic, stroma activated, pure basal-like		Pure basal-like tumors had the worst outcome.
Maurer et al., 2019	2019	Primary resected PDAC (*n* = 60)	RNA-seq	High	Cryo samples underwent LCM	Tumor and stroma transcriptional profiles	Tumor: classical, basal-like Stroma: immune-rich, ECM-rich		Basal-like/ECM-rich tumors had worse outcome than classical/immune-rich tumors
Dijk et al., 2020	2020	Primary resected PDAC (*n* = 90)	RNA-seq		Cryo bulk tissue, PDXs	Tumor transcriptional profiles	Secretory, epithelial, compound pancreatic and mesenchymal	KRAS (highest transcript level in mesenchymal subtype)	Mesenchymal and secretory tumors had worse outcome than epithelial and compound pancreatic tumors.
Chan-Seng-Yue et al., 2020	2020	Primary resected PDAC (*n* = 206) and advanced PDAC (*n* = 111)	WGS, RNA-seq, single cell analysis	High	Cryo samples underwent LCM, single cell	Tumor transcriptional profiles	Basal-like-A, basal-like-B, hybrid, classical-A, classical-B	SMAD4 (high in basal-like-A tumors and low in classical-A tumors), CDKN2A and TP53 (completely loss in basal-like-A/B tumors), GATA6 (high in Classical-A/B tumors)	Classical-A/B tumors were more frequent in early stage, while basal-like tumors in late stage; in resectable disease, basal-like-B and hybrid tumors identified two prognostic subgroups considered to be uniformly aggressive before; in advanced disease, basal-like-A was highly chemo-resistant and trended toward worse survival.

Squamous: is characterized by enrichment for TP53 and KDM6A mutations; upregulation of the TP63ΔN transcriptional network; hypermethylation of pancreatic endodermal cell-fate determining genes and is associated with a poor clinical prognosis.
Pancreatic progenitor: is defined by preferential expression of genes involved in early pancreatic development notably FOXA2/3, PDX1, and MNX1 and also by gene programs involved in metabolism.
Immunogenic: is classed by the enrichment of genes associated with specific immune cell populations, including T-cells and B-cells.
Aberrantly differentiated endocrine exocrine (ADEX): is featured by upregulation of genes that regulate networks involved in KRAS activation, and exocrine (NR5A2 and RBPJL) and endocrine differentiation (NEUROD1 and NKX2-2).

The Squamous subtype overlaps with the Quasi-mesenchymal subtype of Collisson but has notable pan-squamous features, including a significant association with adenosquamous PDAC histology (Bailey et al., 2016). There is a marked epigenetic shift, with changes in DNA methylation down-regulating key transcription factors controlling pancreatic cell fate determination (PDX1, MNX1, GATA6, HNF1B), and the activation of subtype-driver multigene programs regulated by ΔNTP63 and c-MYC, leading to a loss of endodermal identity (Bailey et al., 2016). In addition, the Squamous subtype was also found to be enriched for mutations in KDM6A, MLL2, and MLL3 chromatin modifying enzymes that belong to the COMPASS complex (COMplex of Proteins Associated with Set1-like) (Bailey et al., 2016).

The Pancreatic progenitor subtype has four key characteristics.

(i) Transcriptional networks containing transcription factors PDX1, MNX1, HNF4G, HNF4A, HNF1B, HNF1A, FOXA2, FOXA3, and HES1, which are pivotal for pancreatic endoderm cell-fate determination toward a pancreatic lineage and are linked to maturity onset diabetes of the young
(ii) Gene programs regulating metabolism notably fatty acid oxidation, steroid hormone biosynthesis, and drug metabolism
(iii) O-linked glycosylation of mucins, notably apomucins MUC5AC and MUC1, but not MUC2 or MUC6, that define the IPMN pancreatobiliary subtype with PDAC-associated IPMN clustering
(iv) TGFBR2 inactivating mutations.

The ADEX subtype was defined by both exocrine and endocrine lineage features in later stages of pancreatic development and differentiation (rather than one or the other as is in normal pancreas development), and could be considered a subclass of Pancreatic progenitor tumors. There are two main transcriptional networks.

(i) Acinar cell differentiation and pancreatitis/ regeneration, transcription factors NR5A2, MIST1 (BHLHA15A) and RBPJL and their downstream targets.

(ii) Endocrine differentiation and maturity onset diabetes of the young, including INS, NEUROD1, NKX2-2, and MAFA.

Puleo et al. (2018) proposed two classifications, one specifically for the transformed neoplastic tumor cells and the other for the complete tumor entity, including the stroma: pure basal-like, stroma activated, desmoplastic, pure classical, and immune classical:

Pure basal-like tumors are composed of poorly differentiated tumors with predominant Gly12Asp and Gly12Val KRAS mutations; they have a low stromal signal.
Stroma Activated tumors are moderately differentiated, specifically enriched in the activated stroma component defined by high a-SMA, SPARC, and FAP.
Desmoplastic tumors are also moderately differentiated with a predominant basal association, characterized by a low tumoral component and a large stromal transcriptomic signal, including immune and inflammatory stroma components and, particularly, a high expression of structural and vascularized stroma components.
Pure-classical and Immune classical tumors are histologically well differentiated with fewer CDKN2A and TP53 mutations than basal-like tumors, and are also enriched with the Gly12Arg KRAS mutation, and associated with hENT1 expression; predicted to be Moffitt–Classical, and Bailey–Progenitor subtypes.

Maurer et al. used laser capture microdissection (LCM) epithelial cell enriched samples for mRNA sequencing to profile the expression of 60 matched pairs of human PDAC malignant epithelial and stroma samples (Maurer et al., 2019). They developed a computational model that could infer tissue composition and generate virtual compartment-specific expression profiles from bulk gene expression cohorts (Maurer et al., 2019). This study was able to provide a clearer understanding on the previous molecular gene signatures built from bulk tumor tissue samples with the following conclusions.

(1) Genes used to define the Collisson–Classical, Moffitt–Classical, Moffitt–Basal-like, and Bailey–Progenitor subtypes predominantly provide information about the malignant compartment regardless of the amount of stromal cell infiltration.
(2) Genes used to define the Moffitt–Activated, Moffitt–Normal, and Bailey–Immunogenic subtypes report on stromal expression that is largely independent of the malignant compartment (but see below).
(3) Gene sets in the Collisson–Quasi-Mesenchymal and Bailey–Squamous subtypes represent a mixture of epithelial and stromal identity, indicative of a more poorly differentiated state.
(4) Most genes that define the Collisson–Exocrine and Bailey–ADEX subtypes are largely derived from bulk tumor tissue samples are arguably mostly absent from LCM samples (but see below).

Chan-Seng-Yue et al. (2020) used LCM-purified pancreatic cancers for whole-genome sequencing in tumors from 314

patients, and whole-transcriptome sequencing of tumors from 248 patients, accompanied by single-cell RNA sequencing on 13 resectable and two metastatic tumors. For this classification, tumors with homologous recombination defects and MMR deficiency were excluded due to their unique mutational signatures (Chan-Seng-Yue et al., 2020).

> **Basal-like A:** these tumors were associated with the epithelial mesenchymal transition (EMT) program; TP53 gene and TGF-β signaling enriched; 5% of stage I/II (resectable); and 24% of stage IV (metastatic) tumors.
> **Basal-like B:** these tumors were associated with the EMT program; TP53 and TGF-β signaling enriched; 9% of stage I/II (resectable), 7% of stage III (locally advanced), and 12% of stage IV (metastatic) tumors.
> **Hybrid:** this subtype was found in 24% of stage I/II (resectable), 43% of stage III (locally advanced), and 18% of stage IV (metastatic) tumors.
> **Classical A:** this subtype was found in 44% of stage I/II (resectable), 43% of stage III (locally advanced), and 36% of stage IV (metastatic) tumors.
> **Classical B:** this subtype was found in 8% of stage I/II (resectable), 7% of stage III (locally advanced), and 10% of stage IV (metastatic) tumors.

This classification split each of the previously defined Basal-like and Classical subtypes into two disease subtypes, while the Hybrid subtype was inconsistently classified by previous systems arising from multiple expression profiles (Chan-Seng-Yue et al., 2020). Single-cell RNA sequencing revealed that both Basal-like and Classical clusters were present in the same tumor found in 13 out of 15 patients (Chan-Seng-Yue et al., 2020). The EMT program was positively correlated with Basal-like signatures and negatively correlated with Classical signatures (Chan-Seng-Yue et al., 2020). Moreover, they found that a major imbalance of allelic states of KRAS ($KRAS^{Ma}$) favoring the mutant allele over the wild-type allele occurred in only 4% of primary tumors compared to 29% in metastatic disease (Chan-Seng-Yue et al., 2020). Basal-like A/B tumors were enriched for the major imbalance $KRAS^{Ma}$ allelic states (44%) compared to metastatic Classical A/B tumors (14%), and $KRAS^{Ma}$ tumors were also more chemoresistant (Chan-Seng-Yue et al., 2020).

They proposed a possible model for the genomic evolution of pancreatic cancer as being a consequence of a gene expression continuum from (a) both Basal-like and Classical cell populations, and (b) linked to allelic imbalances in mutant (mt) KRAS, with metastatic tumors being more copy number-unstable compared to primary tumors (Chan-Seng-Yue et al., 2020). In primary tumors, the Basal-like phenotype is linked to minor mtKRAS allelic imbalances, whist in metastatic tumors, it is linked to major mtKRAS allelic imbalances (Chan-Seng-Yue et al., 2020).

Potential Influence of Tumor Cellularity on Transcriptomic Subtypes

Raphael and Cancer Genome Atlas Research Network (2017) performed genomic, transcriptomic, and proteomic profiling of 150 PDAC specimens, including samples with low neoplastic cellularity, provided by the Cancer Genome Atlas Research Network. They applied clustering techniques to reproduce the four-group classification of Bailey et al. (2016; Squamous, Immunogenic, Pancreatic Progenitor, and ADEX), the three-group classification (Classical, Quasi-mesenchymal, and Exocrine-like) of Collisson et al. (2011) and the two-group classification (Basal-like or Classical) of Moffitt et al. (2015). They found that while the Basal-like and Classical subtypes were independent of cancer cell purity, the Collisson Exocrine-like and Quasi-Mesenchymal subtypes, and the Bailey ADEX and Immunogenic subtypes were all associated with lower tumor purity (Raphael and Cancer Genome Atlas Research Network, 2017). Raphael and Cancer Genome Atlas Research Network (2017) also found that, among low purity tumors, a higher estimated leukocyte fraction was associated with the Immunogenic subtype and that the ADEX subtype was a subset of the Collisson Exocrine-like subtype.

Puleo et al. (2018) using formalin-fixed and paraffin-embedded tissues also concluded that the ADEX tumor subtype largely resulted from contamination with pancreatic acinar cells. As few as 39 of the most highly expressed genes of normal acinar cells from healthy pancreas single-cell transcriptomes can alone constitute 50% of the total number of expressed transcripts such that even a low level of normal pancreas contamination can materially affect any otherwise presumed subtype (Puleo et al., 2018).

Maurer et al. (2019) also suggested that the Collisson–Exocrine and Bailey–ADEX subtypes might be a function of the degree of tumor cellularity rather than being a distinct subtype as most of the subtype defining genes are largely derived from bulk tumor tissue samples and are mostly absent from LCM epithelial cell enriched samples.

Nevertheless, the assertion that the Collisson–Exocrine and Bailey ADEX and Immunogenic subtypes were all associated with lower tumor purity cannot be entirely true, since the same gene expression signatures seen in patient clinical PDAC tumors are identified in derived cell lines—cell lines and xenografts from these same tumors, and specifically the Classical, Quasi-mesenchymal and Exocrine-like gene expression profiles (Jones et al., 2008; Knudsen et al., 2018). Moreover, most clinical PDAC tumors have low cellularity, so these too should be included to avoid observer bias. The Bailey Immunogenic subtype as well as containing gene expression profiles derived from tumor stroma immune infiltration predominantly related to B and T cells also contains an underlying Pancreatic progenitor-like gene expression character (Bailey et al., 2016). Both cytotoxic ($CD8^+$) and regulatory T cells ($CD4^+CD25^+FOXP3^+$ Tregs) are predominant (Bailey et al., 2016). It has been suggested that a distinct Immunogenic subtype does not exist as distinct since immune infiltrates are enriched across all tumor-intrinsic subtypes, and their prevalence is primarily driven by tumor cellularity of the sequenced samples (International Cancer Genome Consortium et al., 2010; Puleo et al., 2018; Maurer et al., 2019). Nevertheless, by allowing for different degrees of cellularity, the strong Pancreatic Progenitor-like signals can still be split into an

immune high signature (Immunogenic subtype) and an immune low signature (Progenitor subtype) indicating that the signals from the underlying epithelium drive and formulate tumor cell immunogenicity (**Figure 1**; Bailey et al., 2016; Bear et al., 2020; Sahai et al., 2020). Hwang et al. using single-nucleus RNA sequencing found an association between Basal-like programs and higher immune infiltration with increased lymphocytic content, whereas Classical-like programs were associated with sparser macrophage-predominant microniches (Hwang et al., 2020).

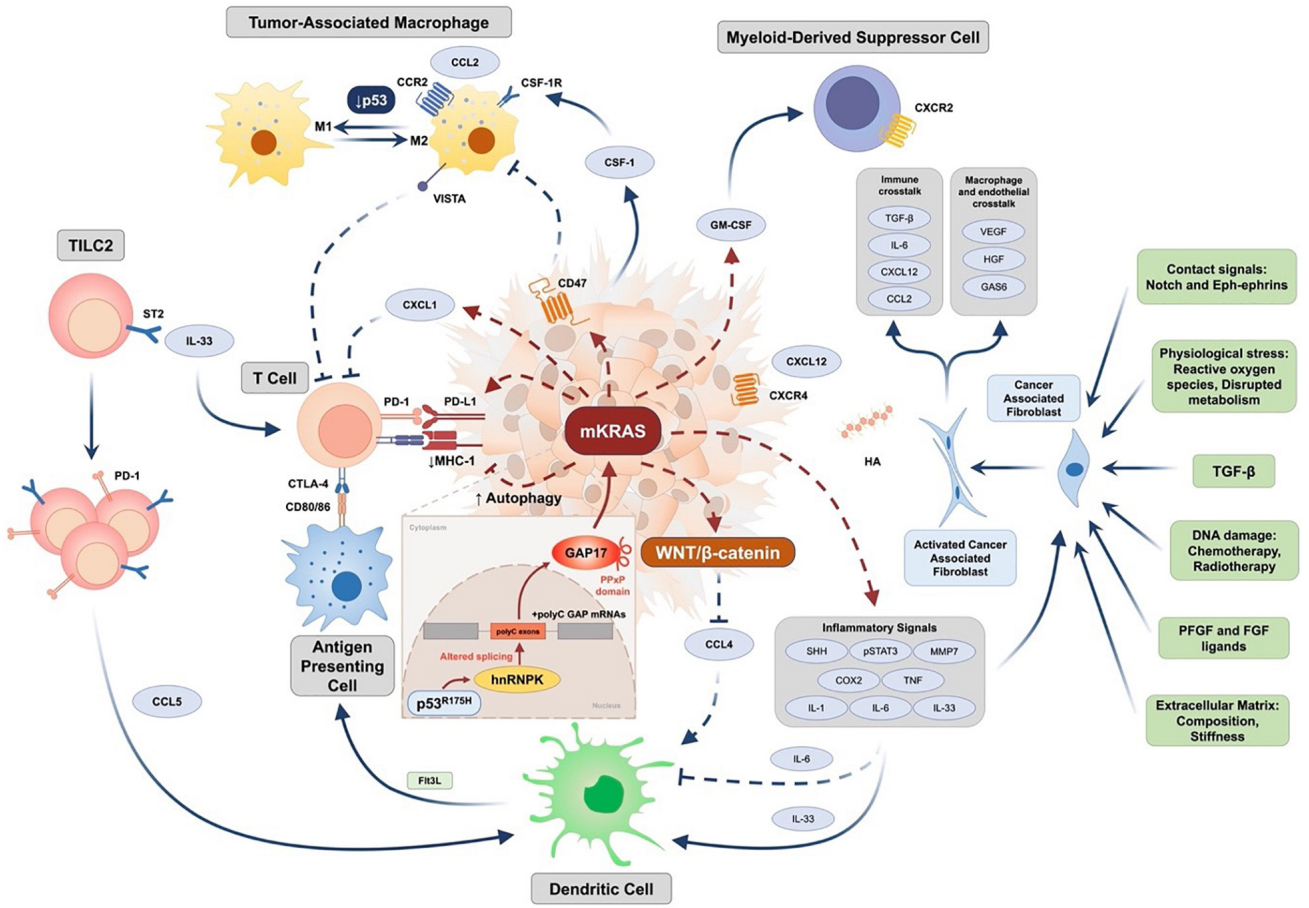

FIGURE 1 | Epithelial and stromal cell interactions in pancreatic cancer (Jones et al., 2008; Waddell et al., 2015; Bailey et al., 2016; Bear et al., 2020; Dominguez et al., 2020; Escobar-Hoyos et al., 2020; Sahai et al., 2020). PDAC immune resistance is driven by complex genetic background. Expression of tumor-intrinsic GM-CSF and CXCL1 is increased by oncogenic KRAS to mediate T cell exclusion and MDSC infiltration. Downstream signaling initiated by mutant KRAS (mKRAS) mediates innate and adaptive immune escape through enhancing autophagy to downregulate MHC-1 expression and upregulate the expression of PD-L1 and CD47. In addition to increased IL-6-mediated systemic dysregulation of conventional type 1 dendritic cell (DC), activation of WNT/β-catenin mediated by mKRAS signaling further downregulates CCL4 expression to inhibit DC recruitment. Tumor group 2 innate lymphoid cells (TILC2s) infiltrate the tumor microenvironment and are activated by IL-33 through binding to the ST2 receptor, further leading to an enhancement of anti-tumor immunity by expressing the inhibitory checkpoint receptor PD-1 and recruiting DCs potentially through CCL5 production. Furthermore, mKRAS signaling enhances chronic inflammation signaling such as Sonic Hedgehog, COX2, and pSTAT3 signaling, and promotes multiple inflammation-associated factors such as IL-1, IL-6, tumor necrosis factor (TNF), and matrix metalloproteinase 7 (MMP) to activate cancer-associated fibroblasts (CAF). Additional factors leading to activation of CAFs include TGF-β, extracellular matrix (ECM) stiffness and composition, RTK ligands such as PDGF and FGF, DNA damage caused by chemotherapy and radiotherapy, physiological stress, and contact signals such as Notch and Eph-ephrins. Activated CAFs further regulate macrophage and endothelial functions by factors such as VEGF, HGF, and GAS6 and participate in immune crosstalk through TGF-β activation, IL-6, CXCL12, and CCL2 production. Deficiency of p53 mediates transition of TAM toward an immunosuppressive M2 phenotype. Mutant p53 (such as R175H) increases expression of the splicing regulator hnRNPK to promote inclusion of cytosine-rich exons (+polyC exons) within GTPase-activating proteins (GAPs), particularly GAP17, leading to enhanced KRAS activity. CCL2/4/5, CC-chemokine ligand 2/4/5; CCR, CC-chemokine receptor; COX2, cyclooxygenase 2; CSF-1, colony-stimulating factor 1; CSF-1R, colony stimulating factor 1 receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CXCL1/12, CXC-chemokine ligand 1/12; CXCR4, CXC-chemokine receptor type 4; DC, conventional type 1 dendritic cell; FGF, fibroblast growth factor; Flt3L, Fms related receptor tyrosine kinase 3 ligand; GAS6, growth arrest-specific protein 6; GM-CSF, granulocyte-macrophage colony-stimulating factor; hnRNPK, heterogeneous nuclear ribonucleoprotein K; HA, hyaluronic acid; HGF, hepatocyte growth factor; IL-1/-6/-33, interleukin-1/-6/-33; MHC-1, major histocompatibility complex 1; PDGF, platelet-derived growth factor; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; SHH, sonic hedgehog; ST2, suppression of tumorigenicity 2; STAT3, signal transducer and activator of transcription 3; TAM, tumor-associated macrophage; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; VISTA, V-domain Ig suppressor of T cell activation.

At the present time, it is not entirely clear that the Collisson Exocrine-like and Quasi-Mesenchymal subtypes, and the Bailey ADEX and Immunogenic subtypes should be discarded, as there is a considerable variation in the way samples have been retrieved, stored, analyzed for mRNA expression, and assessed for epithelial cell purity by direct and indirect methodologies (**Table 2**; International Cancer Genome Consortium et al., 2010; Collisson et al., 2011; Carter et al., 2012; Moffitt et al., 2015; Bailey et al., 2016; Williamson et al., 2016; Cancer Genome Atlas Research Network, 2017; Connor et al., 2017; Raphael and Cancer Genome Atlas Research Network, 2017; Knudsen et al., 2018; Mateo et al., 2018; Puleo et al., 2018; Wartenberg et al., 2018; Maurer et al., 2019; Bear et al., 2020; Chan-Seng-Yue et al., 2020; Dijk et al., 2020; Escobar-Hoyos et al., 2020; Hong et al., 2020; Hwang et al., 2020). The validity of the Collisson Exocrine-like and Quasi-mesenchymal subtypes, and the Bailey ADEX and immunogenic subtypes requires further investigation.

Commonality of Transcriptomic Signatures

There is a strong alignment between the Classical/Pancreatic Progenitor and Quasi-mesenchymal/Basal-like/Squamous subtypes signatures of Moffit, Collinson, Bailey, Puleo and Chan-Seng-Yue (**Figure 2**; Moffitt et al., 2015; Bailey et al., 2016; Cancer Genome Atlas Research Network, 2017; Puleo et al., 2018; Chan-Seng-Yue et al., 2020).

Classical/Pancreatic progenitor tumors have a better prognosis with pancreatic specific transcription factors, such as GATA6, PDX1, and HNF1A, that act to specify and maintain pancreatic identity.

Basal-like/Squamous tumors are associated with a poor prognosis, with increased mtKRAS allelic imbalance and changes in DNA methylation that ultimately repress pancreatic identity and activate characteristic multigene programs (International Cancer Genome Consortium et al., 2010; Lomberk et al., 2018; Puleo et al., 2018; Chan-Seng-Yue et al., 2020).

STROMAL IMMUNE CELL AND CANCER-ASSOCIATED FIBROBLAST INFILTRATE

Signals from the stroma play an important role in disease progression (Bailey et al., 2016; Ligorio et al., 2019; Bear et al., 2020; Sahai et al., 2020). PDAC is characterized by a complex and dense microenvironment with an extensive desmoplastic stromal reaction. Typically, around 5–30% of cells in pancreatic tumors are epithelial cancer cells. Activation of pancreatic stellate cells and cancer-associated fibroblasts (CAF), along with inflammatory and immune cell accumulation, occurs during early pancreatic tumorigenesis, creating an immunosuppressive

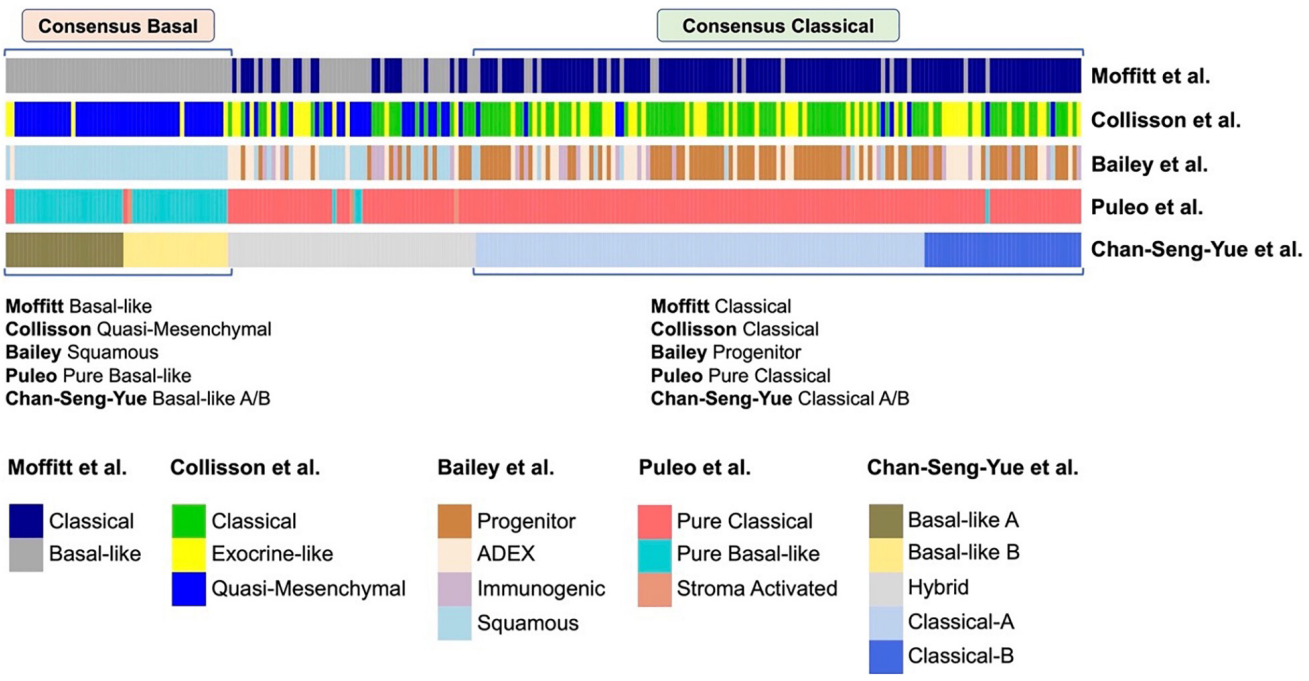

FIGURE 2 | Comparison of different transcriptional classifications of PDAC. Comparison of previously published transcriptional classifications of PDAC, two major consensus subtypes have been identified (Chan-Seng-Yue et al., 2020): **(A)** Consensus Classical, which is named as "Classical" in the classifications of Collisson et al. (2011) and Moffitt et al. (2015), "Progenitor" by Bailey et al. (2016), "Pure Classical" by Puleo et al. (2018), and "Classical-A/-B" by Chan-Seng-Yue et al. (2020); **(B)** Consensus Basal, which is named as "Basal-like" by Moffitt et al. (2015), "Quasi-Mesenchymal" by Collisson et al. (2011), "Squamous" by Bailey et al. (2016), "Pure Basal-like" by Puleo et al. (2018), and "Basal-like A/B" by Chan-Seng-Yue et al. (2020).

microenvironment that restricts immune surveillance and supports tumor growth and invasiveness (**Figure 1**; Bear et al., 2020; Sahai et al., 2020). Oncogenic driver mutations promote immunosuppression from the earliest stages of tumor inception that accompanies oncogenesis. Beyond immunogenic prognostic subtypes, patient-specific immune changes should be considered in combination immune-modulatory therapies targeting roadblocks in antitumor immunity. An immune-signature-based stratification may guide personalized therapy of PDAC patients and enable the design of novel combinatorial treatments with improved clinical efficacy (Kandimalla et al., 2020).

Pancreatic cancer has relatively few coding mutations and thus only few neo-antigenic targets, and is embedded in an immunosuppressive cold tumor microenvironment, which impedes intratumoral $CD8^+$ T cell infiltration and activation (Bear et al., 2020). Therefore, endogenous PDAC-reactive T cells are limited in quantity and quality and single agent immunotherapies with immune checkpoint inhibitors, which unleash pre-existing T cell immunity, are mostly ineffective in pancreatic cancer (Bear et al., 2020). Yet, exceptionally high neoantigen numbers, with robust antitumor $CD8^+$ T cell responses have been associated with long-term survival in pancreatic cancer patients, and immune checkpoint blockade has shown clinical responses in patients with hypermutated MMR-deficient tumors (Balachandran et al., 2017; Le et al., 2017). To induce specific antitumor adaptive immune responses, tumor-derived antigens must not only be taken up by innate immune cells; they must also be efficiently processed and cross-presented to $CD8^+$ T cells in the presence of a costimulatory signal. This mechanism is impeded in the vast majority of PDAC tumors by immunosuppressive mechanisms. $CD8^+$ lymphocytes are trapped in peritumoral compartments and mostly display an exhausted gene expression profile (Steele et al., 2020). Th1-polarized CD4 + T cells are less frequent at the tumor site compared to Th2-polarized $CD4^+$ T cells (De Monte et al., 2011). Tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSCs), which thwart the generation of cytotoxic T cell responses, are predominant over dendritic cells (DCs) that are largely dysfunctional, and immune checkpoint ligands are upregulated on myeloid cells (Hegde et al., 2020; Steele et al., 2020). In order to overcome the web of immune resistance and achieve durable antitumor effects, immunotherapeutic regimens need to target different steps in the cancer-immunity cycle, combining ideal antigen presenting cell (APC) activation that mediates priming of tumor-specific T cells, with strategies that enhance T cell effector function, and disrupt immunosuppressive myeloid cell programs. Thus, immune-modulatory strategies must be multi-modal aiming to (1) enhance endogenous T-cell function, (2) adoptively transfer tumor-specific T-cell immunity, and (3) attempt to devise an immunologically hot tumor microenvironment (Bear et al., 2020).

Moral et al. have shown that group 2 innate lymphoid cells (ILC2s) infiltrate PDACs to activate tissue-specific tumor immunity, inferring another novel immunoregulatory target (Moral et al., 2020). Enhanced anti-tumor immunity ensued blockade of the T cell checkpoint receptor programmed death (PD) receptor-1, which released ILC2 cell-intrinsic inhibition to expand and activate the tumor ILC2s to produce CCL5, thereby resulting in $CD103^+$ dendritic cell expansion and then CD8 + T-cell activation (Moral et al., 2020). Tumor infiltrating ILC2s which express the programmed cell death protein (PD-1) receptor were enriched in long-term survivors with an immunologically hot tumor microenvironment containing abundant activated $CD8^+$ T-cells, and containing higher bulk tumor RNA expression of the ILC2-activating cytokine IL33 (Moral et al., 2020). Pre-clinical studies in KPC mouse models have also suggested that specific targeting of macrophages and neutrophils using small molecule inhibitors, specific for either macrophage receptor CSF1R or the neutrophil receptor CXCR2, might facilitate better outcomes by enhancing endogenous T-cell cancer killing functions and the reprogramming of tumor cell intrinsic phenotypes (Steele et al., 2016; Candido et al., 2018). The PD-1 antibody pembrolizumab has FDA approval to for the treatment of MSI-H solid tumors, although this is present in only 1–3% of pancreatic cancers (Marcus et al., 2019; Marabelle et al., 2020). Inhibition of the CXCR4–CXCL12 pathway in pancreatic cancer also enhances tumor sensitivity to anti-PD-1 ligand-1 treatment. In the two-cohort phase IIa, COMBAT study (NCT02826486) pembrolizumab was combined with BL-8040 (a CXCR4 antagonist) in metastatic pancreatic cancer with promising responses and survival rates (Bockorny et al., 2020).

TAKING MOLECULAR SUBTYPING INTO CLINICAL TRIALS

Targeted therapies for advance pancreatic cancer based on next generation sequencing has been disappointing, with only 3–5% showing any clinical benefit in terms of actionable mutations, and limited to only a few months of additional survival (Pishvaian et al., 2020; Cobain et al., 2021). In reality the greatest sensitivity of pancreatic cancer to systemic therapies is chemotherapy, with increasing interest being shown in developing treatment response transcriptomic signatures to different agents (Tiriac et al., 2018). Moreover, it is important to distinguish treatment response signatures from the two main molecular subtypes as the Basal-like subtype appears to be more chemoresistant compared with reduced patient survival to the Classical subtype (Bailey et al., 2016; Aung et al., 2018; Chan-Seng-Yue et al., 2020; O'Kane et al., 2020). Several groups have now established informatic approaches that proport to accurately stratify patients based on PDAC subtype for clinical use. These include PuRIST, a single sample classifier that can stratify patients into two tumor-cell intrinsic subtypes based on the Moffitt classification scheme (Rashid et al., 2020). While PuRIST and other similar approaches promise better patient selection for chemotherapy, they have not been assessed in clinical trials.

Noll et al. identified hepatocyte nuclear factor (HNF)-1A and KRT81 that enabled stratification of tumors into different molecular subtypes by using immunohistochemistry (Noll et al., 2016). The two-marker combination identified the

QM-PDA ($KRT81^+/HNF1A^-$) subtype, which was associated with the shortest survival; the Exocrine-like ($KRT81^-/HNF1A^+$) subtype which was associated with the longest survival; and the Classical ($KRT81^-/HNF1A^-$) subtype, which was associated with intermediate survival (Noll et al., 2016). Exocrine-like subtype tumors were resistant to tyrosine kinase inhibitors (erlotinib and dasatinib) and paclitaxel, which induced cytochrome P450 (CYP) 3A5 (CYP3A5) in the tumors, leading to the metabolism of these compounds (Noll et al., 2016). CYP3A5 expression was correlated positively with $HNF1A^+$ and negatively with $KRT81^-$, and also contributed to acquired resistance in the QM-PDA and Classical subtypes (Noll et al., 2016).

Hwang et al. (2020) performed single-cell RNA sequencing on 26 flash-frozen pancreatic cancers from patients who underwent surgical resection, with upfront surgery in 11 and in 15 after neoadjuvant chemoradiotherapy. Following chemoradiation, there was a relative increase in Basal-like cells (including the master transcription factor ΔTP63 for the Squamous subtype), and a decrease in Classical-like cells (including the hallmark transcription factor GATA6) (Hwang et al., 2020). Thus, there appears to be a commonality with the effects of chemotherapy such as FOLFIRINOX which will also enrich for the Basal-like subtype (Aung et al., 2018; Porter et al., 2019). Following chemoradiotherapy, there was enhanced expression of genes needed to maintain the Wnt/β-catenin niche, which is critical for treatment resistance and can be mediated by autocrine signaling of the epithelial cells and/or paracrine interactions with CAFs (Hwang et al., 2020). Squamous/Basal-like programs facilitate immune infiltration compared with the Classical-like programs (Hwang et al., 2020; Somerville et al., 2020). Importantly, the immune infiltrates associated with Basal-like and Classical-like malignant cells are distinct, pointing to differential strategies choosing checkpoint inhibitors for the Basal-like subtype, and for the Classical-like subtype choosing myeloid directed therapies such as CD40 agonists and TGF-β modulators (Hwang et al., 2020). The study by Hwang et al. (2020) has yet to be published following review and the findings and conclusions will need further evaluation. Other approaches to subtyping may be required to understand more fully the extent of interpatient heterogeneity such as differential DNA methylation, associated with interferon (IFN) signaling (Espinet et al., 2021).

The encouragement from COMBAT along with other immunotherapy approaches currently being tested will expand the armamentarium against pancreatic cancer. A detailed understanding of the individual patient's response to the different forms of treatment will be necessary to further improve the prognosis of pancreatic cancer patients. The Bailey Immunogenic subtype is associated with immune gene programs involving B-cell signaling pathways, antigen presentation, $CD4^+$ T-cell, $CD8^+$ T-cell, and Toll-like receptor signaling pathways (Bailey et al., 2016). Acquired tumor immune suppression pathways through upregulation of the T cell checkpoint receptor PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in this Immunogenic subtype may offer therapeutic opportunities (Bailey et al., 2016). Puleo et al. (2018) found that the expression of CTLA-4 was higher in the Immune classical and Desmoplastic subtypes and, to a lesser extent, in the Pure basal-like subtypes, making these subtypes potentially sensitive to anti-CTLA-4 therapy such as ipilumumab. Also other promising therapeutic targets identified were the inhibitory checkpoint membrane receptors CD276 (B7-H3) and HAVCR2 (TIM3), both of which were highly expressed in the Desmoplastic, Stroma activated, and Pure basal-like subtypes (Wartenberg et al., 2018). Immune classical and Desmoplastic subtypes also showed high expression of the T-cell checkpoint inhibitor receptor CTLA-4, the costimulatory T-cell receptor CD27, and the tumor inhibitory $T4^+$ cell subset CD26 marker protein; Basal-like tumors were enriched in CD276 (B7-H3) and HAVCR2 (TIM3); and PDL-2 (PDCD1LG2) was expressed in all but the Pure classical subtypes, which overall do not up-regulate any of the immune checkpoints (Puleo et al., 2018).

In an effort to improve outcomes in pancreatic cancer through the use of more effective therapeutics, large-scale efforts are required with multiple centers and cooperating disciplines. Multiple clinical trials have been launched, pursuing better treatment schemes and ideal medication regimens based on molecular profiling and subtyping including COMPASS, PREDICT-PACA, Precision Promise, Know Your Tumor, ESPAC6/7, PANCuRx, and PASS1 (**Table 3**).

The EPPIC (Enhanced Pancreatic Cancer Profiling For Individualized Care Study) study based in Canada aims to sequence metastatic pancreatic tumors of 400 patients through two clinical trials (COMPASS and PanGen), both of which are generating molecular and phenotypic signatures of individual tumors in a clinically relevant timeframe and related to chemotherapy responses[1] [Aung et al., 2018; O'Kane et al., 2020].

Precision Promise was created by the Pancreatic Cancer Action Network and 15 USA clinical academic sites, in cooperation with the FDA and pharmaceutical partners. Precision Promise is an active adaptive Phase II/III clinical trial platform (NCT04229004) that allows rapid evaluation of novel therapeutic options in patients with metastatic pancreatic cancer. The protocol utilizes an adaptive randomization design and includes several trial designs and statistical innovations. All patients undergo pre- and on-treatment biopsies with state-of-the-art genomic, transcriptomic, and immune analysis, along with collection of blood research samples throughout the study. Focused on both first- and second-line treatment of metastatic PDAC, 30% of patients are randomized to one of the two common control arms (gemcitabine plus nab-paclitaxel and FOLFIRINOX), while 70% of patients are randomized to an experimental treatment arm. The platform currently has one experimental arm open (SM-88, Tyme), with two additional experimental arms to be added in 2021. Compared to traditional trial designs, Precision Promise has several advantages: multiple investigational treatments can be evaluated simultaneously using common controls; only 175 patients per experimental arm are required to initiate a regulatory registration, and it is expected that this platform will significantly accelerate the time to evaluate a new therapy, with an anticipated cost savings of 30–50%. With

[1]https://www.tfri.ca/our-research/research-project/enhanced-pancreatic-cancer-profiling-for-individualized-care-(eppic)

TABLE 3 | Translational clinical trials with molecular subtyping.

Trial name	Year	Type of study	Country	Tumor stage	Methodology	Treatment	Aims/Results
COMPASS (Aung et al., 2018; O'Kane et al., 2020) NCT02750657	2018	Cohort study	Canada	Locally advanced or metastatic PDAC	RNA-seq	156 Classical vs. 39 Basal-like subtypes, 91 mFFX vs. 63 GnP	ORR: 33% vs. 10%; Classical mFFX 29.6%, Basal-like mFFX 10%, Classical GA 39%, Basal-like GA 11%. mOS: 9.3 vs. 5.9 mths; Classical mFFX 10.6 mths, Basal-like mFFX 6.5 mths, Classical GA 8.19 mths, Basal-like GA 8.12 mths.
ESPAC-6 EudraCT: 2020-004906-79	2021	Phase III	Europe	Resectable PDAC	LCM, organoid generation, RNA-seq, WGS/WES	Adjuvant chemotherapy, mFFX vs. Gemcitabine with capecitabine	Patients randomized to allocation of oxaliplatin- or gemcitabine-based chemotherapy by standard clinical criteria or by a transcriptomic treatment specific stratification signature after surgery. (1) Primary outcome: disease free survival, time from randomization to disease recurrence or death from any cause (2) Secondary outcomes: overall survival; survival based on targeted signatures in test versus control arms; response to targeted therapies
ESPAC-7	2022	Randomized phase II	Europe	Non-metastatic locally advanced PDAC	Organoid generation, RNA-seq, WGS/WES	Neoadjuvant chemotherapy, mFFX vs. GnP	Patients randomized to allocation of oxaliplatin- or gemcitabine-based chemotherapy by standard clinical criteria or by a transcriptomic treatment specific stratification signature before surgery. (1) Primary outcome: resection rate following neoadjuvant therapy Disease free survival, time from randomization to disease recurrence or death from any cause (2) Secondary outcomes: resection rates and disease-free survival based on targeted signatures in test versus control arms; overall survival; response to targeted therapies
PASS-01 NCT04469556	2019	Randomized phase II	United States, Canada	Metastatic PDAC	Organoid generation, RNA-seq	mFFX vs. GA	(1) To determine the PFS benefit, ORR, DOR, and OS of mFFX compared to GA in patients with metastatic PDAC. (2) To evaluate chemotherapy sensitive signatures. (3) To determine if organoid transcriptomic profiles parallel patient transcriptomic profiles. (4) To explore biomarkers as responders to mFFX/GA (including GATA6 validation).
Know Your Tumor (Pishvaian et al., 2020)	2020	Registry study	USA	Pancreatic cancer	Genomic testing and tailored treatment recommendation based on the molecular profiling.	Molecularly matched therapy group vs. unmatched therapy group vs. no molecular marker group	mOS 2.58 vs. 1.51 vs. 1.32 y; patients with 2/more lines of therapy: mOS 1.81 vs. 0.85 vs. 0.73 y, mPFS 10.93 vs. 4.53 vs. 5.37 mths;
Precision Promise NCT04229004	2020	Phase II/III trials	United States	Metastatic PDAC	Genomic, transcriptomic and immune analysis	SM-88 vs. SOC (GA/mFFX)	(1) To compare each investigational arm in OS in 1st/2nd line metastatic pancreatic cancer patients and to determine which patients benefit from which regimen. (2) To determine short-/long-term safety signals of each investigational arm. (3) To determine clinical benefit (PFS, OS, CR, and PR) and the duration of it.
PREDICT-PACA DRKS00022429	2021	Co-clinical validation cohort study	Germany	Metastatic PDAC	Organoid generation/chemosensitivity testing, PDX analyses, NGS, transcriptomics,	Gemcitabine monotherapy, GnP, 5-FU with oxaliplatin	(1) To validate molecular signatures predicting response to specific chemotherapy regimens and establish point-of-care diagnostics. (2) To establish PDO-based chemosensitivity testing for individual tumors. (3) To evaluate the influence of the tumor microenvironment's composition on chemotherapy response.
PANCuRx	2021	Cohort studies	Canada	PDAC	WGS, RNA-seq	Translational Research Initiative	(1) To seek solutions to the high fatality rate of PDAC by generating genetic and biologic knowledge of the cancer, the mechanisms of how tumors grow and tailored treatment options. (2) To expand the COMPASS trial into the other cancer centers across Canada.

COMPASS, Study of Changes and Characteristics of Genes in Patients with Pancreatic Cancer for Better Treatment Selection; ESPAC, European Study Group for Pancreatic Cancer; PASS-01, Pancreatic Adenocarcinoma Signature Stratification for Treatment; PREDICT-PACA, Integrated Biopsy-Based Approach to Predict Response to Chemotherapy for Patients with Stage IV Pancreatic Cancer; NGS, next-generation sequencing; mFFX, modified FOLFIRINOX; GnP, gemcitabine plus nab-paclitaxel; GA, gemcitabine-abraxane; SOC, standard of care; mths, months; y, year; ORR, overall response rate; (m)OS, (median) overall survival; PFS, progression-free survival; DOR, duration of response; CR, complete response; PR, partial response; PDO, patient-derived organoid.

its unique design and novel method of data sharing, Precision Promise serves as a new clinical trial ecosystem to accelerate drug development for PDAC.

The ESPAC-6 adjuvant trial in resectable patients and the ESPAC-7 neoadjuvant trial in locally advanced patients are evaluating oxaliplatin- or gemcitabine-based chemotherapy response of PDAC patients that will be randomized according to standard clinical criteria (control arms) or by transcriptomic stratification signatures (experimental arms).

The PREDICT-PACA co-clinical trial funded by the German Cancer Aid is being conducted as a biopsy-based approach to predict response to chemotherapy for patients with metastasized pancreatic cancer. The consortium has established robust and highly predictive transcriptomic signatures linked to specific chemotherapy response profiles. Using microfluidic card-based qRT-PCR marker panels, clinical utility of the signatures is validated in a prospective collection of core biopsies from metastases of stage IV PDAC patients that receive one of the current chemotherapies upon clinical decision. In parallel, potential alternative therapies for third-line treatment are identified by high-throughput drug screening using patient-derived organoids and by next-generation sequencing-based detection of actionable mutations.

A major area of discussion is whether to discard the tumor microenvironment for molecular classification and replace this with a tumor-cell intrinsic classification system, but this may seem short-sighted as the stroma is a uniquely powerful biological phenomenon in pancreatic cancer. The relationship between molecular subtypes, most notably the immunogenic subtype as described by Bailey et al. (2016), and the responsiveness to evolving multimodality and immunotherapeutic strategies needs further investigation. Single-cell and spatial transcriptomic approaches now allow single-cell profiling of tumor and immune cell populations resident in patient tumors (Kuboki et al., 2019; Hwang et al., 2020; Moncada et al., 2020). These approaches are providing unparalleled insights into immune-tumor interactions and offer new opportunities for targeted immunotherapeutic intervention.

AUTHOR CONTRIBUTIONS

ZX, KH, PB, CS, SR, TG, MB, and JN prepared the initial drafts of the manuscript which were then critically reviewed by RK, BB, JA, KW, TP, MWB, and TH. All of the authors approved the final version of the manuscript.

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REVIEW
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Pancreatic Cancer and Platelets Crosstalk: A Potential Biomarker and Target

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Platelets have been recognized as key players in hemostasis, thrombosis, and cancer. Preclinical and clinical researches evidenced that tumorigenesis and metastasis can be promoted by platelets through a wide variety of crosstalk between cancer cells and platelets. Pancreatic cancer is a devastating disease with high morbidity and mortality worldwide. Although the relationship between pancreatic cancer and platelets in clinical diagnosis is described, the interplay between pancreatic cancer and platelets, the underlying pathological mechanism and pathways remain a matter of intensive study. This review summaries recent researches in connections between platelets and pancreatic cancer. The existing data showed different underlying mechanisms were involved in their complex crosstalk. Typically, pancreatic tumor accelerates platelet aggregation which forms thrombosis. Furthermore, extracellular vesicles released by platelets promote communication in a neoplastic microenvironment and illustrate how these interactions drive disease progression. We also discuss the advantages of novel model organoids in pancreatic cancer research. A more in-depth understanding of tumor and platelets crosstalk which is based on organoids and translational therapies may provide potential diagnostic and therapeutic strategies for pancreatic cancer progression.

Keywords: pancreatic cancer, platelets, TCIPA, platelet-derived factors, platelet extracellular vesicles, angiogenesis, organoids, biomarker

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INTRODUCTION

Significant interaction of tumor cells with platelets is a longstanding concept. Preclinical and clinical studies showed that tumorigenesis, progression, angiogenesis, and metastasis can be promoted by platelets through a wide variety of crosstalk between platelets and cancer cells. Correlations between high platelet counts and poor prognosis are often described for lung, colon, breast, kidney, ovarian, and pancreatic cancers. Platelet-based biomarkers as liquid biopsy for cancer patients will be a potential platform for improving diagnosis. High risk of venous thrombosis and metastasis have close interrelations with platelets in patients with pancreatic cancer (Sylman et al., 2017). However, studies focused on crosstalk between pancreatic cancer and platelets in different ways are not fully explored compared with other tumors. For example, platelet α-granules contain many different bioactive factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), endostatin, angiostatin, platelet factor-4 (PF4), or thrombospondin, the exact mechanisms of granule release and whether they can be selectively manipulated in pancreatic cancer requires more study. In addition, platelet derived-microparticles and RNA profile alteration are prospective directions for pancreatic cancer

diagnosis and treatment (Thaler et al., 2012; Best et al., 2015). At the same time, multiple drugs have been developed to interfere with cancer growth or metastasis by inhibiting the functions of platelets. These drugs are in pre-clinical development or already in clinical treatment, which target platelet receptors, inhibit platelet granule release, or interfere with platelet-related enzymes (Dovizio et al., 2013; Elwood et al., 2016; Sun et al., 2019). In this review, we summarize recent discoveries in the field of pancreatic cancer and platelets. Compared with other cancer research with platelets, we discuss the potential exploration in pancreatic cancer. Moreover, we propose a suitable research model for pancreatic cancer and platelets investigation, which provides an insight for further study.

OVERVIEW OF PANCREATIC CANCER

Pancreatic cancer remains one of the most lethal neoplasms worldwide. There are three histological classifications of pancreatic cancer. The pancreatic ductal adenocarcinoma (PDAC) comes from the duct cells of exocrine tissue, which represents the most common (> 90%) type. There is one subtype that exhibits both characteristics of adenocarcinoma and squamous carcinoma. It is adenosquamous carcinomas concerning 1–4% of the exocrine malignancies. The other types are acinar and neuroendocrine tumors. According to Globocan estimates, there were more than 495,773 new patients diagnosed and about 466,000 deaths of pancreatic cancer in 2020.

From precursor to invasive cancer, there are three well-defined PDAC precursor lesions: Intraductal papillary mucinous neoplasms (IPMNs), mucinous cystic neoplasms (MCNs), and pancreatic intraepithelial neoplasm (PanIN), the latter being the most frequent precursor lesion which further divided into PanIN-1, 2, and 3 (Hezel et al., 2006; Hruban et al., 2007).

Pancreatic cancer often does not cause symptoms in the early stages, which makes it difficult to diagnose. Common symptoms include tummy pain or back pain, weight loss, indigestion, losing appetite, diarrhea, constipation, jaundice, blood clots, and fatigue. These symptoms can have many causes, and are unlikely to be pancreatic cancer. Thus, different kinds of tests are essential for diagnosis. The tests used to diagnose pancreatic cancer include 1. Blood test, such as blood cell count, liver and kidney function, or tumor markers (such as CA19-9, CEA, B72.3); 2. Ultrasound scan of the abdomen. 3. Computerized tomography (CT) scan or Positron emission tomography (PET-CT) scan. 4. Magnetic resonance imaging (MRI) scan or Magnetic resonance cholangio-pancreatography (MRCP). 5. Endoscopic ultrasound scan (EUS) or with/without Biopsy. 6. Endoscopic retrograde cholangio-pancreatography (ERCP) is usually used if the bile duct is blocked. 7. Laparoscopy. According to these tests, the information about tumor size, burden, and local vessel involvement can be achieved that necessary to determine the TNM (Tumor, Nodes, Metastases) stage (van Roessel et al., 2018).

Currently, the treatments for pancreatic cancer are chemotherapy, surgery, radiation therapy, targeted therapy, and immunotherapy. Only about 20% of people diagnosed with pancreatic cancer are eligible for surgical treatment because most are found after the disease has already spread. The kinds of surgery that are performed depend on the purpose of the surgery. For example, the Whipple procedure, which is carried on if the tumor is located only in the head of the pancreas. However, a Distal pancreatectomy is commonly done if the cancer is in the tail of the pancreas. Usually, surgery will combine with systemic therapy or/and radiation therapy. Adjuvant therapy is given after surgery. Sometimes, a few treatments are used to shrink a tumor before surgery, this is called neoadjuvant therapy or pre-operative therapy. Radiation therapy is performed by high-energy x-rays or other particles to destroy cancer cells such as traditional radiation therapy, stereotactic body radiation (SBRT) or cyberknife, proton beam therapy. Typically, chemotherapy will combine with radiation therapy at the same time, which is named radiosensitization. Chemotherapy is the main type of systemic therapy and includes an intravenous tube placed into a vein by a needle or orally. One type or a combination of different medications are used in patients. Currently, the drugs approved for pancreatic cancer are: Fluorouracil (5-FU), Capecitabin, Gemcitabine, Erlotinib, Leucovorin, Irinotecan, Nab-paclitaxel, Nanoliposomal irinotecan, and Oxaliplatin. In addition, there are some targeted treatments that focus on the specific genes of the cancer, proteins, or the tissue environment. For example, Erlotinib blocks epidermal growth factor receptor (EGFR), Olaparib influences a hereditary *BRCA* mutation, and Larotrectinib can be used for *NTRK* fusion (Wang et al., 2015; Filippi et al., 2021; Tutt et al., 2021). Moreover, Immunotherapy has become popular in recent years. Immune checkpoint inhibitors are an option for treating pancreatic cancer with high microsatellite instability (MSI-H), which include anti-PD-1 antibodies such as pembrolizumab (Diaz et al., 2017). Different treatment options are dependent on the stage of the tumor. For instance, resected patients' chemotherapy will be gemcitabine or 5-FU based treatment, however, metastatic cancer will use Gemcitabine plus Nab-paclitaxel or a combination of 5-FU, Leucovorin, Irinotecan, and Oxaliplatin called FOLFIRINOX. Nevertheless, surgery is not the main method of treatment. The appropriate therapeutics are referenced to European Society for Medical Oncology (ESMO) guideline (Ducreux et al., 2015).

PLATELET FUNCTION

Platelets derive from megakaryocytes, which exist in circulation for 5–7 days. The size is approximately 2–4 μm and their volume is about 7 μm^3. A normal number of platelets ranges between 150,000 and 450,000 per microliter of blood. They are removed from blood vessels by macrophages and neutrophils and, leave the body by the spleen.

The primary role of platelets is to maintain hemostasis, by the formation of a "platelet clot" (Tomaiuolo et al., 2017). Following vascular damage, initial platelet tethering is mediated by the interaction between the GPIbα in the platelet receptor GPIb-IX-V and A1 domain of Von Willebrand factor (vWF) deposited in the subendothelial matrix of the injured vessel wall. After platelet tethering, GPVI and αIIβ1 receptors promote platelet adhesion

and activation (Ruggeri and Jackson, 2013; Welsh et al., 2014). GPVI has a low affinity for collagen. The αIIβ1 maintains stable adhesion to collagen and reinforces GPVI-collagen interaction. Subsequent stable adhesion occurs *via* binding of fibronectin, αIIbβ3, laminin, and vWF. In addition, platelet adhesion will form positive feedback to initiate circulating platelets activation. The final step is platelet aggregation by the binding of fibrinogen or vWF to αIIbβ3.

Apart from hemostasis and thrombosis, platelets also play an important role in immune activities. Platelets are able to recognize and interact with microbial pathogens including bacteria, viruses, and parasites. Platelet bounding shifts fee *L. monocytogenes* from "fast" clearance into CRIg-dependent "slow" clearance pathways (Broadley et al., 2016). In addition, different platelet receptors have various effects on cancer progression (**Supplementary Table 1**). Platelets express toll-like receptors from TLR1 to TLR9 which identify molecular motifs called pathogen-associated molecular patterns (PAMPs) (Cognasse et al., 2015). Interaction between platelets and leukocytes, monocytes, and granulocytes are evidenced, which through different receptor-ligands such as P-Selectin, PSGL-1. Platelets are involved in angiogenesis. Their activation facilitates release, eliciting potent angiogenic responses. Moreover, the release of platelet-derived phospholipids and microparticles are as synergistic regulators of angiogenesis (Walsh et al., 2015). However, tumor growth can be aggravated by uncontrolled angiogenesis. Luminal breast cancer cells secret cytokines absorbed by platelets, which help vessel formation (Kuznetsov et al., 2012). Recently, it has been established that platelets play a crucial role in cancer cell metastasis. Platelets make contact with tumors by direct surface interactions, such as surface receptors and glycoproteins, indirect platelet growth factors, such as VEDF, TGF-β, and microparticles (MP) (Goubran et al., 2014). Platelet-derived signals, for example, CXCL5 and CXCL7 are required for the rapid recruitment of granulocytes to tumor cells to form early metastatic niches (Labelle et al., 2014). Colorectal cancer cell interaction with platelets produces chimeric extracellular vesicles like three types of microparticles that promote metastasis through EMT and endothelial activation (Plantureux et al., 2020). Activated platelets P-selectin interact with circulating tumor cell P-selectin ligands, which form aggregation and prevent shear force-induced tumor membrane damage (Coupland and Parish, 2014; Egan et al., 2014). Accordingly, platelets and tumor cell interaction promote tumor cells extravasation (Haemmerle et al., 2018; Marcolino et al., 2020).

CROSSTALK BETWEEN PANCREATIC CANCER AND PLATELETS

Pancreatic Cancer Influences Platelets

Tumor Cell-Induced Platelet Aggregation and Thrombopoiesis

Tumor cell-induced platelet aggregation (TCIPA) is not a new concept, which can be traced back to the late nineteenth century. For pancreatic cancer cells, it was evidenced by six human cell lines, which can induce platelet aggregation *via* activation of thrombin (Heinmöller et al., 1995). Several molecular pathways are involved in pancreatic TCIPA. Activated phospholipase A2 enzymes release arachidonic acid (AA) which is a precursor of thromboxane A2 (TXA2). Cyclooxygenase 1 (COX-1) catalyzes the transformation of AA into TAX2, which is important for platelet aggregation. TAX2 can activate the thromboxane receptor-induced changes of platelet shape, activation of integrins, and degranulation. Moreover, the expression of COX-2 is reported as increased in tissues of pancreatic cancer (Ding et al., 2003; Sangkuhl et al., 2011). P-selectin and tissue factor (TF) accumulation are associated with platelet activation and platelet-rich thrombus (Wang et al., 2012; Mezouar et al., 2015; Hisada et al., 2017). The increased von Willebrand Factor (VWF), a large polymeric glycoprotein, is involved in the adhesion and aggregation of platelets. Cancer patients are associated with a higher risk of venous thrombosis. Especially, high VWF levels were observed in pancreatic, lung, brain, stomach, and colorectal cancer patients (Obermeier et al., 2019). Podoplanin on the surface of cancer cells induces platelet aggregation. Pancreatic cancer-associated fibroblasts (CAF) reportedly express podoplanin (Takemoto et al., 2017; Suzuki-Inoue, 2019). In addition, a recent study showed pancreatic cancer (Paca) cells can stimulate the rapid release of neutrophil extracellular traps (NETs) and promote thrombus formation (Abdol Razak et al., 2017).

Changes of Phenotype

In patients diagnosed with pancreatic cancer at the head of the pancreas, platelet count and concentration of vascular endothelial growth factor (VEGF) released from platelets were significantly increased (Sabrkhany et al., 2017). Mean platelet volume (MPV) is changed in different stages of pancreatic cancer (PC). MPV was elevated in PC patients with synchronous liver metastases and stage III-IV (Yin et al., 2018). However, MPV was decreased in resectable PC patients with poor prognoses (Yagyu et al., 2021). Best et al. (2015) evidenced mRNA profiles of tumor-educated blood platelets (TEPs) were different between *KRAS* mutant pancreatic cancer and *KARS* wild-type. TEPs RSL24D1 mRNA was negatively related to early pancreatic cancer compared to healthy controls (Xue et al., 2018). Moreover, the platelet proteome of patients with head of pancreas cancer (stage I-II) is significantly different from that of healthy individuals of equivalent sex and age (Sabrkhany et al., 2017, 2018).

Extracellular Vesicles Release and Alteration

Extracellular vesicles (EVs) are a means that facilitate the exchange of a broad array of molecules between adjacent or distant cells. Platelet EV cargo includes lipids, protein, nucleic acids, and organelles, which can enter lymph, bone marrow, and synovial fluid. EVs are classified into exosomes (30–150 nm), microvesicles—also referred as microparticles or ectosomes—(100–1,000 nm), and apoptotic bodies (1,000–3000 nm) (Ferreira et al., 2020). Platelet-derived extracellular vesicles (PDEVs) are the most abundant type of EVs in the circulation. Microparticle (MP) refers to particles released from the surface of cells, especially in the field of platelet research

(**Supplementary Table 2**). Patients with pancreatic cancer had significantly increased levels of MP-associated TF activity compare with healthy controls (Tesselaar et al., 2007; Tilley et al., 2008). MP-TF activity had a strong association with mortality in pancreatic cancer, which could be a marker for aggressive cancer phenotype (Thaler et al., 2012). In addition, circulating microvesicles (MVs)-associated thrombin generation is different between patients and healthy control (Hellum et al., 2017).

RNA Profiles Alteration

Platelet messenger RNA (mRNA) profile is currently emerging as a new potential biomarker in cancer diagnosis. A study demonstrated platelets isolated from glioma or prostate cancer patients contained the cancer-associated mRNA transfer EGFRvIII and PCA3 (Nilsson et al., 2011). Recent studies highlighted pancreatic tumors can alter platelet RNA profiles (Best et al., 2015). Platelets from cancer patients contained tumor-associated RNA biomarkers, indeed, mRNA sequencing of tumor-educated platelets can identify pancreatic cancer patients with 96% accuracy.

Platelets Are Involved in Pancreatic Tumor Progression

Platelets and Platelet-Derived Factors Are Biomarkers for Diagnosis and Prodiagnosis

MPV is associated with the overall survival of pancreatic cancer patients (Yin et al., 2018). Particularly, large platelet size is associated with poor outcomes in patients with metastatic pancreatic cancer (Lembeck et al., 2019). However, reduced MPV levels predict shorter survival in patients after surgery (Yin et al., 2020). There is a novel scoring system based on hemostatic parameters that showed platelet count was an independent prognostic factor in advanced pancreatic cancer (Zhang et al., 2019). In many studies, CA19-9 decrease during treatment has been related to longer survival of pancreatic cancer. Moreover, the correlation of CA19-9 decreases; overall survival was stronger in advanced pancreatic cancer with fewer platelets (Chen et al., 2019). The preoperative platelet to lymphocyte ratio (PLR) was reported to be a significant independent prognostic factor in patients (Song et al., 2017). In addition, the increased PLR is also related to a poor long-term prognosis in resected pancreatic cancer (Yu et al., 2018; Negoi et al., 2019). Combining PLR and CA19-9 values could allow earlier diagnosis of pancreatic cancer patients with type 2 diabetic patients (Qin et al., 2019). Preoperative platelet-to-albumin ratio (PAR) was reported as a novel significant independent prognostic index for disease-free survival (DFS) and overall survival (OS) in patients after pancreatic resection (Shirai et al., 2017). Moreover, platelet receptors and platelet-derived factors are also involved in cancer progression (**Supplementary Table 1**). The platelet-derived growth factor (PDGF) signaling pathway plays an important role in the progression of pancreatic cancer. Duan et al. (2018) analyzed three published genome-wide association study datasets to observe genetic variants in the PDGF subunit B gene associated with pancreatic cancer risk in European populations. High levels of circulating PDGF-AA serve as a predictor of poor cancer-specific survival, whereas high levels of PDGF-BB are associated with a favorable prognosis (Rahbari et al., 2011; Kahlert et al., 2014). In addition, PDGFR beta (PDGFRβ) is more frequently expressed in primary endocrine pancreatic tumors (EPTs) and metastases as compared to normal endocrine pancreatic tissue (Fjällskog et al., 2007). PDGFRβ is a marker of activated pancreatic stellate cells (PSCs) which play a vital role in desmoplasia. Higher expression of PDGFRβ matched shorter prognosis as well as lymphatic invasion and lymph node metastasis (Yuzawa et al., 2012).

Platelets Accelerate Proliferation and Angiogenesis

Recent researches evidence that platelets have a direct effect on tumor cell proliferation. PANC-1 cancer cell proliferation was potentiated by human platelets in a manner dependent on the upregulation and activation of the oncoprotein c-MYC (Mitrugno et al., 2017). Platelet-derived growth factor (PDGF) is an important cytokine in pro-proliferative and invasion signaling, which plays a key role in the regulation of interactions between pancreatic cancer cells and adjacent stroma (Haqq et al., 2016). Moreover, dual-specificity phosphatase 28 (DUSP28) regulates chemo-resistance and migration in pancreatic cancers. PDGF-AA was evidenced in a public microarray database and *in vitro* assay, which is a critical role in pancreatic cancer malignancy. In addition, DUSP28 and PDGF-AA formed an acquired autonomous autocrine-signaling pathway. Targeting DUSP28 inhibited the tumor growth and migratory features through the blockade of PDGF-AA expression and intracellular signaling (Lee et al., 2017). Mucin 1 (MUC1), a transmembrane mucin glycoprotein, regulates PDGFA expression and secretion in pancreatic cancer cells, accordingly, influences the proliferation of pancreatic tumors (Sahraei et al., 2012). There are some treatments for targeting PDGF to decrease the proliferation of pancreatic cancer cells (Inoue et al., 2017; Salem et al., 2020). PDGF-BB mediates pancreatic cancer growth via regulation of the Hippo/Yes-associated protein signaling pathway (Li et al., 2021). NF-κB is the downstream stage of the AA pathway. NF-κB and activator protein 1(AP-1) can bind to COX-2, lipoxygenases (LOXs), and phospholipase A2 (PLA2), which play a pro-tumorigenic role in pancreatic cancer (Gong et al., 2014; Matejcic et al., 2018). COX-2 is only expressed in pancreatic islets and has no expression in normal exocrine pancreatic tissues. Numerous clinical studies reported that mRNA and protein expression of COX-2 are up-regulated in pancreatic cancer. The use of aspirin, the major pharmacological inhibitor of COXs, was negatively related to the incidence risk of pancreatic cancer (Sun et al., 2019). Moreover, low-dose aspirin (81–162 mg orally daily) is relatively selective for COX-1 inhibition, interaction with circling tumor cells, and platelet aggregation. However, two large cohort studies reported regular aspirin or non-aspirin NSAID use was not associated with future risk of pancreatic cancer. There is only a possible reduction in patients of pancreatic cancer with diabetes (Khalaf et al., 2018). Pancreatic cancer cells express the purinergic receptor P2Y12, that is an ADP receptor found mainly on platelets. Ticagrelor, a P2Y12 inhibitor, decreases the survival signals initiated in cancer cells by platelet-derived ADP and ATP (Elaskalani et al., 2017). Arachidonate 12-lipoxygenase

(ALOX12) and 12-hydroxyeicosatetraenoic acid contribute to stromal aging-induced progression of pancreatic cancer (Sarsour et al., 2020). 5-lipoxygenases (5-LOX) were found overexpressed in the tissues of pancreatic cancer, the inhibition of 5-LOX induces apoptosis in pancreatic cancer cells (Zhou et al., 2015). In addition, P-selectin deficiency and soluble P-selectin abolish platelet deposition within tumors, decreasing the secretion of vascular endothelial growth factor and angiogenesis, thereby suppressing tumor growth (Qi et al., 2015). Rivipansel inhibits selectins and decreases the recruitment of plasma cells in multiple myeloma (Azab et al., 2012). Crizanlizumab, a selective blocking antibody of P-selectin, also is indicated as a potential treatment option for patients with pancreatic cancer in the future. Selected clinical trials of glycobiology-targeted therapeutics for pancreatic cancer are in Phase I, for example MVT-5873 and MVT-10775 (Smith and Bertozzi, 2021). The first study for platelets involved in tumor angiogenesis is by Pinedo et al. (1998) They proposed platelets as a rich source of stimulators and inhibitors of angiogenesis and their interaction with the endothelium. Starlinger et al. (2011) compared different methods to get circulating platelet-stored angiogenesis factors in pancreatic cancer patients, and a significant increase of thrombospondin 1(TSP-1) and platelet factor 4 (PF-4) were determined. The overexpression of VEGF and platelet-derived endothelial cell growth factor (PD-ECGF) protein significantly correlated with high microvessel density (MVD) in patient tissues of pancreatic cancer (Fujimoto et al., 1998). VEGF is a chemotactic vascular permeability factor stored in α-granules and released from activated platelets. The study evidenced that VEGF expression correlated significantly with increased intratumoral microvessel density (IMD), which are important regulators of pancreatic tumor angiogenesis and predictive of benefit from adjuvant therapy (Khorana et al., 2005). Platelet factor 4 (PF-4) inhibits angiogenesis *in vivo* and *in vitro* (Maione et al., 1990). PF-4 modulates fibroblast grow factor 2 (FGF-2) activity (Perollet et al., 1998). However, a study reported that FGF-1 and FGF-2 treatment led to the induction of phosphorylation of E-cadherin and β-catenin on tyrosine residues, resulting in angiogenesis in pancreatic cancer cells (El-Hariry et al., 2001). Accordingly, FGFR also plays a key role in tumor angiogenesis, downregulations of FGFR-2 led to decreased phosphorylation of ERK and VEGF-A in PDAC cells after FGF-2 stimulation (Compagni et al., 2000; Kang et al., 2019). Platelets are the sole source of EGF in circulation, however, inhibition of EGFR tyrosine kinase activity suppresses pancreatic tumor angiogenesis (Bruns et al., 2000). Interleukin-8 (IL-8) is a chemokine related to PF-4, which activates G-protein coupled receptors as a pro-angiogenic factor (Le et al., 2000). Platelets contain 40–100 times more TGF-β than other non-neoplastic cells (Assoian et al., 1983). Although TGF-β is a pro-angiogenic factor in many cancers, the role in PDAC is controversial. TGF-β interferes with a soluble TβRII, which suppresses pancreatic cancer angiogenesis (Rowland-Goldsmith et al., 2002). Contrarily, other studies evidenced TGFβ1 is overexpressed in PDAC, and it induces plasminogen activator inhibitor-1(PAI-1) expression in pancreatic cancer cells, promoting angiogenesis *in vivo* (Andreasen et al., 2000). TGF-β can promote stromal activation, which induces angiogenesis and attenuates a productive anti-tumor immune reaction (Hinz et al., 2007; Kano et al., 2007). Similarly, because pancreatic cancer has a highly hypoxic microenvironment, in which hypoxia-inducible factor-1α (HIF-1α) is activated. HIF-dependent pathways subsequently activate HGF/c-MET signaling pathway in pancreatic tumor cells, and induce angiogenesis (Kitajima et al., 2008). HDF can also be anti-angiogenic due to alternative splicing of the α-chain (Date et al., 1998). Platelets can induce several matrix metalloproteinase (MMPs) in platelet-tumor cell interactions. Integrin engagement leads to the secretion of MMP-2, MMP-9 and surface expression of MT1-MMP. MMP-9 is essential to angiogenesis in pancreatic cancer mice model (Nakamura et al., 2007). Expression of MMP-2 and MMP-9 mRNA are associated with microvessel density in pancreatic cancer patients (Xiang et al., 2017). Platelet-derived microparticles (PMP) can enhance tumor growth by the release of potent growth factors in the tumor micro-environment (Goubran et al., 2015).

Platelets Facilitate Metastasis

That platelets promote tumor metastasis is not a new idea. Activation of platelets and the TF-thrombin-PAR-1 pathway are reported to promote metastasis of PDAC cells (Yang et al., 2019). The activation of platelets increases lysophosphatidic acid (LPA) release from platelets, LPA in turn enhanced tumor cell invasiveness and cell migration (Yoshikawa et al., 2013). In addition, vWF-platelet interactions also promote a number of metastases (Patmore et al., 2020). Tumor cell-derived MMPs can elicit platelet activation, accordingly, platelet-activating factors can induce an increased MMP expression, and inhibition of MMP reduces both growth of pancreatic cancer metastases and the death rate (Jimenez et al., 2000). High TGF-β induced expression in PDAC patients is associated with pancreatic cancer cell migration (Costanza et al., 2019). Moreover, TGF-β has a role as a suppressor of stromal promotion or through alterations in pancreatic stellate cell MMP profiles with subsequent inhibition of pancreatic cancer cell migration (Tjomsland et al., 2016). Platelets secrete a mountain of growth factors and chemokines, such as VEGF, PDGF, CXCL5, to help establish metastatic foci, neovessel formation, and metastasis. In addition, inhibition of platelets activation prevents the P-selectin and integrin-dependent accumulation of pancreatic cancer cell microparticles and reduces metastasis (Mezouar et al., 2015). ADAM9 contributes to vascular invasion and is involved in metastasis (Oria et al., 2019). Epithelial-mesenchymal transition (EMT), a transient and reversible process, promotes cell motility, invasion, and dissemination of cancer cells out of the tumor microenvironment. Extravasated platelet aggregation is associated with the first step in the formation of EMT. Primary tumor cells surrounded by platelets exhibited characteristics of EMT in pancreatic cancer (Miyashita et al., 2015).

Platelets Improve Pancreatic Cancer Chemotherapy Resistance

A few studies have shown platelets can influence the efficacy of chemotherapy. Platelets increase the resistance of colon

and ovarian cancer to 5-fluorouracil and paclitaxel (Radziwon-Balicka et al., 2012). Platelet factors hinder the cytotoxicity of sorafenib and regorafenib in hepatocellular carcinoma by increasing the phosphorylation of ERK and p38 (D'Alessandro et al., 2014). Elaskalani et al. (2017) evidenced that platelet-derived ADP and ATP promote pancreatic cancer cell survival

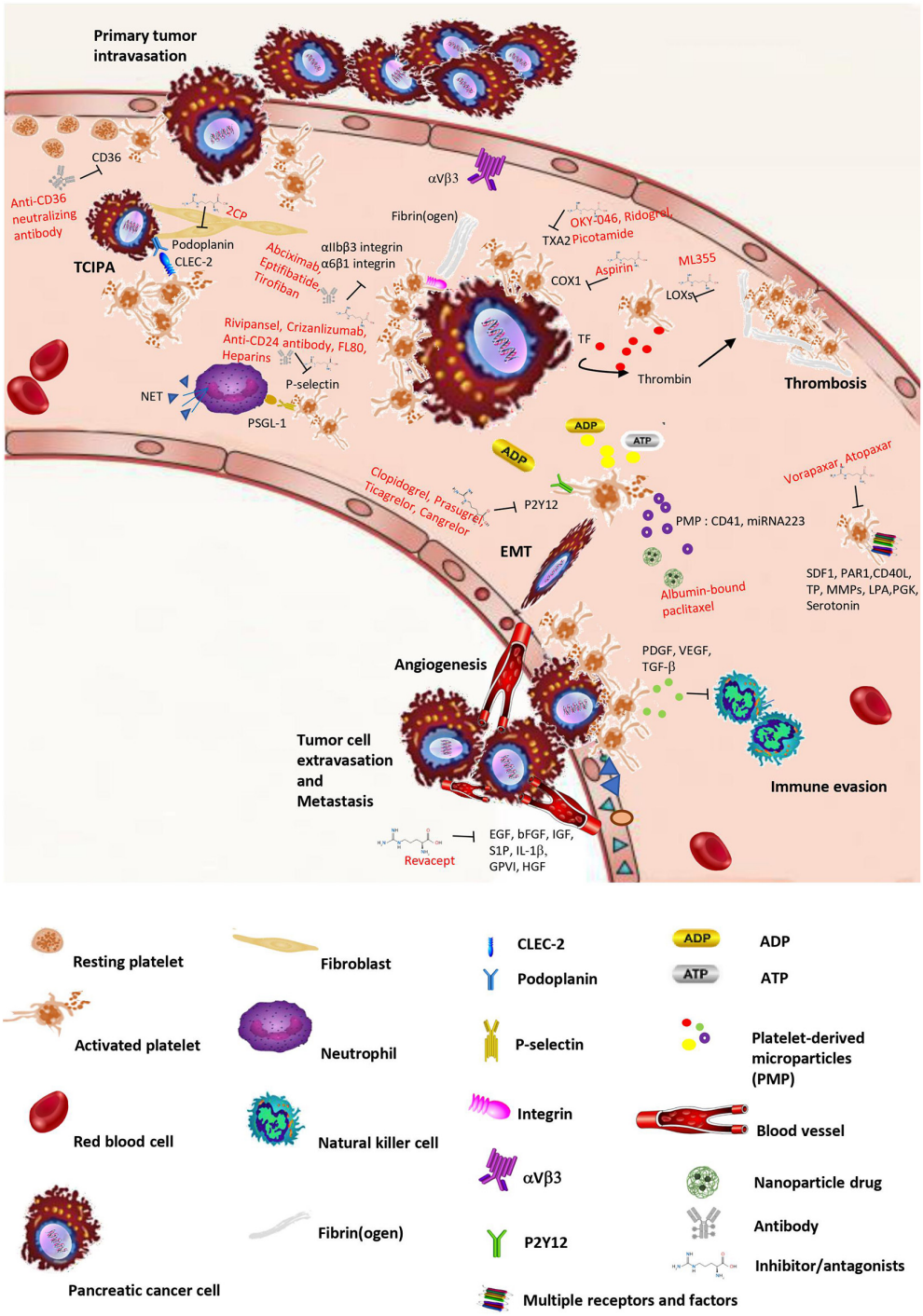

FIGURE 1 | Schematic overview of crosstalk between platelets and pancreatic cancer. Pancreatic cancer can stimulate platelet activation and aggregation (TCIPA), change platelet shapes, and increase platelets' release of microparticles with loading factors. However, platelet activation releases different kinds of factors, small molecules, and microparticles, which facilitate tumor growth, invasion, and metastasis. In the bloodstream, the tumor cell is surrounded by platelets that protect the encircled tumor cell from immune attack. In addition, platelets increase the properties of cancer cell adhesion and extravasation, thereby supporting cancer cell transmigration and metastasis. The intervention or inhibition of potential drugs in crosstalk between cancer and platelets are shown in the process.

and gemcitabine resistance. Ticagrelor, an inhibitor of ADP-P2Y12 axis, enhances chemotherapeutic efficacy in pancreatic cancer cells by targeting the Novel P2Y12-AKT pathway (Elaskalani et al., 2020). In addition, platelets also drive EMT to promote chemotherapy resistance. β1 integrin-dependent interaction within the tumor microenvironment may alter tumor response to chemotherapy.

CONCLUSION AND POTENTIAL DIRECTION

This review highlights evidence for crosstalk between pancreatic cancer and platelets. Cancer influences platelets in different ways, such as TCIPA, thrombopoiesis, phenotype, and extracellular vesicles release. However, platelets are not only bystander cells in circulation, but also play a vital role in primary tumor growth and in the whole metastatic process (**Figure 1**). Although these relationships between platelets and cancer are reported in various kinds of tumors, they are still not full evidenced in pancreatic cancer. Platelet-related factors, chemokines, signaling pathways, and even microparticles are less explored in pancreatic cancer compared with other neoplasms (**Supplementary Tables 1, 2**).

Pancreatic cancer can manifest as exocrine or endocrine tumors, depending on the cell of origin. PDAC represents 90% of pancreatic malignancies. Patients are often diagnosed in the advanced staged, leading to an overall 5-year survival rate below 10%. To find a suitable model is necessary for pancreatic cancer fundamental research and clinical application. There are different *in vitro* and *in vivo* models to study the process of pancreatic cancer (**Supplementary Table 3**). Compared with the advantages and disadvantages of these models, three-dimensional organoid culture will be an optimal choice to study pancreatic cancer progression. Organoids can maintain cell polarity, closely resemble molecular features, and interact with an extracellular matrix (Boj et al., 2015). Platelets as biomarkers are to be a platform for detecting pancreatic cancer (Sabrkhany et al., 2021). Cancer-associated platelets represent a liquid biopsy for diagnosis. In addition, platelets play an important role in thrombocytosis, cancer dissemination, immune surveillance, angiogenesis, recruitment of neutrophils/monocytes, and tumor cell extravasation. Establishing a co-culture model of platelets and pancreatic cancer is inevitable. Some research exists that develops co-culture pancreatic cancer organoids with immune cells, cancer-associated fibroblasts (CAF) (Tsai et al., 2018; Holokai et al., 2020), however, the 3D culture environment for platelets is still in a very early development stage. Rothan et al. (2014) found a 3D culture environment increases the efficacy of platelet-rich plasma release in prompting skin fibroblast differentiation and extracellular matrix formation. Because platelets were defined as one part of blood cells, a perspective on applications of human blood cell culture and organoids is born (Seghatchian and Amiral, 2020). In fact, xenograft modes involving the transplantation of pancreatic tumor organoids have been shown to generate the full spectrum of tumor progression (Miyabayashi et al., 2020). Nevertheless, the development of co-culture pancreatic cancer organoids with platelets can imitate crosstalk between pancreatic cancer, extracellular matrix and platelets in tumor microenvironment. Accordingly, the underlying mechanism of interplay between pancreatic cancer and platelets will be uncovered. Furthermore, clinical and preclinical use of antiplatelet therapies in cancer will be improved, for example, aspirin, dipyridamole, RA-23 (cAMP-PDE inhibitor), clopidogrel (Tzanakakis et al., 1993; Mezouar et al., 2015). Using platelets as a drug delivery system (DDS) was demonstrated by Lyde et al. (2015). Synthetic nanoparticles through coating with platelet membranes or platelet mimicry approach could be a smart DDS against cancer in the future (Burnouf et al., 2018). However, the double-sided effect of tumor microenvironment on platelets targeting nanoparticles is reported that the homing nanoparticles could realize the targeting ability, photo-thermal effect, and tumor immunotherapeutic ability in the accessible tumor but not the hypovascular tumor such as pancreatic cancer (Chen et al., 2018).

In summary, pancreatic cancer has effects on phenotype, aggregation, factors release, RNA profile of platelets, conversely, platelets influence pancreatic cancer progression. To fully reveal the crosstalk between platelets and pancreatic cancer is a prospective direction for clinical diagnosis and treatment.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Deregulation of Transcription Factor Networks Driving Cell Plasticity and Metastasis in Pancreatic Cancer

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Pancreatic cancer is a very aggressive disease with 5-year survival rates of less than 10%. The constantly increasing incidence and stagnant patient outcomes despite changes in treatment regimens emphasize the requirement of a better understanding of the disease mechanisms. Challenges in treating pancreatic cancer include diagnosis at already progressed disease states due to the lack of early detection methods, rapid acquisition of therapy resistance, and high metastatic competence. Pancreatic ductal adenocarcinoma, the most prevalent type of pancreatic cancer, frequently shows dominant-active mutations in *KRAS* and *TP53* as well as inactivation of genes involved in differentiation and cell-cycle regulation (*e.g. SMAD4* and *CDKN2A*). Besides somatic mutations, deregulated transcription factor activities strongly contribute to disease progression. Specifically, transcriptional regulatory networks essential for proper lineage specification and differentiation during pancreas development are reactivated or become deregulated in the context of cancer and exacerbate progression towards an aggressive phenotype. This review summarizes the recent literature on transcription factor networks and epigenetic gene regulation that play a crucial role during tumorigenesis.

Keywords: ADM—acinar to ductal metaplasia, PanIN—pancreatic intraepithelial neoplasia, PDAC—pancreatic ductal adenocarcinoma, transcription factors (TFs), cellular plasticity, epigenetics (chromatin remodelling), development

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1 INTRODUCTION

Patients suffering from pancreatic cancer (PaCa) have the lowest overall survival rate compared to other cancer types in Europe, with roughly 7% surviving over 5-years (European Comission, 2020). Although rated as the ninth most common cancer in Europe, it is currently the fourth most common cause of cancer-related deaths and expected to rank even higher by 2025 (Ferlay et al., 2016). Despite the emergence of new treatment regimens, average survival rates only marginally increased in the past decades. The most prevalent form of PaCa is pancreatic ductal adenocarcinoma (PDAC), accounting for 90% of all diagnosed cases. Different PDAC precursor lesions have been identified with pancreatic intraepithelial neoplasia (PanINs) accounting for the major lesions which continuously progress through distinct stages (Hruban et al., 2007; Macgregor-Das and Iacobuzio-Donahue, 2013). Lineage tracing in mice revealed that acinar cells undergoing acinar-to-ductal metaplasia (ADM) have the greatest propensity to form PanINs, whereas an ADM-PanIN-PDAC route in human PaCa is still controversial (Kopp et al., 2012; Storz, 2017). Mutational events driving PDAC formation have been identified, such as genetic alterations in the proto-oncogenic *KRAS* in early PanIN lesions, inactivation of the tumor suppressor gene *CDKN2A* in intermediate/

late lesions, and mutations in *TP53* and *SMAD4* during the transition to carcinoma (Goggins et al., 2000; Wilentz et al., 2000; Lüttges et al., 2001). Unfortunately, none of these genetic mutations have yet been proven targetable.

The main problem of PDAC is its early propensity towards metastasis together with the lack of early-stage diagnosis and limited treatment options due to rapid acquisition of therapy resistance. Besides the described genetic alterations, early malignancy and resistance are dependent on dysregulated epigenetic and transcriptional networks. These deregulations promote cellular plasticity, which helps tumor cells to adapt to novel environmental challenges during the metastatic cascade, to evade intrinsic control mechanisms, and dampen therapeutic efficacy (Orth et al., 2019). For a better prediction of disease progression and stratification of patient treatments, transcriptional profiling of resected PDAC tumors led to the identification of different molecular PDAC subtypes. Of those, two major subtypes with high tumor cellularity were described: pancreatic progenitor/classical and squamous/quasi-mesenchymal/basal-like (Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016; Puleo et al., 2018). Among these subtypes, the squamous type confers the most dismal prognosis and is associated with loss of endodermal cell fate (Bailey et al., 2016). In addition, this subtype is poorly-differentiated and highly chemoresistant (Chan-Seng-Yue et al., 2020). In contrast, the pancreatic progenitor subtype shows enrichment for the corresponding endodermal markers with a slightly better prognosis and is well-to-moderately differentiated (Bailey et al., 2016). Different samples from the same patient indicated that the pancreatic progenitor and squamous subtype can co-exist within the same tumor (Hayashi et al., 2021). Moreover, these subtypes are highly plastic and can interconvert, making it even more challenging to identify specific markers and subtype-specific treatment regimens (Lomberk et al., 2018; Brunton et al., 2020)

Transcription factors (TFs) are important actors in the spatio-temporal regulation of gene expression by directly binding *cis*-regulatory genomic elements (promoters and enhancers), recruiting cofactors (activators or repressors), and the core transcriptional machinery (Lee and Young, 2013). Together with other gene regulatory mechanisms, they drive cellular gene expression to orchestrate vital biological processes such as development, differentiation, cell cycle progression, tissue homeostasis, and cellular identity in a complex and tightly controlled manner. Deregulation of the delicate TF networks is a major cause of cancer and many other human diseases (Furney et al., 2006; Lee and Young, 2013). Specifically, TFs play a central role in all six hallmarks of cancer, *i.e.* sustained angiogenesis, endless replication, resisting cell death, insensitivity to anti-growth signals, self-sufficiency in growth signals, and activating invasion and metastasis (Hanahan and Weinberg, 2000, 2011). Of note, a staggering 20% of oncogenes encode TFs and TFs are terminal effectors in oncogenic signaling, thus representing important mediators in cancer (Lambert et al., 2018).

Several TFs orchestrating pancreatic organogenesis and driving pancreatic cell identity are deregulated in PDAC, strongly contributing to disease onset and progression. In the current review, we present an overview of our current understanding of transcriptional regulatory networks crucial in pancreas development, tissue homeostasis, and focus on recent findings illustrating how dysregulation of transcriptional networks promotes PDAC pathogenesis. In addition, we discuss the status of therapeutic strategies to target deregulated transcriptional networks and promising perspectives for the future.

2 TRANSCRIPTION FACTORS THAT ORCHESTRATE PANCREAS ORGANOGENESIS

The pancreas in the adult is comprised of an exocrine and endocrine compartment. Acini make up 90% of the cells in the mature organ and secrete nutrient-digestive zymogens, that are collected by a branched network of intralobular ducts for the release into the duodenum (Larsen and Grapin-Botton, 2017; Atkinson et al., 2020; Lorberbaum et al., 2020). The endocrine cells comprise 1–2% of the organ, are organized in islets of Langerhans and synthesize peptide hormones. They are essential for regulating blood glucose levels, produced by α- and β-cells, the main endocrine cell types that produce Glucagon and Insulin, respectively (Pan and Wright, 2011; Bastidas-Ponce et al., 2017; Larsen and Grapin-Botton, 2017). Pancreas organogenesis in mice starts at embryonic day (E)8.5 when the pancreas anlage is emerging as two independently forming dorsal and ventral buds that later fuse. This process is identified by *Pdx1* expression, which induces another key TF for pancreas formation, Ptf1α (p48) (Burlison et al., 2008; Shih et al., 2013). Two phases of pancreas organogenesis can be distinguished, starting with a primary transition (E8.5-E12.5) to specify pancreatic cell types and a secondary transition (E12.5-E17.5) to establish spatial organization of the tissue and cell maturation for generating numerous endocrine and exocrine cells (Bastidas-Ponce et al., 2017; Larsen and Grapin-Botton, 2017; Dumasia and Pethe, 2020). Initiation and maturation depend on an orchestrated network of TF activities.

Analyses of gene expression patterns by *in situ* hybridization and immunofluorescence labeling revealed that the pancreas is specified by combined activities of Activin, Fgf2, retinoic acid, Bmp, Shh, and Notch pathways. The morphogenetic events involve the underlying mesoderm, endothelium and notochord (Deutsch et al., 2001; Chung et al., 2008; Pan and Wright, 2011; Shih et al., 2013; Xuan and Sussel, 2016; Lorberbaum et al., 2020). Pancreas identity is specified by increasing Pdx1 levels established by a feedback loop induced by Ptf1α (Ahlgren et al., 1996; Wiebe et al., 2007). Expression maintenance of these genes is controlled by a network orchestrated by Sox9, Hnf1β and Foxa2 (Shih et al., 2013; Bastidas-Ponce et al., 2017). Moreover, Sox9 is important to reinforce pancreatic identity by blocking *Cdx2* expression combined with activation of the Notch target *Hes1,* which in turn supports progenitor cell proliferation and repression of the endocrine cell inducer Ngn3 (**Figure 1A**) (Jensen et al., 2000; Ahnfelt-Rønne et al., 2012; Shih et al., 2015). This network generates a pool of multipotent progenitor cells (MPCs) that expand by combined activation of genes encoding Nkx6.1, Mnx1,

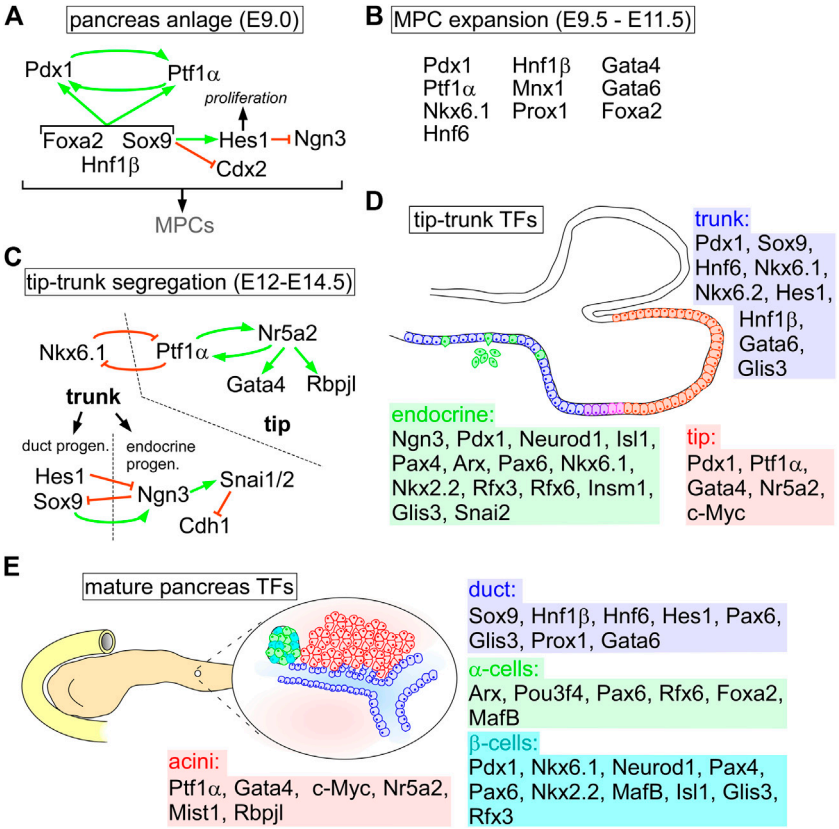

FIGURE 1 | Transcription factor networks orchestrating pancreas specification and homeostasis. **(A)** Early events to form the pancreas anlage and MPC specification. **(B)** TFs involved in expansion of the MPC pool during primary transition. **(C)** TF networks to define tip and bipotent trunk domains. In the trunk additional networks are established for endocrine and duct specification. **(D)** Sketch of tip-trunk cell distribution in the pancreas progenitors highlighting TFs active in each domain's networks. **(E)** overview on TFs of each cell compartment in the adult pancreas during homeostasis.

Hnf1β, Hnf6 (Onecut1), Prox1, Foxa2, and Gata4/6 (**Figure 1B**) (Gittes, 2009; Pan and Wright, 2011). Many of these TFs have a pivotal role in early pancreas specification, as their loss results in organ agenesis or severe hypoplasia, including Ptf1α, Sox9, Mnx1, Gata4/6, Hnf1β, and Hes1 (Jonsson et al., 1994; Offield et al., 1996; Harrison et al., 1999; Li et al., 1999; Jensen et al., 2000; Haumaitre et al., 2005; Seymour et al., 2007, 2012; Watt et al., 2007; Shaw-Smith et al., 2014; Xuan and Sussel, 2016).

Once pancreas identity is established, branching morphogenesis in MPCs leads to separation into tip and trunk cells, precursors of acinar and ductal structures, respectively. Initially co-expressed between E10.5 and E13.5, *Nkx6-1* becomes restricted to trunk and *Ptf1a* to tip cells. Tip cells initiate *Myc* (c-Myc) expression, whereas trunk cells are defined by *Hnf1b*, *Sox9*, *Hnf6*, and *Hes1* gene activities (Bastidas-Ponce et al., 2017; Larsen and Grapin-Botton, 2017). Furthermore, expansion and maintenance of the exocrine compartment is further supported by inhibition of the Hippo pathway to repress endocrine specific TF genes, including *Pax6*, *Ngn3*, *Isl1,* and *Nkx6-1,* as well as *Gcg* and *Ins1/2* (Gao et al., 2013; Dumasia and Pethe, 2020). Consequently, active Hippo signals antagonize Yap activity promoting an endocrine fate (Rosado-Olivieri et al., 2019). In tip cells, Ptf1α induces *Nr5a2*, crucial for acinar identity, as Nr5a2 directly regulates *Ptf1a* in a feedback loop as well as *Gata4* and *Rbpjl* (**Figures 1C,D**) (Hale et al., 2014; von Figura et al., 2014). In addition to future acinar and duct cell fates, the endocrine compartment emerges in a few individual cells within the trunk that activate *Ngn3*, presumably by lateral inhibition orchestrated by the Notch pathway, as shown by lineage tracing in mice (Gu et al., 2002; Murtaugh et al., 2003; Magenheim et al., 2011). $Ngn3^{+}$ cells delaminate from the trunk epithelium, subsequently cluster and form islets of Langerhans in the proximity of the tubular epithelium (**Figure 1D**) (Johansson et al., 2007; Pan and Wright, 2011; Shih et al., 2013). This process is reminiscent of epithelial-mesenchymal transition (EMT), by which epithelial cells lose the epithelial identity and apical-basal polarity to gain cell motility (for more details, see **Box 1**) (Johansson et al., 2007; Pan and Wright, 2011; Shih et al., 2013; Bastidas-Ponce et al., 2017). It involves coordinated expression of *Snai1* (Snail) and *Snai2* (Slug), EMT-TFs that are directly activated by Ngn3 and repress *Cdh1* (E-cadherin) (**Figures 1C,D**) (Rukstalis and Habener, 2007; Gouzi et al., 2011). Interestingly, another EMT-TF, Zeb1, is also expressed at low levels in the epithelial compartment of the developing pancreas.

BOX 1 | Epithelial-mesenchymal transition.
EMT is an embryonic program that is essential for establishing the three germ-layers and other key morphogenetic events during development, but also becomes activated during wound healing. Besides its physiological function, EMT is hijacked during progression towards metastasis in various cancers (Nieto et al., 2016; Lu and Kang, 2019). The activation of EMT governs changes in cell fate, allowing (partial) transition of stationary epithelial cells towards a motile, invasive mesenchymal state (Johansson et al., 2007; Pan and Wright, 2011; Shih et al., 2013; Bastidas-Ponce et al., 2017). Recent findings show that the process of EMT is highly dynamic, representing a spectrum of intermediary states (Jolly et al., 2015; Nieto et al., 2016; Lambert et al., 2017; Aiello et al., 2018). Moreover, the reverse process mesenchymal-epithelial transition (MET) promotes metastatic colonization and outgrowth, highlighting the need for cellular plasticity during the metastatic cascade (Brabletz, 2012; Takano et al., 2016; Aiello et al., 2018). Various intrinsic and extrinsic signals can mediate the induction of EMT in cancer, often involving the activation of major signaling pathways, including TGFβ, HGF, BMP, PDGF, EGF, SHH, Notch, Integrin, WNT/β-catenin, and NF-κB (Taipale and Beachy, 2001; Heldin et al., 2012; Espinoza and Miele, 2013; McCormack and O'Dea, 2013; Gonzalez and Medici, 2014; Mihalko and Brown, 2018; Tam et al., 2020; Xu et al., 2020). Activation of EMT by any of these cascades often converges in the activation of a core set of EMT-TFs, including ZEB1/2, Snail (*SNAI1*), Slug (*SNAI2*), and Twist (Nieto et al., 2016; Stemmler et al., 2019). Consequently, EMT-TFs directly or indirectly downregulate genes that promote epithelial identity with apical-basal polarity, including *CDH1*, *EPCAM*, Claudins, and miR-200 family members (Brabletz and Brabletz, 2010; Dongre and Weinberg, 2019). Simultaneously, they activate mesenchymal genes that promote migration, invasion, and a front-rear polarity, including *CDH2*, *VIM*, *ACTA2* (α-SMA), *FN1*, and MMPs (Dongre and Weinberg, 2019).

In contrast to the role of Snail and Slug during endocrine cell delamination, Zeb1 is crucial for proper lineage specification in correct ratios and for tissue homeostasis in the adult pancreas (Lasierra Losada et al., 2021). Temporal waves of TF expression initiate maturation of endocrine cells to ensure unidirectional unique cell type specification, including Neurod1, Insm1, and Rfx6, whose loss compromises islet cell identity and function (Bastidas-Ponce et al., 2017; Larsen and Grapin-Botton, 2017). Endocrine specification depends on repeated, transient rises in Ngn3 expression in the bipotent progenitor cells, that is regulated by *Pax6* activation, while maintained Pdx1 and Nkx6.1 levels are crucial for β-cell identity in the mature pancreas (Gannon et al., 2008; Schaffer et al., 2013) (**Figure 1D**).

In the adult pancreas, mature duct cells are maintained by continuous expression of trunk cell TFs, including Hnf6, Hnf1β, Sox9, Hes1, Pax6, Gata6, and Glis3, whereas mature acini express Ptf1α, Gata4, Mist1, and Nr5a2 (Shih et al., 2013; Bastidas-Ponce et al., 2017; Larsen and Grapin-Botton, 2017). Terminally differentiated β-cells are positive for Pdx1, Nkx6.1, Neurod1, Pax4/6, Rfx3, Nkx2.2, and MafA, whereas α-cells are defined by Arx, Pou3f4, Pax6, Rfx6, Foxa2, and MafB expression (**Figure 1E**) (Gittes, 2009; Pan and Wright, 2011; Shih et al., 2013; Cano et al., 2014; Dassaye et al., 2016; Bastidas-Ponce et al., 2017; Larsen and Grapin-Botton, 2017; Dumasia and Pethe, 2020; Jennings et al., 2020). Besides these regulatory circuits of TFs, correct pancreas progenitor formation, MPC identity, islet specification, and maintenance of individual cell types require epigenetic regulation and the activity of PcG proteins (Dumasia and Pethe, 2020). Deregulation of the established networks is an inevitable event in tumorigenesis and fosters disease progression.

3 DEREGULATED EXPRESSION OF TRANSCRIPTION FACTORS IN TUMORIGENESIS

3.1 Transcription Factors Driving Pancreatic Ductal Adenocarcinoma Initiation

PDAC is considered to emerge from a sequential progression of pre-neoplastic precursor lesions. Different histological types of putative precursor lesions have been described: PanIN, intraductal papillary mucinous neoplasia (IPMN), and pancreatic mucinous cystic neoplasm (MCN) (Hruban et al., 2000, 2007). PanIN lesions represent the most extensively studied precursors of PDAC and are categorized from PanIN1 to PanIN3, that accumulate progressive features reflecting increasing dysplastic morphology and acquisition of genetic alterations (Hruban et al., 2000, 2001; Maitra et al., 2003; Hezel et al., 2006; Guerra et al., 2007). Nevertheless, the cell of origin responsible for the initiation and early progression of PDAC remains undetermined. Despite the phenotypic similarity of these benign precursor lesions to ducts, mutant *Kras* expression in adult mouse ductal cells driven by *CK19* failed to induce PDAC, challenging the ductal origin of PDAC (Brembeck et al., 2003; Ray et al., 2011). Data from genetically engineered mouse models (GEMMs) suggest that the expression of oncogenic *Kras* in acinar cells induces transdifferentiation to duct-like cells during ADM. Although still debated, several lines of evidence suggest that this process precedes the formation of PanIN lesions and ultimately causes PDAC (Carriere et al., 2007; Guerra et al., 2007; Zhu et al., 2007; De La et al., 2008; Habbe et al., 2008; Morris et al., 2010; Kopp et al., 2012; Reichert et al., 2016). For example, analyses of patients with familial pancreatic cancer show that PanIN lesions, as well as ADM, and atypical flat lesions, can be found in human specimens (Brune et al., 2006; Zhu et al., 2007; Shi et al., 2009; Mazur et al., 2010; Aichler et al., 2012; Hidalgo-Sastre et al., 2016). Moreover, besides the classical PanIN-to-PDAC progression model, PDAC initiation was demonstrated to evolve separately from acinar or duct cells in a PanIN-independent mechanism (Ferreira et al., 2017). Likewise, expression of $Kras^{G12D}$ in combination with haploinsufficiency of *Smad4* leads to a sequential progression of MCN lesions towards a distinct class of PDAC (Izeradjene et al., 2007). Based on oncogenic mutations, TF networks become deregulated and cells start to transdifferentiate in multiple ways in favor of tumor progression.

Transdifferentiation or loss of cellular identity is a crucial feature at the onset of cancer formation (Slack, 2007; Stanger and Hebrok, 2013; Xiong et al., 2019). Upon injury or inflammation (pancreatitis) in mice, acinar cells can dedifferentiate towards a duct progenitor-like state, transiently expressing acinar, ductal, or early precursor markers to replenish the pancreas during tissue regeneration (Parsa et al., 1985; Song et al., 1999; Miyamoto et al.,

2003; Jensen et al., 2005; Means et al., 2005; Kopp et al., 2012; Stanger and Hebrok, 2013; Storz, 2017). This involves the re-expression of progenitor and lineage-specific TFs and subsequent re-differentiation, demonstrating cellular plasticity induced upon injury. Acinar cell identity in mice is maintained by several cooperating TFs, such as Ptf1α and Mist1 (Pin et al., 2001; Rose et al., 2001; Pan and Wright, 2011; Martinelli et al., 2013; Bastidas-Ponce et al., 2017; Larsen and Grapin-Botton, 2017). Downregulation of these TFs results in the acquisition of progenitor cell characteristics and increased ADM and PanIN formation, highlighting the importance of maintained expression of these identity factors to prevent tumor initiation (Miyamoto et al., 2003; De La et al., 2008; Shi et al., 2009; Flandez et al., 2014; von Figura et al., 2014; Krah et al., 2015). In line with that, oncogenic *Kras* expression prevents the acinar re-differentiation and helps to maintain a ductal phenotype after acute inflammation, *e.g.* during pancreatitis. This suppressed re-differentiation promotes PanIN progression (Morris et al., 2010; Collins et al., 2012). Furthermore, during the ADM process in PDAC GEMMs, TFs involved in MPC specification or in ductal identity maintenance are (re-)expressed, including Pdx1, Hes1, and Sox9 (Song et al., 1999; Miyamoto et al., 2003; Jensen et al., 2005; Seymour et al., 2007; Kopp et al., 2012). Examples of the role of specific TFs that become deregulated during the early event of transdifferentiation and tumorigenesis are discussed in more detail.

3.1.1 Gata6

Initially, Gata6 was presented as an important regulator of early pancreas specification and cell type differentiation, showing a partially overlapping expression with Gata4 (Ketola et al., 2004; Decker et al., 2006). Recently, Gata6 was demonstrated to be required for terminal differentiation and homeostasis of acinar cells and establishment of polarity (Martinelli et al., 2013). Evidently, *Gata6* inactivation induces massive loss of acinar cells and fosters ADM in the pancreas (Martinelli et al., 2013). In addition, *Gata6* ablation accelerates $Kras^{G12D}$ driven tumorigenesis, demonstrating that Gata6 maintains acinar differentiation by driving expression of acinar master TFs and suppressing ectopic programs in the pancreas. Hence, in this context, Gata6 functions as a tumor suppressor (Martinelli et al., 2016). In fact, *GATA6*, among other genes encoding endodermal cell-fate determination TFs, is silenced via promoter hypermethylation in the squamous subtype of PDAC (Bailey et al., 2016; Seino et al., 2018). In line with that, *GATA6* expression was preferentially detected in well-differentiated low-grade tumors upon transcription profiling (Collisson et al., 2011; Moffitt et al., 2015; Diaferia et al., 2016). Interestingly, silencing of *Gata6* and the subsequent loss of acinar differentiation was observed during nicotine administration in mice, providing a possible link to cigarette smoking, which is a major risk factor contributing to pancreatitis and PDAC initiation (Hermann et al., 2014; Weissman et al., 2020). These findings altogether emphasize the importance of Gata6 maintenance to prevent tumor initiation and progression towards PDAC.

3.1.2 Mist1

Mist1 is another acinar specification TF that is crucial for acinar cell maturation, function, stability, and identity and is involved in establishing granule organization and exocytosis pathways (Pin et al., 2001; Johnson et al., 2004; Direnzo et al., 2012). In the absence of Mist1 in pancreata with a $Kras^{G12D}$ mutation, destabilization of the acinar phenotype leads to acceleration of PanIN formation (Shi et al., 2009). Furthermore, in cell culture models Mist1 was shown to reduce acinar cell proliferation rates by activating p21 (CIP1/WAF1) (Jia et al., 2008). Data from a 3D ADM culture model revealed that forced expression of *Mist1* attenuates $Kras^{G12D}$-induced ADM and PanIN formation (Shi et al., 2013). Activation of *Mist1* upon orthotopic transplantation of murine PDAC cells rescues the acinar gene expression program (Jakubison et al., 2018). Overall, the maintenance of a differentiated acinar identity by Mist1 protects acinar cells from early tumorigenesis.

3.1.3 Ptf1α

Ptf1α maintains acinar cell identity and restrains Kras-mediated tumorigenesis (Rose et al., 2001; Thompson et al., 2012; Krah et al., 2015; Hoang et al., 2016). Nevertheless, *Ptf1a* is downregulated during inflammation-induced ADM and in acinar cells transformed by $Kras^{G12D}$ and Notch co-activation (Molero et al., 2007; De La et al., 2008). Specifically, downregulation of *Ptf1a* is a necessary and rate-limiting step in ADM and neoplastic progression to PanINs and PDAC to overcome the Ptf1α-mediated maintenance of acinar gene signatures and quiescence in mice (Krah et al., 2015). Additionally, *Ptf1a* was shown to be epigenetically silenced in murine ADM and PDAC cells harboring an oncogenic *Kras* allele (Benitz et al., 2016). Moreover, the sustained expression of *Ptf1a* prevents and reverts Kras-driven pancreas tumorigenesis, rescues the acinar gene program in PDAC cells, and can inhibit tumor growth (Jakubison et al., 2018; Krah et al., 2019). These examples highlight the role of Ptf1α as a key transcriptional regulator of acinar cell identity rendering differentiated acinar cells less sensitive for cancer initiation.

3.1.4 Pdx1

In the adult pancreas, the primary function of Pdx1 is the specification and maintenance of mature β-cells (Ahlgren et al., 1998; Gannon et al., 2008; Gao et al., 2014). During tumor formation, *Pdx1* is upregulated in ADM and PanINs upon overexpression of TGFα or expression of oncogenic *Kras* (Song et al., 1999; Hingorani et al., 2003; Park et al., 2011). In addition, gain- and loss-of-function analyses in human PDAC cell lines resulted in increased proliferation and invasion potential in the presence of PDX1. In contrast, its loss decreases cell survival and tumor growth *in vivo*, suggesting that PDX1 acts as an oncogene (Liu et al., 2008). In line with that, persistent Pdx1 expression in the normal pancreas promotes ADM induction via Stat3 activation. Simultaneous depletion of Stat3 blocks ADM formation (Miyatsuka et al., 2006). Despite its oncogenic function, *Pdx1* often becomes downregulated by hypermethylation during progression towards the squamous

and more aggressive subtype of PDAC. Conversely, PDX1 is part of a transcriptional network determining pancreatic endoderm cell fate and its presence results in a better prognosis in the pancreatic progenitor subtype (Bailey et al., 2016). In line with this conflicting data, Pdx1 was demonstrated to act as context-dependent TF during PDAC initiation and progression. Pdx1 switches from a safeguard of acinar cell identity during early tumorigenesis to an oncogene after the establishment of ADM (Roy et al., 2016). In summary, Pdx1 has two opposing functions that are activated in a context- and progression-dependent manner, emphasizing the necessity for more detailed analyses to better understand its bipartite function and prognostic value.

3.1.5 Sox9

Expression of Sox9 in the adult pancreas is restricted to cytokeratin-positive duct cells including centroacinar cells (Miyamoto et al., 2003; Seymour et al., 2007; Furuyama et al., 2011; Shroff et al., 2014). During tumor formation, it was shown that *Sox9* is induced in ADM and PanINs and is maintained in the pancreatic progenitor PDAC subtype (Morris et al., 2010; Kopp et al., 2012; Prevot et al., 2012; Meng et al., 2014; Grimont et al., 2015). Importantly, ADM and PanINs originating from the acinar compartment require ectopic induction of *Sox9*. Specific depletion of Sox9 from acinar cells efficiently blocks Kras-mediated PanIN formation in mouse models (Kopp et al., 2012). Furthermore, co-expression of oncogenic *Kras* and wild-type *Sox9* promotes induction of precursor lesions from the acinar compartment (Kopp et al., 2012). Mechanistically, efficient repression of acinar genes and activation of ductal/progenitor genes in cells that undergo ADM is dependent on the combined expression of *Sox9* and *Hnf6*, as *Hnf6* overexpression also triggers ADM in mouse acinar cell lines and upon adenoviral gene delivery *in vivo* (Prevot et al., 2012). During pancreatitis, inflammation-induced EGFR signaling was shown to induce *Nfatc1* and *Nfatc4* expression, leading to ADM and PDAC progression due to upregulation of *Sox9* (Chen et al., 2015; Hessmann et al., 2016). In addition, SOX9 may play a role in the IPMN-PDAC route, however, conflicting evidence have been observed. Some studies identified a gradual decrease in SOX9-positive cells in IPMNs during progression, while others report constant or even elevated SOX9 expression in both low-grade and high-grade IPMNs compared to the normal pancreas (Tanaka et al., 2013; Shroff et al., 2014; Gnerlich et al., 2019). In mice, *Arid1a* deficiency in the $Kras^{G12D}$ pancreas results in reduced Sox9 expression and less PanINs, but increased IPMN and PDAC formation. Simultaneous *SOX9* overexpression does not affect IPMN incidence, but reduces PDAC formation, demonstrating that Sox9 is a major downstream target of Arid1a and prevents tumor progression by promoting ductal differentiation (Kimura et al., 2018). Conclusively, Sox9 is a crucial mediator of ductal- or progenitor-like identity. Due to its embedding in multiple signaling pathways and feedback loops in cell-type specification, its deregulated expression is ultimately linked to early tumorigenesis.

3.1.6 Hes1

In the adult pancreas, the expression of the Notch target *Hes1* is limited to centroacinar and ductal cells associated with progenitor cell function (Miyamoto et al., 2003; Kopinke et al., 2011). Upregulation of *Hes1* by active Notch signaling was observed during ADM and PanIN formation (Hingorani et al., 2003; Miyamoto et al., 2003; Jensen et al., 2005; De La et al., 2008; Plentz et al., 2009). Moreover, Notch-induced Hes1 was suggested to control the expansion of an undifferentiated precursor cell population, thereby promoting Kras-mediated tumor initiation and progression (Miyamoto et al., 2003; Jensen et al., 2005; De La et al., 2008). In fact, acinar-specific expression of mutant *Kras* induces *Hes1* expression along with ADM and PanIN formation. In this context, Notch activation was shown to sensitize acinar cells to mutant *Kras*-induced ADM/PanIN initiation and progression (De La et al., 2008; Nishikawa et al., 2019). Interestingly, Elastase-mediated Hes1 depletion blocks the progression from ADM to PanINs, combined with a re-differentiation to acinar cells (Nishikawa et al., 2019). However, the role of Hes1 is likely more complex as in another mouse model using Ptf1α-mediated *Hes1* ablation and oncogenic *Kras* induction, loss of *Hes1* displayed increased ADM formation and accelerated PDAC tumorigenesis. Reduced numbers of high-grade PanINs were detected in this model, hinting towards tumor formation from a direct ADM-to-PDAC route that skips precancerous PanIN lesions (Hidalgo-Sastre et al., 2016). These findings convey that context-specificity and maintained activity of Notch and Hes1 during homeostasis are essential regulators of tumor initiation.

In summary, PDAC formation depends on early pre-neoplastic events like ADM, which relies on the downregulation of TFs that control acinar cell identity, including Gata6, Mist1, and Ptf1α, and a gain of TFs that promote duct or MPC-like specification, including Pdx1, Sox9, and Hes1 (**Figure 2**). However, some controversies and the incomplete understanding of the cellular origin of PDAC warrant further analyses to decipher the TF networks that are active during early tumorigenesis.

3.2 Transcription Factor Alterations Driving Pancreatic Ductal Adenocarcinoma Progression and Metastasis

Profiling of human PDAC specimens led to the identification of several PDAC subtypes (Collisson et al., 2011; Moffitt et al., 2015; Raphael et al., 2017; Puleo et al., 2018). The classification in the different studies largely overlap with one another (Collisson et al., 2019). Unsupervised clustering of PDAC tumors with high tumor cellularity identified the pancreatic progenitor and squamous subtype, suggesting that only these subtypes define the tumor compartment (Moffitt et al., 2015; Bailey et al., 2016; Puleo et al., 2018). Histopathologic evaluation revealed that tumors belonging to the pancreatic progenitor subtype are moderate-to-well differentiated, whereas the squamous subtype is poorly-differentiated (Puleo et al., 2018). Transcriptional network analysis of resected human PDAC specimens identified that the pancreatic progenitor subtype is enriched for TF transcripts pivotal for specifying pancreas cell-fate (*e.g. PDX1*, *HNF4A*, *HNF1B*, *HNF1A*, *FOXA2*, *FOXA3*, *HES1*, and *MNX1*) (Bailey et al., 2016). The squamous subtypes shows enriched gene

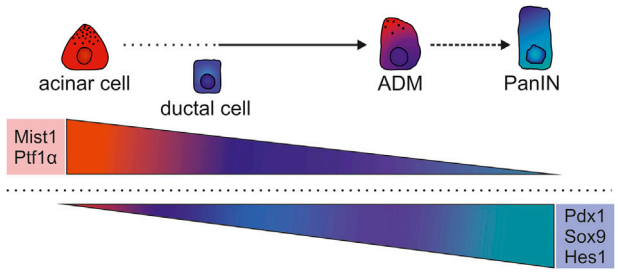

FIGURE 2 | Modulated expression of key TFs of the process of transdifferentiation during ADM. Differentiated acinar cells are specified and maintained by Gata4, Ptf1α, Mist1, and others, whereas duct cells depend on Sox9, Hes1, and others. During ADM several duct-specific TF networks are induced including Sox9 and Hes1, while acinar-specific networks involving Ptf1α and Mist1 are collapsing. ADM cells also adopt non-duct like features by activation of Pdx1, gaining more progenitor-like characteristics. Although Gata6 is required for acinar specification, but absent in mature acinar cells, its continuous expression prevents ADM.

networks involved in TGFβ signaling, *MYC* activation, inflammation, metabolic programming, and the upregulation of ΔNp63 and its targets. Multi-omics analyses of 24 patient-derived xenografts (PDXs) recapitulated the presence of the pancreatic progenitor and squamous subtype (Lomberk et al., 2018). Activated genes in the pancreatic progenitor subtype are mainly involved in pancreas development (*e.g. GATA6*, *BMP2*, *PDX1*, and *SHH*) and Ras signaling (*e.g. KITLG* and *RASA3*). The squamous subtype shows enrichment for pathways with strong oncogenic potential (*e.g.* PI3K-AKT, Hippo, and WNT), EMT (*e.g.* TGFβ signaling) (**Box 1**) and deregulation of genes involved in cell proliferation, differentiation and apoptosis (*e.g. YAP1*, *CD44*, *MYC,* and *E2F7*). These findings connect PDAC subtypes and differentiation states to deregulated signaling pathways. The deregulation of a subset of TFs play a pivotal role in facilitating a subtype switch to the more aggressive squamous phenotype by altering transcriptional regulatory networks. We will discuss these TFs and their effects upon deregulation.

3.2.1 Subtype-specific Transcription Factors

The oncogenic *KRAS* mutation is found in over 90% of PDAC patients and results in the persistent stimulation of downstream signaling leading to sustained cell proliferation, transformation, migration, and survival (Biankin et al., 2012; Buscail et al., 2020). Although Ras signaling is enriched in the pancreatic progenitor subtype, the activation of certain KRAS downstream mediators is able to foster the transition towards the squamous subtype. Elevation of Etv1, a downstream target of Kras, promotes stromal expansion and metastases through *Sparc* and *Has2* activation in tumors generated by orthotopic transplantation of KPC cells (Kar and Gutierrez-Hartmann, 2013; Heeg et al., 2016). *Etv1* overexpression induces all core EMT-TFs (**Box 1**) and molecular markers associated with the mesenchymal phenotype (*e.g. Vim*, *Mmp3*, and *Mmp9*), whereas knockdown of *Etv1* reduces Zeb1 levels. Using human PaCa cell lines *in vitro* it was shown that elevation of HAS2 is able to fuel a self-enforcing feedback loop of CD44 and ZEB1 that involves differential splicing of CD44 by ESRP1, further promoting EMT (Preca et al., 2015, 2017). In addition, EMT and enhanced invasion can be activated by increased *MAZ* expression in human PaCa cell lines. MAZ acts downstream of KRAS and facilitates CRAF-MAPK signaling involving PAK and suppression of AKT/PKB (Maity et al., 2018). Moreover, the upregulation of MAPK or inactivation of *TP53* leads to the overexpression of *KLF7*, promoting tumor growth and metastasis in mice (Gupta et al., 2020). Expression of KLF7 activates IFN-stimulated genes and stabilizes Golgi integrity and thus protein glycosylation to enhance the secretion of cancer-promoting growth factors. In cooperation with *Myc* Yap1 maintains the expression of metabolic genes required for proliferation and survival (Murakami et al., 2019). Ablation of *Yap1* in a PDAC mouse model leads to the downregulation of *Myc*, inducing growth arrest and apoptosis (Murakami et al., 2019). Interestingly, a subset of tumor cells was able to restore Myc levels allowing cell survival through the induction of genes encoding EMT-TFs Snail, Zeb2, Twist2, and the stemness factor Sox2, thus compensating for *Yap1* loss.

Multiple studies show that the pancreatic progenitor subtype is KRAS-dependent, whereas the squamous subtype is less dependent on KRAS (Singh et al., 2009; Collisson et al., 2011; Ischenko et al., 2021). Moreover, CRISPR/Cas9-mediated *Kras* knockout in tumor cells derived from the KPC mouse model showed pathway enrichment for EMT and TGFβ signaling, hinting that ablation of *Kras* drives a subtype switch towards the squamous subtype (Ischenko et al., 2021). Secondary ablation of $Kras^{G12D}$ in established tumors of a GEMM with doxycycline-inducible $Kras^{G12D}$ and conditional *Tp53* inactivation leads to complete regression (Kapoor et al., 2014). Although these initial results are promising, the majority of mice show relapse and exhibit poorly-differentiated pancreatic tumors. The survival of tumor cells in this model in the absence of *Kras* is mediated by the upregulation of the transcriptional coactivator Yap1, a downstream mediator of the Hippo signaling cascade, and Tead2, forming Yap1/Tead2 complexes coordinating downstream gene expression. Other compensatory mechanisms have been identified, including the induction of the transcriptional repressor *Gli2*, a downstream mediator of the Shh pathway, upon *in vitro* $Kras^{G12D}$ ablation (Adams et al., 2019; Ischenko et al., 2021). *Gli2* induction rescued viability and induced upregulation of squamous-specific gene signatures (*e.g. Vim* and *Zeb1*). Moreover, *GLI2* induction in human PaCa cell lines promotes a gene signature switch from the pancreatic progenitor towards the squamous subtype, accompanied by a decrease in epithelial identity markers (E-cadherin, ESRP1, GATA6, and SHH) and enrichment in expression of EMT/stemness markers (ZEB1, VIM, CK14, SOX2, and CD44). Primary tumor growth and metastatic outgrowth can be suppressed by ablation of *SPP1*, a downstream target of GLI2, emphasizing its role in promoting tumor aggressiveness. These findings demonstrate that aberrant activation of several TFs exacerbate PDAC progression (**Table 1**) with various degrees of KRAS-dependency. Interestingly, these deregulations frequently mediate the indirect upregulation of EMT-TFs (ZEB1/2, Snail, Slug, and Twist) and stemness factors

TABLE 1 | Overview of the individual TFs and their effects upon elevation in primary PDAC tumors. Influence on cellular identity, subtype, tumor characteristics and biological processes are highlighted. An upward pointing arrow (↑) indicates promoting effects, a downward pointing arrow (↓) inhibitory effects, a minus symbol (–) nor promoting nor inhibitory effects. Blank cells reflect that the process was not analyzed or no conclusion could be drawn from the indicated studies.

		Cellular identity		Subtype		Tumor characteristics		Biological processes					
TF	**Context**	**Epithelial**	**Mesenchymal**	**Pancreatic progenitor**	**Squamous**	**Growth/ Progression**	**Metastasis**	**Proliferation**	**Stemness**	**EMT**	**Invasion**	**Migration**	**References:**
Yap1					↑	↑		↑					Kapoor et al. (2014), Lomberk et al. (2018)
GLI2		↓	↑	↓	↑				↑	↑			Adams et al. (2019)
ETV1		↓	↑			↑	↑	-		↑	↑		Heeg et al. (2016)
MAZ		↓	↑						↑	↑	↑	↑	Maity et al. (2018)
KLF7						↑	↑				↑	↑	Gupta et al. (2020)
SMAD4	$SMAD4^{+/+}$	↓	↑			↓				↑			Bardeesy et al. (2006), Ischenko et al. (2021)
	$SMAD4^{-/-}$	↑	↓	↑	↓	↑		-		↓			
RUNX3	$SMAD4^{+/-}$					↑	↓	↑			↓	↑	Whittle et al. (2015)
	$SMAD4^{-/-}$					↑	↑			↓			
TGIF1		↑	↓			↓	↓	-	↓	↓	↓	↓	Weng et al. (2019)
PDX1		↑	↓	↑	↓	↑	↓	↑		↓	↓		Collisson et al. (2011), Roy et al. (2016), Lomberk et al. (2018)
GATA6		↑	↓	↑	↓	↓	↓	↑		↓	↓		(Martinelli et al., 2016, 2017, Lomberk et al. (2018)
FOXA1		↑	↓	↑		↑	↑	↑		↓	↑	-/↓	Song et al. (2010), Diaferia et al. (2016), Martinelli et al. (2017), Roe et al. (2017)
FOXA2		↑	↓	↑				↑		↓		↓	Song et al. (2010), Bailey et al. (2016), Martinelli et al. (2017)
HNF4α		↑	↓	↑	↓	↓	-	↓					Bailey et al. (2016), Camolotto et al. (2021)
HNF1α								↓					Hoskins et al. (2014), Luo et al. (2015)
					↓	↑		↑	↑		↑	↑	Abel et al. (2018), Subramani et al. (2020)
SIX1				-	-	↑		↑					Camolotto et al. (2021)
SIX4				↓	↑	↑		↑					
BACH1		↓	↑			-	↑	-		↑	↑	↑	Sato et al. (2020)
ZEB1		↓	↑	↓	↑	-	↑	↑	↑	↑	↑		Krebs et al. (2017)
SNAI2		↓	↑		↑	↑	↑			↑	↑	↑	Recouvreux et al. (2020)
SOX2		↓	-					↑	↑	↑			Herreros-Villanueva et al. (2013)
PRRX1A		↑	↓	↑	↓		↑	↑		↓	↓		Takano et al. (2016)
PRRX1B		↑	↓	↓	↓		↑			↑	↑		

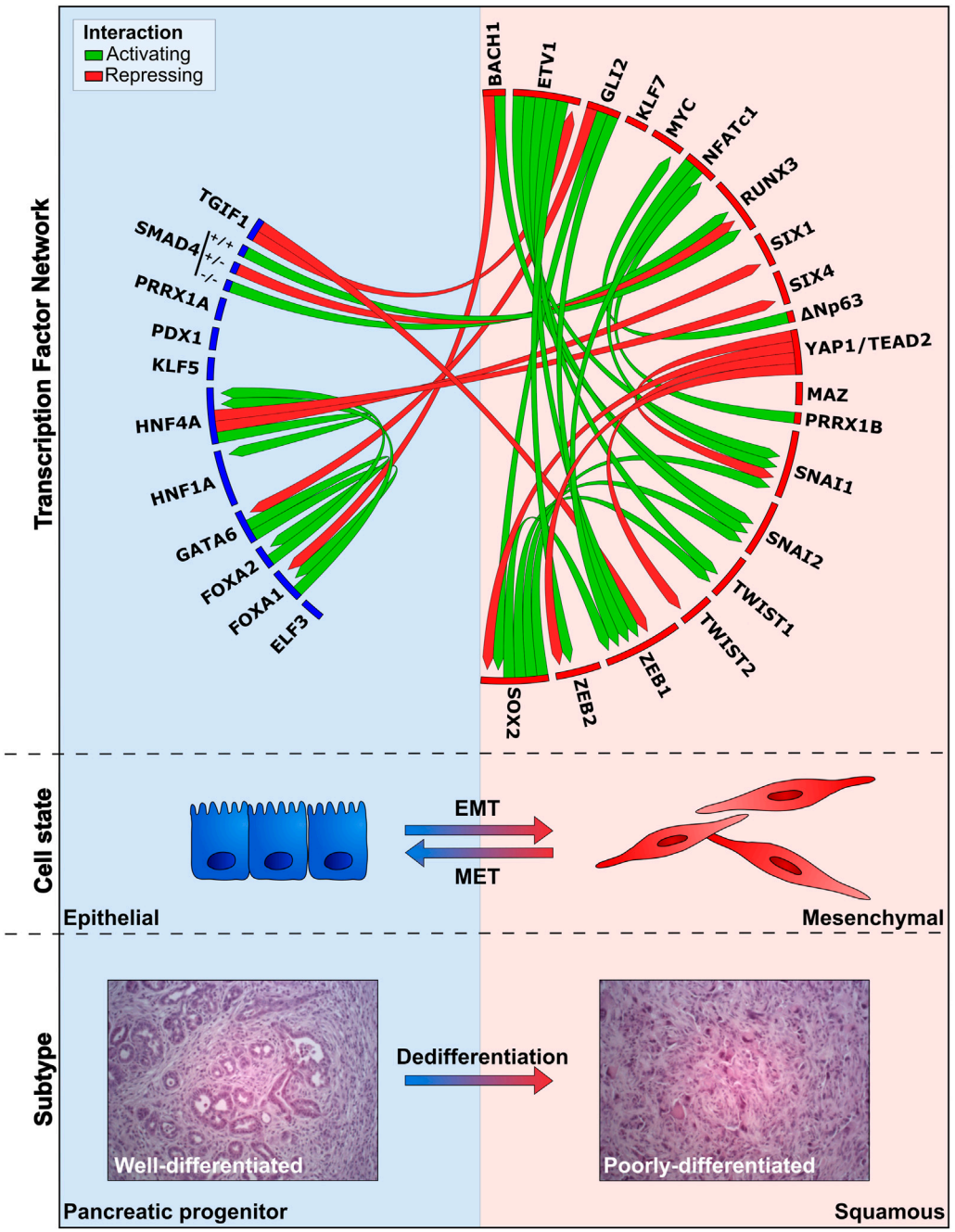

FIGURE 3 | Overview of TF networks active during PaCa progression, EMT, and metastasis. The deregulation directly or indirectly affects the expression of other TFs, thereby promoting or inhibiting the differentiation/EMT state. TFs depicted in the blue box are associated with the epithelial cell-state/pancreatic progenitor subtype (E-PP), whereas the red box shows TFs linked to the mesenchymal-state/squamous subtype (M-S). Links resulting in activation and repression of TFs in the network are indicated by green and red lines, respectively. Expression of E-PP TFs in the blue box promote the differentiated endoderm/epithelial identity, block dedifferentiation, and prevent activation of stemness/EMT/dedifferentiation TFs. Activation of M-S TFs is associated with a mesenchymal identity, promotes dedifferentiation and activation of EMT and stemness. TFs without connections in the network have been associated with specific subtypes, but how they integrate into the network is poorly understood. H&E images depict well-differentiated and poorly-differentiated tumors, derived from KPC primary pancreatic tumors, reflecting E-PP and M-S phenotypes (adapted from Krebs et al. (2017)).

(SOX2 and CD44) (**Figure 3**, **Box 1**). These findings corroborate that the induction of the reversible EMT program promotes an aggressive PDAC phenotype by enabling cellular plasticity, metastasis formation, chemoresistance, and the acquisition of CSC properties in PDAC (Satoh et al., 2015; Wang et al., 2015; Zheng et al., 2015; Krebs et al., 2017; Aiello et al., 2018; Recouvreux et al., 2020).

Apart from the activation of signaling cascades downstream of KRAS driving the subtype transition, the squamous subtype shows enrichment for TGFβ signaling (Bailey et al., 2016; Lomberk et al., 2018). In pre-malignant cells TGFβ acts as a tumor suppressor by inducing cell-cycle arrest, differentiation, and apoptosis (Massagué, 2008). Disruption of TGFβ signaling in PDAC prevents the tumor-suppressive effects, while activation of TGFβ signaling in a progressed state is a potent inducer of EMT (Dardare et al., 2020). A central player in canonical TGFβ signaling is SMAD4, whose inactivation or loss occurs as a late event during PDAC progression (Wilentz et al., 2000). The role of Smad4 in progression and metastasis remains controversial, as *Smad4*-deficiency attenuates EMT, leads to upregulated E-cadherin protein levels, and promotes a well-differentiated PDAC phenotype in the $Kras^{G12D}$;$Ink4a/Arf^{\Delta}$ mouse model (Bardeesy et al., 2006; Ahmed et al., 2017; Shichi et al., 2019). Moreover, simultaneous knockout of *Smad4* and *Kras* reduces EMT-related genes and promotes a Ras signaling signature (Ischenko et al., 2021). In contrast, other studies show that the loss of *SMAD4* is associated with shorter overall survival and a squamous phenotype (Blackford et al., 2009; Yamada et al., 2015). Interestingly, the TF Runx3 promotes metastatic colonization but is dependent on the *Smad4* state (Whittle et al., 2015). Heterozygous *Smad4* inactivation in the KPC mouse model promotes progression and growth of the primary tumor, while loss of the remaining wild-type allele leads to a highly metastatic disease. Furthermore, the expression of TGFβ target genes can be repressed by elevated TGIF1, potentially decreasing PDAC progression as demonstrated by HEK-293 cell transfection experiments and in PDAC mice (Seo et al., 2004; Weng et al., 2019). In summary, the intricate balance among several TFs involved in TGFβ signaling and their mutational status determine the impact on progression.

On the other hand, several endodermal lineage specifiers promote the pancreatic progenitor molecular subtype in PDAC, such as GATA6, FOXA1/A2, and HNF4α, identified *in silico* (Bailey et al., 2016; Roe et al., 2017; Brunton et al., 2020). Although *PDX1* expression is often increased during ADM and tumor onset, its downregulation or loss is mainly observed in poorly-differentiated tumors correlated with EMT and metastasis (Roy et al., 2016). Hence, high expression of *PDX1* is observed in the pancreatic progenitor subtype and well-differentiated tumors (Ischenko et al., 2014; Roy et al., 2016; Lomberk et al., 2018). Dysregulation of these TFs by inactivating mutations or repression can affect the EMT-equilibrium towards a more squamous identity. As an example, loss of *Gata6* in a mouse PDAC model decreases the *Cdh1* inducers Foxa1/a2 and de-represses EGFR signaling in favor of dedifferentiation (Martinelli et al., 2016, 2017). In contrast, FOXA1 elevation was identified in patients' primary lesions and well-differentiated low-grade tumors, in part by activating *HNF4A* and other endodermal lineage specifiers (Duncan et al., 1998; Diaferia et al., 2016; Roe et al., 2017). Together with Gata6, Foxa1/a2 block EMT and promote epithelial differentiation in GEMM PDAC models (Song et al., 2010; Martinelli et al., 2017). Hence, direct transcriptional repression of *FOXA1* by BACH1 is required for metastatic colonization of AsPC-1 PaCa cells in an orthotopic implantation model (Sato et al., 2020). Interestingly, loss of *FOXA1/A2* is frequently detected in the squamous subtype and is sufficient to induce EMT in human PaCa cell lines (Song et al., 2010; Roe et al., 2017). Apart from repressing *FOXA1*, BACH1 activates *SNAI2,* which further promotes EMT, assessed by gene inactivation in human cell lines (Sato et al., 2020). Transcriptomic analysis on primary tumors and patient-derived cell lines revealed that during tumorigenesis HNF4α directly activates *HNF1A*, and loss of the former enables a transition towards a more squamous phenotype (Brunton et al., 2020; Camolotto et al., 2021). Moreover, HNF4α directly represses the mesodermal and neural differentiation TFs *SIX1/4*, whose elevated expression was linked to the squamous subtype (Camolotto et al., 2021). Downregulation of *HNF1A* is observed in the tumor vs. normal pancreas, suggesting that decreased HNF1α levels are important for PDAC tumor progression (Hoskins et al., 2014; Luo et al., 2015). Overexpression of *HNF1A* decreases cell-doubling times, while its knockdown significantly increases proliferation *in vitro*. HNF1α downregulates apoptosis inhibitors and modulates the expression of cell cycle genes. However, whether HNF1α acts through the AKT/mTOR pathway requires additional investigation, since silencing of *HNF1A* activates AKT/mTOR signaling, but may also result in reduced expression of PI3K, AKT and mTOR (Hoskins et al., 2014; Luo et al., 2015; Subramani et al., 2020). Other studies indicate that *HNF1A* is an oncogene necessary for the regulation of cancer stem cell (CSC) populations in PDAC, promotes anchorage-independent growth, proliferation, as well as invasive and migratory capacities (Abel et al., 2018; Subramani et al., 2020). These contradictory findings could be explained by the hypothesis that cellular plasticity and thus the ability to induce partial-EMT is indeed necessary to acquire stemness, whereas reversal to an epithelial phenotype is crucial for metastatic outgrowth at secondary sites. Conclusively, the expression of several TFs involved in specifying pancreatic cell-fate maintain the pancreatic progenitor subtype by (in)directly promoting epithelial-identity markers and inhibiting EMT/dedifferentiation (**Table 1**). Their downregulation abrogates these effects, allowing a switch towards the more aggressive squamous subtype (**Figure 3**).

3.2.2 Induction of Epithelial-Mesenchymal Transition and Metastasis

Comparisons between primary PDAC tumors and matched metastasis revealed no specific metastasis-inducing genetic mutations, hinting towards gene regulatory mechanisms affecting late PDAC progression and metastasis (Campbell et al., 2010; Yachida et al., 2010; Makohon-Moore et al., 2017). The involvement of EMT-TFs in PDAC invasion and metastasis was initially questioned due to experimental challenges to observe EMT and the metastatic cascade *in vivo*. In particular, depletion of either Twist or Snail in the KPC mouse model of PDAC is not affecting metastasis formation, indicating that they are dispensable for this process (Zheng et al., 2015). However, depletion of Zeb1 in the same mouse model suppresses metastasis formation as well as experimental lung colonization capacity, stemness, and cell and metabolic plasticity (Krebs et al.,

BOX 2 | Epigenetic regulation of gene expression.
Epigenetic mechanisms control the accessibility for TFs and the transcription machinery to selective regions of the genome. Consequently, depending on the state of the epigenetic landscape, TFs can bind to *cis*-regulatory elements to regulate gene transcription (Shen and Laird, 2013; Klemm et al., 2019). These mechanisms can be broadly divided into: post-translational histone modifications, DNA/RNA modifications (*e.g.* methylation) and non-coding RNAs (Shen and Laird, 2013; Lu et al., 2020). Histone modifications at specific regulatory regions include methylation and acetylation predominantly at histone H3 sites K4, K9, and K27 and are associated with active genes/promoters (H3K4me3), active/poised enhancers (H3K4me1), polycomb-repressed regions (H3K27me3) or heterochromatin (H3K9me3). These post-translational marks are set by a group of histone modifications enzymes, which are reviewed elsewhere (Bannister and Kouzarides, 2011). Super-enhancers (SEs) are a special type of enhancer which have been first identified in embryonic stem cells with clusters of TF binding sites for Sox2, Oct4 and Nanog (Hnisz et al., 2013; Whyte et al., 2013). SEs have also been identified in cancer and represent large regions of chromatin (up to 20 kb) that are densely clustered with enhancers, highly enriched for TF binding sites (Hnisz et al., 2013; Whyte et al., 2013). Their function is crucial in shaping cellular identity by regulating cell-type specific gene expression in both normal and diseased states (Hnisz et al., 2013).

2017). Moreover, glutamine depletion promotes metastasis of orthotopically and intravenously injected KPC cells through induction of EMT by upregulation of *Snai2* via ERK signaling and ATF4 activation (Recouvreux et al., 2020). Collectively, these findings and research on core EMT-TFs in other cancers show that their individual contribution to invasion and metastasis is highly dependent on the cellular context (Elloul et al., 2005; Imamichi et al., 2007; Caramel et al., 2013; Denecker et al., 2014; Fischer et al., 2015; Zheng et al., 2015; Krebs et al., 2017; Stemmler et al., 2019; Recouvreux et al., 2020).

Liver metastases in KPC mouse models show elevated expression of Foxa1 and Prrx1a, whereas the expression of these factors decreases when the primary tumor acquires more squamous-associated features during progression (Takano et al., 2016; Roe et al., 2017). The decrease during progression and elevated expression in liver metastases suggests that re-expression of TFs associated with the pancreatic progenitor subtype is essential for successful liver colonization. Moreover, it was shown that Prrx1a enhances self-renewal, decreases invasiveness, and promotes metastatic outgrowth (Takano et al., 2016). Isoform b, on the other hand, fosters invasion, EMT, and dedifferentiation by promoting *Hgf* expression, suggesting that both isoforms distinctively regulate EMT and MET to form overt metastases. In addition, these two isoforms can form homo- and heterodimers, affecting transcriptional activity in human PaCa cell lines (Marchand et al., 2019). Simultaneous inactivation of *Snai1* and *Twist* induces a shift of the EMT-equilibrium to a more epithelial-like state in the primary tumor of KPC mice while enhancing liver metastases (Carstens et al., 2021). Sca1 and Pdx1 levels are also regulating metastatic capacities: $Sca1^-$ cell lines derived from the KPC mouse model express elevated levels of Pdx1 and successfully metastasize in lungs and lymph nodes upon tail vein injections, whereas $Sca1^+$ cells with lower levels of Pdx1 fail to metastasize (Ischenko et al., 2014). These findings support the idea that the reinforcement of epithelial features and thus cellular plasticity are required for metastatic competence (**Figure 3**).

3.3 Chromatin Dynamics and Epigenetic Regulation of Pancreatic Ductal Adenocarcinoma

In addition to the deregulation of established TF networks in tumor progression, epigenetic mechanisms of gene regulation are altered and become hijacked by cancer cells resulting in global changes in gene expression (**Box 2**). Genomic analyses in human PDAC revealed that up to 10% of mutations are identified in chromatin remodeling genes (Hayashi et al., 2021). Moreover, the epigenetic landscape of PDXs revealed that the squamous and pancreatic progenitor subtype can also be classified by patterns in DNA methylation and gene regulatory elements (Nicolle et al., 2017; Lomberk et al., 2018). Deregulation of specific histone modification enzymes in PDAC can lead to the transition towards the more aggressive squamous subtype by altering the chromatin states. Specifically, mutations in histone lysine demethylase 6a (*KDM6A*) combined with p53 alterations were associated with the squamous subtype of PDAC (Bailey et al., 2016). Loss of *KDM6A* alone is sufficient to induce a squamous-like subtype through activation of ΔNp63 (*TP63*), *MYC*, and *RUNX3* enhancer regions (Andricovich et al., 2018). Interestingly, upregulation of ΔNp63 alone is able to reprogram the enhancer landscape towards the squamous subtype by installing H3K27ac near genes promoting this subtype (Somerville et al., 2018). Upregulation of the histone methyltransferase *Nsd2* increases the global accumulation of the activation mark H3K36me2, thereby enriching the squamous gene signature in the KPC model. In contrast, loss of *Nsd2* decreases H3K36me2, resulting in enrichment of markers of the pancreatic progenitor subtype (Yuan et al., 2020). These findings suggest that the accumulation of dimethylation at H3K36 is necessary for cells to undergo EMT. Moreover, H3K36me2 may induce alterations in the enhancer landscape as its decrease leads to loss of H3K27ac in the same domains. Interestingly H3K36me2 transcriptionally affects the enhancer activity and thus the expression of most EMT-TF genes (*Zeb1/2*, *Snai1*, and *Twist2*) and of other metastasis-promoting TFs (Yuan et al., 2020). Histone methyltransferase EZH2 is part of the polycomb repressor complex 2 (PRC2) to set H3K27 methylation marks (Viré et al., 2006; Völkel et al., 2015). During pancreas regeneration, EZH2 transcriptionally represses *NFATC1,* whereas during tumorigenesis it induces *NFATC1* to drive KRAS-mediated PaCa plasticity (Chen et al., 2017). Making use of uncoupling Ezh2-NFATc1 regulation by combining conditional *Nfatc1* activation with *Ezh2* inactivation in $Kras^{G12D}$ mice, Patil et al. recently showed that partial loss of *Ezh2* leads to more differentiated PDAC tumors and fewer liver metastases in line with higher EZH2 protein expression in human high-grade tumors (Patil et al., 2021). Strikingly the most abundant negatively regulated target of Ezh2 is *Gata6*, a key regulator of endodermal identity. Moreover, re-expression of

wild-type *Ezh2* abrogates *Gata6* expression in *Ezh2*-deficient cells. Conclusively, alterations in histone modification enzymes affect the chromatin states and aid in PDAC progression by inducing or repressing genes involved in PDAC progression.

Very recently, a strong contribution to PDAC progression was observed by the regulation of so-called super-enhancers (SEs) (**Box 2**) (Andricovich et al., 2018; Lomberk et al., 2018; Somerville et al., 2018). Comparisons between healthy cells and related cancer cells revealed that these SEs accumulate close to the loci of oncogenes during cancer progression, thus playing an important role in tumorigenesis (Hnisz et al., 2013). Profiling of the SE landscape revealed that several TFs transcriptionally regulate the expression of genes associated with the pancreatic progenitor subtype by binding upstream of these SEs (*i.e.* GATA6, FOS, FOXP1, FOXP4, KLF4, ELF3, NFIX, CUX1, and SSBP3) (Lomberk et al., 2018). These SEs mainly regulate genes of TFs associated with pancreas development (including *HNF1A*, *HNF4A,* and *PDX1*) and lipid metabolism. Epigenomic mapping of PDXs and PaCa cell lines showed that ΔNp63 activates squamous-specific SEs, including those near *FAT2*, *NECTIN1,* and *HIF1A* loci (Hamdan and Johnsen, 2018). Depletion of ΔNp63 reduces the H3K27ac at those SEs, indicating the dependency of ΔNp63 for writing these H3K27 acetylation marks. Loss of *KLF5* leads to a reduction in H3K27ac and H3K4me1 near these (super-)enhancers, inducing activation of stem cell- and mesenchymal-associated genes (Diaferia et al., 2016). *KLF5* was shown to be selectively expressed in well-differentiated human PDAC tumors and is required to maintain the expression of epithelial identity genes. Moreover, binding of KLF5 to enhancers increased the binding of ELF3 and FOXA1, which are both associated with low-grade PDAC tumors, demonstrating how KLF5 contributes to the regulation of pancreatic progenitor identity (Diaferia et al., 2016).

Progressive loss of repressive marks in large heterochromatin domains (H3K9 and H4K20) and increased H3K9, H3K27, and H4K16ac were found in distant metastases in comparison to the primary tumor, which helped to follow the acquisition of malignant traits (McDonald et al., 2017). These traits included resistance to oxidative stress, promoting a poorly-differentiated state, upregulation of DNA repair genes, and downregulation of oncogenic signal transduction in distant lung metastases. Comparisons of the epigenetic landscape from matched tumor and metastasis-derived organoids of the KPC model revealed that metastatic transition is accompanied by prominent changes in H3K27 acetylation, predominantly in enhancer regions. These changes are controlled by Foxa1, which is upregulated in metastases and was shown to cooperate with Gata5 for enhancer activation (Roe et al., 2017). Epigenetic regulation is also required to overcome the tumor-suppressive effects of TGFβ signaling, *i.e.*, the induced senescence and apoptosis before it can act as a trigger of EMT induction. Strikingly, NFATc1 elevation is crucial to overcome TGFβ-induced growth arrest by antagonizing H3K27ac and activation of TGFβ target genes including *Birc5*, *Ccnd1,* and *Plk1* (Hasselluhn et al., 2019).

Altogether, these findings indicate that epigenetic states define the molecular subtypes of PDAC in a highly dynamic process. Alterations in the epigenetic landscape including SEs are key features in PDAC progression towards malignancy, supporting the acquisition of cellular plasticity.

4 NOVEL THERAPEUTIC APPROACHES TO TARGET TRANSCRIPTION FACTORS

Despite the advances in therapies, non-metastatic local PDAC eligible for surgical resection followed by adjuvant chemotherapy remains the sole curative option, applicable for only 10–20% of patients (Gillen et al., 2010; Werner et al., 2013; Benassai et al., 2015; Orth et al., 2019). First-line treatment options for patients with locally advanced or distant metastatic PDAC are usually limited to conventional chemotherapies. Despite changes in treatment regimens from monotherapies to multi-agent chemotherapies, the survival rates of PaCa patients remain largely unchanged and success is severely limited due to *de novo* acquisition or pre-existing resistance. Various intrinsic and extrinsic tumor feature alterations have been proposed contributing to drug resistance, including the microenvironment, altered metabolism, EMT, and the presence of CSCs (Grasso et al., 2017; Swayden et al., 2018; Tuerhong et al., 2021). The lack of blood vessels and the abundant desmoplasia create a hypoxic and nutrient-scarce environment, forcing PDAC cells to alter their metabolism to sustain proliferation (Sousa and Kimmelman, 2014; Yang et al., 2020). In addition, these microenvironmental features impede therapeutic delivery (Neesse et al., 2011; Dufort et al., 2016). In general, global efforts are made to design precision therapies to combat PDAC, including therapeutic targets to inhibit tumor-intrinsic pathways such as KRAS, PI3K, AKT, mTOR, JAK/STAT, SHH, NOTCH, and WNT signaling cascades (Chandana et al., 2019). These strategies predominantly target mediators in oncogenic signaling cascades upstream of TFs, thereby indirectly affecting the expression of deregulated TFs. So far, the only precision medicine approved for PDAC treatment is erlotinib, a potent inhibitor of EGFR-related kinase, used in combination with gemcitabine (Moore et al., 2007; Sinn et al., 2017).

As summarized before, PDAC is highly plastic, and inhibition of certain kinases can be compensated by the dysregulation of other effectors (as seen for KRAS) and often converge to the same set of TFs (*e.g.* ZEB1/2, Snail, Slug, Twist, and SOX2) (**Figure 3**). Hence, these TFs are attractive therapeutic targets. For example, silencing of *ZEB1* restores the expression of epithelial markers and resensitizes PaCa cells to standard chemotherapy (Arumugam et al., 2009; Wellner et al., 2009; Meidhof et al., 2015). Similarly, treatment with the HDAC inhibitor Mocetinistat *in vitro* and upon xenotransplantation upregulates ZEB1-repressed target genes, particularly miR-200 and miR-203, reducing ZEB1 protein expression and restoring drug sensitivity (Meidhof et al., 2015). Moreover, knockout of *Snai1* or *Twist* increased the sensitivity to erlotinib and gemcitabine (Zheng et al., 2015). Although initially thought to be undruggable, recent attempts to design and identify drugs that target TFs are promising (Henley and Koehler, 2021). Strategies to inhibit TFs directly and indirectly include targeting the

expression level, modulating proteasomal degradation, disrupting protein/protein interactions, and ligand/DNA binding abilities (Lambert et al., 2018; Bushweller, 2019).

Multiple clinical trials are currently ongoing to evaluate therapeutics that indirectly target TF expression levels in PDAC. Studies on triptolide (TPL), a diterpenoid triepoxide, shows promising results in PDAC cell lines and orthotopic pancreatic cancer models (Borja-Cacho et al., 2010; Chugh et al., 2012; Zhao et al., 2020). TPL binds to XPB, a subunit of TFIIH, thereby inhibiting transcription globally (Vispé et al., 2009; Titov et al., 2011). Moreover, treatment with TPL (Minnelide) leads to a rapid downregulation of *MYC* gene expression and protein levels (Titov et al., 2011). Phase II clinical trials are currently ongoing to study the effect of TPL on non-responsive PDAC tumors. In addition, clinical attempts to target epigenetic deregulation are currently under evaluation. These include treatment with Azitidine and/or Romideposin, in combination with immuno- as well as standard chemotherapeutic treatments in patients with surgically resected and advanced PDAC. The inhibition of effectors upstream of TFs may pose severe problems in non-neoplastic cells, as these pathways are often indispensable for proper cell functioning. It is hypothesized that direct TF-targeting approaches minimize the side effects by precisely modulating their deregulated transcriptional programs (Henley and Koehler, 2021). The drug COTI-2, a thiosemicarbazone, has been shown to directly convert mutant p53 to the wild-type 3D structure. *TP53* is approximately mutated in 72% of all PDAC (Raphael et al., 2017). Gain-of-function mutations in *TP53* increase the aggressiveness in PaCa and promotes metastasis (Morton et al., 2010; Weissmueller et al., 2014). Additionally, it negatively regulates the PI3K/AKT/mTOR pathway (Salim et al., 2016; Robertson et al., 2020). Phase I clinical trials are currently ongoing to study the effect of COTI-2 as monotherapy or with combinations for the treatment of malignancies. Moreover, bi-weekly treatment of pretreated metastatic PDAC patients with the STAT3 inhibitor BBI608, shows promising activity (Bekaii-Saab et al., 2016).

The advent of targeted therapies to target TF together with the advances in other therapeutic strategies (*e.g.* immunotherapy, targeting receptors, membrane transporters, and enzymes) and rise of precision medicine bear the promise to improve PDAC patient outcomes. Although the field is still evolving, these combinational treatments offer valuable options for PaCa patients to overcome acquired therapy resistance and aggressive phenotypes.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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GLOSSARY

ΔNP63 deltaNp63 alternate variant of the tumor protein p63

3D three dimensional

ACTA2 (αSMA) actin alpha 2, smooth muscle

ADM acinar-ductal metaplasia

AKT/PKB AKT serine/threonine kinase 1/protein kinase B

ALDH1 aldehyde dehydrogenase 1

ATF4 activating transcription factor 4

BACH1 BTB domain and CNC homolog 1

BIRC5 baculoviral IAP repeat containing 5

BMP bone morphogenetic protein

BRCA2 breast cancer type 2 susceptibility protein

CCND1 cyclin D1

CDH1 cadherin 1/(epithelial) E-cadherin

CDH2 cadherin 2

CDKN2A cyclin dependent kinase inhibitor 2A

CDX2 caudal type homeobox 2

CK14 cytokeratin 14

CK19 cytokeratin 19

CRAF/Raf1 Raf-1 proto-oncogene, serine/threonine kinase

CSC cancer stem cell

CUX1 cut like homeobox 1

DNA deoxyribonucleic acid

E2F7 E2F transcription factor 7

EGF epidermal growth factor

EGFR epidermal growth factor receptor

ELF3 E74 like ETS transcription factor 3

EMT epithelial-mesenchymal transition

EPCAM epithelial cell adhesion molecule

ERK extracellular signal-regulated kinases

ESRP1 epithelial splicing regulatory protein 1

ETV1 ETS variant transcription factor 1

EZH2 enhancer of zeste 2 polycomb repressive complex 2 subunit

FAT2 FAT atypical cadherin 2

FBP1 fructose-bisphosphatase 1

FGF2 fibroblast growth factor 2

FN fibronectin 1

FOS Fos proto-oncogene, AP-1 transcription factor subunit

FOXA1 forkhead box protein A1

FOXA2 forkhead box protein A2

FOXP1 forkhead box P1

FOXP4 forkhead box P4

GATA4 GATA binding protein 4

GATA6 GATA binding protein 6

GCG glucagon

GEMM genetically engineered mouse model

GLI2 GLI family zinc finger 2

GLIS3 GLIS family zinc finger 3

HAS2 hyaluronic acid synthase 2

HDAC histone deacetylase

HES1 hairy and enhancer of split 1

HGF hepatocyte growth factor

HIF1A hypoxia inducible factor 1 subunit alpha

HKDC1 hexokinase domain containing 1

HNF1α hepatocyte nuclear factor 1 alpha

HNF1β hepatocyte nuclear factor 1 beta

HNF4α hepatocyte nuclear factor 4 alpha

HNF6 (Onecut1) hepatocyte nuclear factor 6 (one cut family member 1)

IFN interferon

INK4A/ARF cyclin-dependent kinase 4 Inhibitor/alternative reading frame

INS1 insulin I

INS2 insulin II

INSM1 insulinoma-associated 1/INSM transcriptional repressor 1

IPMN intraductal papillary mucinous neoplasia

ISL1 islet 1/ISL LIM homeobox 1

JAK janus kinase

KDM6A lysine demethylase 6A

KITLG KIT ligand

KLF krüppel-like factor

KRAS KRAS proto-oncogene, GTPase

MAFA musculoaponeurotic fibrosarcoma oncogene homolog A/MAF bZIP transcription factor A

MAFB musculoaponeurotic fibrosarcoma oncogene homolog A/MAF bZIP transcription factor B

MAPK mitogen-activated protein kinase

MAZ MYC associated zinc finger protein

MCN mucinous cystic neoplasm

MET mesenchymal-epithelial transition

miRNA microRNA

MIST1 muscle, intestine and stomach expression 1/basic helix-loop-helix family member A15 (BHLHA15)

MMP matrix metalloprotease

MNX1 motor neuron and pancreas homeobox 1

MPC multipotent progenitor cell

mTOR mammalian target of rapamycin

MYC MYC proto-oncogene, bHLH transcription factor

NECTIN1 nectin cell adhesion molecule 1

NEUROD1 neuronal differentiation 1

NFATC1 nuclear factor of activated T cells 1

NFATC4 nuclear factor of activated T cells 4

NFIX nuclear factor I X

NF-κB nuclear factor kappa B

NGN3 neurogenin 3

NKX2.2 NK2 homeobox 2

NKX6.1 NK6 homeobox 1

NR5A2 nuclear receptor subfamily 5 group A member 2

NSD2 nuclear receptor binding SET domain protein 2

p21(CIP/WAF1) cyclin-dependent kinase inhibitor 1/wild-type p53-activated fragment 1

p53/TP53 tumor protein p53

p63/TP63 tumor protein p63

PaCa pancreatic cancer

PAK p21 activated kinase

PanIN pancreatic intraepithelial neoplasia

PAX4 paired box 4

PAX6 paired box 6

PcG polycomb group

PDAC pancreatic ductal adenocarcinoma

PDGF platelet-derived growth factor

PDX patient-derived tumor xenograft

PDX1 pancreatic and duodenal homeobox 1

PI3K phosphoinositide 3-kinases

PLK1 polo like kinase 1

POU3F4 POU class 3 homeobox 4

PRC2 polycomb repressor complex 2

PROX1 prospero homeobox 1

PRRX1 paired-related homeodomain transcription factor 1

PTF1A/p48 pancreas associated transcription factor 1a

RASA3 RAS p21 protein activator 3

RBPJL recombination signal binding protein for immunoglobulin kappa J region like

RFX3 regulatory factor X3

RFX6 regulatory factor X6

RNA ribonucleic acid

RUNX3 RUNX family transcription factor 3

SCA1 stem cell antigen 1

SE super-enhancer

SHH sonic hedgehog

SIX1 SIX homeobox 1

SIX4 SIX homeobox 4

SLUG/SNAI2 snail family transcriptional repressor 2

SMAD4/DPC4 SMAD family member 4/deleted in pancreatic cancer 4

SNAIL/SNAI1 snail family transcriptional repressor 1

SOX2 SRY-box transcription factor 2

SOX9 SRY-box transcription factor 9

SPARC secreted protein acidic and cysteine rich

SPP1 secreted phosphoprotein 1

SSBP3 single stranded DNA binding protein 3

STAT3 signal transducer and activator of transcription 3

TF transcription factor

TFIIH transcription factor IIH

TGFα transforming growth factor alpha

TGFβ transforming growth factor beta

TGIF1 TG-interacting factor 1

TJP1/ZO-1 tight junctional protein 1/zonula occludens 1

TPL triptolide

TWIST1 twist-related protein 1

VIM vimentin

WNT portmanteau from wingless and int-1 (locus of frequent mouse mammary tumor virus (MMTV) integration)

XPB/ERCC3 xeroderma pigmentosum type B/ERCC excision repair 3, TFIIH core complex helicase subunit

YAP Yes1 associated transcriptional regulator

ZEB1 zinc finger E-box binding homeobox 1

ZEB2 zinc finger E-box binding homeobox 2

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REVIEW
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Targeting Tumor-Stromal Interactions in Pancreatic Cancer: Impact of Collagens and Mechanical Traits

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Pancreatic ductal adenocarcinoma (PDAC) has one of the worst outcomes among cancers with a 5-years survival rate of below 10%. This is a result of late diagnosis and the lack of effective treatments. The tumor is characterized by a highly fibrotic stroma containing distinct cellular components, embedded within an extracellular matrix (ECM). This ECM-abundant tumor microenvironment (TME) in PDAC plays a pivotal role in tumor progression and resistance to treatment. Cancer-associated fibroblasts (CAFs), being a dominant cell type of the stroma, are in fact functionally heterogeneous populations of cells within the TME. Certain subtypes of CAFs are the main producer of the ECM components of the stroma, with the most abundant one being the collagen family of proteins. Collagens are large macromolecules that upon deposition into the ECM form supramolecular fibrillar structures which provide a mechanical framework to the TME. They not only bring structure to the tissue by being the main structural proteins but also contain binding domains that interact with surface receptors on the cancer cells. These interactions can induce various responses in the cancer cells and activate signaling pathways leading to epithelial-to-mesenchymal transition (EMT) and ultimately metastasis. In addition, collagens are one of the main contributors to building up mechanical forces in the tumor. These forces influence the signaling pathways that are involved in cell motility and tumor progression and affect tumor microstructure and tissue stiffness by exerting solid stress and interstitial fluid pressure on the cells. Taken together, the TME is subjected to various types of mechanical forces and interactions that affect tumor progression, metastasis, and drug response. In this review article, we aim to summarize and contextualize the recent knowledge of components of the PDAC stroma, especially the role of different collagens and mechanical traits on tumor progression. We furthermore discuss different experimental models available for studying tumor-stromal interactions and finally discuss potential therapeutic targets within the stroma.

Keywords: pancreatic cancer, PDAC—pancreatic ductal adenocarcinoma, stroma, collagen, mechanical traits, extracellular matrix

1 INTRODUCTION

Pancreatic cancer is one of the most fatal malignancies with a 5-years survival rate below 10% (Europe 2018) (Siegel et al., 2021). The poor survival rate in pancreatic cancer is mainly due to its asymptomatic progression in early stages, resulting in late diagnosis, and the lack of effective treatment regimens against the advanced stages of the disease. More than 90% of all pancreatic cancer patients present exocrine tumors where an overwhelming majority of these tumors are pancreatic ductal adenocarcinoma (PDAC) which can originate from either the epithelial cells lining pancreatic ducts or the acinar cells of the pancreas (Flowers et al., 2021) (Grant et al., 2016).

One of the distinctive characteristics of PDAC, as compared to other solid tumors, is its highly fibrotic stroma which can contribute up to 80% of the tumor volume (Erkan et al., 2012). PDAC stroma primarily consists of extracellular matrix (ECM) and cancer-associated fibroblasts (CAFs) (**Figure 1**) (Hosein et al., 2020). ECM, which is the three-dimensional (3D) structural non-cellular portion of the tumor, is synthesized and secreted by the cells in the tumor microenvironment (TME) to provide mechanical and biochemical support to the tumor (Frantz et al., 2010). High levels of ECM production in reaction to a pathological state such as a wound, or tumor such as in PDAC, is known as a desmoplastic reaction or desmoplasia. Desmoplasia is further characterized by continuous ECM remodeling which is the constant degradation and deposition of ECM molecules, such as collagens, into the TME. A high amount of collagen deposition in the TME increases tumor density, thus altering its mechanical traits compared to normal pancreatic tissue. These alterations increase solid stress, interstitial fluid pressure, and stiffness in the tumor and collectively bring about changes in the tissue microarchitecture (Nia et al., 2020). These deviations in the mechanical traits of the tumor affect the tumor vasculature resulting in hypoxia and play a significant role in disease progression, invasiveness, metastasis, and treatment resistance (Jain et al., 2014).

In a disease with late-onset symptoms such as PDAC, studies of the precursor lesions become crucial to enable early detection and for improving patient survival. Precursor lesions of PDAC develop as a result of the accumulation of genetic mutations in epithelial cells and include pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasm (IPMN).

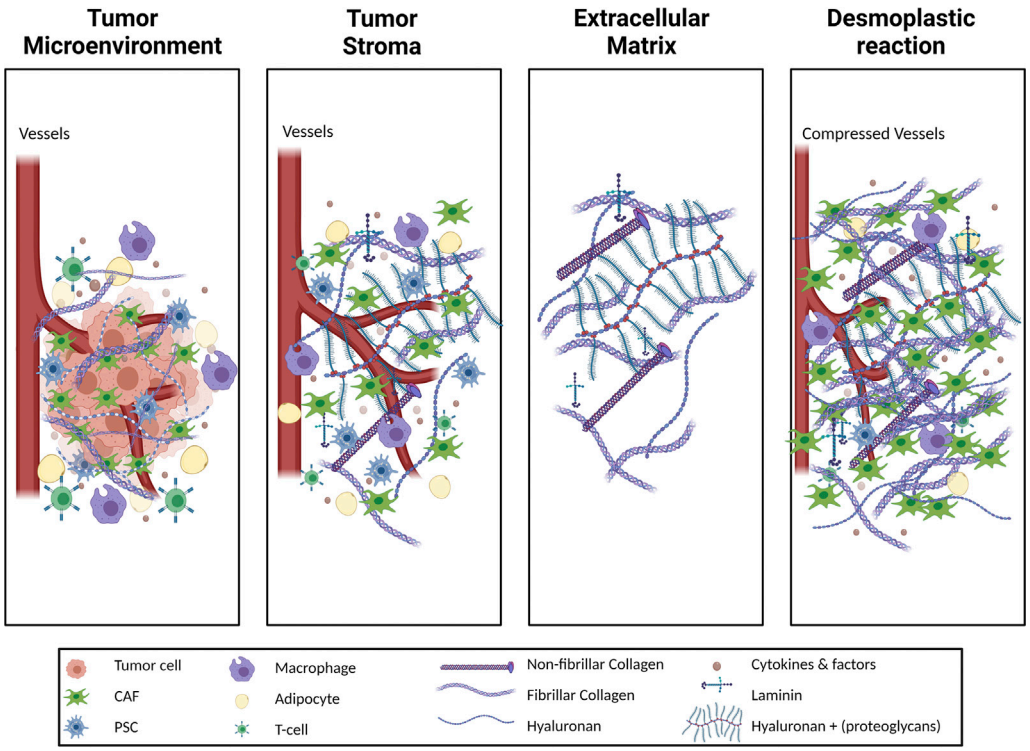

FIGURE 1 | Schematic illustration of different terms used to describe the tumor stroma. The tumor microenvironment (TME) of pancreatic adenocarcinoma (PDAC) includes the tumor cells in addition to the stroma. The stroma is a complex milieu of non-tumor cells, cytokines, growth factors and the proteins of extracellular matrix (ECM). The ECM consists of the non-cellular structural arrangement of the tissue and is highly proteinaceous. Cellular components of PDAC stroma include immune cells such as T-cells and macrophages, as well as stromal cells such as adipocytes and pancreatic stellate cells (PSCs) which are a source of cancer associated fibroblasts (CAFs). CAFs and, to a lesser extent, tumor cells alter the ECM composition by means of increased collagen, both fibrillar and non-fibrillar, deposition as well as laminins, hyaluronan and other proteoglycans. The pathophysiological increase of ECM protein content in the TME results in a desmoplastic reaction, generating denser, stiffer tissue than in the healthy pancreatic setting. Consequences of the desmoplastic reaction are a constrained blood vessels, infiltration around the tissue, and a corresponding reduction of fluid perfusion into the tissue, which in turn cultivates a hypoxic TME.

PDAC is presumed to develop primarily from PanIN lesions (Hruban et al., 2004) which share several genetic mutations with PDAC such as a gain of function mutation in the KRAS gene which is found in up to 95% of all PDAC cases (Hruban et al., 2000) and is also found in around 55% of early PanIN lesions and more than 80% of late PanIN (Yonezawa et al., 2008). As with PDAC, desmoplasia is observed in PanIN lesions (Pandol et al., 2012).

The shared characteristic of stromal aberrations in PDAC and in benign ailments which may then develop into PDAC indicates the possibility that stromal aberrations may support or promote tumor initiation and future malignancy. Since desmoplasia is one of the major stromal aberrations common to several precursor lesions as well as PDAC, it is prudent to study the formation of the desmoplastic stroma and its interaction with pancreatic cells in order to understand the role of stroma and changes thereof in the early stages of disease progression. Moreover, it can be surmised that comparing the composition of pancreatic stroma at different stages of the disease might help in identifying unique molecular indicators for the onset of the disease and its progression.

One stromal component that can be studied in detail between disease stages is the matrisome (Tian et al., 2019). The matrisome consists of the full repertoire of ECM proteins, including the core ECM proteins such as collagens, glycoproteins, and proteoglycans, as well as ECM-associated proteins, like ECM regulators, ECM-affiliated proteins, and other secreted metabolites (Naba et al., 2012). It has been shown that collagens are the most prominent group of proteins in the stroma during PDAC development and chronic pancreatitis, accounting for more than 90% of the ECM proteins at all stages (Tian et al., 2019). Collagens are primarily deposited by CAFs into the ECM and provide a hospitable microenvironment for the tumor cells (Bachem et al., 2005) (Olivares et al., 2017). Collagens also play an important role in altering the mechanical traits of tumor tissue (Riegler et al., 2018).

Finding new biomarkers for early PDAC diagnosis in high-risk individuals is under intense exploration. It is suggested that PDAC can take almost two decades to develop from initial mutation to metastatic disease, indicating a significant window of opportunity for early diagnosis (Yachida et al., 2010). Despite this, no PDAC-specific biomarker with high sensitivity for the early disease has yet reached the clinic. One reason for this can be that initial cancer clones stay dormant for a long period of time, and the exponential increase of cancer cells is a late event in disease progression (Hruban et al., 2000) (Notta et al., 2016). However, the remodeling of the stroma starts early and is pronounced already in preinvasive precursor lesions (Erkan et al., 2012) which suggests that stromal fragments in the circulation might be easier to detect than biomarkers released from cancer cells themselves, which is an idea that has shown promising results in initial studies. For example, a fragment of collagen type XVIII, endostatin, has been found in the blood of PDAC patients at higher levels than normal and these levels return to normal after surgery (Öhlund et al., 2008). Another study has shown elevated levels of collagen type IV in the peripheral circulation of PDAC patients and that persisting high levels of collagen type IV after curative surgery is correlated with poor survival and a quick relapse in patients (Öhlund et al., 2009). Moreover, circulating levels of matrix metalloproteinase (MMP)-degraded fragments of collagens type I, III, and IV and a pro-peptide of collagen type III have been used as biomarkers for PDAC (Willumsen et al., 2019). A recent report has also shown the applicability of combined analysis of stroma-derived markers, including collagen type IV and endostatin with conventional tumor markers such as CA 19-9, TPS, CEA, and Ca 125, in detecting PDAC (Franklin et al., 2015).

Taken together, the desmoplastic stroma of PDAC is found early in the development of the disease and plays important and complex pathophysiological roles throughout disease progression. Here, we aim to review recent literature on components of the PDAC stroma, with a focus on the role of different collagens and mechanical traits on tumor progression. Moreover, we discuss different experimental models available for studying tumor-stromal interactions and finally discuss potential therapeutic targets within the stroma.

2 THE DESMOPLASTIC REACTION IN PANCREATIC DUCTAL ADENOCARCINOMA

The desmoplastic reaction is the formation of a fibrotic, stiff, and collagen-rich connective tissue with high solid stress around the cancer cells (**Figure 1**). It also creates a special microenvironment that facilitates tumor growth and metastasis while acting as a physical barrier to drug penetration resulting in chemo-resistance (Schober et al., 2014). The complexity and abundance of the ECM increases with an increasing level of desmoplasia as PDAC progresses. (Tian et al., 2019).

The major producers of desmoplasia are fibroblasts. Fibroblasts are the most abundant cell type within the connective tissue with an important role in homeostasis and wound healing (Öhlund et al., 2014). It is hard to define fibroblasts due to their lack of specific markers which are not shared with other lineages, however, they are often identified by their location, morphology and lack of epithelial, endothelial, or leucocyte specific markers (Sahai et al., 2020). They proliferate and differentiate into myofibroblasts in response to tissue injury or cancer, which resembles a "chronic wound" in many ways (Dvorak 1986). This process is marked by increased expression of alpha-smooth muscle actin (α-SMA), a contractile stress fiber protein (Tomasek et al., 2002). CAFs generally refer to all the non-cancerous fibroblastic cells embedded in the ECM of tumor tissue (Öhlund et al., 2014). They are derived from different sources (**Figure 2**) such as resident fibroblasts (Kojima et al., 2010), bone marrow-derived mesenchymal stem cells (Quante et al., 2011) (Mishra et al., 2008), neighboring adipose tissue (Kidd et al., 2012), or arise from the transdifferentiation of other cell types such as pancreatic stellate cells (PSCs) (Bachem et al., 1998), bone marrow-derived fibrocytes (Reilkoff et al., 2011), epithelial cells (Iwano et al., 2002), and endothelial cells (Zeisberg et al., 2007). Stellate cells are a form of retinoic acid (a metabolite

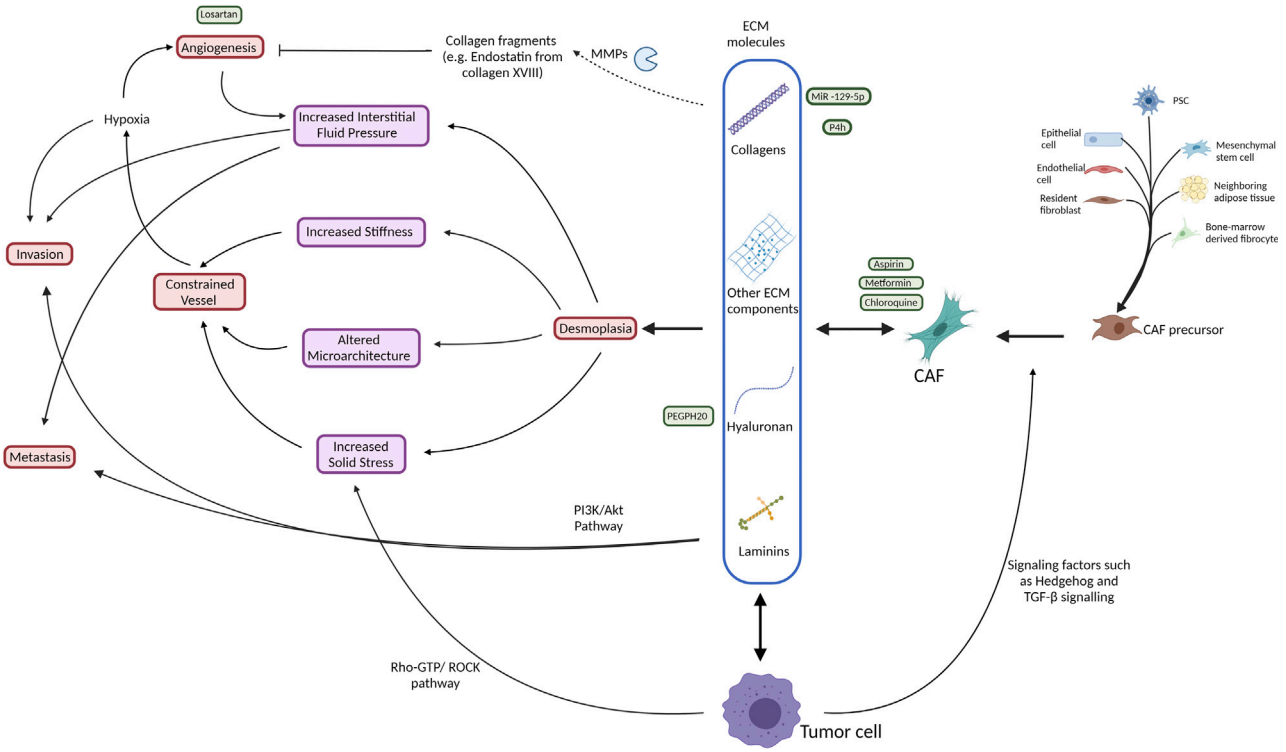

FIGURE 2 | Schematic illustration of the interactive network of the tumor stroma with respect to ECM production (blue box) and its effects on biomechanical (purple) traits and induction of physiological processes (red) within the tissue. Additionally, indicated are various approaches to therapeutic intervention (green). Broadly, both tumor cells and CAFs, which have several cell types of origin, produce ECM proteins that contribute to desmoplasia, consequently increasing tissue stiffness, pressure and architecture in a manner that constrains vessels, leading to a hypoxic environment. This hypoxic environment promotes angiogenesis and cell invasion. The increased angiogenesis results in an increased interstitial fluid pressure that acts to promote invasion and metastasis of the tumor cells. The tumor cells themselves also contribute directly by means of their proliferation to increase the solid stress of the tissue that also supports a feedback loop by constraining vessel access to the tissue. Attempted therapeutic interventions include reduction of CAF formation, degradation of ECM collagens and hyaluronan, MMP inhibition and anti-angiongenic treatments. Akt, protein kinase B; CAF, cancer associated fibroblast; COL, collagen; ECM, extracellular matrix; GTP, guanosine triphosphate; HA, hyaluronan; LAMB3, laminin subunit beta-3; MMP, matrix metalloproteinase; PDAC, pancreatic ductal adenocarcinoma; PEGPH20, PEGylated human recombinant PH20 hyaluronidase; PI3K, phosphatidylinositol 3-kinase; PSC, pancreatic stellate cell; ROCK, Rho associated protein kinase; TGF-β, transforming growth factor β; TME, tumor microenvironment.

of vitamin A) and lipid-containing cells in their quiescent state that reside in the pancreas among other organs and share some characteristics with fibroblasts (Kordes et al., 2009). Upon activation in response to tissue damage signals or in tumor conditions, PSCs lose their reservoir of retinoic acid and promote desmoplasia by differentiating into α-SMA expressing CAFs that secrete large amounts of collagens into the TME (Kocher et al., 2020) (Bachem et al., 1998). Indeed, the level of PSC activation in the stroma is strongly correlated with collagen deposition (Apte et al., 2004) (Bachem et al., 1998).

Desmoplastic areas within the TME also show high levels of collagenase membrane-type 1 MMP (MT1-MMP), a major ECM-degrading enzyme (Shields et al., 2012). This indicates that collagens are not only produced but also degraded in the tumors, which is reflected by the fact that fragments of collagens can be detected in the blood of PDAC patients (Öhlund et al., 2008) (Willumsen et al., 2019). Besides MMPs, collagens are also degraded by other ECM-modifying enzymes produced by CAFs, such as lysyl oxidases (LOX) and LOX-like proteins which catalyze crosslinking between deposited collagen fibers in the tumors, thereby increasing complexity in the 3D framework of the matrix (Myllyharju and Kivirikko, 2004).

The significance of desmoplasia in influencing cancer cell activity is well accepted, but the evidence on whether these factors have a suppressive or supporting role in PDAC is multifaceted (Ligorio et al., 2019). Incubation of PSCs with conditioned media from cancer cells leads to a significant increase in their proliferation (Apte et al., 2004). Similarly, CAF conditioned media has also led to a significant increase in invasive potency of the already invasive PDAC cell lines *in vitro* (Ligorio et al., 2019), indicating an important paracrine crosstalk between cancer cells and the surrounding CAFs. In addition, using a mouse orthotopic xenograft model, a significant increase in the metastatic burden was observed with *in vivo* transplantation of PDAC cell lines together with CAFs compared to PDAC cell lines alone (Ligorio et al., 2019).

Even though most studies on tumor stroma have demonstrated the pro-tumorigenic effects of desmoplasia, newfound evidence has revealed tumor-suppressive features of the stroma. The controversial effects of the stroma on PDAC

progression and pathogenesis can be linked to the phenotypic variation of different subtypes of CAFs. We have previously identified two phenotypically distinct, mutually exclusive, and spatially separated CAF subtypes, myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs) (Öhlund et al., 2017). It is hypothesized that some CAF subtypes may be able to restrain cancer progression while others facilitate it. *In vivo* studies in a mouse model of PDAC have shown that depletion of the α-SMA positive CAFs in PanIN or PDAC, while reducing the desmoplasia, resulted in increased hypoxia, invasiveness, epithelial-mesenchymal transition (EMT), and reduced survival (Özdemir et al., 2014). Moreover, deletion of the *Col1a1* gene, which encodes for the alpha 1 subunit of collagen type I, in α-SMA positive CAFs led to a significant decrease in total stromal collagen type I deposition which results in acceleration of disease and reduced overall survival due to the suppression of $CD8^+$ T cells (Chen et al., 2021). In addition, inhibition of a G-protein coupled receptor, smoothened (SMO), in the hedgehog signaling cascade in PDAC tumors led to reduced desmoplasia, decreased collagen type I content, and a transient increase in vascularization that improved the delivery of chemotherapy and immunotherapy to the tumors resulting in longer median survival in genetically engineered animal models of PDAC (Olive et al., 2009) (Wang et al., 2019). On the other hand, despite the ability of Sonic hedgehog (SHH) to drive the formation of a CAF-rich stroma, its deletion also leads to low-stroma containing tumors that are more aggressive, have more undifferentiated histology, enhanced vascularity, and higher proliferation (Rhim et al., 2014). Further, a recent study has suggested that tumor-promoting behavior of the stroma when inhibiting the hedgehog pathway may be due to the increase in iCAF numbers and the immunosuppressive TME that is exhibited via the lower number of $CD8^+$ T cells and higher number of regulatory T cells (Steele et al., 2021). Nevertheless, these observations illustrate the complex and multifaceted role of stroma in PDAC progression.

Transforming growth factor β1 (TGF-β1)-regulated pathways also have a similar multifaceted effect on stroma-mediated PDAC progression as well as suppression (Tod et al., 2017). TGF-β1 regulates the highest number of overexpressed matrisome proteins in PDAC (Tian et al., 2019) hence, is a major driver of fibrosis. TGF-β is produced by stromal cells and its overexpression in the tissue microenvironment has been found in several precancerous disorders as well as in advanced tumors (Pickup et al., 2013) (Verrecchia, and Mauviel 2007) (Gordon, and Blobe 2008). Due to its crucial role in maintaining cellular homeostasis, TGF-β is expressed as an inactive precursor formed by the noncovalent association of mature TGF-β homodimer with the latency-associated peptide (LAP). LAP is encoded by the *TGFB1* gene, upstream to the region encoding mature TGF-β. Upon expression, LAP is processed from the N-terminal part of the TGF-β proprotein but remains linked to the precursor (Annes et al., 2003) (McGowan et al., 2003). LAP binds to the receptor-binding site on the mature TGF-β thereby hindering its binding to cell-surface receptors and inhibiting downstream signaling (Young and Murphy-Ullrich, 2004). Several stromal factors participate in the activation of TGF-β which involves the release of LAP from its receptor-binding site and thus facilitating a plethora of downstream signaling. Human PDAC samples with defective epithelial TGF-β signaling show higher epithelial STAT3 activity which results in the production of a rigid stroma, increased tension, and decreased patient survival (Laklai et al., 2016) (Winkler et al., 2020).

Integrins, as the principal receptors involved in cell-ECM interactions, adhesion, and mechanotransduction, are influenced by TGF-β signaling (Barczyk et al., 2010). Loss of TGF-β signaling and increased β1-integrin mechanosignaling in mice results in promoting tumor development by increasing matricellular fibrosis and tissue tension via STAT3 signaling (Laklai et al., 2016). On the other hand, epithelial STAT3 ablation slowed tumor development by decreasing stromal stiffness and epithelial contractility caused by TGF-β signaling loss (Laklai et al., 2016). Time course mass-spectrometry-based protein analysis of the PDAC cell lines incubated with CAF conditioned media showed an early activation of the MAPK pathway that was followed by overexpression of the STAT3 pathway and revealed a substantial enrichment of proliferation and EMT protein networks (Ligorio et al., 2019). Furthermore, a combined single-cell RNA sequencing of PDAC cells co-cultured with CAFs showed a significantly higher EMT and proliferation gene signature of the PDAC cells compared to when they were cultured alone. The gene signatures for proliferation and invasion of PDAC cells seem to be driven by CAF-secreted TGF-β (Ligorio et al., 2019). Collectively, these observations point out the complex and multifaceted roles of TGF-β signaling in tumor development and tumor-stroma interactions.

Studies have illustrated some differences between the ECM in the primary tumor and the tumor at the metastatic site. However, due to the low accessibility of metastatic tissue from patients and the lack of proper preclinical models, these studies are very limited. ECM components, including collagen and hyaluronan, are identified in high concentrations in both primary tumors and metastatic lesions according to a study analyzing patient samples from both primary and metastatic sites (Whatcott et al., 2015). No significant difference was seen in the extent of desmoplasia between primary tumors and metastatic lesions. Interestingly, solid stress is increased in liver metastases compared to the primary tumor for pancreatic cancer (Nia et al., 2017). However, this relationship is reversed for colorectal cancer and its liver metastases (Nia et al., 2017), demonstrating that mechanical abnormalities such as solid stress vary between tumors and are dependent on the cancer cell type and specific microenvironment. Despite efforts made to indicate the differences between tumor ECM at primary and metastatic sites, a comprehensive understanding remains elusive.

In summary, the desmoplastic reaction in PDAC has an important multifactorial yet incompletely understood role in disease progression and clinical outcome. To some extent, the non-linear correlation between the desmoplastic stroma and PDAC progression in part can be attributed to the contrary contributions from different subtypes of CAFs present in the tumor microenvironment.

3 EXTRACELLULAR MATRIX

The ECM appears in the body in two distinct types: basement membrane and interstitial matrix. The basement membrane is a thin layer of highly crosslinked specialized ECM proteins that is found in all tissues in the body and separates layers of epithelial or endothelial cells from the underlying connective tissue (LeBleu et al., 2007) (Jayadev, and Sherwood 2017). This membrane matrix maintains the polarization and functionality of the cells by retaining them in a permeable and loose structure (Karsdal 2019). Major components of the basement membrane include collagen type IV and laminin amongst others. The basement membrane owes its sheet-like architecture to two distinct polymeric structures which are formed by self-assembly of collagen type IV and laminin molecules, respectively. Although these collagen type IV and laminin networks are assembled independently, they are extensively linked by other ECM proteins such as nidogen and perlecan, increasing their mutual stability and ultimately providing structural integrity to the basement membrane (LeBleu et al., 2007) (Karsdal 2019) (Jayadev, and Sherwood 2017).

Laminin is the most abundant non-collagenous protein in the basement membrane that is essential for its proper organization and function (Rasmussen, and Karsdal 2019). Laminins are heterotrimer proteins, each containing one α chain, one β chain, and one γ chain (α1-5, β1-3, and γ1-3) which are linked in different combinations to comprise the 15 members of the laminin protein family (LeBleu et al., 2007). Laminins play important roles in the pathophysiology of cancer due to their influence on differentiation, adhesion, migration, and key steps in carcinogenesis (Maltseva, and Rodin 2018). Laminin subunit beta-3 (LAMB3) has been shown to be upregulated in the ECM of many malignancies including stomach, colon, lung, and pancreas (Tian et al., 2019) (Zhang et al., 2019). A recent study has shown that LAMB3 promotes invasion and metastasis in PDAC cells via the activation of the PI3K/Akt signaling pathway (Zhang et al., 2019). In contrast, another study has shown that culturing PDAC cell lines on laminin-coated plates slows down the cell migration compared to the uncoated, FN1 coated, and type I collagen-coated plates (Procacci et al., 2018). However, the type of laminin used in this study has not been indicated. Therefore, this apparent contradiction might be a result of using different laminin types.

The interstitial matrix, on the other hand, has proteoglycans and fibrous proteins as its two major macromolecule groups. Proteoglycans form a hydrated gel that fills most of the interstitial space within the tissue. The primary fibrous proteins in the interstitial matrix are fibrillar collagens (such as collagen type I and III), elastin, and fibronectins, which form long parallel polypeptide chains (Frantz et al., 2010) (Karsdal 2019). As with basement membranes, collagens are also the most common fibrous protein in the interstitial matrix (Frantz et al., 2010). Given the importance of different types of collagens, in constituting ECM and due to their significance in facilitating biochemical as well as biomechanical interactions in the PDAC tumors, they are discussed in detail in a subsequent subsection of this review.

The desmoplastic stroma of PDAC also shows an abundance of a large glycosaminoglycan called hyaluronan. Hyaluronan is a high molecular weight polymer synthesized by three isoforms of a membrane-bound enzyme, hyaluronan synthases (HAS1-3), located at the intracellular side of the plasma membrane (Liu et al., 2019). HAS isoforms catalyze hyaluronan synthesis in both stromal and carcinoma cells and high levels of these enzymes are linked to the accumulation of hyaluronan in the desmoplastic stroma (Auvinen et al., 2014) (Sato et al., 2016). Dysregulated hyaluronan buildup in the stroma severely affects its mechanical and biochemical characteristics and a hyaluronan-rich stroma is linked to poor prognosis in several carcinomas including PDAC (Amorim et al., 2021). In a recent study, pretreatment serum hyaluronan levels were found to be significantly higher in PDAC samples than in benign tumors or normal pancreata. It was also indicated that higher serum hyaluronan levels could be associated with metastasis and that this level could be used as a predictor of overall survival in PDAC patients (Chen et al., 2020). In ECM, hyaluronan and collagens interact in a complex manner that results in increased solid stress and interstitial fluid pressure (Provenzano et al., 2012) (Stylianopoulos et al., 2012) (Alexandrakis et al., 2004). ECM contains a range of hyaluronan polymers of different molecular weights, generated by enzymatic fragmentation of large hyaluronan polymers by hyaluronidase 1, 2, and 3 (HYAL1-3) and free radicals (Liu et al., 2019). Due to the diversity in their sizes, these fragments perform distinct functions in cell proliferation, migration, and differentiation. Hyaluronan molecules with high molecular weights can occupy the ECM in a way that leads to an increase in the compressive force in the TME resulting in reduced drug delivery (Sato et al., 2016).

Hyaluronan is primarily produced by fibroblasts present in the stroma and interacts with the cell surface receptor CD44 to regulate a cascade of pathways involved in cell proliferation, migration, and invasion, and thus plays an important role in cancer progression and metastasis (Bourguignon 2019) (Misra et al., 2015). CD44 is particularly important in pancreatic carcinogenesis since apart from being the principal cell-surface receptor for hyaluronan, it also provides anchorage for several other ECM components such as MMPs, collagens, laminin, and an important PDAC biomarker, osteopontin, all of which are upregulated in PDAC (Rychlíková et al., 2016) (Goodison et al., 1999). Incidentally, the binding of osteopontin to CD44 stimulates CD44 overexpression, providing additional binding sites for hyaluronan and other ligands and further aiding cancer progression (Marroquin et al., 2004).

Whilst 90% of the ECM proteins in malignant tumors are produced by stromal cells, such as fibroblasts, a fraction of the ECM-proteins originates from the cancer cells (Tian et al., 2019) (Naba et al., 2012). Many of these cancer-cell-derived ECM proteins promote tumor progression and metastasis in PDAC. Detection of elevated levels of these cancer cell-derived proteins in PDAC tumors has been frequently correlated with poor survival. These proteins regulate different cellular mechanisms to promote tumor growth and metastasis, thus leading to poor survival in preclinical PDAC models as well as in patients (Tian

et al., 2019). On the other hand, some cancer-cell origin proteins (such as fibrillar collagens) have been shown to suppress tumor growth and metastasis (Tian et al., 2021). It is also interesting to note that these anti-tumorigenic properties were exclusive to cancer-cell-derived fibrillar collagens. Further, a recent study hints at the multifaceted characteristics of myCAFs in the tumor development showing that tumor-promoting myCAF-derived secreted factors can reverse the tumor-restricting effects of collagen type I produced by myCAFs in the stroma which complicates the stroma-targeting therapeutic strategies even further (Bhattacharjee et al., 2021). Another recent study, identifies two distinct subsets of pancreatic fibroblasts that are distinguished by the expression of CD105 (Hutton et al., 2021). It suggests that $CD105^{+}$ pancreatic fibroblasts have tumor-promoting characteristics while their $CD105^{-}$ counterparts have tumor-suppressive effects mediated with the functional adaptive immunity. Interestingly, both $CD105^{+}$ and $CD105^{-}$ cell populations could exhibit either iCAF and myCAF characteristics upon stimulation which challenges the previously thought paradigm of iCAFs being the pro-tumorigenic subtype and myCAFs being the anti-tumorigenic one.

Mass-spectrometry-based proteomics characterization of ECM in different pancreatic lesions and PDAC has shown that many overrepresented proteins in PDAC ECM are also overexpressed in several precursor lesions. For example, the cancer-cell derived matrisome proteins, AGRN, CSTB, and SERPINB5, which are overexpressed in PDAC stroma, are also expressed at elevated levels in early and late PanINs as compared to the normal pancreas (Tian et al., 2020), indicating that these proteins may have relevance in PDAC initiation and thus could potentially serve as early PDAC progression signatures (Tian et al., 2019).

Besides the chemical composition of ECM, its biophysical features also play a significant role in the pathophysiology of PDAC. Resident fibroblasts can significantly influence the organization and structure of stromal fibers, such as fibrillar collagens, by exerting tensile forces on the matrix and reorganizing collagen fibrils into sheets and cables, thereby influencing the ECM rigidity (Frantz et al., 2010). A higher proportion of randomly arranged ECM fibers were found in large and metastatic tumors, where randomly arranged ECM fibers were linked to poor prognosis. In contrast, parallel arrangement of ECM fibers in PDAC tumors was shown to be linked with better overall survival in patients (Bolm et al., 2020). However, contrary to the above report, another study by Drifka *et al.* suggested a more active role of stromal collagen topology in PDAC tumor progression. Based on the comparative structural analysis of stromal fibers in PDAC and non-malignant pancreatic manifestations using second-harmonic imaging microscopy, a unique spatial configuration of stroma was observed around the malignant ducts in PDAC which was characterized by increased alignment and cross-linking of collagen fibers (Drifka et al., 2015).

These diverse functions carried out by paralogous ECM proteins based on their cellular origin further signify the intricate nature of communication between cancer cells and the ECM.

3.1 Collagens and Pancreatic Ductal Adenocarcinoma

Collagens, being the major constituent of ECM, evidently play a crucial role in the interactions within the PDAC tumor microenvironment. They affect cell adhesion, migration, ECM remodeling, and EMT partly through the activation of their main class of receptors, discoidin domain receptor (DDR) family (reviewed elsewhere) (Orgel, and Madhurapantula 2019) (Huang et al., 2019). Collagens participate in different pathological conditions of the pancreas, including PDAC, and essentially involve the action of the major matrix processing enzyme, MMP. Collagens represent a large family of extracellular matrix molecules that play an important role in tissue assembly and maintenance. The collagen superfamily has 29 different known members (collagen type I - collagen type XXIX) in vertebrates (Kadler et al., 2007) (Gordon, and Hahn 2010) (Söderhäll et al., 2007). Being the principal structural components of the ECM, collagens contribute to a plethora of tissue functions, such as providing tensile strength, cell adhesion regulation, chemotaxis and migratory support, and tissue development directions. They also have a wide variety of roles in the functioning of the basement membrane (Kadler et al., 2007). Collagens, being secreted into the ECM by fibroblasts, are involved in cell-stromal interactions through different receptor families, the most common of which are integrins which also serve as receptors for laminins and fibronectin (FN1) (Nam et al., 2009). Most collagens are made of 3 α chains in either distinct (heterotrimers) or identical (homotrimer) combinations that fold together to form a triple helix. These triple-helical subunits systemically bind together to form collagen fibrils which cohere to form collagen fibers. Prior to the formation of the triple helix, collagens undergo substantial post-translational modifications which are mediated by several enzymes and molecular chaperones to ensure proper folding and trimerization of these proteins (Kadler et al., 2007).

The desmoplasia in PDAC stroma abundantly employs fibrillar collagens, such as collagens type I, III, and V as well as non-fibrillar collagens such as collagens type IV and VI. An *in vitro* wound-healing assay using PDAC cell lines also revealed the significance of collagen type I in cell migration and metastasis of PDAC cell lines *in vitro* compared to no coating and FN1 coating (Procacci et al., 2018). Collagen type I and collagen type III collectively account for approximately 90% of total collagen mass in different pancreatic pathologies and increase to nearly 2.6-fold during malignant transformation of the normal pancreas to PDAC (Tian et al., 2019). A recent study showed increased serum levels of collagen type III propeptide (PRO-C3) in patients with pancreatic malignancy. Moreover, the higher serum PRO-C3 levels resonated with more advanced stages of the disease and metastasis (Chen et al., 2020). Moreover, collagen type IV, which is an abundant network-forming collagen that accounts for nearly half of the basement membrane, is highly expressed and secreted

by cancer cells. Being an important autocrine factor, it regulates the growth and migration of PDAC cells (Öhlund et al., 2013).

Besides the more abundant collagen types I, III, and IV, other collagen types, including collagen type VI, VIII, and XIV have also been shown to be upregulated in PDAC, as well as in chronic pancreatitis and PanIN, indicating common changes occurring in the stroma during the onset and progression of pancreatic malignancy (Tian et al., 2019). Collagen type VI has a beaded-filament structure and is of stromal origin (Tian et al., 2019). It forms a unique microfibrillar network in the basement membrane and interstitial matrix interface of many tissues (Sun et al., 2019). Apart from PDAC, collagen type VI is also upregulated in other malignancies, such as cancers of the colon, prostate, breast, and high-grade ovarian cancer amongst others (Xie et al., 2014) (Thorsen et al., 2008), where it promotes tumor progression, metastasis, and chemoresistance (Chen et al., 2013). Structurally, collagen type VI is a heterotrimer of a large tandem α-chain protein (*COL6A3*) and its two relatively smaller partners (*COL6A1* and *COL6A2*). The α3 chain of the collagen type VI protein (COL6A3) is particularly significant in the etiology of PDAC since the desmoplastic stroma in PDAC shows elevated levels of PDAC-specific isoforms of COL6A3. These PDAC-specific isoforms are a product of alternative splicing involving exon 3, 4 and 6, and are not detected in precursor lesions (Arafat et al., 2011). While PDAC-specific splicing of exons 3 and 6 of COL6A3 has been observed in 97% of the paired tumor-adjacent pancreas tissue samples, the presence of COL6A3 transcripts with alternate splicing of exon 4 is almost exclusive to the tumor tissue.

The importance of COL6A3 in different malignancies has been extensively studied. A microarray-based co-expression study using different gastrointestinal cell lines has shown that COL6A3 is co-expressed with a set of 62 genes, most of which are well-characterized oncogenes (Xie et al., 2014). At the core of this COL6A3 co-expression network lies FN1, which has been implicated for its role in cell adhesion, differentiation, and migration in many gastrointestinal tumors (Xie et al., 2014) (Pankov, and Yamada 2002). Another study, involving immunohistological comparison of a cohort of resected PDAC and adjacent pancreatic tissue, linked moderate to high levels of COL6A3 in PDAC tissue with negative prognostic factors, such as tumor differentiation, lymph node metastasis, perineural invasion, and microvascular invasion (Svoronos et al., 2020).

Apart from COL6A3, the α1 chain of collagen type VI (COL6A1) has also been shown to be involved in PDAC. A significant increase in COL6A1 expression was observed in a highly metastatic subset of cells harvested from the human pancreatic cancer cell line, BxPC-3 (Owusu-Ansah et al., 2019). Invasive and metastatic abilities of this invasive subset of cells decreased after *COL6A1* knockdown. Furthermore, retrospective IHC staining of paraffin-embedded PDAC tissue showed significantly higher levels of COL6A1 in cancerous tissue as compared to the para-cancerous regions. Also, increased expression of COL6A1 has been demonstrated as an independent predictor of decreased overall survival (Owusu-Ansah et al., 2019).

Collectively, collagens play a significant role in tumorigenesis and disease development owing to their interactions with different components of the stroma and their structural volume. They also provide a platform for other elements of the stroma to interact with each other, making them one of the most potential drug targets in regulating cancer.

3.2 Matrix Metalloproteinases

Molecular interactions within the ECM are tightly regulated and involve several matrix processing enzymes. MMPs, a group of zinc-dependent endopeptidases, significantly contribute to the regulation of stromal networking owing to their role in the catabolism of collagen and other ECM proteins (Knapinska et al., 2017) (Ricard-Blum 2011). Aggressive tumors recruit MMPs (especially membrane-bound MMPs, such as MT1-MMP) to remodel the tumor stroma and facilitate their invasion and metastasis (Jacob, and Prekeris 2015) (Nguyen et al., 2016). While MMPs are mostly secreted by stromal cells like inflammatory cells and fibroblast, they are also secreted by cancer cells (Kessenbrock et al., 2010). Due to their critical role in controlling tissue invasiveness, the expression of active MMPs in the tissue is tightly regulated. MMPs are synthesized as inactive zymogens and their activation is dictated by tight allosteric regulations (Sternlicht, and Werb 2001). Several cellular and ECM components influence MMP activation, where the furin-like proteinases are important players (Sternlicht, and Werb 2001).

Interestingly, *in vitro* studies on cultured PDAC cell lines showed that collagen type I significantly induce secretion and activation of MMPs, especially MMP-2 and MMP-9 in PDAC (Procacci et al., 2018). Both MMP-2 and MMP-9 are considered as important biomarkers for EMT in PDAC (Procacci et al., 2018). Retroactive analysis of a cohort of resected PDAC samples has also shown a strong correlation between elevated levels of MMP-8 and MMP-9, with poor patient survival (Hu et al., 2018). Although MMP-9 is overexpressed in PDAC (Qian et al., 2001) and has been suggested as a potential PDAC biomarker, its systemic depletion results in more invasive and metastatic tumors (Grünwald et al., 2016).

As mentioned above, MT1-MMP has been identified as a key modulator of desmoplastic reaction in pancreatic cancer progression since it is upregulated in PDAC and can directly activate TGF-β1 (Nguyen et al., 2016). By processing latent TGF-binding protein-1 (LTBP-1), MT1-MMP can also release latent TGF-β1 from the ECM (Tatti et al., 2008). Enhanced collagen synthesis by PDAC stellate cells as a result of MT1-MMP activation of TGF-β1 resulted in an increased fibrotic microenvironment (Krantz et al., 2011).

As mentioned previously, several stromal factors participate in the activation of TGF-β which involves the release of LAP from its receptor-binding site either mechanically or by proteolysis of LAP (Annes et al., 2003). Yu et al., showed that proteolytic cleavage of LAP by cell surface localized matrix metalloproteinases MMP-2 and MMP-9 releases mature TGF-β in the milieu. The secreted TGF-β effects downstream signaling in distant effector cells and can promote their proliferation and migration (Yu and Stamenkovic, 2000). Taken together, MMPs have an important role in the desmoplastic reaction as a result of their involvement in ECM turnover and in regulating critical signaling pathways.

4 MECHANICAL TRAITS

The desmoplastic reaction leads to stiffening of the stroma resulting in increased mechanical stress and rearrangement of collagen fibers within the ECM in a way that facilitates tumor progression and dissemination of cancer cells to metastatic sites (Freeman et al., 2017). Besides collagens, several factors contribute to make up of the mechanical microenvironment of PDAC tumors. Integrins, the predominant collagen receptors in the stroma, also modulate biophysical traits of the tumor through multiple mechanisms. The epithelium-specific integrin αvβ6 is upregulated during epithelial remodeling but is not expressed in healthy tissue. It interacts with a variety of ligands such as FN1, tenascin, vitronectin, LAP, and TGF-β3. Integrin αvβ6 is involved in the non-proteolytic activation of TGF-β by inducing conformational changes in the precursor complex which renders LAP unable to inhibit binding of TGF-β to its cell surface receptors (Annes et al., 2003). This mechanical activation of TGF-β is orchestrated by several stromal components (Munger et al., 1999). The secreted precursor LAP-TGF-β covalently associates with an ECM-glycoprotein, the latent TGF-β-binding protein (LTBP1) to form a tripartite complex also known as large latency complex (LLC). LTBP1 anchors itself in the ECM via fibronectin (Dallas et al., 2005). At the cell surface the transmembrane integrin αvβ6 binds to the LLC at the integrin-binding domain of LAP while its cytoplasmic domain binds to the actin cytoskeleton (Wipff and Hinz, 2008) (Annes et al., 2004). The mechanical stress in the stroma creates traction between ECM and the cells. This traction counters the strong non-covalent binding between LAP and TGF-β by inducing conformational changes in LAP and thus, results in the release of active or mature TGF-β (Wipff et al., 2007) (Robertson and Rifkin, 2016). Activation of TGF- β1 by integrin αvβ6 binding results in modulation of collagen fibril thickness. Integrins have also been shown to endorse invasion and metastasis in different carcinomas, including PDAC, partially by controlling the activity of MMP-2, MMP-9, and MMP-13 (Tod et al., 2017).

PDAC, like other solid tumors, shows a very high intratumoral interstitial pressure. The rapid proliferation of cancer cells together with angiogenesis increases the stromal pressure in the tumor which in turn causes tumor vessel leakiness and thus contributes to further increase the intratumoral interstitial pressure (McDonald and Baluk, 2002). The dynamics of these biomechanical events are further influenced by the outward interstitial fluid flow from the tumor core to its margins (Evje and Waldeland, 2019).

The term coined for this mutual relationship between these escalating mechanical forces within the tumor and their contributory factors is mechanoreciprocity, where tumor cells in the TME, when mechanically challenged, reciprocate by exerting a proportional cell-generated force (Paszek and Weaver, 2004). While this phenomenon pertains to the cell-generated forces at the single-cell level, it also considers the mechanical changes in the TME since each cell is constantly mechanically challenged by its surrounding microenvironment. The cell-generated mechanical forces originate at the cytoskeleton and are transmitted to the microenvironment via focal adhesion contacts on the cytoskeletal-integrin-ECM interface. This is carried out generally through myosin-dependent contractility that depends largely on the Rho-GTPase transduction cascade and Rho-Rock signaling (Shiu et al., 2004).

Rho-Rock signaling regulates motility and invasion of tumor cells by remodeling both the cytoskeleton as well as the tumor ECM (Rath et al., 2017). This is mainly achieved through TGF-β-induced EMT (Ungefroren et al., 2018). EMT involves a series of morphological changes that lead to motility and invasiveness of cancer cells, a prerequisite for metastasis (Ungefroren et al., 2018) (Safa 2020). The cytoskeleton-modulating activity of the Rho family of small GTPases involves regulation of actin filament nucleation, elongation, capping, and depolymerization (Lee and Dominguez, 2010). Reportedly, adhesion to the extracellular matrix in conjunction with the accompanying changes in cell shape and cytoskeletal tension is necessary for GTP-bound RhoA to activate its downstream effector, ROCK. Hence, it has been surmised that biophysical signals, such as cytoskeletal stress and adhesion maturation, and the intrinsic ROCK signaling form a feedback loop that contributes to a variety of mechanochemical activities in tumor tissue (Bhadriraju et al., 2007).

Different cells show varying degrees of sensitivity to biophysical changes depending on the tissue type. For example, neutrophils are sensitive to forces under 1 Pa while chondrocytes, which are constantly under mechanical load, show tolerance up to 20 MPa (Paszek, and Weaver 2004). It is interesting to note that normal epithelial cells, such as mammary cells, are sensitive to small changes in ECM stiffness and they reciprocally exert relatively small forces (Yeung et al., 2005). This contrasts with the strong response that is exhibited by mechanically active fibroblasts and cancerous mammary cells (Yeung et al., 2005). Limited data is available on biophysical sensitivity and cellular response in the pancreas and pancreatic tumors. However, PDAC tumors are known to exhibit a highly cross-linked desmoplastic reaction like what characterizes the mammary tumors and is considered as the main contributor to the latter's high tensile strength and intratumoral pressure (Di Maggio, and El-Shakankery 2020).

In a study, Freeman et al. showed that the stress-induced invasion, which can be initiated by applying tensional loads on 3D collagen gel matrices in culture, is dependent on Rap1 GTPases. Rap1 GTPase belongs to the Ras-family of GTPases which has pleiotropic cellular functions including regulation of cell adhesion, proliferation, apoptosis, and cytoskeleton remodeling (Jaśkiewicz et al., 2018). Rap1 activity stimulates the formation of focal adhesion structures that align with the tensional axis (Freeman et al., 2017). Incidentally, Rap1-GTPases have also been implicated in EMT and metastasis in different cancer types (Freeman et al., 2010) (Zhang et al., 2017).

Mechanical characteristics of the tumors can be categorized into 4 major traits: solid stress, interstitial fluid pressure, stiffness, and tissue microarchitecture (Nia et al., 2020). While the former two are extrinsic by nature and depend on environmental cues, tissue stiffness and microarchitecture are intrinsic properties of the tissue. The degree of invasiveness of tumor cells is determined

by an interplay between different mechanical traits exhibited by the tumor (Nguyen et al., 2016).

Solid stress is generated due to the excessive number of proliferating cells that apply chronic compressive stress on the neighboring tissues. Elevated solid stress within tumors triggers the expression of Growth Differentiation Factor 15 (GDF15). GDF15 is a TGF-β-family ligand whose cellular expression also takes cues from other prevailing cellular stresses such as hypoxia and nutritional stress (Patel et al., 2019) (Wang et al., 2021). GDF15 activates the Akt-signaling pathway in the cancer cells leading to invasiveness, and migration (Kalli et al., 2019) (Nia et al., 2020). On the other hand, it has been shown that solid stress suppresses tumor growth in spheroids independent of the tissue of origin, host species, or differentiation status (Helmlinger et al., 1997). An earlier study measured this suppressive aspect of solid stress within the tumor to be 45–120 mm Hg (~6–16 kPa) which is substantially higher than the blood pressure in the tumor vasculature and is sufficient to cause the collapse of the vasculature which explains the impaired blood flow to the tumor (Helmlinger et al., 1997). Another study measured the compressive solid stress exerted by tumor cells to be as high as 3.8 kPa (~30 mm Hg) in the center of murine pancreatic tumors (Nia et al., 2017) while the measured solid stress in normal soft tissues such as kidney and liver was negligible (Nia et al., 2017). These studies have emphasized that solid stress or the intrinsic intertumoral stress due to accelerated cell proliferation, is a fundamental aspect of cancerous tumors that severely affects the tumor vasculature and as a result the intertumoral drug distribution.

The interstitial pressure within tumors depends on extrinsic factors and is controlled by circulatory systems. It has been shown that compression of lymphatic vessels increases interstitial fluid pressure while blood vessel compression decreases blood flow and consequently the interstitial pressure. The core of nearly all solid tumors exhibits chronically elevated levels of interstitial pressure that abruptly decreases to almost zero around the edge of the tumor creating a radial fluid flow towards the neighboring tissue that provides an escape route for disseminating cancer cells and consequently leads to invasion and metastasis via flow-induced shear stress (Nia et al., 2020). In addition, reduced blood flow because of high solid stress results in a hypoxic TME. Tumor cells residing in this hypoxic microenvironment are forced to switch their metabolic activity from oxidative phosphorylation to glycolysis (the Warburg effect) in order to survive (Hanahan, and Weinberg 2000). This metabolic change, together with other contributing factors results in tumor progression, immunosuppression, inflammation, and eventually invasion and metastasis. These changes also render most anti-cancer therapies ineffective (Stylianopoulos et al., 2012).

Increased stiffness is another mechanical alteration that is developed in tumors and has been observed in many solid tumor types including pancreatic cancer (Nia et al., 2020). Matrix stiffening is mainly caused by increased matrix deposition rates and crosslinking of collagens. Moreover, high levels of tensile stress in the microenvironment can also lead to increased stiffness through "strain stiffening", a phenomenon representing non-linear changes in the elasticity of collagen fibrils (Storm et al., 2005). Higher stiffness activates proliferative, invasive, and metastatic signaling pathways within the tumor cells and is correlated with chemoresistance (Deville, and Cordes 2019). Recent studies have proposed using tissue stiffness as a diagnostic marker and predictor of poor prognosis (Nia et al., 2020).

Microarchitecture in normal tissue determines homeostasis and provides signals for the transformation of the tissue. High level of proliferation, matrix deposition, and increased stiffness in tumor tissue results in altered microarchitecture that alters the interactions between individual cells in the TME and their surrounding matrix. This may result in the reprogramming of the cells, which in turn will activate signaling pathways associated with invasion and metastasis (Mayer et al., 2018) (Nia et al., 2020).

Interestingly, despite having the same stiffness, pancreatic primary tumors have higher solid stress compared to their derivative metastatic counterparts in the liver showing that stiffness and solid stress are two distinct mechanical anomalies in tumors that should be targeted separately (Nia et al., 2017). Since carcinogenesis is linked to substantial changes in tissue tension as well as modifications in force sensing by transformed cells, it seems very likely that altered mechanotransduction and the loss of tensional homeostasis are important factors in epithelial tumor pathogenesis (Paszek and Weaver, 2004). Collectively, these observations strongly correlate PDAC progression and invasiveness with substantial changes in the biophysical traits of the tumor tissue.

5 MODELS TO STUDY INTERACTIONS WITH EXTRACELLULAR MATRIX AND TENSION/PRESSURE

It is clear, then, that the effects of the ECM on tumor progression are simultaneously multifaceted, operating at multiple levels of analysis (physically and chemically), and are often both instructive and consequential with respect to disease progression. In order to disentangle the biomechanical and biochemical roles that the ECM plays in PDAC, effective tools and models are required. Avenues to explore the effect of tissue tension are relatively limited with traditional, two-dimensional, cell culture, however, tools are available such as culture on elasticated tissue culture surfaces that can be put under uni- or bi-directional tension (Kamble et al., 2016). Although the direct application of these devices in the context of PDAC does not appear to have been performed, such devices have been used to identify that breast cancer cells undergo increased proliferation and migration in response to mechanical strain and that exosomes derived from these breast cancer cells induce an immunosuppressive phenotype (Wang et al., 2020). Alteration in tumor cell migration has also been identified in other tumor cell lines via induction of Rap1 activity both in two- and three-dimensional (3D) cultures (Freeman et al., 2017). Critically, two-dimensional models result in monolayer cell cultures, where cell morphology is altered because of the format, inducing artefactual pressures on the cell, such as a forced polarity and a flatted

nucleus which can alter cell signaling, compared to a 3D counterpart (reviewed elsewhere) (Yamada and Cukierman, 2007) (Kim 2005) (Kapałczyńska et al., 2018).

Recently, 3D organoid cultures of primary cell lines derived from a variety of organs have been developed (Barker et al., 2010) (Sato et al., 2011) (Huch et al., 2015) (Rosenbluth et al., 2020), including those of the pancreatic ductal epithelium and their tumor counterparts (Boj et al., 2015) (Baker et al., 2016). A contemporary review comprehensively explores developments of PDAC culture models for broad application (Heinrich et al., 2021), however, typically PDAC organoid cultures are maintained in a 3D hydrogel-based scaffold comprised of a variety of basement membrane proteins such as Matrigel (Baker et al., 2016). Matrigel is derived from basement membrane extracts of murine Engelbreth-Holm-Swarm sarcoma, and contains laminin, collagen type IV, and entactin (Kleinman and Martin, 2005).

One advantage of 3D culture in hydrogel scaffolds is that they are biomechanically more representative of tissue than traditional two-dimensional cultures on plastic and permits examination of cell interactions with a pre-established matrix (Öhlund et al., 2014). Here we present some examples of hydrogel composition and construction, however, a comprehensive examination on hydrogel construction and developments is well beyond the scope of this review but is deftly addressed elsewhere (Karoyo and Wilson, 2021) (Zhu and Marchant, 2011) (Caliari and Burdick, 2016) (Gyles et al., 2017). Hydrogels are highly customizable in composition and can be formed from a variety of substrate polymers both natural and synthetic as well as a differential contribution to chemical cell signaling. Alginate and polyethylene glycol, for instance, are relatively inert whereas collagens, laminin, and/or FN1 can contribute directly to cell signaling (Miroshnikova et al., 2011) (Caliari and Burdick, 2016). This ability to tailor matrix composition can be utilized to assist in decoupling the mechanical and biochemical signaling effects of the ECM on the growth of tumor organoids (Chang and Lin, 2021). Indeed, seaweed-derived alginate, which lacks the native ligands that permit biological signaling with mammalian cells can be utilized to alter matrix mechanics without contributing to biological matrix signaling (Rowley et al., 1999). Further, alginate has been used in an *in vitro* breast cancer model where fine-tuning the elasticity of the gel was sufficient to determine an inverse correlation between tumor cell proliferation and tissue hardness (Cavo et al., 2016).

Hydrogel stiffness is dependent on several factors such as the polymer used, concentration, and cross-linking method. Stowers *et al.* developed an alginate-based system of tunable stiffness that is dependent upon the concentration of ionic calcium chelators that promote polymer cross-linking (Stowers et al., 2015). The concentration of calcium available could also be modulated photo-dynamically, thus allowing characterization of cell cultures over time under altering matrix stiffness. In this example, the migration mode of fibroblasts altered in response to differing matrix stiffness, with an inverse relationship between matrix stiffness and cell elongation and migration (Stowers et al., 2015). Alternatively, synthetic polyacrylamide (PA) gel stiffness can be modified by the concentration of the monomer, cross-linker, and the temperature of gelation, permitting robust and reproducible investigation of matrix stiffness on cultured cells (Denisin, and Pruitt 2016). Further, there are several examples where hydrogels composed of the synthetic polymer polyethylene glycol (PEG) have their stiffness modulated photodynamically, which offers the opportunity to examine the effect of inducing ECM stiffness on cellular behavior (Mabry et al., 2015) (Liu et al., 2018) (Günay et al., 2019) (Kalayci et al., 2020).

Regardless of how the matrix is constructed *in vitro*, effective quantification of its mechanical and biochemical properties is essential for hypothesis testing. With respect to biochemical properties, composition and alteration can be readily examined by means of immunohistochemistry and immunofluorescence, or destructively by means such as mass spectrometry. Additionally, histological analysis by Masson's trichrome and picrosirius red can be used to examine collagen structure, linearization, and orientation. Further, dynamic techniques that can acquire such data over time can be applied to live cultures and tissue explants and thus capture complex spatiotemporal dynamics of matrix remodeling. Such techniques include second harmonic generation (SHG) that can also be used to examine collagen fiber alignment (Fuentes-Corona et al., 2019).

In contrast, when it comes to measuring the mechanical properties of the ECM there are several methods to measure the mechanical properties from the cellular to tissue scales. Techniques such as atomic force microscopy (AFM) can measure sample stiffness on tissue down to the nanometer range and have demonstrated utility as a diagnostic tool in other cancer types such as breast and liver (Stylianou et al., 2018) (Tian et al., 2015) (Plodinec et al., 2012) (Rother et al., 2014). Additionally, tensile and compression testing can be used to assess the viscoelastic properties of soft tissue samples, and so can rheometry for fluid samples (Buckley et al., 2009) (Griffin et al., 2016). In addition, techniques such as mesoscale indentation are well suited for the examination of nuanced regional mechanical heterogeneity in tissue (Rubiano et al., 2018). This approach applied to human healthy and PDAC pancreatic samples identified that PDAC tissue exhibited a higher steady-state modulus than normal tissue on average (Rubiano et al., 2018). Furthermore, isolated tumor-associated stromal cells cultured in collagen hydrogels formed stiffer hydrogels when exposed to conditioned medium from PDAC cells than when in control medium (Rubiano et al., 2018).

There exist a range of tools by which the effect of matrix properties on PDAC behavior can be examined and the majority of consideration in the literature on this topic has concentrated on the effects of biochemical matrix composition (Biondani et al., 2018) (Begum et al., 2017) (Tian et al., 2019). In contrast, comparatively little has been elucidated on how the physical properties of the matrix affect PDAC behavior. Whilst *in vivo* murine models have determined that though alleviating solid stress within the tumor by means of removing the dense stromal tissue of the tumor may improve drug perfusion to the tumor site, doing so also leads to increased invasiveness of the cancer cells which are no longer constrained by the surrounding tissue (Rhim

et al., 2014). However, in this example, the role of physical properties is not disentangled from the other signaling of the stromal cells present. Experiments culturing established PDAC cell lines in polyacrylamide gels of differing stiffnesses (1 kPa, 4 kPa, and 25 kPa) identified that vimentin expression and nuclear β-catenin localization, both markers for EMT, increased with matrix stiffness (Rice et al., 2017). Of therapeutic relevance, these same experiments demonstrated that matrix stiffness promoted resistance of the cells to paclitaxel, however, cells remained sensitive to gemcitabine regardless of stiffness (Rice et al., 2017). The role of cell stiffness in PDAC invasion potential has also been explored by models utilizing cell lines in both traditional scratch wound healing and transwell migration assays which identified that stiffer pancreatic cell lines tend to be more invasive (Nguyen et al., 2016).

The effect of matrix stiffness on tumor cells does not yield a comprehensive understanding of the effects of the mechanical properties of the matrix on overall tumor development as CAFs, for example, are a non-malignant cell type that are critical in modulating tissue stiffness. CAF matrix production is influenced by matrix pressure, whereby CAFs under pathological tissue stiffnesses (~7 kPa) produce a matrix resembling pathological ECM (Malik et al., 2019). However, when cultured under normal physiological stiffnesses in polyacrylamide gel (~1.5 kPa) CAFs generate an ECM that is similar to that of normal fibroblasts (Malik et al., 2019). Further, the culture of human CAFs in 3D conditions identified that the mechanical quiescence of PSCs can be restored by means of all-trans retinoic acid (ATRA) in addition to the culture medium (Chronopoulos et al., 2016). Additionally, pancreatic cancer cells readily migrated into matrices remodeled by control PSCs but demonstrated minimal invasion into matrices remodeled by ATRA-treated PSCs (Chronopoulos et al., 2016). An alternative study also demonstrated that modulation of ECM stiffness itself was a key factor in PSC activation to CAFs by means of culture on polyacrylamide gels of varying stiffnesses (from 1 kPA–25 kPA), where high stiffness matrices induced a myofibroblastic-like phenotype in the PSCs (Lachowski et al., 2017). In this example, it should be observed that 25 kPa is much stiffer than typical for the upper quartile range for PDAC of 4 kPa, although stiffnesses of up to 10 kPa are observed (Rice et al., 2017). An alternative examination of PSC density and matrix stiffness identified that increased PSC cell density increased tissue stiffness up to 1 kPa (Robinson et al., 2016). Further, it was identified that PSC-mediated re-modeling of the collagen matrix was dependent on the cellular contractile apparatus of the PSCs since when actomyosin contraction was blocked by means of blebbistatin, collagen fiber alignment was severely inhibited compared to the untreated control (Robinson et al., 2016). Similar results were also identified when PSCs were treated with ATRA, inhibiting their activation, indicating again the requirement of PSC activation for matrix remodeling (Robinson et al., 2016).

Overall, the field is ripe for addressing the question of the mechanical influences of the matrix on PDAC. The development of robust and relevant 3D organoid models of PDAC, coupled with the availability of tunable matrices both in terms of stiffness and composition provides a great opportunity for exploration. Furthermore, the development of 3D *in vitro* co-culture models of PDAC whereby PDAC tumor organoids are cultured together with PSCs leads to the formation of multiple CAF subtypes and allows for the examination of the signaling loops between these various cell types (Öhlund et al., 2017). These models could similarly be utilized to examine the interplay between CAFs, tumor cells, and their matrix context, for example by culture in matrices of differing initial stiffness. Further, as CAFs are themselves a key modulator of tissue stiffness, examination of co-culture models could provide clues as to how to interfere with CAF-mediated modulation of matrix stiffness. Rubiano *et al.* have worked on generating an *in vitro* model of PDAC that mimics the pathology with respect to the stiffness of PDAC tumors in conjunction with PDAC cells co-cultured in collagen gels with patient-matched PSCs (Rubiano et al., 2018). Whilst this model does not use organoids, it would be encouraging to explore whether this work could be adapted to this end.

6 CLINICAL IMPLICATIONS

Traditional diagnosis and treatment strategies against PDAC target cancer cells and cancer cell-derived biomarkers. However, since desmoplastic reaction accounts for up to 80% of the tumor volume (Erkan et al., 2012), characterizing PDAC tumor stromal components for identifying potential biomarkers and drug targets would not only improve the chances for early detection but also enhance the effectiveness of current treatment strategies aimed against advanced stages of the disease. As explained above, desmoplasia causes excessive extracellular matrix turnover, which includes constant breakdown and biosynthesis of collagens resulting in the concurrent discharge of collagen fragments into circulation (Willumsen et al., 2019). Nevertheless, desmoplasia is one of the main culprits in failed systemic treatments against PDAC. It not only hampers drug delivery, owing to its role in increasing pressure and decreasing circulation within the tumor, but also contributes in treatment resistance through its influence on intracellular signaling and biomechanical properties of the tumors (Saini et al., 2018). In this section, we review the current knowledge and discussions around the clinical implications of stromal contents in PDAC and the possibilities of establishing them as potential drug targets for future treatment strategies.

6.1 Diagnostic Implications

Conventional tumor-derived biomarkers, such as CA 19-9, TPS, CEA, and Ca 125 together with circulatory tumor cells, have long been used for diagnosis, monitoring disease progression, and prognosis in PDAC (Franklin et al., 2015) (Martini et al., 2019). However, owing to their origin from cancer cells, these biomarkers usually appear in circulation during advanced stages of the disease leading to late diagnosis, one of the hallmarks of PDAC, and limiting the scope of a successful treatment regimen. In recent years, measuring circulating ECM components in the blood has emerged as a new strategy to identify a novel source of potential biomarkers which can be

used in the early detection of the disease (Franklin et al., 2015). This strategy holds an immense potential since the detection of differentially expressed levels of stromal cell-derived proteins and other biomolecules, pertaining to the earlier stages of the disease, could lead to a better diagnosis and, in turn, prognosis of the disease. Moreover, detection of stroma-derived biomarkers from precancerous lesions could pave the way for establishing a successful preventive medicine system for PDAC, thus further signifying the importance of studying stromal-derived ECM components (Tian et al., 2019). CAFs, being the main contributors to desmoplasia, can detect and react to cues from cancer cells even from early stages and provide an opportunity for early detection. Many groups have studied the precision of different stromal components and their specificity in diagnosis and predicting patient outcomes. Collagens, being the main components of the ECM, have justifiably garnered considerable attention from PDAC researchers. Since collagens in the desmoplastic ECM are constantly being produced and degraded, measuring both intact and degraded collagen-derived epitopes in the circulation could be used to determine the collagen as well as ECM turnover in the pancreas. Deviations from the carefully maintained ECM homeostasis of healthy stroma could indicate an impending anomaly such as the onset of PDAC (Willumsen et al., 2019).

As the main fibrous proteins of the tissue, collagens type I and III have been analyzed often. In a phase-III clinical study, measured pre-treatment serum levels of several different MMP-degraded collagens including type I (C1M), type III (C3M), type IV (C4M), and pro-peptide of type III (PRO-C3), in patients with stage III or IV PDAC were found to be above the reference range for 67–98% of patients with median values being almost two-fold as compared to the healthy controls (Willumsen et al., 2019). High levels of all the collagen fragments were associated with shorter survival while a higher degradation/formation ratio of collagen type III (C3M/PRO-C3) was associated with better overall survival (Willumsen et al., 2019). It can be surmised that a similar patient-sample-based approach for detecting levels of circulating-collagen turnover, that corresponds to specific stages of disease progression, could help in establishing a credible circulatory collagen-based tool for monitoring progression of PDAC and/or response to treatment.

As a result of the distinctive structure and role of collagen type VI in the ECM, researchers have often investigated it as a potential biomarker for several metabolic diseases including PDAC (Sun et al., 2019). Retrospective analysis of serum samples of patients has shown significantly higher levels of COL6A3 protein in the serum of patients with PDAC compared to serum from healthy subjects or patients with benign lesions (IPMN and cystic lesions) (Kang et al., 2014). The elevated level of COL6A3 did not have a significant association with overall survival but it was able to predict survival better than the CA19-9 level, a carbohydrate biomarker that is currently the most frequently used biomarker to monitor tumor progression in PDAC patients (Kang et al., 2014). A recent study, analyzing different fragments of collagen type VI, found elevated levels of MMP-cleaved fragments of COL6A1 and COL6A3 in serum of patients with different stages of solid tumors, including PDAC, than in the serum obtained from subjects with either a healthy or non-cancer pancreas. This suggested that MMP-mediated collagen type VI alteration is crucial in carcinogenesis and that measuring collagen type VI fragments in serum might be used as a tumor biomarker (Willumsen et al., 2019). Although endotrophin, a collagen type VI-derived peptide, was previously measured to be elevated in PDAC (Sun et al., 2017), it did not show any significant difference in this study (Willumsen et al., 2019).

While individual stromal components such as collagens and their cleavage products, are widely accepted as potential biomarkers, most preclinical approaches and clinical trials have emphasized the importance of using diagnosis panels with a combination of different biomarkers that include both stromal and tumor-derived markers in order to have a holistic view of the disease characteristics in patients (Balasenthil et al., 2017). A recent study demonstrated this notion by showing that when tested using a panel of eight biomarkers, including both tumor and stroma-derived biomarkers, some patient samples presented with elevated levels in only one or two biomarkers which in few cases were only stroma-derived biomarkers (Franklin et al., 2015).

6.2 Therapeutic Implications

Different components of the stroma have also been studied to find new therapeutic opportunities. Many anti-desmoplastic agents have been used to target these components. These treatment options either target the mechanical properties of the stroma, such as high interstitial pressure and solid stress, or target specific molecular components regulating tumorigenesis, such as TGF-β, Sonic Hedgehog. Recently, Neesse et al., have extensively reviewed the clinical significance of biophysical and molecular aspects of PDAC stroma (Neesse et al., 2019). Here, we highlighted selected stroma-associated targets that have been used in clinical trials or had promising preclinical results.

6.2.1 Targeting the Mechanical Properties of the Tumor

Recent decades have seen more focused research on developing treatments that target different mechanical traits of tumors. As mentioned previously, four major mechanical characteristics of tissue that are altered during malignancy include solid stress, interstitial fluid pressure, stiffness, and tissue microarchitecture. Although treatments targeting stiffness and solid stress in tumors have been previously explored, fewer studies have targeted interstitial fluid pressure and altered microarchitecture of a tumor.

The main influencers of the increased stiffness in the tumor tissue are the high levels of matrix deposition in the desmoplastic stroma and increased matrix cross-linking (Nia et al., 2020). Moreover, since solid stress is mainly exhibited in ECM, many of its adverse physical effects such as compressed vasculature, can be reversed by relieving the stress in ECM (Nia et al., 2020). Therapeutic agents that primarily act by selective depletion of one or more of the abundant ECM components often reduce the total stromal volume of the tumor. This, in turn, decreases the

solid pressure and stiffness and alleviates compression on the vasculature, allowing increased intratumoral circulation and perfusion and thus improve drug delivery to the tumor. Most therapeutic strategies to counter solid stress in the tumor involve individual or combined targeting of cancer cells, CAFs, collagen, or hyaluronan (**Figure 2**).

As the major component of the ECM, collagen types I, II, and III have been the focus of several clinical trials. These trials include drugs that neutralize collagens or accelerate their degradation, such as anti-inflammatory drugs or collagen-neutralizing antibodies (Choi et al., 2013) those which interfere with collagen expression directly, such as micro-RNA based drug MiR-129-5p (Wang, and Yu 2018), or those which target enzymes regulating collagen biosynthesis and stability, such as 4-hydroxylase which is involved in stabilizing the collagen triple helix (Gorres, and Raines 2010) (Vasta, and Raines 2018). Conventional multi-drug regimens which use chemotherapeutic agents such as nab-paclitaxel and gemcitabine have also been shown to affect collagen content of the ECM. Tumor samples from patients treated with this combination-treatment showed decreased collagen type I content coupled with disorganized collagen networks and fewer CAFs compared to untreated samples (Alvarez et al., 2013).

An important class of tumorigenic components of desmoplastic stroma is MMPs, which regulate collagen metabolism. Despite several reports linking the MMPs with PDAC carcinogenesis and evidence of the effect of their inhibition in survival (Bramhall et al., 2001), their broad-based inhibition in PDAC did not yield encouraging results in clinical trials mainly due to adverse side effects such as musculoskeletal toxicities (Bramhall et al., 2002). Most of these drugs showed a very low therapeutic index, where both higher and lower doses induced toxicity (Peterson 2006). This suggests the significance of maintaining equilibrium in MMPs' activity for tissue homeostasis. It has also been surmised that the success of any MMP inhibitor would also greatly depend on the stage of the disease at the time of commencing the drug regimen since both premature and late delivery of the drug would result in adverse results, thus leaving a very narrow therapeutic window corresponding to a specific premetastatic stage of the tumor (Knapinska et al., 2017). Nevertheless, rapidly accumulating evidence on the role of aberrant MMP activity in different aspects of PDAC progression and metastasis has necessitated the need for reassessment of strategies targeting these MMPs.

Another component of the ECM that contributes significantly to stromal stiffness is hyaluronan. Combined biochemistry of hyaluronan and collagens in the TME has been shown to increase solid stress in the tumors (Stylianopoulos et al., 2012). One study has shown that collagens and hyaluronan work in tandem to compress intratumoral vessels. An angiotensin inhibitor, Losartan, which acts by reducing the production of hyaluronan and collagen in CAFs, has been found effective in reducing solid stress and improving drug and oxygen delivery in PDAC tumors (Chauhan et al., 2013). The activity of PEGylated human recombinant PH20 hyaluronidase (PEGPH20), a hyaluronan degrading enzyme developed as an anticancer drug, has also been found to enhance doxorubicin and gemcitabine chemopermeability. A combination drug regimen that used PEGPH20 in conjunction with gemcitabine in a preclinical mouse model resulted in suppression of pancreatic tumor development and better survival compared to gemcitabine alone (Jacobetz et al., 2013), however, results from clinical trials have not been conclusive (Hingorani et al., 2018) (Van Cutsem et al., 2020). The phase I/II study (NCT01839487) resulted in better prognosis for patients who had high pre-treatment levels of hyaluronan. However, the phase III study (NCT02715804) was stopped due to the ineffectiveness of the combined PEGPH20 with gemcitabine and nab-paclitaxel to improve the overall survival, progression-free survival, and the duration of response compared to gemcitabine and nab-paxitacel alone which resulted in the withdrawal of interest in the drug by the sponsor company (Tucker 2019) (Halozyme Therapeutics 2019). Another strategy to reduce desmoplasia and remove excess ECM is to target the cells producing ECM, namely CAFs. However, this strategy has presented challenges as well. Several novel and repurposed small-molecule drugs have been tested for their anti-desmoplasia effects in preclinical as well as clinical trials (Nandi et al., 2020). Some of the most intriguing, repurposed candidate drugs include Aspirin, Metformin and Chloroquine which are tested for their anti-desmoplasia activity by targeting mainly CAFs, pancreatic stellate cells (PSCs) and cancer stem cells (CSCs), respectively (Takada et al., 2004) (Incio et al., 2015) (Balic et al., 2014).

Despite encouraging results at the pre-clinical stage, many promising anti-desmoplasia drug candidates targeting either CAFs or other stromal components, fail to give conclusive results in clinical trials, a trend they share with many anti-cancer drugs. The main reason for this chemoresistance being desmoplasia itself. To circumvent this issue, in a recent study Nicolásnicolás-Boluda *et al.* tried to deplete CAFs in cholangiocarcinoma by using gold nanoparticle-mediated photothermal therapy and successfully decreased tumor stiffness which subsequently resulted in tumor regression (Nicolásnicolás-Boluda et al., 2020). Similar unconventional approaches are also required to negate the effect of desmoplastic reaction in PDAC and normalize the tumor mechanics to increase the effectiveness of both conventional and novel drug therapies.

6.2.2 Targeting Tumor-Promoting Stromal Components

Depletion of desmoplastic reaction in stroma has been found effective in eliciting initial drug response in tumors, however, targeting abundant non-neoplastic tumorigenic components of the stroma, such as CAFs, CSCs, and regulatory T cells, is required for effective and long-lasting treatment strategies and better prognosis. Targeting the tumorigenic components in TME, especially CAFs, has shown encouraging results for reducing relapse and metastasis (Sunami et al., 2021). One of the most significant events in tumorigenesis is the activation of quiescent PSCs to form CAFs (Apte et al., 2012). The process is governed by the cancer cells and involves several signaling pathways, such as hedgehog signaling and TGF-β signaling, as well as specific environmental cues, such as

hypoxia and malnutrition. Many of these CAF-activation factors have been exploited by researchers to find efficient anti-cancer therapies (Wu et al., 2021).

Patients with PDAC present with deficiencies in fat-soluble vitamins such as A, D, and K which mainly stem from exocrine pancreatic insufficiency (Min et al., 2018). Vitamin A plays a key role in maintaining pancreatic homeostasis and its deficiency leads to the activation of PSCs. In a preclinical study, Froeling *et al.*, exposed activated PSCs to a vitamin A homolog, ATRA. Incubation with ATRA resulted in altered gene expression in PSCs which caused their phenotype to revert to the quiescent state and suppressed genes involved in proliferation and motility (Froeling et al., 2011). ATRA has also been repurposed as a stromal targeting agent to be used in a phase-I clinical trial and after being declared safe to use in combination with gemcitabine-nab-paclitaxel it has been approved for phase II clinical trials (Kocher et al., 2020). Likewise, vitamin D receptor has been identified to act as a master transcriptional regulator for PSC quiescence, suggesting that priming the pancreas with calcipotriol (a vitamin D analog) could support PDAC therapy (Sherman et al., 2014). Tests in murine models of PDAC where gemcitabine treatment was supplemented with calcipotriol showed reduced stromal activation, together with an increased intratumoral concentration of gemcitabine and longer median survival. Further, since paricalcitol (another vitamin D analog) has been demonstrated to be effective in a pre-clinical setting (Schwartz et al., 2008), it is currently being tested in several clinical trials (NCT03331562) (NCT03415854) (NCT03520790) (Sherman et al., 2014). However, an earlier clinical trial on patients who had their treatment regimen supplemented with seocalcitol (another vitamin D analog) failed to show an objective response to treatment (Evans et al., 2002). With respect to vitamin K, an inverse association between dietary intake of phylloquinone (vitamin K1), but not menaquinones (vitamin K2), and risk of pancreatic cancer has been recently identified, however little seems to be known about this association at the molecular level (Yu et al., 2021).

In addition to environmental cues, cellular communication between cancer cells and quiescent PSCs plays an important role in stromal remodeling during PDAC tumorigenesis(Apte et al., 2012). Important paracrine signals from cancer cells include hedgehog signaling ligands such as SHH which bind to its receptor Patched1 (PTCH1) on the PSCs and via uncoupling of SMO, activates the signaling cascade (Saini et al., 2019). SHH is a key development regulator which is absent in the normal pancreas but has been shown to be overexpressed in PDAC (Kayed et al., 2006). SHH-mediated hedgehog signaling plays an important role in regulating differentiation of PSCs and proliferation and motility of CAFs and is considered an important tumorigenic factor (Bailey et al., 2008). Immuno-blocking of SHH by monoclonal antibody 5E1 has led to the depletion of desmoplasia and formation of smaller tumors in a PDAC mouse model (Bailey et al., 2008). Similar effects were observed when SMO was inhibited on PSCs with cyclopamine, a naturally occurring alkaloid and inhibitor of SHH signaling (Li et al., 2014) (Ma et al., 2013).

Another core signaling pathway involved in PDAC tumorigenesis is TGF-β signaling. Downregulation of TGF-β signaling in PSCs is considered a crucial molecular event during PDAC progression owing to its tumor-suppressive effect (Neuzillet et al., 2014). On the other hand, TGF-β signaling also regulates PSC activation, and TGF-β activated CAFs have been implicated in tumor growth and metastasis (Calon et al., 2014). Despite the role of regulatory T cells (Treg) in immune suppression, their depletion in a PDAC mouse model led to the unexpected result of accelerated tumor progression due to their ability to reprogram the fibroblast population and deplete αSMA^{+} tumor suppressive CAFs via the loss of TGF-β ligands (Zhang et al., 2020). Regardless of its dual role in PDAC tumorigenesis, several small-molecule TGF-β signaling blockers such as Vactosertib (TGF-β type 1 receptor kinase inhibitor) and Luspatercept (allosteric inhibitor of TGF- β ligand) have been shown effective in targeting PDAC progression and metastasis (Kim et al., 2021). However, inadvertent side effects, such as inhibition of other tumor-suppressive pathways, have limited clinical applications of many TGF-β signaling antagonists (Ciardiello et al., 2020).

Another key event in PDAC tumorigenesis is angiogenesis which is driven by an intricate balance of several pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factors, epidermal growth factor, in antagonism with anti-angiogenic factors, such as angiostatin and endostatin. Several anti-angiogenic drugs have been used to target angiogenesis in PDAC. Most of these drugs such as Axitinib, Bevacizumab and Nintedanib (Kindler et al., 2011) (Bill et al., 2015) (Crane et al., 2009), target VEGF and cause depletion of the tumor stroma followed by decreased tumor growth (Bergers, and Benjamin 2003). However, long-term use of these drugs targeting the tumor vasculature may lead to hypoxia in the TME which re-activates VEGF expression and triggers angiogenesis resulting in more aggressive tumor growth (Awasthi et al., 2015). Given the heterogeneity in the regulation of angiogenesis, multidrug treatments targeting different angiogenic factors have been suggested as a more prudent approach than monotherapy to counter angiogenesis-mediated tumor growth (Rhim et al., 2014).

7 CONCLUSION

In this review, we described the current knowledge regarding the PDAC stromal components with special reference to collagens and mentioned their multifaceted role in disease development even from early stages. We emphasized the importance of these early changes in the ECM and their potential for being early detection markers in the blood. We also mentioned the desmoplastic reaction that takes place in the primary tumor and the metastatic site and explained its importance in tumor characteristics. We then discussed the role of acquired mechanical traits of the ECM and mechanoreciprocal interactions taking place within the TME and their function in the disease progression of PDAC. Furthermore, we pointed out

the therapeutic opportunities that arise by studying the physical interactions within the TME. Moreover, we discussed the advancements of the models to study tumor-stromal interactions and their abilities to capture the physical characteristics of the TME.

Taken together, biochemical and physical interactions in the TME, affect the course of the disease and have been under the spotlight of research in recent years. Studies of these physical interactions can lead to the development of new tools for early detection and late-stage therapy options in the near future. Indeed, measuring circulating collagen fragments is an effective means by which to monitor disease progression (Willumsen et al., 2019), it also indicates that the composition and regulation of the ECM are dynamic and alter throughout disease progression. It would be interesting and potentially extremely useful to further study whether the composition of these secreted collagen fragments demonstrated any specificity to early PDAC or indeed for any specific fibrotic disease.

With respect to therapeutic avenues, it is clear that the desmoplastic reaction is a key hurdle to overcome for therapeutic perfusion into the tumor. An idea to explore in this content might be to target the cross-linking of the collagens in the tumor stroma in order to reduce the stiffness and improve drug delivery. Another idea might be to block the HAS enzymes in order to reduce the production of hyaluronan, however, its depletion has shown controversial results in the past (Hingorani et al., 2018) (Van Cutsem et al., 2020). Similarly, though it is encouraging to see that there are approaches that alleviate the severity of desmoplasia by targeting CAFs such as in cholangiocarcinoma (Nicolásnicolás-Boluda et al., 2020), it seems that PDAC is a case where ablating α-SMA expressing-CAFs results in worse outcomes (Rhim et al., 2014) (Özdemir et al., 2014) despite a corresponding reduction in fibrosis and associated desmoplasia. There ultimately remains a need to better understand the role of the ECM in the PDAC TME in order to explain this counter-intuitive finding and ultimately develop effective therapeutics for PDAC.

The effects of desmoplasia at the tissue scale are reasonably characterized (**Figure 2**), however how these biomechanics influence PDAC at the cellular level remains relatively poorly characterized. That said, the recent development of 3D PDAC cell and organoid culture models in tunable and dynamic hydrogel systems provide the perfect opportunity to decouple the biochemical and biomechanical properties of the ECM and identify any specific pathways ripe for therapeutic intervention.

Overall, the stroma is clearly an important component from potentially even prior to tumor initiation, through tumor development and eventual metastasis. Looking forward, with the development of investigative and analytical tools and approaches, the ECM and the TME provide much potential for the discovery of both diagnostic and therapeutic approaches. Further, it should be stressed that a focus on ablation of aspects of the PDAC TME has so far not been entirely fruitful, however there has been identification of CAF subtypes that have a tumor restraining phenotype. Perhaps in the future a focus on potentiation of tumor suppressive functions, rather than ablation, of the stroma will be an avenue to tame and treat the disease.

AUTHOR CONTRIBUTIONS

PM and DÖ designed the concept and planned the outline of the review. PM, JM, and MD performed the literature search, compiled the references and wrote the manuscript. PM designed the figures. MD and DÖ revised the manuscript. DÖ supervised the project. All authors read, discussed, and approved the final manuscript.

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REVIEW
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Cell Lineage Infidelity in PDAC Progression and Therapy Resistance

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Infidelity to cell fate occurs when differentiated cells lose their original identity and either revert to a more multipotent state or transdifferentiate into a different cell type, either within the same embryonic lineage or in an entirely different one. Whilst in certain circumstances, such as in wound repair, this process is beneficial, it can be hijacked by cancer cells to drive disease initiation and progression. Cell phenotype switching has been shown to also serve as a mechanism of drug resistance in some epithelial cancers. In pancreatic ductal adenocarcinoma (PDAC), the role of lineage infidelity and phenotype switching is still unclear. Two consensus molecular subtypes of PDAC have been proposed that mainly reflect the existence of cell lineages with different degrees of fidelity to pancreatic endodermal precursors. Indeed, the classical subtype of PDAC is characterised by the expression of endodermal lineage specifying transcription factors, while the more aggressive basal-like/squamous subtype is defined by epigenetic downregulation of endodermal genes and alterations in chromatin modifiers. Here, we summarise the current knowledge of mechanisms (genetic and epigenetic) of cell fate switching in PDAC and discuss how pancreatic organoids might help increase our understanding of both cell-intrinsic and cell-extrinsic factors governing lineage infidelity during the distinct phases of PDAC evolution.

Keywords: PDAC, pancreatic ductal adenocarcinoma, organoid culture, cell lineage, progression, therapy resistance

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INTRODUCTION

During embryonic development, cells progress into specialised biological units that need to perform distinct functions within their designated tissues. A cell's "lineage" details its developmental history, which includes tightly regulated division and differentiation processes to ensure each cell meets its "fate", i.e., differentiates into its physiologically relevant type (Furlong, 2010). Throughout this journey, cells gradually lose their potential to differentiate into alternative cell types and eventually end up in a fully differentiated state. Strict control over the processes that develop and maintain cells' identity is crucial to ensure normal physiological functions (Lander et al., 2009). Deregulation of the programmes that maintain phenotype can lead to infidelity to cell fate and lineage conversion, with differentiated cells losing their identity and, accordingly, the expression of type/function-specific genes. Cells can either revert back to a state with increased developmental potential (de-differentiation) or switch phenotypes entirely, within or across embryonic germ layers (*trans*-differentiation) (Sancho-Martinez et al., 2012). However, cells can also *trans*-differentiate by undergoing de-differentiation first. In some situations (e.g.: response to injury), certain flexibility over the cell lineage (i.e.: plasticity) can be beneficial. For example, biliary epithelial cells

(i.e., cholangiocytes) can change fate and become hepatocytes following liver damage (Deng et al., 2018). In cancer, however, the transcriptional programmes that maintain cell identity can be disrupted and eventually hijacked to drive uncontrolled proliferation (O'Brien-Ball and Biddle, 2017). Notably, suppression of cell-identity specific genes is often associated with cancer initiation and progression (Roy and Hebrok, 2015). Moreover, the ability of cancer cells to switch phenotypes can give them an evolutionary advantage that allows them to survive therapy (Yuan et al., 2019). In summary, infidelity to cell fate is an extremely important driver of cancer progression.

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease, with the lowest 5-years survival rate of all cancers (Siegel et al., 2021). Cell type infidelity seems to play an important role in PDAC initiation and progression and might even drive therapy resistance (Collisson et al., 2011; Moffitt et al., 2015; Bailey P. et al., 2016; Camolotto et al., 2018). Here, we briefly discuss the mechanisms leading to cell fate commitment within the normal exocrine pancreas (where PDAC arises from), the role of cell infidelity in cancer progression and therapy resistance and how these concepts fit within the challenging clinical context of PDAC. Finally, we focus on the role of the 3D organoid culture system and how it can contribute to elucidating the mechanisms of lineage infidelity in PDAC.

TRANSCRIPTION FACTORS GOVERNING PANCREAS DEVELOPMENT

To understand cell lineages in PDAC, it is important to first appreciate the cell fates in the normal and developing pancreas. The specification and maintenance of the pancreatic cell fate during embryogenesis is a highly complex and coordinated process that relies on the stepwise interplay between cell extrinsic (i.e., growth factors and morphogens) and cell intrinsic factors (i.e., transcription factors). The mature pancreas is made up of two specialised compartments: endocrine and exocrine. The endocrine compartment is composed of five types of hormone-producing cells, whose main function is to regulate nutrient homeostasis. On the other hand, the exocrine compartment is composed of acinar and ductal cells, whose main role is to produce and transport digestive enzymes, respectively.

Most of our knowledge of the embryonic development of the pancreas is based on mouse models owing to the ethical concerns and practical difficulties in obtaining suitable human samples, as well as to the wealth of genetic tools that can be used to study organs' development in mice. However, the key cell fate decisions and regulators involved in the pancreas development appear to be evolutionary conserved between mice and humans (Pan and Wright, 2011). The pancreas is an endoderm-derived organ that develops from the embryonic foregut, in a region adjacent to the liver, and it is first evident in mice around embryonic day (E) 9.5 and in humans at E26 (Pan and Wright, 2011; Jennings et al., 2013; Pan and Brissova, 2014; Ghurburrun et al., 2018). In mice, pancreas development is divided into primary and secondary transitions. The primary transition takes place between E8.5 and E12.5 and includes the formation of pancreatic dorsal and ventral buds from the foregut (**Figure 1**). The dorsal and ventral buds contain multipotent pancreatic progenitor cells (MPCs), which can give rise to both acinar and bipotent progenitors (ductal and endocrine precursors) (**Figure 1**). During the primary transition, the MPCs undergo rapid proliferation and generate a stratified epithelium which, in turn, forms microlumens (**Figure 1**) (Villasenor et al., 2010; Pan and Wright, 2011). At E11.5, the gut tube begins to coil, bringing the two buds closer and causing them to eventually fuse and form the pancreas. During the secondary transition, between E12.5 and E15.5, the pancreatic epithelium branches and forms tip and trunk domains (**Figure 1**). At this stage, cell lineage allocation to the main pancreatic fates (endocrine, acinar, and ductal) begins (Zhou et al., 2007; Pan and Wright, 2011). The tip domains will end up producing acinar progenitors, whilst the trunk will produce bipotent ones (**Figure 1**) (Zhou et al., 2007; Pan and Wright, 2011; Villamayor et al., 2020). After E16.5, expansion of the acinar tissue is mainly driven by acinar cell replication rather than *de novo* formation of acini. Postnatally, tissue maintenance is ensured mainly by the proliferation of differentiated endocrine and exocrine cells with the replication of insulin-expressing (Dor et al., 2004; Teta et al., 2007) as well as of acinar cells (Dor et al., 2004; Hezel et al., 2006; Teta et al., 2007; Murtaugh and Keefe, 2015) gradually decreasing.

Molecularly, there are multiple extrinsic signals from the neighbouring mesoderm that control pancreas development, including fibroblast growth factors (FGFs), Wnt, retinoic acid, bone morphogenic proteins (BMPs), as well as suppression of sonic hedgehog (Shh) signalling (Hebrok et al., 1998; Martín et al., 2005; Xu et al., 2011). These signals instruct the expression of transcription factors (TFs) that confer cell fate and aid in maintaining cell identity throughout adulthood (Puri et al., 2015). The mouse genetic toolkit has helped identifying the TFs involved in the different stages of pancreatic development, including patterning of the endoderm, the specification and maintenance of the pancreatic fate, and the determination of different pancreatic cell lineages. Here, we will focus on the relevant TFs, which are causally associated with PDAC molecular subtypes, in addition to those used to generate autochthonous models of pancreatic cancer.

All pancreatic cell types derive from MPCs that are marked by the expression of *PDX1* and *PTF1A* (**Figure 1**) (Kawaguchi et al., 2002; Burlison et al., 2008). Of those, *PDX1* (Pancreatic and duodenal homeobox 1) is recognised as the earliest TF, expressed in the pancreas primordia (Ohlsson et al., 1993; Ahlgren et al., 1996). Nevertheless, there are TFs known to precede both PDX1 and PTF1A, and neither of the two TFs is necessary for the initial pancreatic buds' formation (Jonsson et al., 1994; Offield et al., 1996; Stoffers et al., 1997; Krapp et al., 1998; Kawaguchi et al., 2002; Sellick et al., 2004). PDX1 can first be detected at E8.5 in mice and between E29 and E31 in humans (Sherwood et al., 2009; Jennings et al., 2013; Pan and Brissova, 2014). Even if the specification of the endoderm to a pancreatic fate does not rely on its function, *PDX1* expression is necessary for the

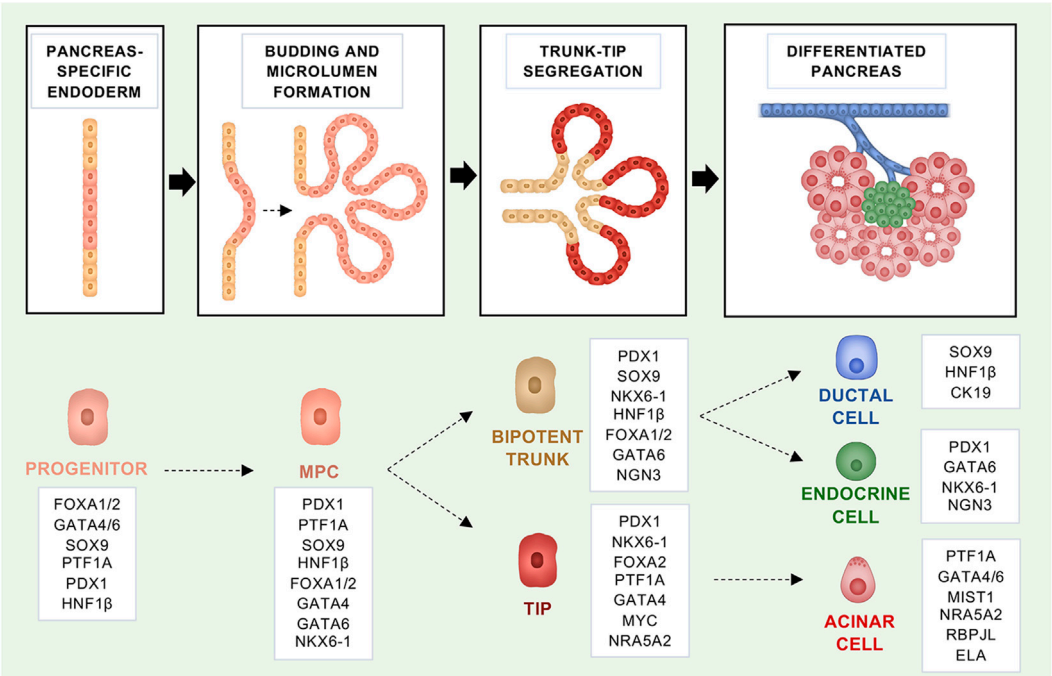

FIGURE 1 | Fate regulators that govern the embryonic development of the mouse pancreas and maintain identity in the adult organ. Schematic representation of the embryonic mouse pancreas development. In boxes, the fate regulators for each developmental stage are highlighted.

formation of all pancreatic cell lineages and its deficiency in mouse and humans results in complete pancreatic agenesis at birth (Jonsson et al., 1994; Offield et al., 1996; Stoffers et al., 1997; Schwitzgebel et al., 2003). Later in life, high PDX1 levels are important to maintain the identity of endocrine β-cells and heterozygous variants of *PDX1* have been linked to the development of Mature Onset Diabetes of the Young (MODY) (Stoffers et al., 1997). In the early stages of pancreas development, the pancreas transcription factor 1a subunit PTF1A (P48) functions as part of a trimeric complex, which includes RBPJ and sustains developmental program of early pancreatic epithelium (**Figure 1**) (Masui et al., 2007). *Ptf1a* is first detected at E9.5 (along with *Pdx1*), and lineage tracing experiments have shown that *Ptf1a* is important for all pancreatic cell fates (Kawaguchi et al., 2002; Pan and Wright, 2011). In mice, full body *Ptf1a* deficiency results in pancreas agenesis and lethality shortly after birth (Krapp et al., 1998). Furthermore, in the absence of *Ptf1a*, cells normally contributing to the ventral pancreas are re-directed to a duodenal fate in mice (Kawaguchi et al., 2002; Burlison et al., 2008). Complementary to that, misexpression of *Ptf1a* in the early endoderm re-directs non-pancreatic endodermal cells into pancreatic precursors and determines the formation of pancreatic tissue at ectopic sites in the embryo (i.e., rostral duodenum, extrahepatic biliary system, and glandular stomach) (Willet et al., 2014). In humans, mutations in the *PTF1A* gene and an associated enhancer region have also been linked to pancreatic agenesis (Sellick et al., 2004; Weedon et al., 2014). Later during development, high *Ptf1a* expression gets restricted to the acinar progenitors and it is maintained in the differentiated acini during adulthood (**Figure 1**) (Puri et al., 2015). In pro-acinar cells, RBPJL replaces RBPJ in the PTF1 complex to drive the expression of the secretory digestive enzymes (**Figure 1**) (Hoang et al., 2016). Moreover, in the transition from MPCs to pro-acinar cells there is a critical downregulation of *c-Myc,* which has been shown to bind and repress the transcriptional activity of PTF1A (Sánchez-Arévalo Lobo et al., 2018). Other critical transcription factors for the acinar maturation are NR5A2 and MIST1 (**Figure 1**). NR5A2 is a nuclear receptor required during early embryonic development and active at more than one stage during pancreas development, including acinar maturation (Hale et al., 2014). *Nr5a2* deficiency results in strong reduction of endocrine cells and acini, as well as disruption in the ductal compartment (Hale et al., 2014). In terms of its role in acinar cells development, NR5A2 interacts with the PTF1 complex and in its absence the remaining acinar cells do not complete differentiation (Hale et al., 2014). The basic helix-loop-helix transcription factor MIST1 is required to complete acinar cell differentiation, acting downstream of PTF1A (Pin et al., 2001; Jia et al., 2008). In mice, *Mist1* deficiency results in acinar cells losing their apical-basal polarity and exocrine disorganisation (Pin et al., 2001).

While specific combinations of TFs are necessary to specify and maintain cell fates, certain TFs have a "pioneer" function: they have the unique ability to bind to closed chromatin and increase the accessibility to multiple regulatory sequences (Drouin, 2014). Members of the fork-head-box DNA-binding proteins (FOXAs) are such TFs, termed "pioneer factors", that can bind heterochromatin and recruit additional TFs to ensure

cell specification (Zaret et al., 2008). FOXA2 is expressed by the endoderm before pancreatic development (E6.5) and it is required for the development of both the liver and pancreas (**Figure 1**) (Lee et al., 2005; Gao et al., 2008). In the pancreas, FOXA1/2 are required to activate the pancreatic specifier *PDX1* and seem to have interchangeable roles (Gao et al., 2008).

Other relevant TFs that ensure maintenance of the pancreatic cell fate are the zinc finger TFs GATA4 and GATA6 (**Figure 1**). *Gata6* and *Gata4* seem to have partly redundant functions in the development of the pancreas. While full-body knockout of either *Gata4* or *Gata6* is embryonically lethal (Kuo et al., 1997; Molkentin et al., 1997; Koutsourakis et al., 1999), the pancreas-specific inactivation of either *Gata4* or *Gata6* has only mild effect on pancreas formation (Carrasco et al., 2012; Xuan et al., 2012). However, the simultaneous inactivation of both genes results in no development of the pancreas and lethality shortly after birth (Carrasco et al., 2012; Xuan et al., 2012). In mice, *Gata4/6* are expressed in the early pancreatic epithelium and throughout pancreas development (Decker et al., 2006). At late stages of pancreas development, expression of *Gata4* gets restricted to the tips of the epithelial branches and then to the acinar cells of the mature gland (Decker et al., 2006). In contrast, *Gata6* continues to be expressed by all types of pancreatic cells (Decker et al., 2006; Martinelli et al., 2013). Moreover, deletion of *Gata6* in the early pancreatic epithelium revealed the importance of the TF in maintaining acinar identity; its deletion results in restrained acinar differentiation, an increased rate of acinar cell apoptosis and acinar-to-ductal metaplasia (Martinelli et al., 2013). In humans, mutations in *GATA6* have been shown to cause pancreatic agenesis and moderate diabetes with or without exocrine insufficiency, whilst *GATA4* mutations have also been linked to neonatal and childhood-onset diabetes with or without exocrine insufficiency (Bonnefond et al., 2012; Shaw-Smith et al., 2014; Villamayor et al., 2018).

Another family of TFs that is important in pancreatic development are the hepatocyte nuclear factors (HNFs) (**Figure 1**). *HNF1*β is first expressed by the MPCs at E9.5 and it is required for expansion of pancreatic progenitor cells, whereas later on its expression gets restricted to ductal cells only (**Figure 1**) (Nammo et al., 2008; De Vas et al., 2015). *HNF1*β is critical for pancreas development and heterozygous inactivating mutations in the gene lead to MODY (Bingham and Hattersley, 2004). In mice, the homozygous deletion of *Hnf1*β in the epiblast results in pancreas agenesis owing to no formation of the ventral bud and failed expansion of progenitor cells from the dorsal bud (Haumaitre et al., 2005). Pancreas-specific inactivation of *Hnf1*β impairs expansion of MPCs by reduced proliferation and increased cell death (De Vas et al., 2015).

In contrast to acinar and endocrine cells, the regulation of the ductal fate is a little bit more elusive. This is also contributed by the heterogeneity of the pancreatic ductal system, which is composed by large ducts, small inter and intra-lobular ducts, and by intercalated ducts that insert into the acini (Flay and Gorelick, 2004; Pandiri, 2014). The use of sophisticated whole-organ 3D imaging technique applied to the adult mouse pancreas has demonstrated the heterogenous morphology of cells composing the large (cuboidal) versus smaller ducts (elongated) (Messal et al., 2019). There is some evidence that distinct developmental programmes distinguish large from intercalated ducts, however more studies are needed to elucidate concretely the lineage determinants of the ductal fate (Krapp et al., 1998; Kawaguchi et al., 2002; Hale et al., 2005; Masui et al., 2007; Nakano et al., 2015). More in general, a *Ptf1a/Nkx6-1* switch determines the tip vs trunk cell fate in MPCs (**Figure 1**). Whilst *Ptf1a* gets restricted to the tip compartment and determines the acinar fate, the homeobox transcription factor *Nkx6-1* becomes restricted to the trunk compartment, giving rise to the bipotent progenitors that eventually generate the endocrine and ductal cells, and it is later required for endocrine cell differentiation (Schaffer et al., 2010; Pan and Wright, 2011). Further on, endocrine and ductal progenitors are differentiated by the transient expression of *Ngn3,* which is required for the differentiation of endocrine cells. In ductal cells, the SRY-Box transcription factor, SOX9, plays a crucial role. It is expressed in mouse MPCs at E10.5 and later also by the bipotent progenitors. In adults, *Sox9* expression is maintained only by the ductal population (**Figure 1**) (Seymour et al., 2007). As we will see below, many of the cell fate regulators discussed so far have been used to generate conditional mouse models of PDAC. Furthermore, expression of some of those transcription factors can be used to distinguish between molecular subtypes of PDAC. In summary, mouse genetic models have allowed to precisely dissect the critical regulators of pancreatic cell type fate; most of the studies in mice have found corresponding evidence for similar roles in humans. Nevertheless, it is conceivable that some species-specific differences exist.

PDAC AND ITS CELL OF ORIGIN

PDAC evolves from non-invasive precursor lesions, which arise from the synergistic action of oncogenic mutations and inflammation. The majority of PDAC is believed to arise from microscopic pancreatic intraepithelial neoplasia (PanIN) (Basturk et al., 2015). However, a significant number of PDACs develop in association with large and radiographically detectable cysts that include intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) (Basturk et al., 2015). Despite encompassing a variety of histological subtypes with specific genetic alterations, comparative sequencing of matched non-invasive neoplasms and invasive cancers has conclusively demonstrated that IPMN are a direct precursor of PDACs that are histologically indistinguishable from non-IPMN-derived tumours (Noë et al., 2020). As for other tumour entities (Chen et al., 2017; Labidi-Galy et al., 2017), early IPMN presented with remarkable heterogeneity in driver gene mutations and progression to invasive carcinoma has been associated with both loss of precancerous mutations and accumulation of further genetic abnormalities (Noë et al., 2020). In PDAC, the earliest oncogenic alteration is usually an activating mutation in *KRAS* which stimulates multiple signalling pathways to promote cell proliferation, survival, and metabolic reprogramming (Bourne et al., 1990; Suzuki et al., 2021) (**Figure 2A**). However, *KRAS* oncogenic activation alone is not

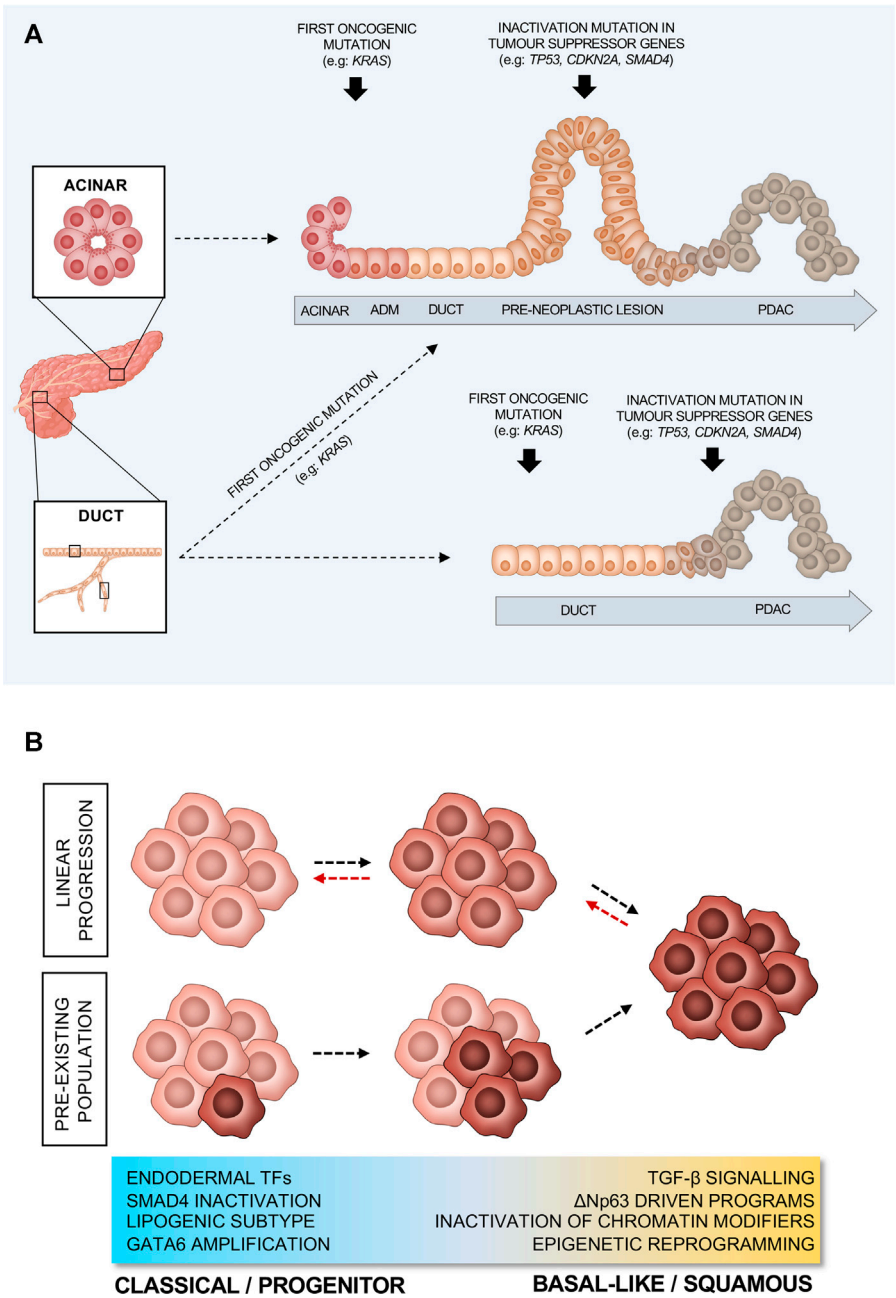

FIGURE 2 | Proposed models of PDAC progression. **(A)** PDAC progression from ductal or acinar cells. **(B)** Schematic representation of different models of PDAC evolution from classical/progenitor to basal-like/squamous subtype. Red arrows indicate switch between subtypes, in response to environmental pressures.

sufficient for the development of pre-neoplastic lesions as mutations in *KRAS* can also be detected in the pancreata of people with no evidence of disease (Yan et al., 2005; Yang et al., 2017) and *Kras* oncogenic induction in adult mouse pancreas does not lead to PDAC formation (Guerra et al., 2007). Coupled with cell insult, however, *Kras* activation in mice results in the lesions that lead to PDAC (Guerra et al., 2007). Nevertheless, progression of those lesions to cancer also requires further inactivating mutations in tumour suppressor genes, such as *CDKN2A* (Maitra et al., 2003; Hezel et al., 2006) (**Figure 2A**). Even though these driver mutations occur in the majority of cases, PDAC tumours are characterised by extensive inter- and intra-tumour heterogeneity, which is a result of a long tail of relatively infrequent events affecting key drivers of tumorigenesis and contributing to the complex biology of this disease (Jones et al., 2008; Biankin et al., 2012; Kanda et al., 2012; Moffitt et al., 2015; Waddell et al., 2015; Bailey P. et al., 2016; The Cancer Genome Atlas Research Network, 2017; Hayashi et al., 2020).

PDAC affects the pancreatic exocrine compartment, which is made up of ductal and acinar cells. Despite the ductal morphology of the neoplastic lesions, there has been a considerable debate regarding the cell of origin of PDAC. Genetically engineered mouse models (GEMMs) have shown that PDAC can arise from pancreatic embryonic precursors, acinar cells, or ductal cells (**Figure 2A**). GEMMs of PDAC rely upon the pancreas-specific expression of mutant alleles and knowledge of the cell- and time-specific expression pattern of certain TFs is crucial to understanding the cell of origin. One of the most used PDAC GEMM is the KPC (*Kras*$^{+/LSL-G12D}$; *Trp53*$^{+/LSL-R172H}$; *Pdx1/p48*-Cre) model (Hingorani et al., 2003). This model is based on the Cre-Lox technology (Kim et al., 2018) that permits the conditional activation of endogenous oncogenic alleles (oncogenic activating *Kras*^{G12D} mutation and a point mutation *Trp53*^{R172H}) in cells that express the Cre recombinase under the control of the pancreatic TFs *Pdx1* or *Ptf1a/p48* (Hingorani et al., 2003). While restricting expression of mutant *Kras* and *Trp53* to the mouse pancreatic epithelium, these models do not allow for the identification of the cell of origin as the Cre-driven recombination of the mutant alleles will happen during embryonic development (at the MPC stage), when the expression of these TFs is not restricted to a specific cell type. Conditional activation of mutant alleles in specific compartments of the adult mouse pancreas can be achieved through the use of a tamoxifen inducible Cre allele (Cre^{ER}) expressed in different cell types (Pimeisl et al., 2013). This gene editing technology has made possible the generation of models, where the activation of the oncogenic alleles can be restricted either to mature acinar or ductal cells. For example, oncogenic mutations can be restricted to the ductal compartment using Cre^{ER} driven from the *Sox9* (Kopp et al., 2012; Lee et al., 2019; Flowers et al., 2021), *Hnf1ß* (von Figura et al., 2014; Bailey J. M. et al., 2016), and *Krt19* (Ferreira et al., 2017) alleles. Conversely, oncogenic insults can be restricted to mature acinar cells using *Ptf1a*- (Kopp et al., 2012; von Figura et al., 2014; Lee et al., 2019; Flowers et al., 2021), *Ela*- (De La O et al., 2008; Habbe et al., 2008; Guerra et al., 2011), or *Mist1*-driven alleles (Tuveson et al., 2006; Habbe et al., 2008; Bailey J. M. et al., 2016). Finally, next generation murine PDAC models have also been developed using a dual-recombinase system that integrates the Cre-Lox and the Flippase (Flp-FRT) recombination technologies (Schönhuber et al., 2014), which allows for sequential and independent manipulation of gene expression (Schönhuber et al., 2014; Chen et al., 2018).

When acinar cells serve as the cell of origin of PDAC, the induction of a ductal-like state is a prerequisite for transformation (**Figure 2A**) (Guerra et al., 2007; Habbe et al., 2008; Kopp et al., 2012; Bailey J. M. et al., 2016; Lee et al., 2019). Indeed, during this process acinar cells downregulate typical acinar markers (e.g.: *Mist1*) whilst upregulating several ductal ones (e.g.: *Sox9*). This process, termed acinar-to-ductal metaplasia (ADM) occurs upon insult (e.g., tissue inflammation), and in the presence of oncogenic *Kras,* it becomes irreversible (**Figure 2A**). *Kras* activation supports and maintains ADM, resulting in the preinvasive neoplasms that lead to PDAC (Liou et al., 2016). It has been shown that the ectopic expression of *Sox9* in acinar cells drives ADM (Kopp et al., 2012). Despite being quite resistant to oncogenic *Kras* induced transformation, in the presence of additional mutations, adult ductal cells have also been shown to give rise to PDAC (**Figure 2A**) (Bailey J. M. et al., 2016; Ferreira et al., 2017; Lee et al., 2019; Flowers et al., 2021). While histologically indistinguishable PDACs originate in mice when the same oncogenic drivers (e.g., oncogenic activation of *Kras* and/or inactivation of *Trp53* and *Fbw7*) are targeted to either acinar or ductal cells, the cell of origin seems to dictate the way the disease progresses (Bailey P. et al., 2016; Ferreira et al., 2017; Flowers et al., 2021). Acinar-derived tumours in transgenic mice exhibit a stepwise PDAC progression from PanIN lesions to frank carcinoma regardless of the type of oncogenic insult (**Figure 2A**) (Bailey P. et al., 2016; Ferreira et al., 2017; Flowers et al., 2021). On the contrary, oncogenic insults into adult ductal cells generate invasive PDACs without clear evidence of PanIN (**Figure 2A**) (Bailey P. et al., 2016; Ferreira et al., 2017; Flowers et al., 2021). However, it cannot be excluded that PanIN lesions can form when pancreatic cancer originates from ductal cells, yet they might be difficult to detect if preinvasive lesions rapidly and invariably progress to frank carcinoma (**Figure 2A**). Furthermore, reflecting the heterogeneity of the ductal system, mice engineered to develop tumours from adult ductal cells present with two different types of lesions growing either away from the ductal lumen (termed exophytic) or into the ducts (termed endophytic) (Messal et al., 2019). In an elegant study, Messal and others applied an innovative 3D whole organ imaging technique (termed FLASH) to the pancreata of mice, where the combination of oncogenic activation of *Kras* and the deletion of either *Trp53* or *Fbxw7* was driven in adult ductal cells by *Krt19* or *Hnf1ß* (Messal et al., 2019). They showed that the morphology of the lesions did not depend upon the specific oncogenic combination, but rather on the diameter of the source epithelium, with endophytic lesions forming from ductal segments with diameter above 17 µm (Messal et al., 2019). Mechanistically, the oncogenic activation of *Kras* in ductal cells, regardless of their position in the ductal system, led to cytoskeleton changes in transformed cells with reduced apical-to-basal tension that is required for endophytic lesions to form, while the high curvature of the duct prevented inward growth in ductal segments with diameter below 17 µm. The type of lesions could be also seen in human specimens and, more importantly, both mouse and human exophytic lesions displayed a more invasive phenotype (Messal et al., 2019).

Despite being widely used to model PDAC initiation and progression, GEMMs also bear limitations, which need to be considered. For example, *Sox9* is expressed in other adult cells and ductal-derived Sox9 models have presented with other types of carcinomas (Flowers et al., 2021). Moreover, restricting *Kras* oncogenic mutation to adult *Mist1*-expressing acinar cells resulted in pancreatic tumours with mixed histological features as well as hepatocellular carcinomas (Tuveson et al., 2006). Yet, regardless of their limitations, GEMMs have shown that PDAC can arise from both acinar and ductal cells and have provided important insights into how the cell of origin affects PDAC progression (extensively reviewed in Grimont et al., 2021). However, we still do not know whether these models reflect

what truly happens in patients. Reconstruction of the lineage relationships in human cancer formation requires the use of "endogenous" barcodes which can be either somatic mutations, gene variants or heteroplasmic mitochondrial DNA variants (Ju et al., 2017; Ludwig et al., 2019; Xu et al., 2019). These genetic markers can also be used in combination with single-cell sequencing technologies to trace cellular hierarchies back to the embryonic state. While still in their infancy, these methods are increasingly being used for lineage reconstruction during human organ development and have the potential of providing conclusive evidence on the cell of origin of pancreatic cancer, as well as whether PDAC predisposing mutations occur in precursor cells during embryonic development.

MOLECULAR DETERMINANTS OF CELL LINEAGES IN PDAC

Several studies have derived various molecular classifications of PDAC, based on bulk transcriptomic data from primary non-treated tumours as well as from cell lines (Collisson et al., 2011; Moffitt et al., 2015; Bailey P. et al., 2016; Puleo et al., 2018). What appears to be common between all classification systems is the existence of a "classical" or "progenitor" PDAC, which exhibits higher expression of pancreatic endodermal cell-fate determinants, such as *GATA6, HNF1A,* and *HNF4A* and shows slightly better prognosis (**Figure 2B**). On the other hand, there is a more aggressive basal-like/squamous subtype that shows loss of pancreatic identity and mostly associates with elevated expression of programmes driven by the master regulator ΔNp63, as well as with upregulation of the TGFβ signalling (**Figure 2B**) (Collisson et al., 2011; Moffitt et al., 2015; Bailey P. et al., 2016; Puleo et al., 2018). Genetic and non-genetic dysregulations of gene expression programmes involved in the maintenance of pancreatic cell identity are integral drivers of PDAC molecular subtypes. In their seminal manuscript, Bailey and others (Bailey P. et al., 2016) showed the association between reduced expression (through gene hypermethylation) of the endodermal cell fate determinants (*PDX1, GATA6*, and *HNF1*β) and the basal-like phenotype. In particular, GATA6 has been demonstrated as a critical regulator of the classical programme and thus a valid surrogate biomarker of the classical subtype (Martinelli et al., 2017; Aung et al., 2018). However, it has been recently shown that, while necessary, GATA6 loss is not sufficient to drive the basal phenotype (Kloesch et al., 2021). Further downregulation of other endodermal fate determinants such *HNF1A* and *HNF4A* is also needed for the complete switch from classical to squamous/basal-like subtype (Kloesch et al., 2021). This is supported further by the fact that HNF4A loss also causes a switch to a squamous metabolic profile in human PDAC cell lines (Brunton et al., 2020). Epigenetic reprogramming, due to alterations in epigenetic modifiers might also favour gradual loss of the endodermal cell fate. This is supported by the fact that basal-like/squamous tumours exhibit alterations in epigenetic modifiers and transcription master regulators, such as *ARID1A* and *MYC* (**Figure 2B**). In a recent multiregional sampling analysis of primary and metastatic PDACs, the integration of histology, expression profiling, and DNA sequencing revealed the enrichment of clonal mutations in chromatin modifiers (e.g., *ARID1A*, *KMT2C*, *KMT2D*, and *KDM6A*) in tumours with basal-like/squamous features (Hayashi et al., 2020). Furthermore, aberrant activation of *MYC,* due to gene amplification, drives PDAC progression by activating cell proliferation, survival programmes (Dang, 1999; Pelengaris et al., 2002), and metabolic reprogramming (Dey et al., 2020). Accordingly, the frequency of *MYC* amplification is higher in advanced stage PDACs and in tumours with basal-like features (Hayashi et al., 2020). However, the effect of *MYC* amplification on cellular lineage seems to be context-dependent as induced overexpression of *MYC* in PDAC cells conferred the basal-like/squamous phenotype exclusively in the background of chromatin modifier genes inactivation (Hayashi et al., 2020). The SWI/SNF subunit AT-rich interactive domain ARID1A regulates the expression of *Sox9* to maintain the ductal fate while its loss drives aggressive PDACs (Kimura et al., 2018). The loss of another epigenetic regulator, the X-chromosome encoded histone demethylase KDM6A, activates gene networks regulated by p63 and MYC that promote squamous-like and poorly differentiated PDAC with sarcomatoid features (Andricovich et al., 2018). Interestingly, Andricovich and others (Andricovich et al., 2018) demonstrated that gene expression changes resulting from the loss of *Kdm6a* are independent from the enzyme's demethylase activity but are rather due to changes in the activity of super-enhancers. Similarly to the loss of *Kdm6a*, the pancreas-specific deletion of *Hnf1a* synergises with mutated *Kras* to induce PDAC lesions with sarcomatoid features as well as a molecular phenotype that aligns with human basal-like/squamous tumours (Kalisz et al., 2020). Mechanistically, HNF1A recruits KDM6A at functional genomic sites in acinar cells to activate differentiation and suppress oncogenic pathways (Kalisz et al., 2020). More evidence for the epigenetic reprogramming of PDAC has been provided by Somerville et al. (Somerville T. D. D. et al., 2020), showing aberrant expression of the transcription factor zinc finger protein (ZBED2), which seems to downregulate the pancreatic progenitor cell fate. In addition to the expression of transcription factors and the genetic inactivation of chromatin modifiers, distinct methylation patterns of repetitive elements can be used to distinguish classical from basal-like PDAC (Espinet et al., 2021). Tumours showing low levels of DNA methylation at these elements (defined as MC2, methylation cluster 2) display increased Interferon-response signatures, a pro-inflammatory microenvironment, and associate with the basal-like phenotype (Espinet et al., 2021). Moreover, through oxidative bisulfite sequencing of archival samples, Eyres et al. (2021) have recently found that the basal/squamous-like phenotype is a direct result of epigenetic silencing of regulator of the classical programme. In basal-like/squamous tumours, the authors found that TET2-maintained levels of 5-hydroxymethylcytosine (5 hmc) are significantly reduced at genetic loci which promote the classical gene programme (such as *GATA6*). This further supports the classical programme as the "default lineage" that is epigenetically

silenced to drive a phenotype switch towards the basal-like/squamous cell lineage.

While the dichotomisation into two subtypes has the perceived advantage of simplifying biomarker and functional studies, there is increasing evidence that cells with classical and basal-like features co-exist in the same tumour (**Figure 2B**) (Puleo et al., 2018; Porter et al., 2019; Chan-Seng-Yue et al., 2020; Hwang et al., 2020; Juiz et al., 2020; Nicolle et al., 2020). This evidence has been generated from analyses of both human tissues (Puleo et al., 2018; Porter et al., 2019; Chan-Seng-Yue et al., 2020; Hwang et al., 2020; Raghavan et al., 2021) and *ex vivo* cultures (Porter et al., 2019; Juiz et al., 2020; Nicolle et al., 2020; Raghavan et al., 2021) and implies that molecular classification systems should account for this phenotypic heterogeneity for a better prediction of patient outcomes. Accordingly, Nicolle and others have recently shown the benefit of classifying patients based on a continuum of phenotypes rather than on two non-overlapping subtypes (Nicolle et al., 2020). Despite the observation of co-existence of subtypes within the same tumour, the question remains as to whether those are two interconverting cell types, different entities, or bear a precursor-to-product relationship. There is some evidence to suggest that PDAC progression is associated with accumulation of basal-like cells (**Figure 2B**). Enrichment of basal-like/squamous cells has been observed in advanced stages of the disease (Chan-Seng-Yue et al., 2020) as well as in post-treatment tumours (Hwang et al., 2020). This is also supported by *in vitro* data showing enrichment of basal state post-treatment with FOLFIRINOX (Porter et al., 2019) and that PDAC cell lines exist on a continuum, suggesting linear evolution from classical to basal-like/squamous PDAC. However, there is also evidence to suggest that the cell of origin affects PDAC's progression. Support to this hypothesis is given by a recent study proposing that, in mice, the cell of origin can also influence subtypes as ductal cell-derived PDAC are enriched for basal-like signatures, whilst the acinar derived ones are enriched for classical gene signatures (Flowers et al., 2021). In keeping with this observation, the MC2 methylation subtype described by Espinet and others (Espinet et al., 2021) and aligning with the transcriptomic basal-like subtype is suggested to derive from ductal cells. Furthermore, a recent manuscript demonstrating the presence of a rare $\Delta Np63^{+}$ ductal cell population in the normal human pancreas raises the possibility that these might represent a cell of origin for tumours with basal-like/squamous features (Martens et al., 2021). Moreover, cell reprogramming, as a driver of basal-like/squamous PDAC, might have a cell-dependent context. For example, in ductal cells only, loss of *ARID1A* appears to promote *MYC* driven gene programmes and the formation of cystic PDAC (Wang et al., 2019).

In summary, basal-like cells appear to accumulate in PDAC as the tumour progresses or under the selective pressure of certain chemotherapeutics (**Figure 2B**). Cells with basal-like features might originate from classical cells via genetic and non-genetic dysregulation of pancreatic transcriptional programmes. Alternatively, classical, and basal-like cells in PDAC might have different ontogeny. Finally, we cannot exclude that in some instances they represent interconverting cell types depending on microenvironmental conditions (**Figure 2B**). None of these hypotheses is necessarily mutually exclusive of the others.

Single cell and spatial transcriptomics promise to provide further insights into the evolution of PDAC. Recent studies have used single-cell RNA sequencing of either biopsies or organoids coupled with multiplex immunofluorescence to reveal that classical and basal programmes co-exist even at the cellular level (Juiz et al., 2020; Raghavan et al., 2021). Finally, these techniques might help elucidate better the role of the microenvironment in influencing PDAC subtypes.

THE INFLUENCE OF THE TUMOUR MICROENVIRONMENT ON PDAC SUBTYPES

PDAC is characterised by a prominent stromal component, which can make up to 80% of the tumour mass (Erkan et al., 2012). *In silico* micro-dissection of transcriptomic data from bulk PDAC tissues by Moffitt and others (Moffitt et al., 2015) identified two major stromal subtypes, namely the "normal" and "activated" subtypes, with the latter enriched for expression of inflammatory cytokines and preferentially associated with the basal-like subtype. Furthermore, single-cell RNA sequencing has also revealed that the tumour microenvironment (TME) appears to be just as heterogenous as the tumour cells themselves, and that it also seems to influence subtypes (Peng et al., 2019; Raghavan et al., 2021).

The TME consists predominantly of cancer-associated fibroblasts (CAFs), but there is also an abundance of immune and endothelial cells (Murakami et al., 2019). CAFs are largely responsible for the desmoplastic reaction in PDAC, as they secrete multiple extracellular matrix (ECM) components (Tian et al., 2019; Sperb et al., 2020). They mainly arise from quiescent pancreatic stellate cells (PSCs) that become activated in response to injury, from tissue-resident fibroblasts, and from mesenchymal stromal cells recruited to the tumour site (Öhlund et al., 2014; Sperb et al., 2020; Gorchs and Kaipe, 2021). CAFs have been invariably associated with pro-tumorigenic functions, and the dense desmoplasia they produce was historically considered as both a physical and a biochemical barrier to the delivery of therapies to tumour cells (Olive et al., 2009; Erkan et al., 2012). However, CAF depletion in experimental mouse models surprisingly led to worse prognosis and higher tumour aggressiveness (Rhim et al., 2014; Özdemir et al., 2014). In mice, depletion of CAFs at different stages of PDAC evolution invariably led to acceleration of the disease, poor differentiation of epithelial cells, and reduced animals' survival (Rhim et al., 2014; Özdemir et al., 2014). In this context, there was substantial remodelling of other relevant microenvironmental features. Indeed, Özdemir et al. showed that genetic depletion of αSMA positive cells increased survival of experimental mice upon blocking of the immune checkpoint receptor CTLA-4 (Özdemir et al., 2014). Blockade of the sonic hedgehog axis, either pharmacologically or genetically (Rhim et al., 2014), led to

tumours with increased vasculature and, accordingly, superior sensitivity to vascular endothelial growth factor (VEGF) inhibition. Finally, myofibroblast-specific deletion of type 1 collagen in a mouse model of PDAC accelerated progression of the disease resulting in more undifferentiated tumours' histology (Chen et al., 2021). These preclinical findings might explain the failure of clinical trials testing the use of sonic hedgehog inhibitors (stroma depleting agents) in association with chemotherapy, which resulted in progression of disease and poorly differentiated tumours (Kim et al., 2014; Ko et al., 2016). Furthermore, they suggest that targeting of certain stromal elements might lead to increased sensitivity of PDAC to therapeutic agents (e.g., immune checkpoint inhibitors) that otherwise have no effects. Overall, these data suggest that the influence of CAFs on the tumour behaviour is much more complex than initially anticipated. In terms of lineage plasticity, CAFs might participate in the process by secretion of growth factors, cytokines, ECM components and other signalling molecules (Hass et al., 2020). For example, CAFs represent a prominent source of TGFβ1, which appears to drive PDAC cells to a more proliferative and undifferentiated phenotype, consistent with the role of TGFβ signalling in the basal-like/squamous subtype (Ligorio et al., 2019). Recent studies on mouse and human PDACs have revealed different CAFs subpopulations with distinct functions (Öhlund et al., 2017; Elyada et al., 2019). Most notably, they found that the inflammatory CAFs (iCAFs), characterised by high expression of inflammatory interleukins, act to promote tumour progression and are located distally from the neoplastic glands (Öhlund et al., 2017; Biffi et al., 2019). Myofibroblast CAFs (myCAF), which are in the vicinity of neoplastic cells, are instead characterised by high expression of αSMA and appear to restrain tumour growth (Öhlund et al., 2017; Biffi et al., 2019; Bhattacharjee et al., 2021). A recent study demonstrated both anti- and pro-tumorigenic function for myCAFs in the context of metastatic PDAC (Bhattacharjee et al., 2021). The pro-tumorigenic effects of myCAFs result from their production of hyaluronan, which promotes cancer proliferation, whilst the type 1 collagen produced by myCAFs acts to suppress the tumour, which is in line with findings from Chen and others (Bhattacharjee et al., 2021; Chen et al., 2021). Moreover, an antigen-presenting CAF subpopulation, characterised by its ability to activate $CD4^+$ T cells has also been found (Elyada et al., 2019). Finally, the secretome of basal-like/squamous PDAC cells can polarise PSCs and fibroblasts towards the iCAF phenotype (Somerville T. D. et al., 2020), but how distinct CAF populations affect tumour subtype is still unclear.

Immune cells, and tumour associated macrophages (TAMs) in particular, are another important component of the PDAC TME. Macrophages are recruited to the tumour via signalling from the cancer cells, where they become TAMs (Yang et al., 2021). However, just like CAFs, resident macrophages can also become TAMs (Yang et al., 2021). TAMs participate in establishing a high immunosuppressive environment and their density within the PDAC TME is correlated with worse prognosis (Habtezion et al., 2016; Hu et al., 2016). In several preclinical studies, TAM depletion has been shown to reduce metastatic burden, improve response to the chemotherapy drug gemcitabine (Buchholz et al., 2020), and alter gene programmes that define the basal-like/squamous subtype (Candido et al., 2018). Using single cell RNA sequencing of patient metastases, Raghavan et al. (2021) have classified TAMs in three different subtypes: monocyte-like, phagocytic, and angiogenesis-associated TAMs. The authors also found an association between basal-like tumours and phagocytic TAMs, and between classical and angiogenesis-associated TAMs, suggesting reciprocal influences between epithelial and stromal subtypes.

Tumour associated neutrophils (TANs) are the other important component of the PDAC immune microenvironment (Jin L. et al., 2021). Neutrophils are recruited by the tumour via chemokines, most notably CXCs (Hosoi et al., 2009; Steele et al., 2016; Nywening et al., 2018). Just like TAMs, they also support the proliferation of neoplastic cells, promote an immunosuppressive environment and facilitate distant metastases (Stromnes et al., 2014; Lianyuan et al., 2020). Depletion of neutrophils in a KPC mouse model reduces metastatic burden and causes a switch from squamous to progenitor subtype (Steele et al., 2016). Furthermore, preventing neutrophil recruitment in a PDAC mouse model led to recruitment of T-cells and tumour-suppression (Chao et al., 2016). Pharmacological suppression of neutrophils also makes mouse PDAC more vulnerable to immune checkpoint blockade (Nielsen et al., 2021). In wound healing and *trans*-well assays, neutrophils from PDAC patients promote tumour cell migration and invasion, whilst neutrophils from healthy individuals cannot (Jin W. et al., 2021). Moreover, it seems that the TME of basal-like/squamous tumours is characterised by an increased infiltration of neutrophils that is at least partially driven by secretion of Cxcl1 by squamous-instructed iCAFs (Somerville T. D. et al., 2020). All these studies show that neutrophils are pro-tumorigenic, yet how they impact on subtypes and lineages in PDAC is unclear.

Finally, endothelial cells are also found in the PDAC TME (Feig et al., 2012). Generally, PDAC is an extremely hypoxic tumour and is poorly vascularised (Zhang et al., 2018). Under hypoxia, hypoxia-inducible factor (HIF-1α) drives VEGF upregulation which promotes tumour angiogenesis, proliferation and metastases (Zhang et al., 2018). Yet, vascular remodelling in PDAC has also been shown to improve delivery of therapies and activation of T cells (Ruscetti et al., 2020). However, the role of endothelial cells and how they support tumour progression and subtypes is still unclear.

LINEAGE INFIDELITY AND THERAPY RESISTANCE

The capability of cancer cells to move across cell states (i.e., fate plasticity) is a source of cell heterogeneity that cancers employ to survive drug treatment (Quintanal-Villalonga et al., 2020). Infidelity to the cell lineage, in particular, has been implicated in therapeutic resistance in multiple solid cancers (Seldin and Macara, 2020). Most prominently, therapy resistance in prostate

adenocarcinoma can be driven by *trans*-differentiation of cancer cells into a neuro-endocrine phenotype (Hu et al., 2015). Similarly, EGFR-positive non-small cell lung tumours acquire resistance to anti-EGFR therapies also by *trans*-differentiation into a neuroendocrine phenotype (Oser et al., 2015). In PDAC, the contribution of lineage infidelity to therapy resistance is less clear. This is due to the difficulties in procurement of tissues from patients undergoing treatment. Thus, most of our knowledge related to mechanisms of escape to treatments relies on preclinical works. Recently, single nucleus RNA sequencing analysis of archival samples from post-treatment tumours has revealed an enrichment of basal-like cells in the post-treatment setting, consistent with the more aggressive nature of basal-like/squamous tumours (Hwang et al., 2020). Furthermore, Hwang and others (Hwang et al., 2020) also reported an enrichment of neuroendocrine transcriptional programmes in post-treatment tumours, suggesting that neuroendocrine *trans*-differentiation might play a role also in this cancer type. Given their profound differences, it is not unexpected that the two main cell lineages of PDAC display different pharmacological sensitivity. In his seminal work, Collisson reported that classical cell lines were more sensitive to the epidermal growth factor receptor (EGFR) inhibitor erlotinib, whilst basal-like (defined as quasi-mesenchymal) cells exhibited sensitivity to gemcitabine (Collisson et al., 2011). The squamous/basal-like tumours exhibit a glycolytic metabolic profile (as opposed to the lipogenic profile of the classical cells) (Bailey P. et al., 2016), which is susceptible to glycogen synthase kinase 3β (GSK3β) inhibition (Brunton et al., 2020). In this study, inhibition of GSK3β eventually resulted in resistance; however, the resistant cell lines were also susceptible to porcupine inhibition (inhibition of WNT ligand production) (Brunton et al., 2020). In addition to preclinical studies, differential sensitivity of PDAC subtypes to available chemotherapeutic regimens has been also demonstrated, with classical tumours reported to be more sensitive to FOLFIRINOX and, in contrast with the findings from Collisson et al., to gemcitabine (Martinelli et al., 2017; Aung et al., 2018; Nicolle et al., 2021). Recently, a transcriptomic signature of elevated replication stress generated from analysis of patients-derived cell lines was found enriched in basal-like/squamous tumours and predicted responses to cell cycle checkpoint inhibitors in cell lines and organoids (Dreyer et al., 2021). These findings will be tested by the ongoing clinical trial PRIMUS004 (ISRCTN16004234).

Even if there is a consensus that the basal-like/squamous subtype is a more aggressive form of the disease in the setting of an early stage and resectable disease (Bailey P. et al., 2016), it should be noted that the classical PDAC subtype is as lethal as the basal-like. Furthermore, this dichotomisation is not informative of patients prognosis in a more advanced setting (Chan-Seng-Yue et al., 2020), where more complex and hybrid cell states also seem to emerge (Hwang et al., 2020). Therefore, it is more likely the ability of cells to switch between lineages and subtypes provides them with a superior advantage to escape from different types of selective pressures (**Figure 2B**). Given the profound biological differences between subtypes and their unique therapeutic vulnerabilities, it is conceivable that a viable strategy to achieve deeper and durable responses in PDAC might be the identification of targets that prevent subtype switching.

While basal-like and classical subtypes in PDAC likely reflect two different epithelial differentiation programs, the epithelial-mesenchymal transition (EMT) refers to a process whereby epithelial cells acquire mesenchymal traits (Kalluri and Neilson, 2003) that favour cell dissemination and metastatization (Wang et al., 2017). The contribution of EMT to therapeutic resistance in PDAC has been investigated more in depth than lineage infidelity. As for other solid malignancies, upregulation of the EMT program in PDAC has been shown to be associated with poor prognosis and therapy resistance (Javle et al., 2007; Arumugam et al., 2009; Weadick et al., 2021). EMT is activated by TGFß signalling, which in turn tends to be upregulated in more aggressive basal-like/squamous PDACs. EMT has also been shown to be regulated by the classical transcription factor GATA6, which directly represses EMT genes while positively regulating pro-epithelial genes (Martinelli et al., 2017). Accordingly, single-cell analysis of human PDAC cells has demonstrated that basal-like and classical programs are positively and negatively associated with EMT, respectively (Chan-Seng-Yue et al., 2020). Whether the switch from classical to basal-like phenotypes precedes the acquisition of a full EMT phenotype by PDAC cells needs to be clarified. EMT might play an important role in therapy resistance as it is associated with gemcitabine resistance both in cell lines and patients (Weadick et al., 2021). This is supported by data in KPC mice showing that EMT inhibition via knockout of the EMT-inducing TFs *Twist1* and *Snai1* improved response to gemcitabine and increased survival (Zheng et al., 2015).

ORGANOIDS: A 3D PLATFORM TO MODEL PDAC INITIATION AND PROGRESSION

While it seems clear that a loss of endodermal commitment is a feature of aggressive PDAC phenotypes, the mechanisms leading to this lineage infidelity are still elusive. We believe that the pancreatic organoids culture system offers a unique opportunity to model the contributions of the cell of origin as well as of tumour intrinsic and extrinsic factors to the definition of PDAC cell fate. Organoids are a 3D culture system, where epithelial cells can be cultured in a semi-solid medium supplemented with growth factors and morphogens that collectively recreate the *in vivo* stromal *niche* (Drost and Clevers, 2018; Seino et al., 2018). Organoids can be derived directly from adult primary cells, either from healthy or diseased pancreata, and from human pluripotent stem cells (iPSCs) permitting expansion of the epithelial compartment even from limited amount of material (**Figure 3A**). In the field of epithelial tumours, this culture system has recently become the alternative to 2D cell lines, as organoids have been shown to preserve better the histological and genetic features of the parental tumours (Weeber et al., 2015; Baker et al., 2016). Organoids can have clinical implications as they mimic patient response and can be used to identify patients that would benefit from certain treatments (Tiriac et al., 2018). Additionally, organoids derived from embryonic pancreatic cells can also

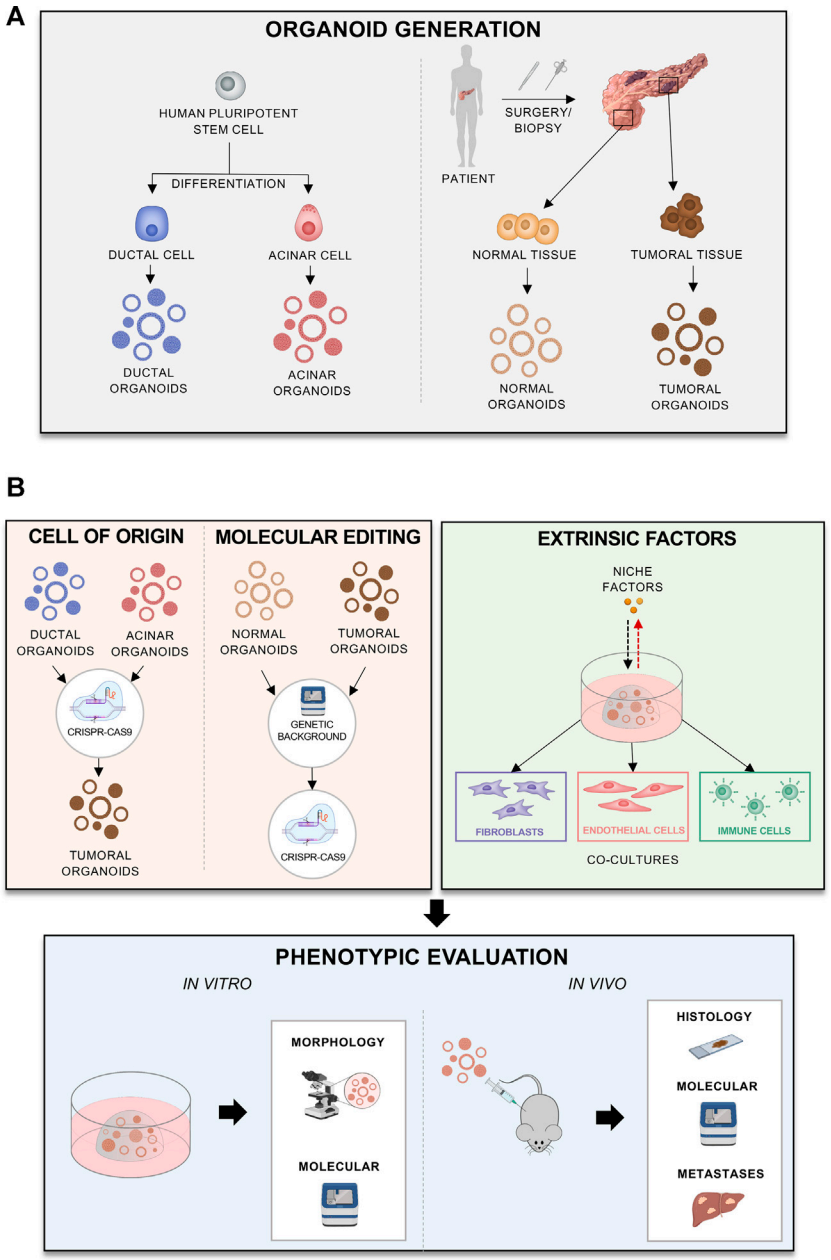

FIGURE 3 | Different applications of organoids to model progression **(A)** Different methods and sources for organoid derivation. **(B)** Generation of different organoid models, reflecting cell of origin, mutations of interest, patients' background, or effects of extrinsic factors. The models can be phenotypically evaluated *in vitro* or *in vivo*.

contribute to understanding better the human pancreatic development and lineage relationships, which are altered in PDAC (Balak et al., 2019). Finally, organoid cultures can be established and propagated (albeit for a limited time) from adult pancreatic exocrine cells, which allows evaluating the contribution of individual genes and their influence on the PDAC tumorigenic process by the stepwise introduction of genetic alterations through genome editing approaches (**Figure 3B**) (Greggio et al., 2013; Huch et al., 2013; Huch and Koo, 2015; Baker et al., 2016). Indeed, the Sato group (Seino et al., 2018) has demonstrated the feasibility of genetically engineering human normal pancreas organoids through the sequential introduction of the typical PDAC alterations (*KRAS, TP53, CDKN2A, SMAD4*) and that only quadruple mutant organoids generated lesions histologically resembling human PDAC when transplanted in immunodeficient mice (Seino et al., 2018).

With regards to the cellular origin of PDAC, organoids might provide a human alternative to GEMMs (**Figure 3B**). Despite the similarities between humans and mice, there are differences between the two species in pancreas anatomy and

development. Furthermore, murine tumours tend to display less genetic diversity and complexity than human cancers. To elucidate the role of the cell origin in the disease progression, human acinar and ductal organoids can be derived from iPSCs and transformed to model PDACs derived from each cell type (**Figure 3A**) (Breunig et al., 2021; Huang et al., 2021). This has shown that different oncogenic mutations, such as *KRAS* and *GNAS*, affect tumorigenesis differently depending on the cellular context (Huang et al., 2021). *In vivo* transplantation of acinar organoids with *KRAS* mutations caused cancer lesions more effectively, whilst *GNAS* mutations in ductal ones caused cystic outgrowths (**Figure 3B**). The study from Huang and others (Huang et al., 2021) also shed some light on how initiating cancer mutations affect cell identity in PDAC. In acinar organoids, *KRAS* mutations caused silencing of acinar-specific genes (*PTF1A*) and upregulation of the ductal *SOX9*. In ductal organoids, however, *KRAS* upregulated *SOX9, NKX6-1* and *PDX1*, suggesting re-direction of cells towards a progenitor state (Huang et al., 2021). In another elegant study, the Kleger's laboratory developed a two-phase protocol to differentiate human PSCs into pancreatic ductal-like organoids (PDLO) which recapitulated features of mature ductal cells (Breunig et al., 2021). PDLOs were then used to explore the cell-context specific effects of oncogenic drivers (either alone or in combination) on the development of dysplastic and cancerous lesions (Breunig et al., 2021). Upon orthotopic engraftments of PDLOs engineered to carry different combinations of oncogenic insults, they found that PDLOs carrying *KRAS* activating mutation generated heterogeneous dysplastic lesions, while PDLOs with simultaneous activation of *KRAS* and loss of *CDKN2A* generated de-differentiated tumours (Breunig et al., 2021). When PSCs were engineered to express the oncogenic $GNAS^{R201H}$ variants, PDLOs formed large cysts *in vitro* and IPMN-like structure upon engraftment (Breunig et al., 2021). This is in line with the prevalence of GNAS mutations in IPMN (Furukawa et al., 2011; Hosoda et al., 2015) and the observations from Huang et al. (Huang et al., 2021). The same *in vitro* and *in vivo* cystic phenotype was observed when PDLOs were established from iPSCs of a patient suffering from McCune-Albright syndrome (MAS), which is caused by postzygotic mosaic *GNAS* mutations (Breunig et al., 2021). This work exemplifies the possibility of using organoids to assess the impact of individual patients' genetic background on inception and progression of the disease and strengthen the evidence that a complex interplay between the oncogenic mutations and cellular context dictate the way disease progresses (**Figure 3B**).

Organoids established from tumour resections also provide a powerful platform to study cell autonomous processes that affect lineage commitment and malignant behaviour (**Figure 3A**). For example, organoids have been used to show how *MYC* copy number gain drives PDAC progression. *MYC* amplification is associated with poor prognosis and advanced disease. To model how MYC affects PDAC, Hayashi et al. (2020) overexpressed *MYC* in patient derived organoids with and without deleterious mutations in chromatin modifier genes, such as *ARID1A*. The authors showed that *MYC* overexpression induces squamous features, only when combined with mutations in the chromatin modifier genes. Moreover, organoids can be genetically modified to study effects of different mutations (**Figure 3B**): for examples Seino et al. (2018) introduced different driver mutations into organoids in order to examine whether cancer driver mutations, alone, can confer niche factor dependency. Thus, organoids provide a personalised platform to study how an individual's genetic background affects functional perturbations, which also include therapeutic treatment.

The TME has a dramatic influence on PDAC progression, and therefore it is important to understand how stromal signals affect tumour cells and vice versa. *In vivo* experiments of organoid transplantation in mice have demonstrated that the local microenvironment drives PDAC subtypes (Miyabayashi et al., 2020). As the PDAC stroma supports the tumour by producing ligands, elucidating appropriate ligand-receptor relationships between the stroma and the tumour might provide novel drug targets. This has been exemplified by a recent study on single cell RNA-Seq data from primary and metastatic tumours, identifying multiple potential ligand-receptor relationships and opening avenues for targeted therapies (Lee et al., 2021).

Organoids can provide a powerful platform to study the interactions between the stroma and tumour cells either through modification of the culture medium or by coculturing neoplastic cells with the different microenvironmental components (**Figure 3B**). The TME can be partially recreated *ex vivo* using organoid-based coculture systems (**Figure 3B**). As an example, Öhlund and others demonstrated that the co-cultivation of mouse tumour organoids with PSCs trigger the deposition of stroma *ex vivo* (Öhlund et al., 2017). Moreover, this co-culture system permits modelling of the different CAF subtypes, which show different functions and influence on tumour behaviour (Öhlund et al., 2017; Biffi et al., 2019). Using organoid-based co-cultures, Feldmann et al. (2021) have shown that CAFs expressing the transcription factor *Prrx1* can induce PDAC cells transition towards a mesenchymal phenotype. Moreover, there appears to be a stromal niche dependency of PDAC organoids that also associates with expression of endodermal transcription factors. Seino et al. (2018) revealed three subtypes of PDAC organoids, with distinct dependency on Wnt ligands. PDAC organoids classified as "classical" were reported to be more dependent for their propagation on the supplementation of Wnt ligands, either exogenously or through cocultivation with CAFs (Seino et al., 2018). Interestingly, suppression of *GATA6* expression rendered organoids less reliant on exogenous Wnt supplementation. Thus, it is likely that stromal niche factors play a role in maintaining the endodermal commitment and that depletion of certain signalling cues from the organoid-rich media would allow for modelling progression associated with depletion of stromal elements (**Figure 3B**).

Organoid co-culture systems can also provide a platform to elucidate the role of the other components of the stroma on the tumour subtype, including immune cells (**Figure 3B**). Co-culture of PDAC organoids with autologous lymphocytes and CAFs showed activation of a myCAFs phenotype and infiltration of the lymphocytes towards the tumour cells (Tsai et al., 2018). Thus, the co-culture system can incorporate multiple cell types

and help modelling the interactions between the human TME and cancer cells (**Figure 3B**).

While there is a considerable excitement about the possibility that tumour organoids facilitate translational research and even become part of the clinical decision-making process, they are models and therefore, are imperfect. The limitations of the culture system, which restrain its wider adoption by the scientific community and the implementation in clinical practice, should be acknowledged. One important bottleneck of the technology is the success rate in the derivation of cultures. While the establishment of organoid cultures is undoubtedly more efficient than 2D culture methods, the methodology still needs optimisation to enable the systematic and timely derivation of *ex vivo* cultures to meet clinical criteria. Furthermore, we do not know whether failure in generating cultures is driven by specific genotypes/phenotypes that cannot be captured efficiently or rather due to characteristics of the specimens, such as the neoplastic cell content. The optimisation of the culture conditions seems necessary also to limit across-laboratory variability due to the use of the animal-derived matrices that suffer from batch-to-batch variability and undefined composition. Beyond the standardisation of the ECM components, considerable attention has been recently given to the growth medium composition due to the presence of elevated concentrations of growth factors and pathways inhibitors, which are potential confounders of functional perturbation experiments and substantially contribute to the elevated costs of the technology. Moreover, single cell sequencing has shown that organoids can drift away molecularly from their original tissue by becoming more "classical", even when derived from basal-like/squamous tumours (Raghavan et al., 2021). Given the differential responses to available chemotherapy regimens reported for the two molecular subtypes in the adjuvant setting (Collisson et al., 2011; Aung et al., 2018; Porter et al., 2019; Brunton et al., 2020; Nicolle et al., 2021), the inability of organoids to faithfully replicate patients' molecular subtypes would limit their use as a forecasting tool. Nevertheless, Tiriac and others have demonstrated that organoids represent an efficient drug-screening platform that could predict responses observed in patients to the common chemotherapy used in PDAC (Tiriac et al., 2018). Interestingly, while classical and basal-like signatures could be identified in the organoids, the authors described organoid-derived gene signatures that are unrelated to the transcriptional phenotypes and that could predict patient's response to specific compound (Tiriac et al., 2018). Further major concern for clinical implementation is the absence of autologous stromal elements (endothelial cells, fibroblasts, immune cells) in most organoid culture systems. Even if this can be partially rescued by a reconstituted TME using patients' derived cells, we still do not know whether culture conditions alter the stability and the phenotypes of stromal cells or even if clonal selection occurs in culture.

There are many ongoing efforts in the field, including our own (https://precode-project.eu/) trying to improve aspects of the organoid technology. For example, a recent report from the Jørgensen' group (Below et al., 2021) described a fully-synthetic hydrogel that supported tumour organoid propagation and co-cultivation with stromal elements, thus promising to be transformative for the field. Moreover, modifying medium formulations can "push organoids back" to a phenotype that more accurately resembles their origins (Raghavan et al., 2021). These recent advances will likely accelerate organoids implementation in clinical practice and promote a wider adoption in the scientific community.

CONCLUDING REMARKS

PDAC is an extremely deadly disease, whose biology, tumorigenesis, and progression we still do not fully understand as reflected by the limited therapy options and poor prognosis. PDAC might progress from a classical to basal-like/squamous phenotype through genetic or epigenetic dysregulations, influenced by intrinsic and extrinsic factors, that cause loss of pancreatic endodermal fate. However, the basal-like programmes might already exist within the normal pancreas and the disease progression might be dependent on pre-existing cell populations, which initiate the cancer. To understand lineage relationships and plasticity in this cancer and how they affect progression and therapy resistance, organoids and organotypic cultures have emerged as a valuable tool that holds promise to offer insights into PDAC, reveal novel targets, and bring tangible changes to patients' management.

AUTHOR CONTRIBUTIONS

AM wrote the manuscript and performed literature search. LV prepared the figures. VC devised the idea. FXR and VC finalised the manuscript.

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REVIEW
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PDAC as an Immune Evasive Disease: Can 3D Model Systems Aid to Tackle This Clinical Problem?

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with a high mortality rate. The presence of a dense desmoplastic stroma rich in fibroblasts, extracellular matrix, and immune cells plays a critical role in disease progression, therapy response and is a distinguishing feature of PDAC. PDAC is currently treated with a combination of surgery, chemotherapy and radiation therapy in selected cases which results in long-term survival only in a small percentage of patients. Cancer therapies that incorporate immunotherapy-based techniques have become increasingly common in recent years. While such a strategy has been shown to be effective for immunogenic, "hot" tumors like melanoma and lung cancer, thus far PDAC patients display poor responses to this therapeutic approach. Various factors, such as low tumor mutational burden, increased infiltration of immunosuppressive cells, like MDSCs and Treg cells promote tolerance and immune deviation, further aggravating adaptive immunity in PDAC. In this review we will elaborate on the ability of PDAC tumors to evade immune detection. We will also discuss various 3D model system that can be used as a platform in preclinical research to investigate rational combinations of immunotherapy with chemotherapy or targeted therapy, to prime the immune microenvironment to enhance antitumor activity.

Keywords: PDAC, immunotherapy, immune evasion, 3D model systems, co-culture

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1 INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with a high mortality rate. Indeed, prognosis for PDAC patients is one of the poorest among all cancers (Dell'Aquila et al., 2020). PDAC is characterized by a rapid progression, a high propensity for metastatic spread and an exceptional resistance to all forms of anticancer treatment (Mizrahi et al., 2020; Park et al., 2021). The 5-year survival rate of PDAC patients has climbed from 6 to 10% between 2014 and 2021 as a result of current therapeutic strategies based on a combination of surgery, chemotherapy and radiation therapy (American Cancer Society, 2021). However, the long-term survival benefit occurs only in a small percentage of patients. Thus, even though this moderate improvement in survival rates demonstrates progress, there is still a pressing clinical need to improve patients' outcome for this devastating disease.

At the histopathological level, PDAC presents with a prominent desmoplastic stroma, which consists of a heterogeneous cell microenvironment that includes fibroblasts, immune and endothelial cells, as well as a rich extracellular matrix of collagen and non-collagen proteins, such as laminins,

fibronectin and other glycoproteins (Santi et al., 2018; Rawla et al., 2019). Such dense stroma represents not only a physical but a biologically functional barrier that limits infiltration and antitumor activity of immune cells as well as proper diffusion of therapeutics, therefore playing a critical role in disease progression and therapy response.

At the genomic level, multiple genetic and epigenetic alterations characterize PDAC. A prevailing genomic feature of PDAC is the high rate of KRAS mutations, found in ~90% of cases (Hezel et al., 2006). Mutations in KRAS occur early in PDAC tumorigenesis and function as an initiating event of the disease (Biankin et al., 2012; Waddell et al., 2015; Witkiewicz et al., 2015; Frappart and Hofmann, 2020). Despite the sequential acquisition of additional genomic alterations that contribute to mold the course of PDAC development (Schneider et al., 2017), KRAS mutations strongly influence tumor maintenance and metastasis (Collins et al., 2012). Thus, KRAS oncoprotein stands as a key molecular target in this malignancy, particularly in the context of advanced disease where therapeutic options are required. Unfortunately, neither targeted therapies against canonical KRAS effectors nor the most recently developed KRAS inhibitors targeting the G12C mutation, which only occurs in 2–3% of all cases, have demonstrated significant benefit for PDAC patients, what emphasizes the need for novel treatment options (Bryant et al., 2014; Brown et al., 2020).

Large-scale analysis of the PDAC genome has revealed remarkable inter- and intra-tumoral heterogeneity and complexity. Several studies on transcriptional profiling of patient PDAC specimens have indicated the existence of multiple tumor subtypes, each with distinct molecular characteristics. Existence of classical and basal-like subtypes have been validated across multiple studies in both primary and metastatic samples (Bailey et al., 2016; Raphael et al., 2017; Cao et al., 2021; Flowers et al., 2021). Classical tumor subtype is characterized by the expression of epithelial markers, whereas basal-like subtype present with more mesenchymal features like the expression of laminin and basal keratin, stem cell and epithelial-to-mesenchymal transition (EMT) markers. The importance of such a classification lies in the fact that the basal subtype tumors are poorly differentiated and correlate with worse prognosis and drug response (O'Kane et al., 2020). However, the complex mechanisms underlying the establishment of a specific subtype are still under investigation. The stromal compartment is another source of intratumoral heterogeneity. Within the tumor microenvironment (TME), several subpopulations of fibroblasts and macrophages can be identified (Elyada et al., 2019). Cancer associated fibroblasts (CAF) are a diversified population of cells with the capacity to modify the TME and influence the fate of tumor cells (Sahai et al., 2020). In PDAC, transcriptionally distinct macrophage subpopulations arise from various sources, including embryonic precursors, adult hematopoietic stem cell (HSC) progenitors, and monocytes (Poh and Ernst, 2021). The presence of macrophages has been negatively correlated with PDAC patient survival (Yu et al., 2019). Furthermore, differential presence and ratio of immune cell populations in the tumor may account for intratumoral heterogeneity. Taken together, there is considerable evidence that these diverse stromal populations play a pivotal role in tumor development, ECM remodeling, and therapy response.

This review provides an overview of factors responsible for immune evasion in PDAC that leads to failure of immunotherapy. We also discuss emerging 3D preclinical models that can be utilized in developing effective treatment strategies.

2 FAILURE OF IMMUNOTHERAPY IN PANCREATIC DUCTAL ADENOCARCINOMA

Cancer treatments that incorporate immunotherapy-based techniques have revolutionized the Oncology field in recent years. However, patient responses vary dramatically across cancers (Nixon et al., 2018). For instance, while immunotherapy has become standard of care in melanoma or lung adenocarcinoma, it has so far been ineffective in some gastrointestinal tumours including PDAC, which is particularly refractory to immune-based therapeutic strategies. Pembrolizumab, an FDA-approved drug that targets the PD-1/PD-L1 pathway for the treatment of solid tumors with a high mutation burden, as well as tumors with high microsatellite instability (MSI-H) or mismatch repair deficiency (dMMR), can only be used in the 1–2% of PDAC patients who have these characteristics (Luchini et al., 2021). A multiparameter analysis of the immune landscape in PDAC revealed heterogeneous expression of immune checkpoint receptors in individual patients' T cells and increased markers of $CD8^+$ T cell dysfunction in the disease stage (Steele et al., 2020). This suggests that a one-size-fits-all approach to immune checkpoint inhibitor therapy may not apply to PDAC. Instead, the therapeutic strategies should be tailored to specific individuals based on their checkpoint expression profile, genomic characteristics and TME populations' profile such (i.e., lymphocyte infiltration). Overall, the use of immunotherapy in PDAC could be improved with the design of rational combinations with chemotherapy and, in this regard, research regarding the unique biology of PDAC should be explored further.

3 FACTORS RESPONSIBLE FOR FAILURE OF IMMUNOTHERAPY IN PANCREATIC DUCTAL ADENOCARCINOMA

Inactivation of the immune response by the immune suppressive TME, as well as impaired effector T cell infiltration contribute to the poor prognosis of PDAC patients. The particular host tissue distinguishes the response of PDAC to immunotherapy from that of other solid cancers. PDAC features an abundance of tumor stroma, where distribution and activity of different immune cell populations are governed by its interactions with other cellular components of the TME and the tumor (Feig et al., 2012). These interactions culminate in a very complex immunosuppressive TME. Here, we outline the key factors responsible for the poor

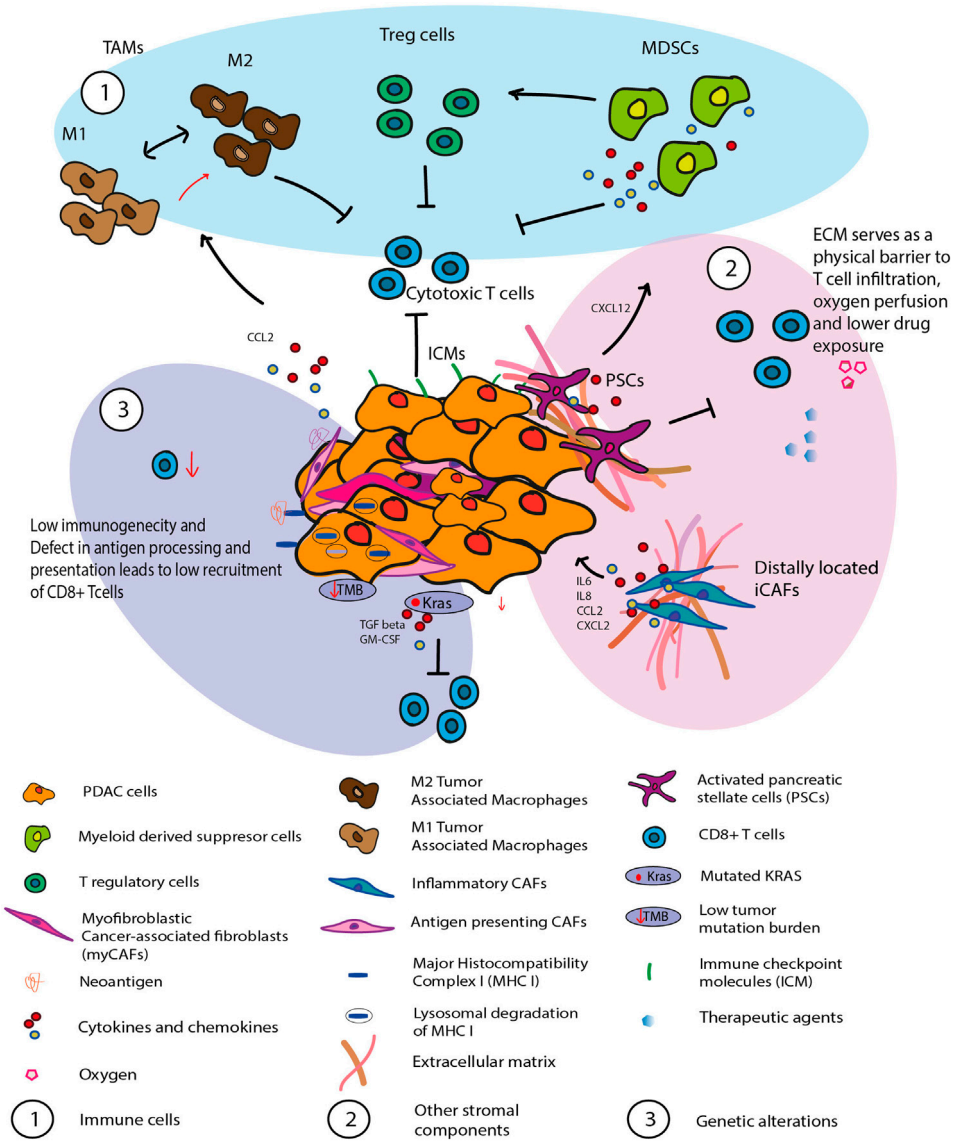

FIGURE 1 | Illustration of the immune evasive and immune suppressive PDAC tumor microenvironment. The interaction between the tumor and the other cellular components of the TME culminate in a very complex immunosuppressive TME. 1) Immune cells such as MDSC, TAM, Treg are implicated in immune evasion and tumor growth in PDAC 2) Other stromal components such as PSCs and inflammatory CAFs has been shown to contribute towards T cells dysfunction. The desmoplastic ECM which is a major component of the PDAC stroma forms a physical barrier which prevents T cell infiltration as well as effective drug exposure. 3) launch of an appropriate immune response is compromised by tumor cell-inherent resistance mechanisms which include tumor mutational load and abnormal expression of oncogenic signatures (i.e., KRAS). Lower level of quality neoantigen and defect in antigen processing and presentation also leads to low recruitment of $CD8^+$ T cells to the tumor site.

therapeutic response, focusing on the immune cell network around cancer cells, additional stromal components, and tumor intrinsic mechanisms (**Figure 1**).

3.1 Immune Cells

PDAC tumor microenvironment shows a highly heterogeneous immune infiltration profile in individual patients (Chakrabarti et al., 2018). In early stages, PDAC's TME is distinguished by the lack of evidence for T cell activation due to its strongly immunosuppressive traits (Stromnes et al., 2017). As the disease progresses, a subset of patients with unresectable late stages of disease had a profile of $CD8^+$ T cells with a more pronounced exhaustion signature (Huber et al., 2020; Steele et al., 2020). By definition, T-cell exhaustion is a T-cell differentiation state caused by persistent antigen exposure, which activates T-cell receptor (TCR) signaling during chronic infections and increases with age (Wherry and Kurachi, 2015). In PDAC, T cells transform into an exhausted differentiation state, which is

characterized by upregulation of inhibitory receptors like PD1 or TIGIT, resulting in loss of effector function (Freed-Pastor et al., 2021). Furthermore, the combinations of immunological checkpoint genes expressed in each patient's $CD8^+$ T cells were distinct, suggesting that immune-modulatory therapies should potentially be targeted to specific individuals based on their tumor checkpoint expression profile (Steele et al., 2020).

Multiple types of tumor-promoting immune cells such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) or regulatory T cells (Tregs) infiltrate tumors and enable immune evasion and tumor growth (Martinez-Bosch et al., 2018; Chen et al., 2020). These myeloid cells are attracted from the circulation to the tumor site via chemokine pathways that tumor cells co-opt to enhance myeloid cell attraction like CCL2 (Schmid and Varner, 2010; Gu et al., 2021). Of note, a recent study demonstrated that tumoral MDSCs can stimulate Treg cell proliferation and/or development in a cell-cell dependent way in mouse models (Siret et al., 2020). Furthermore, they discovered that Treg cells influence the survival and/or proliferation of MDSCs in PDAC. Paradoxically, another study using murine models found that reducing Tregs did not improve immunosuppression, but rather promoted tumor growth (Zhang et al., 2020). The authors observed Treg cell depletion reprogramed the fibroblast population, with loss of tumor-restraining, smooth muscle actin-expressing fibroblasts (myCAFs), similar to what was described in a previous study (Rhim et al., 2014). Interestingly, Zhang et al. (2020) also observed an increase in chemokines Ccl3, Ccl6, and Ccl8, which resulted in enhanced myeloid cell recruitment, immune suppression and tumor progression. TAMs are one of the most abundant immune population in the TME. TAMs can originate from either monocytes or tissue-resident macrophages of embryonic origin (Zhu et al., 2017). They can further differentiate into functionally distinct M1 and M2 macrophages depending on the polarizing signals present in the microenvironment. M1 macrophages are known to be pro-inflammatory with anti-tumor activity, whereas M2 macrophages secrete anti-inflammatory signals aiding tumor progression (Lankadasari et al., 2019). TAMs have a well-recognized role of immune suppression as evidenced by a study lead by Nywening (Nywening et al., 2018). They reported that targeting $CCR2^+$ TAMs along with tumor associated $CXCR2^+$neutrophils (TAN) launched a robust antitumor immune response as well as better chemotherapeutic response in PDAC. Another interesting study using orthotopic and genetically engineered mouse models of PDAC found that PI3Kγ selectively drives immunosuppressive transcriptional programming in macrophages inhibiting adaptive immune responses and promotes tumor cell invasion and desmoplasia (Kaneda et al., 2016).

The existence of a delicate balance between the populations of $CD4^+$ and $CD8^+$ subsets determines whether the environment is anti- or pro-tumorigenic (Clark et al., 2007; Saka et al., 2020). Notably, regulating the differentiation of naïve $CD4^+$ T cells into Th1, Th2, Th17, Th9, Th22, and Tregs is essential for eliminating immunosuppressive restrictions from the tumor environment and boosting effector T-cell activity (Knochelmann et al., 2018). It is possible that the disruption of the correct ratio of these cell populations causes immune evasion in cancer and even the failure of several immune cell targeted therapies.

3.2 Other Stromal Components

Phenotypically, the dense ECM present in the PDAC composed of collagen I, laminin and hyaluronan (HA) alone accounts for up to 90% of the total tumor volume making up the stromal components (Murphy et al., 2021). Because of their dense tumor architecture, PDAC has poor perfusion compared to normal tissues and even other cancers. Such particular architecture causes a distorted blood vessel network, which obstructs oxygen perfusion and causes hypoxia, which in turn promotes tumor progression (Jacobetz et al., 2013). Such poor tissue perfusion will inevitably result in a significant reduction in total treatment exposure as well affecting its efficacy. Furthermore, immune suppressive myeloid derived cells have been demonstrated to be more infiltrating in such a milieu than lymphocytes, contributing to the failure of numerous immunotherapies. To complicate matters, hypoxia is known to trigger the activation of pancreatic stellate cells (PSC), which are thought to be PDAC's "partners in crime" (Yamasaki et al., 2020).

PSCs secrete a variety of soluble cytokines that has been shown to contribute towards T cell exhaustion and dysfunction (Ho et al., 2020). Activated PSC are known to regulate T-cell migration. They sequester anti-tumor $CD8^+$ T-cells, preventing them from infiltrating juxtatumoral stromal compartments and therefore limiting access to cancer cells (Ene-Obong et al., 2013). Furthermore, they have been shown to recruit myeloid-derived suppressor cells (MDSCs) to the tumor site *via* the CXCL12/CXCR4 axis. Activated PSCs also promote M1 macrophage development into a pro-tumor M2 phenotype (Puré and Lo, 2016). It is well known that PSCs are responsible for producing the desmoplastic ECM within PDAC, such an ECM also forms a physical barrier which prevents T cell infiltration as well as effective drug exposure (Di Maggio et al., 2016; Fu et al., 2018). Cancer-associated fibroblasts (CAFs) originating from activated PSC's form a major cellular component of the TME. CAFs can be further characterised into functionally distinct subtypes: α-SMA + myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs) and fibroblasts with antigen presenting ability (apCAF) (Pereira et al., 2019). Studies have shown that myCAFs restrain tumor cell growth, whereas iCAFs display a more pro-tumorigenic function. iCAFs secrete inflammatory factors such as interleukin (IL)-6, IL-8, CCL2, and CXCL2 that promote tumor growth and also promote T-cell dysregulation by promoting expression of immune checkpoint inhibitors (PD-1, TIM-3) (Gorchs et al., 2019; Gorchs and Kaipe, 2021).

3.3 Genetic Alterations

Antitumor immunity is also hampered by tumor cell-inherent resistance mechanisms, which include tumor mutational load and unusual expression of oncogenic signatures (Tang et al., 2021). PDAC is regarded as a "cold tumor" with a low T cell infiltration and low tumor mutation burden (TMB) with few neoantigens. This makes successful application of immunotherapy in these cancers very difficult. Neoantigens are the consequence of

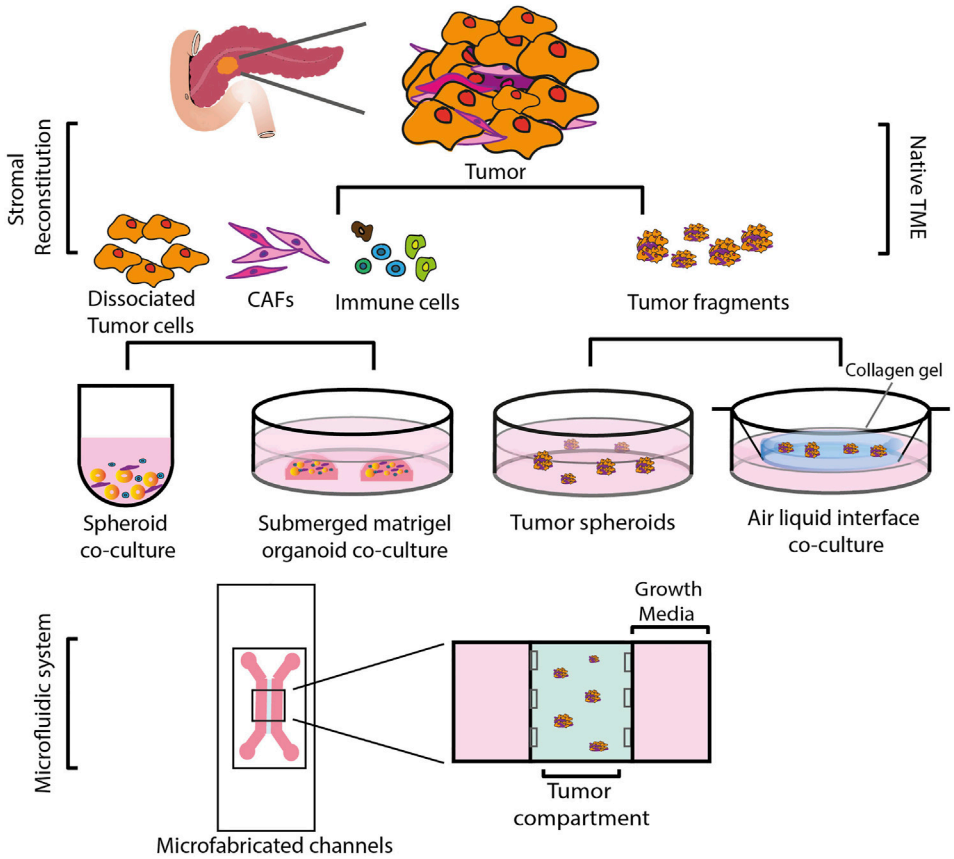

FIGURE 2 | Schematic representation of various 3D co-culture systems. These could be broadly divided into two types: i) Reconstituted TME, in which cells are mechanically and enzymatically dissociated from the primary tumor tissue and sorted and expanded into different cell populations. Tumor cells grown as spheroids or organoids are then reconstituted with stromal cells of choice. ii) Native TME, where primary tumor tissue is mechanically fragmented and grown as tumor spheroids on low attachment plates or cultured in an air-liquid interface, embedded in a collagen gel in an inner transwell dish. The culture media from an outer dish diffuses into the inner dish via a permeable transwell, and the top of the collagen layer is exposed to air via an ALI, allowing cells to oxygenate. Both of these methods can be incorporated into a specifically designed microfluidic system.

mutations that overwrite the coding sequence and cause proteins to be transcribed that are not present in the normal proteome (Chen et al., 2020). These proteins can activate the immune system and are the basis of cancer immunity. In a recent study in long-term survivors of PDAC, the highest number of quality neoantigen load in combination with abundant $CD8^+$ T-cell infiltrates within the tumor correlated with survival (Balachandran et al., 2017). The researchers have also identified MUC16 as apparent neoantigenic hotspot in rare long-term surviving patients. This is an exciting development as there is great potential to harness such neoantigens therapeutically.

Some tumor cells have devised a variety of methods to prevent identification by host immune cells, allowing them to evade immune regulation and continue cancer growth. PDAC cells can evade immune recognition by downregulating expression of antigen processing and presentation molecules, like the major histocompatibility complex (MHC) I proteins, TAP (transporter associated with antigen processing) protein and latent membrane proteins (Pandha et al., 2007; Martinez-Bosch et al., 2018; Hiraoka et al., 2020; Yamamoto et al., 2020). The loss of neoantigens due to the inherent genetic instability of the tumors has also been reported (Mardis, 2019). Another level of immune evasion relates to expression of dominant oncogenic drivers in PDAC. KRAS oncogene imparts its pro-tumoral activity *via* regulation of cell (proliferation, migration, invasion, apoptosis blockade, and metabolic adaptation) and non-cell autonomous (tumor microenvironment remodelling and immune suppression) mechanisms (Zhang et al., 2014). Mutant KRAS induces expression of cytokines such as transforming growth factor β (TGFβ) and granulocyte macrophage colony-stimulating factor (GM-CSF) via the classical Raf/MAPK and PI3K signaling pathways (Cullis et al., 2018). These secreted immunomodulatory factors play dominant roles in shaping the immune microenvironment. For instance, KRAS oncogene dependent upregulation of GM-CSF has shown to recruit of Gr1+CD11b + MDSCs and hinder antitumour T cell activity (Pylayeva-Gupta et al., 2012). Another study demonstrates the immune suppressive role of KRAS by its genetic ablation in a mouse model. The authors noted

TABLE 1 | Overview of different 3D organoid coculture system.

Features	Spheroids	Organoids		Microfluidic system
		Reconstituted TME	Native TME	
Cell source	Established cell lines	Patient derived cells, established cell lines	Patient derived tissues	Patient derived cells, established cell lines
Co-culture method	Reconstitution with stromal cells	Reconstitution with stromal cells	Tumor cells, stroma from native tissue - fibroblasts, tumor-infiltrating lymphoid and myeloid cells, including DCs, MDSCs	Reconstitution with stromal cells or maintain stromal components from the native tissue
Advantages	Easy to establish and maintain; captures the essential pathobiology of PDAC, like the presence of hypoxia, nutrient gradient, a necrotic core and soluble factor distribution; can simulate chemoresistance in 3D with a more matrix-rich phenotype	Recapitulates molecular and morphological features of the original tumor; enables study of tumor-stroma interaction; can potentially be used to study patient specific drug response	Recapitulates molecular and histological features of the original tumor; retains stromal components from the native tissue; Long term culture; enables study of tumor–stroma interactions; can be used to study patient specific drug response	Requires small amount of tissue and medium; Both Reconstituted and Native TME organoids can be used; enables study of tumor–stroma interactions; can be used to study patient specific drug response; Can be modified to increase throughput
Disadvantage	Lacks native stromal components; depends on cell self-aggregation, which restricts control over the 3D culture environment and its architecture	Lacks native stromal components; collaboration between the lab and Clinicians needed to obtain patient derived tissues;	Contains only tumor infiltrating T cells and not circulating tumor cells; difficult to visualize tumor-stroma interaction in real time; contacts between the lab and Clinicians needed	Specialized devices are required
References	Longati et al. (2013), Ware et al. (2016), Courau et al. (2019), Nunes et al. (2019); Norberg et al. (2020)	Jenkins et al. (2018), Kopper et al. (2019), Tiriac et al. (2019), Delle Cave et al. (2021)	Ootani et al. (2009), Li et al. (2014, 2016), Neal et al. (2018)	Zervantonakis et al. (2012), Aref et al. (2018), Jenkins et al. (2018), Aung et al. (2020), Palikuqi et al. (2020)

increased influx of immune cells into the tumor and tumor regression upon oncogenic inactivation, and identified BRAF and MYC as key mediators of KRAS-induced immune evasion (Ischenko et al., 2021). The ability of mutant KRAS to modulate tumor immunity highlights the importance of adopting a combinatorial treatment approach with KRAS inhibition and simultaneous stimulation of the immune system.

4 3D MODELS TO INVESTIGATE IMMUNO-ONCOLOGY IN PANCREATIC DUCTAL ADENOCARCINOMA

Anticancer drug activity has traditionally been assessed in two-dimensionally (2D) cultured cancer cell lines. However, it is now recognized that 2D-cultured cells are incapable of simulating the complex microenvironment of the tumors *in vivo* (Duval et al., 2017). This might be one of the reasons why many drugs proven to be effective in 2D preclinical models failed in the clinic. A large body of research now focuses on the development of alternate, three dimensional (3D) models as a way to overcome some of the drawbacks of 2D-culture models (**Figure 2**) (Suri et al., 2020). Transplantable mice models in which PDAC cells are injected either orthotopically or ectopically result in tumors that are histologically different from human PDAC, with a higher vascularity and a lower desmoplasia, presenting with increased drug sensitivity (Olive et al., 2009). Genetically modified animal models, on the other hand, more accurately reflect the stroma of PDAC. These models, however, are resource-intensive and time-consuming to develop (Lee et al., 2016). Furthermore, observing tumor progression and its response to treatments over several time periods is challenging in such a model, as studies frequently offer only single endpoint data. Because of these factors, getting mechanistic and temporally resolved data while examining tumor-stromal interactions in PDAC is difficult. In order to better understand cell-stromal interactions and make accurate treatment predictions, 3D models offer a better alternative compared to 2D systems as they more closely recapitulate processes such as cell-cell, cell-matrix interaction, tumor heterogeneity and gradient formation of nutrients, oxygen, and drugs (**Table 1**). When compared to mouse models, 3D models are considerably more accessible and amenable to genetic manipulation (Heinrich et al., 2021). Here we describe different 3D models developed that have application in immune oncology.

4.1 Spheroids

Spheroids are cell aggregates growing in suspension in 3D with or without an extracellular matrix. Unlike 2D models, spheroid models are able to capture the essential pathobiology of PDAC, like the presence of hypoxia, nutrient gradient, a necrotic core and soluble factor distribution (Ware et al., 2016). It is worth noting that the spheroid size can be defined by fine-tuning the technique, making this model extremely reproducible. Spheroids with a diameter of 150 μm have been shown to display cell–cell and cell–matrix interactions, as well as an altered expression profile. A tumor spheroid of size 200–500 μm displays oxygen, nutrition, and other soluble factor gradient development (Hirschhaeuser et al., 2010). At a diameter of >500 μm, cells in the perimeter are actively proliferating, while cells in the interior are quiescent and eventually die by apoptosis or necrosis, resulting in the formation of a necrotic core. Spheroid assays are highly

reproducible and relatively low cost (Nunes et al., 2019). Furthermore, they can simulate chemoresistance in 3D with a more matrix-rich phenotype. This experimental approach may be helpful for drug testing as it more closely simulates the *in vivo* scenario (Longati et al., 2013).

Spheroid models that incorporate the tumor cells with stromal components are an attractive model to investigate the efficacy of tumor stroma targeting immunotherapies in the preclinical setting. In a triple co-culture with PDAC and cancer associated fibroblasts, myeloid cell infiltration was observed within spheroid compartment (Kuen et al., 2017). Also, an increase in immunosuppressive cytokines and polarization of monocytes into M2 polarized macrophages was found, highlighting the importance of the presence of stromal components in the preclinical models. A simple scaffold-free 3D spheroid model of direct PDAC and PSC co-cultures has been reported to facilitate the study of cellular cross-talk (Norberg et al., 2020). The authors observed a shift in the phenotypes of both the cell populations, tumor cells to be more mesenchymal and the activated pancreatic stellate to a myofibroblast like phenotype, indicating a tumor-stroma crosstalk. They also employed an interesting interspecies approach where human PDAC is co-cultured with mouse PSC to investigating cell-type specific gene expression in intact spheroids, using species-specific primers. In another example, infiltration of NKG2D expressing T cell and NK cell in a primary colorectal spheroid model was seen (Courau et al., 2019). Using this approach, targeting MICA/B molecules of the NKG2D axis resulted in increased NK cell infiltration and cytotoxicity. Collectively, these studies demonstrate the potential advantages of utilizing a simple but reliable 3D model to evaluate therapy response in the setting of immuno-oncology. One limitation of this technique is the dependency on cell self-aggregation, which restricts control over the 3D culture environment, and its defined architecture, what may be critical for systematic investigation of certain TME characteristics as well as their response to drug treatment.

4.2 Organoid System

Organoid technology has grown in popularity during the last decade. Organoids are 3D structures formed from tissues with multiple cell lineages, including stem cells and differentiated cells (Delle Cave et al., 2021), which retain the ability to self-renew and self-organize in a mini-organ-like structure that resembles the architecture and the cellular heterogeneity of the tissue of origin (Broutier et al., 2017; Shi et al., 2020). Organoids also preserve the genetic stability of the cells, allowing for improved modelling of tissue processes. Recently, organoid technology has grown in popularity in PDAC research. It is now possible to recapitulate disease-specific alterations *in vitro*, allowing researchers to mimic the various phases of tumor formation (Boj et al., 2015b). In fact, technology has improved to the point that organoids can be grown from very small biopsies, what permits to examine patients with tumors that are localized, advanced, or metastatic as shown for example by Tiriac et al. (2019), in PDAC samples.

Patient-derived organoids (PDO) are generated by embedding a single cell suspension of tumor cells isolated from primary tissue digestion or fine needle biopsy in a matrix such as matrigel or collagen (Gao et al., 2014; Kopper et al., 2019). These matrices are then supplemented with tumor-selective medium containing a well-defined mix of growth factors, such as R-spondin (RSPO), WNT3A, epidermal growth factor (EGF) and bone morphogenetic proteins (BMP) inhibitor Noggin. These factors help stem cells to maintain their ability of differentiation and self-renewal (Sato et al., 2009).

Previously, the use of clonally derived organoids to reveal patient-specific sensitivities to new medicinal drugs has been reported (Huang et al., 2015). A heterogeneous response to EZH2 inhibition in PDOs was observed, which correlated with H3K27me3 expression in both tumor organoids and matched patient tumors, demonstrating organoids' capacity to maintain the original tumor's epigenetic signatures. More recently, a well-characterized biobank of 30 patient-derived organoids was used to undertake comprehensive drug screenings, revealing distinct drug sensitivity profiles (Driehuis et al., 2019). These findings highlight the enormous potential of PDOs in precision medicine. Although co-clinical trials in PDO have been described with success in gastrointestinal malignancies and PDAC, in general access to patient tissue is restricted due to the need of performing this type of studies in a safe environment for the patients (clinical trial or similar) and the need of the tissue for a correct diagnosis (Vlachogiannis et al., 2018; Seppälä et al., 2020). Moreover, the success rate of PDO depends on tumor type, amount of starting material (resection versus fine needle aspiration) and treatment history (Busslinger et al., 2020).

As an alternative to PDO, pancreatic organoids generated from wild-type mice and genetically modified mouse models have been demonstrated *in vitro* to precisely replicate physiologically relevant features of PDAC development (Boj et al., 2015a). When compared to PDOs, mouse organoids can be obtained considerably more easily in terms of sample accessibility and amount. Additionally, mouse organoids are far more amenable to various genetic manipulations, facilitating mechanistic studies. Furthermore, results obtained from the organoid model may be easily transferred to an *in vivo* model with the same mutational background in an immunocompetent model. Working with mouse organoids also allows us to study different stages of PDAC development (Boj et al., 2015a). This has resulted in better understanding of the mechanisms underlying the development and progression of PDAC, and it is an excellent tool for the study of immuno-oncology.

Organoid culture represents a novel approach to investigating the immunobiology of PDAC tumors. As previously stated, epithelial-only submerged Matrigel organoids can aid in predicting a patient's response to therapy and selecting individualized treatment methods; nevertheless, these organoids do not fully recreate the TME due to the absence of stromal components. Organoid technology, is fast adapting to incorporate stromal cells, allowing for the research of diverse immunotherapy strategies as well as studying immune evasion (Bar-Ephraim et al., 2020; Fitzgerald et al., 2020).

4.2.1 Reconstitution of Stromal Components

Organoids may now be co-cultured with exogenously provided stromal components such as cancer-associated fibroblasts (CAFs)

and immunological populations, including peripheral blood mononuclear cells (PBMCs), leukocytes, tumor-associated macrophages (TAMs), and DCs (Boucherit et al., 2020). These stromal cells can be isolated either from the tumor fragments (i.e., Tumor infiltrating lymphocytes) or from peripheral blood mononuclear cells (PBMCs). Co-culture of human PDAC organoids with pancreatic stellate cells, a precursor population of CAFs, has led to better understanding of CAF heterogeneity. A pioneer study by Öhlund et al. (2017) using a tumor organoid-CAF co-culture revealed the presence of two spatially separated, mutually exclusive, dynamic, and phenotypically distinct CAF subtypes: alpha-SMA expressing myofibroblasts (myCAFs) and inflammatory cytokine secreting CAFs (iCAFs). Another study reported that squamous *trans*-differentiation in an aggressive p63-expressing squamous PDAC was associated with neutrophil infiltration and other markers of inflammation (Somerville et al., 2020). Using *in vitro* PDAC organoid models and *in vivo* mouse models, the authors discovered p63-induced IL-1 secretion which in turn promotes iCAF formation. It is apparent that such co-culture models are an excellent tool for understanding CAF heterogeneity, which is an important factor to consider when designing stroma targeting immunotherapies.

A well characterized multi-cell type organotypic co-culture model of the tumor microenvironment has been reported (Tsai et al., 2018). Human PDAC organoids grown with matched tumor-associated fibroblasts and immunological components of the tumor microenvironment reveal the emergence of a sophisticated disease-representative model. In a recent study, Freed-Pastor et al. (2021) demonstrate the use of mouse models and organoid/$CD8^+$ T cell co-culture system to model neoantigen expression. The use of organoid co-culture offered flexibility and genetic tractability to investigate new and diverse neoantigens. Using two such complementary model systems, the authors identified a central role of CD155/TIGIT axis in mediating immune evasion in PDAC (Freed-Pastor et al., 2021). Using a more complex cellular set up, Chakrabarti et al. (2018) reported the use of a co-culture of gastric tumor organoids from mouse models with cytotoxic T lymphocytes (CTLs) and bone marrow-derived DCs, to potentially predict the efficacy of immune-checkpoint inhibition for the treatment of gastric cancer. They observed an increase in CD8 lymphocyte mediated cell killing with the inhibition of PDL1. This indicates that this approach may also be extended to PDAC as a platform for the study of immunotherapy (Chakrabarti et al., 2018). Along the same lines, a recent study demonstrated the use of murine and human pancreatic ductal adenocarcinoma (PDAC) autologous organoid-immune cell co-culture to test efficacy of a combinatorial immunotherapy involving PD-1 inhibition and MDSC depletion (Holokai et al., 2020). The authors observed that PDAC co-culture with MDSCs promoted tumor growth and suppressed T cell proliferation, and when treated with the combination therapy rendered the organoids susceptible to anti-PD-1/PD-L1-induced cancer cell death. This demonstrates the value of pre-clinical organoid models in predicting the success of targeted therapies to enhance patient outcomes. However, such a model has its disadvantages, most notably the lack of native stromal components. Immune cells are isolated from blood that has not been constantly exposed to tumor antigens. Also, obtaining pure populations of primary cells on a regular basis from matched donor is relatively more difficult, especially when isolating low abundance cells. Another important factor to consider is the cell culture medium used. Organoid media contains very particular growth factors that may influence T cell activity. More research into the effects of each component on T cell activity and optimizing culture conditions would be extremely advantageous to set up appropriate co-culture systems.

4.2.2 Air-Liquid Interface

Air-liquid interface (ALI) 3D culture was originally reported by the Kuo lab in 2009, who described an application of this approach for a sustained 3D *in vitro* intestinal epithelial culture (Ootani et al., 2009). Later, this strategy was adapted to model PDAC-tumor immune microenvironment using patient derived organoids (Neal et al., 2018). To establish an ALI culture, minced primary tissue fragments are embedded in a collagen gel in a compartmentalized chamber with a porous membrane, similar to a transwell dish, to physically separate from the underlying medium (Yuki et al., 2020). The culture media in an outer dish diffuses into the inner dish via the permeable transwell, while the top of the collagen layer is exposed to air *via* an ALI, providing cells access to an adequate oxygen supply. Additionally, this system allows for vigorous expansion of primary epithelium for a long-term culture as organoids with multilineage differentiation, including endogenous native stromal and immune components *via* improved oxygenation *in vitro* (Li et al., 2016). This technique successfully retains the original tumors' genetic alterations as well as the TME's complex cellular composition and architecture. This method allows primary pancreatic ductal epithelium to be cultured in close apposition to myCAFS and iCAFS, known to be antitumorigenic and pro-tumorigenic respectively, hence influencing PDAC growth *in vitro* (Neal et al., 2018). Furthermore, the potential inclusion of all immune components, including tumor-infiltrating lymphocytes and myeloid cells, makes this a suitable system for precision medicine to examine patient-specific drug response.

PDAC ALI 3D culture is a relatively new technique, with only one published report on establishment and characterization of the culture system. Hence extensive study is required to understand its full potential in immuno-oncology. As it is, a major drawback of this technique is the inability to monitor changes in real-time of the cellular and molecular features. Moreover, this culture system is less compliant to genetic manipulation due to its "*en bloc*" nature.

Although the organoid models described above reproduce the complexity observed in the 3D tissue architecture of living organs to a certain extent, they fail to incorporate the mechanical forces that can substantially influence cancer cell behavior, for instance, fluid shear stress and hydrostatic pressure. Furthermore, neither

of these systems model a functional vasculature which is perfused by nutrient-rich medium, which results in an inability to study recruitment of circulating immune cells and also bioavailability of the test therapeutic agents.

4.3 Microfluidic System

Microfluidic culture, also known as organ-on-a-chip, combines the benefits of 3D culture in a dynamic and controlled environment. This system can be utilized to examine many aspects of carcinogenesis, as well as to perform drug screening and forecast response treatments. Both spheroids and organoids have been reported to be used in microfluidic systems. In essence, the 3D culture is deposited in a microfluidic chip, and the medium is dynamically perfused with or without therapeutic drugs, this allows to offset many of the limitations mentioned above. Additionally, microfluidic methods provide a set of unique capabilities for real-time monitoring of cellular processes, which is critical for the investigation of dynamic tumor-stroma interactions (Zervantonakis et al., 2012). Furthermore, the technology may now be scaled to achieve a larger throughput, increasing the prospect of precision medicine (Schuster et al., 2020). In this study, the authors describe an automated, high-throughput, microfluidic PDAC PDO platform to screen combinatorial and dynamic drug treatments on hundreds of cultures. This integrated platform designed to mirror real patient treatment, combined with real-time analysis of organoids has a great potential in the field of immuno-oncology.

A microfluidic co-culture of pancreatic tumor spheroids with stellate cells has been reported to investigate epithelial to mesenchymal transition and drug resistance (Lee et al., 2018). They embedded 3D tumor spheroids and PSCs separately in type 1 collagen and loaded into each designated channel with intermittent feeding of media. With this system, activation of PSCs under co-culture conditions which in turn influenced the migratory ability of cancer cells was observed. There are multiple reports on microfluidic systems of tumor-immune cell co-culture to study cytotoxic activity as well as resistance to immunotherapy in a 2D system (Aref et al., 2018; Rothbauer et al., 2018). In comparison, 3D models in a microfluidic system are a relatively recent advance. In an interesting proof of concept study reported by Aung et al. (2020), the development of a multicellular tumor-on-a-chip platform was described. Herein, the authors studied the effect of the tumor microenvironment, especially the presence of monocytes and hypoxia have on breast cancer spheroids. They observed increase recruitment of T cells in the hypoxic condition. Moreover, the addition of monocytes to the cancer cells improved T-cell recruitment. Highlighting their potential application in studying recruitment of cells by the tumor cells. A study led by Jenkins demonstrated the use of an organotypic 3D microfluidic culture from murine and patient derived tumor tissue, which retained the native immune TME, in assessing response and resistance to immune checkpoint blockade (Jenkins et al., 2018). They discovered that targeting the TBK1/IKK axis improved responsiveness to PD-1 blockade using their model system, highlighting its application in immuno-oncology. An organoid co-culture in a blood-perfusable pericyte-coated microfluidic chamber has also been reported (Palikuqi et al., 2020). In this system, the endothelial cells could functionally arborize patient derived colorectal cancer organoids and respond to microenvironment stimuli. A system like this has a lot of potential as a tissue-specific *in vitro* platform for the evaluation of administration and response to modified immune cells like CAR-T cells and chemotherapeutic drugs.

Microfluidic systems heavily rely on microfabrication technologies. Such resources and the expertise are not readily available to all researchers. Moreover, this technique also suffers from the same limitations as organoid system with respect to the cells/tissues used. The decision to adopt any of the given model systems should be strongly based on the research objective and the system providing benefits that would outweigh the limitations.

5 CONCLUSION AND FUTURE DIRECTIONS

Our understanding of PDAC tumor biology and the tools used for its research have advanced significantly over the years, however, patients' prognosis remains very poor. A rational treatment strategy that considers the intricate tumor cell-cell and cell-ECM interactions, as well as the tumor-drug interaction may help to improve this adverse scenario. 3D models may offer a great deal of promise as surrogate tumor models, but their choice should be strictly based on the question under investigation.

To improve the predictive response of a model system for studying treatment response, one factor to consider may be tumor subtype. As previously stated, PDAC has multiple subtypes, each with a varied response to different treatments. In fact, the basal subtype is shown to have an altered metabolism favouring glycolysis (Daemen et al., 2015) and a high lactate content in these tumors might impair the immune response and cause treatment resistance (Husain et al., 2013; Manoharan et al., 2021). Hence, these factors should be included in the 3D model systems used for identification of novel drug sensitivities and resistance mechanisms in PDAC. To date, no reports of immunotherapy responses stratified by tumor subtype have been published. 3D cultures might be an alternate method of reanalysing current preclinical data. The use of PDAC patient-derived organoids that represent each subtype should be a suitable foundation for creating valid models for molecular characterisation and response prediction.

In addition, a predictive model should consider the complex tumor-stroma interaction and future therapeutics should be targeted towards the pro-tumorigenic population. For example, of the various fibroblast subtypes present in the tumor mass, targeting the inflammatory subtype found distal to the tumor is beneficial, whereas targeting the SMA positive population adjacent to the tumor mass is counterproductive. Diffusible small molecule metabolites may be involved in the interaction of tumor cells with distant stromal cells. Thus, an innovative approach that combines an efficient 3D model with technologies to resolve the role of soluble factors, such as mass spectrometry imaging-based metabolomics, may be uniquely suited to elucidate this phenomenon. Combining an optical image of a co-culture

containing the desired cellular population with an averaged mass spectrum of the sample's molecular components, the spatial distribution of metabolic signals involved in the bidirectional communication may be visualized and a proper understanding of the cellular crosstalk may be achieved.

Several clinical trials are now underway in PDAC that employ immunotherapy combinations with standard-of-care chemotherapy (Desai et al., 2019; Bockorny et al., 2020). The design of clinical trials should be guided by solid preclinical data that highlight optimal synergistic combinations to boost anti-tumor immune activity in order to get the best possible outcome. Current chemotherapy regimens need a high dosage which in addition to the apparent toxicity may have a negative effect on the overall immune function. A preclinical study using the 3D models to determine lower concentrations of these drugs that could synergize with immunotherapy would be extremely beneficial. Using an array of organoids that differentially express immune checkpoint inhibitors to screen for effective ICI-Chemotherapy combinations may result useful for designing treatment strategies. A well-characterized 3D model system integrating all aspects aforementioned would stand as an ideal proxy to determine the drug combination that is most effective. Furthermore, it could potentially be utilized to create a therapy-resistant model in order to better understand the mechanism of action.

Another way to look at leveraging 3D models is to generate a set of predictors that can assist in clinical decision making. For example, integrating data from genetically and multi-omically defined PDOs treated with a spectrum of immunochemotherapy combinations, with medical imaging obtained at diagnosis, may possibly uncover predictors of therapeutic response. Large multi-center level studies may aid to facilitate this relevant task.

Given the growing body of evidence supporting the importance of the tumor microenvironment in immune evasion and immunotherapy failure, further research is necessary to fully elucidate the crosstalk between tumor and stroma. 3D models are a tremendous advance and could be used to gain improved understanding of the tumor supportive, immune evading role of stroma. Based on the continued integrative profiling of PDAC, it is likely that the repertoire of 3D models that accurately depict PDAC pathology will expand and be integrated into the treatment decision making process in the next years.

AUTHOR CONTRIBUTIONS

SN, SV and MP-S conceived the work, designed the outline of the review and supervised all aspects of the manuscript. SN, SV and MP-S participated in the literature search, scrutiny and interpretation, as well as in writing and editing all contents of the manuscript.

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Microfluidic Platforms for High-Throughput Pancreatic Ductal Adenocarcinoma Organoid Culture and Drug Screening

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Pancreatic Ductal Adenocarcinoma (PDAC), the most common pancreatic cancer type, is believed to become the second leading cause of cancer-related deaths by 2030 with mortality rates of up to 93%. It is often detected at a late stage due to lacking symptoms, and therefore surgical removal of the tumor is the only treatment option for patients. Only 20% of the tumors are resectable, mainly due to early metastasis. Therefore, for 80% of cases chemotherapeutic treatment is the leading therapy for patients. PDAC is characterized by high-density stroma which induces hypoxic conditions and high interstitial pressure. These factors impact carcinogenesis and progression of PDAC and support the formation of an immunosuppressive microenvironment that renders this tumor type refractory to immunotherapies. Most *in vitro* PDAC models have limited translational relevance, as these fail to recapitulate relevant aspects of PDAC complexity. Altogether, there is an urgent need for novel and innovative PDAC modeling platforms. Here, we discuss the relevance of microfluidic and organoid technologies as platforms for modeling bio- and physicochemical features of PDAC and as translational models that enable high-throughput phenotypic drug screenings, while also allowing for the development of novel personalized models used to identify treatment responsive patient subsets.

Keywords: PDAC, tumor microenvionment, drug screening, organoids, organ-on-a chip

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PANCREATIC DUCTAL ADENOCARCINOMA

The pancreas consists of three main cell types: acinar cells, which secrete digestive enzymes, duct cells secreting bicarbonate and hormone-secreting endocrine islet cells (Kleeff et al., 2016). The most common pancreatic cancer type involves the exocrine part and is known as pancreatic ductal adenocarcinoma (PDAC). This disease is hard to predict, detect, diagnose, and treat. In 90% of these tumors KRAS codon 12 is mutated, whereas TP53 ("the gatekeeper"), CDKN2A and SMAD4 are mutated with an incidence rate of 50–80% (Kleeff et al., 2016). In addition, epigenetic and copy number variations of genes, as well as somatic and germline mutations including the repair pathways BRCA1/2, ATM and PALB2 are characteristics of PDAC. The TGF- β, WNT, NOTCH, and DNA damage repair pathways are potential drug targets as these are also activated in PDAC. Aerobic glycolysis and pentose phosphate pathways are upregulated in pancreatic cancer stem cells, which represent a minority in the tumor microenvironment making therapeutic targeting of these metabolic pathways difficult, as apart from their self-renewing characteristics these are also of heterogenous nature (Huang et al., 2015).

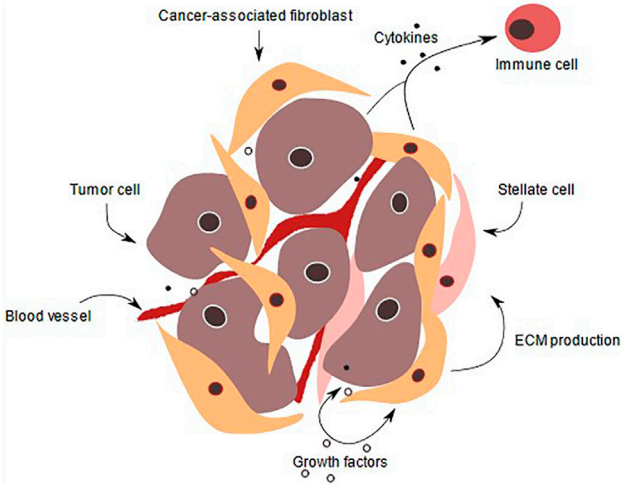


FIGURE 1 | PDAC tumor microenvironment. In a normal pancreas, the basal lamina is highly organized, and apical-basal polarity is present. Tissue is vascularized and ECM supports pancreatic cells. Also, pancreatic stellate cells produce ECM proteins and remain quiescent. During pancreatic intraepithelial lesion, the ductal cells start to transform, their morphology as well as gene expression change. Immune cells are recruited and fibroblasts as well as pancreatic stellate cells become activated, thereby secreting a variety of signaling factors, that are received by the transformed cells. ECM production is enhanced and PDAC is initiated, transformed cells start to form niches and colonize. ECM deposition increases and the stroma amount increases up to 90% of the whole tumor volume. The cancer cells proliferate due to various growth factors secreted by immune cells such as $CD8^+$ T-cells, tumor-associated macrophages and pancreatic stellate cells. In addition, they invade other tissue and intravasate leading to metastasis to other organs (Kota et al., 2017).

PDAC is furthermore characterized by resistance to conventional treatments and rapid metastasis to liver, lung, and peritoneal cavity. Both liver and pancreas arise from endoderm and control metabolism by secreting enzymes. Their common developmental origin and function is thought to be one of the reasons pancreatic tumors first metastasize to the liver (Hindley et al., 2016). This propensity to metastasize originates from paracrine and autocrine signals of guiding cells towards other tissue. During metastasis, cancer cells from the primary tumor invade foreign microenvironment facilitated by *KRAS, TP53, p16, CDKN2A* and *SMAD4*. These intravasate into the bloodstream, disseminate, extravasate through the endothelia, enter and colonize a distant organ (Thomas et al., 2020). Epithelial-to-mesenchymal-transition (EMT) plays a role in metastasis, however, only a small number of cells in pancreatic cancer have been shown to undergo EMT and thus the contribution of EMT is not fully understood in PDAC metastasis (Tan et al., 2014).

A COMPLEX TUMOR MICROENVIRONMENT

PDAC cells are supported by a complex microenvironment (**Figure 1**) that is composed of approximately 90% stroma. This dense stroma creates a hypoxic environment, and mainly consists of collagen, fibronectin, fibrillar collagen and hyaluronic acid (HA). Increased HA content promotes cancer-cell migration and increases the interstitial pressure, which limits drug availability. Enhanced HA production is a prognostic factor in PDAC but also laminin expression correlated with poor patient prognosis, as it increases drug resistance due to promoting high cell adhesion (Miyamoto et al., 2004). Several strategies for targeting HA, such as synthesis inhibition, signal blockage and HA depletion in the stroma have shown beneficial effects in PDAC treatment (Jacobetz et al., 2012; Jiang et al., 2015; Nagy et al., 2015). In addition, the tumor microenvironment (TME) comprises mesenchymal derived cells such as fibroblasts, pericytes, endothelium and immune cells such as T-cells, B-cells, macrophages, dendritic cells (DCs), eosinophils, myeloid-derived suppressor cells (MDSCs) and natural killer cells (NK-cells) (Palucka et al., 2016).

In particular, tumor associated macrophages (TAMs) play a role in PDAC as these suppress antitumor responses, promote metastasis and angiogenesis. TAMs are classified as either classical activated (M1) or nonclassical activated (M2). M1 cells have a proinflammatory and cytotoxic behavior, whereas M2 have the opposite functions and are thus pro-tumoral. M2 type TAMs and regulatory T-cells are accumulated in PDAC (Bulle et al., 2020). Pancreatic stellate cells (PSCs) are myofibroblasts and are responsible for fibrosis and desmoplastic reactions in PDAC. In a diseased pancreas, PSCs become activated, produce laminin, collagen, and fibronectin, and promote immunosuppression. In addition, an imbalance between PSCs that secrete matrix metalloproteinases (MMPs) for fibrosis repair, inhibitors of those metalloproteinases (TIMPs) and increased ECM production for tumor proliferation is observed. Fibroblasts present in the PDAC TME differentiate into cancer-associated fibroblasts (CAFs) upon TGF-β, EGF, FGF or TNFα secretion, hypoxia and oxidative stress. In turn CAFs secrete factors, which promote tumor growth and act as a barrier for drug delivery into the tumour site. There is a dynamic exchange of supportive signaling factors between CAFs and tumor cells. Two distinct subtypes of CAFs can be distinguished: myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs). myCAFs express α-smooth muscle actin (α-SMA) and are located in the acinus, while iCAFs lack α-SMA expression, but express IL-6 and are more distantly located (Li et al., 2020). IL-6 can activate fibroblasts for ECM production. Blocking IL-6 in combination with chemotherapy, induced apoptosis of tumor cells and increased survival in mice (Long et al., 2017). Therefore, distinguishing between iCAFs and myCAFs is necessary before including these into drug screening tumor models due to different gene expression profiles and antigen presentation (Oehlund et al., 2017; Elyada et al., 2019). CAFs also support exosome release, which in turn increase chemoresistance-inducing factor when exposed to chemotherapeutics and secrete fibroblast activation protein α (FAPα) leading to angiogenesis and invasion (Kawase et al., 2015). Costa-Silva and colleagues demonstrated that exosomes derived from

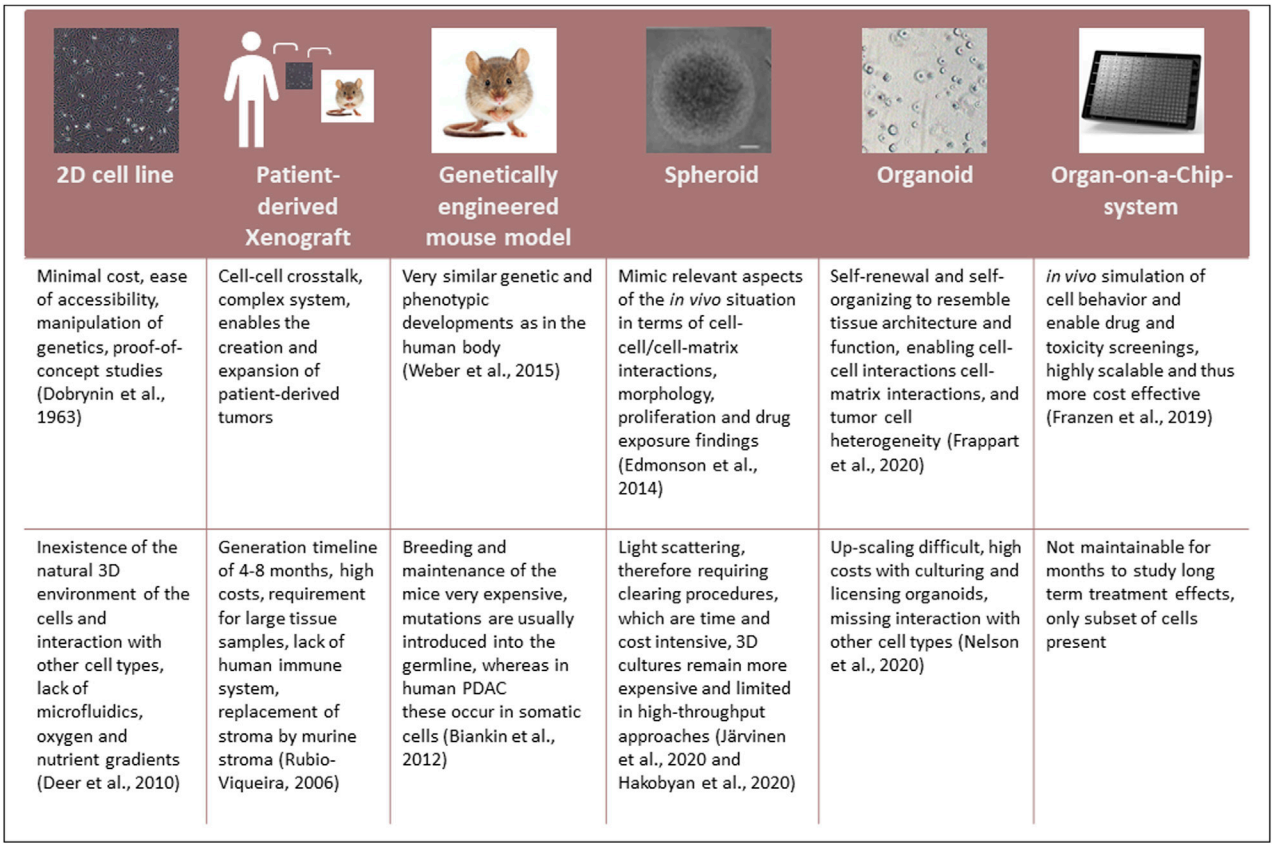
lesions are involved in liver niche formation. Uptake of the exosomes by liver Kupffer cells (KC) support the inflammatory state found in metastasis. High TGFβ level in patients is associated with poor prognosis (Costa-Silva et al., 2015), however when depleting CAFs, the TME was characterized by decreased angiogenesis, collagen deposition, increased cancer stem cell and regulatory T-cell numbers, and hypoxia, which resulted in worse patient survival (Özdemir et al., 2014). However, Ware et al. showed, that gemcitabine effect was impaired in stroma rich PDAC spheroids compared to spheroids without stroma (Ware et al., 2016). On the one hand studies suggest the need for targeting the tumor stroma in addition to cancer cells for a successful PDAC treatment, while on the other hand contradictory studies show that the complexity of the tumor stroma and that experimental design must be carefully taken into consideration.

Altogether, recapitulating the complexity of this disease in one single *in vitro* model is challenging, and it is more likely that multiple versions of PDAC models that serially include different components will together aid to reveal and translate processes influencing PDAC growth and progression. Therefore, platforms that enable the gradual incorporation of different bio- and physicochemical features are relevant for the development of translational PDAC models.

CURRENT *IN VITRO* MODELS USED FOR DRUG SCREENINGS

The 5 years survival rate of PDAC is less than 7% and there are limited *in vitro* models to study pancreatic cancer. PDAC radiation treatment trials have been halted as no clinical benefit was observed. Targeted therapy of patient specific mutations of KRAS, TP53, SMAD4, MLL3, TGFBR2 and CDKN2A has not worked but needs further investigation. Apart from the mentioned therapies, NTRK fusion inhibitors seem to provide an alternative as well. Although progress is being made, there is no treatment for patients that relevantly improves outcomes (Roth et al., 2020). Chemotherapy remains the gold standard in treatment, when surgical resection is not possible. The standard of care chemotherapeutics are Gemcitabine, Gemcitabine/nab-Paclitaxel, Oxaliplatin and FOLFIRINOX (5-Fluorouracil, Leucovorin, Irinotecan, Oxaliplatin). However, treatment with these chemotherapeutics prolongs the life of patients only with a few months and the average survival remains less than 1 year (Frappart et al., 2020).

Immunotherapy has increasingly become a treatment option for various cancer types. Despite several trials, PDAC remains unresponsive to immunotherapies. Strategies to combine immunotherapeutics with chemotherapeutics are currently being evaluated in clinical trials (Nywening et al., 2016; Pfirschke et al., 2016). Furthermore, numerous approaches such as cytokine therapy (IL-2, IFN, IL-15), therapeutic vaccines, agonistic and antagonistic antibodies, small molecule agonists, adoptive cell therapy and chimeric antigen receptor were tested in PDAC (Brahmer et al., 2012; Zambirinis et al., 2015; Yuki et al., 2020). However, all of these treatments have either failed in clinical trials due to adverse effects, or due to ineffectiveness of the drug in the complex human PDAC tumor microenvironment. Therefore, it is necessary to screen for new drugs for finding new hits, which not only prolong the life of patients, but also cure this disease. Several approaches have been made in PDAC cancer research: Hou et al. studied the effect of FDA/EMA-approved drugs on pancreatic cancer primary cells and identified 14 drugs, which had an effect in four types of cells in 3D culture models (e.g., Bortezomib, Carfilzomib, Romidepsin, Homoharringtonine, and Trametinib). Two CAF lines and two PDAC cell lines were grown as monocultures in 2D, but also grown separately as spheres under the absence of exogenous ECM components in 3D to determine treatment differences in 2D and 3D. It was shown that 3D culture models were more resistant to chemotherapy than 2D cultures, indicating the importance of selecting the correct model for drug screenings (Hou et al., 2018). Phan et al., 2020 used the mini-ring method upon plating single cells mixed with Matrigel to generate organoids in a ring shape around a rim in a 96-well plate and to test 240 protein kinase inhibitors. This mini-ring approach allows drug testing on a very low number of cells, and they could discern different treatment behavior between patients. (Phan et al., 2020). Moreover, Driehuis et al. studied the effect of 76 therapeutics on PDAC organoids, and showed that some drugs are only effective in a subset of patients with the same or similar mutation pattern. These authors proposed the necessity of a personalized approach for achieving effective tumor killing (Driehuis et al., 2019). These studies suggest the necessity of stratified drug treatment and therefore promote the use of organoids, which can be harvested from each patient and subjected to drug screening prior to patient treatment for finding the most effective therapeutic. Frappart et al. (2020) used patient derived organoid (PDO) and Patient derived xenograft (PDX) models to screen several FDA-approved drugs and proved in *in vivo* studies, that PDOs derived from PDX serve as an alternative to PDX (Frappart et al, 2020). All these experiments with improved model systems raise hope for patients to screen for new drug candidates, as all previously in clinical trials tested drugs have failed so far. However, a high-throughput screening of drugs has not been feasible yet, due to the limited amount of patient material and organoids. Thus, a preliminary sequencing of the DNA of patients and mutation status could give an indication of the type of drugs that should be tested on organoids and afterwards prescribed to a given patient. Moreover, Haque et al. recently discussed the impact of the heterogenous TME in PDAC and concluded that extensive model development is still needed for establishing efficient drug pipelines (Haque et al., 2021). Therefore, current *in vitro* model systems are lacking in translational value, and novel PDAC models that allows for rapid initial drug testing that provide a truly translational outcome for identifying novel patient treatment options are called for.



2D cell line	Patient-derived Xenograft	Genetically engineered mouse model	Spheroid	Organoid	Organ-on-a-Chip-system
Minimal cost, ease of accessibility, manipulation of genetics, proof-of-concept studies (Dobrynin et al., 1963)	Cell-cell crosstalk, complex system, enables the creation and expansion of patient-derived tumors	Very similar genetic and phenotypic developments as in the human body (Weber et al., 2015)	Mimic relevant aspects of the *in vivo* situation in terms of cell-cell/cell-matrix interactions, morphology, proliferation and drug exposure findings (Edmonson et al., 2014)	Self-renewal and self-organizing to resemble tissue architecture and function, enabling cell-cell interactions cell-matrix interactions, and tumor cell heterogeneity (Frappart et al., 2020)	*in vivo* simulation of cell behavior and enable drug and toxicity screenings, highly scalable and thus more cost effective (Franzen et al., 2019)
Inexistence of the natural 3D environment of the cells and interaction with other cell types, lack of microfluidics, oxygen and nutrient gradients (Deer et al., 2010)	Generation timeline of 4-8 months, high costs, requirement for large tissue samples, lack of human immune system, replacement of stroma by murine stroma (Rubio-Viqueira, 2006)	Breeding and maintenance of the mice very expensive, mutations are usually introduced into the germline, whereas in human PDAC these occur in somatic cells (Biankin et al., 2012)	Light scattering, therefore requiring clearing procedures, which are time and cost intensive, 3D cultures remain more expensive and limited in high-throughput approaches (Järvinen et al., 2020 and Hakobyan et al., 2020)	Up-scaling difficult, high costs with culturing and licensing organoids, missing interaction with other cell types (Nelson et al., 2020)	Not maintainable for months to study long term treatment effects, only subset of cells present

FIGURE 2 | Comparison of the currently available model systems used in PDAC research.

MODELLING PDAC: *IN VITRO* AND *IN VIVO*

Current drug screenings are mainly performed using conventional 2D grown PDAC cell lines, which were first generated in 1963 and remain used in research to date (Swayden et al., 2020). Nevertheless, several limitations influence the relevance of these models. Alternative model systems to cell lines grown in 2D have been established (**Figure 2**):

Every model has advantages and disadvantages. However, the use of organoids, especially in the context of Organ-on-a-Chip systems, is currently being widely regarded as potentially the models that most closely mimic the *in vivo* setting.

Organoids are three-dimensional structures recapitulating tissue geometry, dynamics, molecular and genomic signatures, thereby enabling *in vivo* like preclinical studies. Tumor organoids maintain patient-specific oxygen consumption, differentiation status and epigenetic marks as different patient samples showed a varied mutation status depending on diet, lifestyle and genetics and can thus contribute to tumor-related interpatient heterogeneity (Juiz et al., 2019). Organoids share stem cell characteristics such as self-renewal and can be maintained for several passages. Furthermore, organoids can self-organize to resemble tissue architecture and function, enabling cell-cell interactions, cell-matrix interactions, and tumor cell heterogeneity. PDX models and derived organoids have shown the same results in proteome analysis, however organoid models have proven less labor-intensive and more time efficient (Frappart et al., 2020). Another approach to use organoids would be to use genome engineering tools such as transcription activator like effectors (TALEN) or clustered regularly interspaced short palindromic repeats (CRISPR) in combination with a CRISPR-associated (Cas) 9 protein to introduce or excise mutations/genes, and to compare the phenotype and genotype of the newly generated organoid with a healthy wildtype organoid to study diseases (Schwank et al., 2013).

Although organoids can provide valuable translational models and potentially enable the development of personalized models, there are also challenges regarding up-scaling, and cost of culturing and licensing (Nelson et al., 2020). In addition, organoid cultures lack interactions with other cell types such as immune cells, vasculature, and stromal cells as well as biomechanical cues. Therefore, translational relevance of organoids is potentially limited when used in monoculture. Another relevant limitation is the variability across different organoid lines, genotypes, batches of organoids and even within the organoids in one culture. Even with these

limitations, organoids have the potential to become the most valuable cellular model, that can be applied for patient stratification and personalized medicine in PDAC. To overcome lacking TME components, Frappart et al., 2020 introduced the term "PDACoids", which entails the co-culture of PDAC organoids with immune cells and CAFs and further prompts the use of Organ-on-a-Chip approaches to incorporate several cell types (Frappart et al., 2020).

ORGAN-ON-A-CHIP SYSTEMS

The majority of 3D cell culture systems do not incorporate multiple cell types such as endothelial, and stromal cells in addition to the PDAC cells and thereby fail to recapitulate the cellular complexity of the PDAC TME (Rothbauer et al., 2019). Thus, Organ-on-a-Chip systems provide novel platform for incorporating diverse cell types and flow conditions present in the human body. Moreover, tissue geometry, dynamics and gradients are recapitulated in specialized cell networks in these platforms (Bhatia et al., 2014). Organs-on-a-Chip are microfluidic systems, which enable the generation of a defined microenvironment for growing cells. As only a small number of cells is needed for experimental studies in Organs-on-a-Chip in comparison to traditional well plate cultures, it would be feasible to use these for patient stratification and for individualization of therapies (Van den Berg, 2019). The ductal tumor-microenvironment-on-chip (dt-MOC) applied by Bradney et al., gave further insight into the relevance of Organ-on-a-Chip systems in order to understand the complexity of intratumoral heterogeneity. EMT was mimicked by creating an epithelial cancer cell duct of KPC2, eKIC and mKIC mouse cell lines within a Collagen I matrix to study heterogenous invasion characteristics. The group thus generated a platform to study tumor cell invasiveness and aggressiveness and further suggested the development of patient-derived tumor-stroma interaction models (Bradney et al., 2020). As PSCs play a role in cancer progression, Lee et al. cultured tumor spheroids with PSCs in microfluidic devices to show, that the number of spheroids increased in co-culture conditions and that EMT related gene expression was increased as well (Lee et al., 2018). Another example of stromal cell incorporation is provided by Bi et al., who showed the successful incorporation of macrophages into a tumor-on-a-chip system, where they combined PDX generated PDAC cell lines with vasculature and immune cells. They demonstrate inhibition of tumor growth, invasion and angiogenesis in response to macrophages in their Organ-on-a-Chip device, which is a crucial step towards recapitulating TME complexity (Bi et al., 2020).

In addition, Organ-on-a-Chip models containing PDAC organoids can also be applied for studying drug efficacy in drug discovery and development programs. Mimetas developed several OrganoPlates (**Figure 3**) to establish 3D *in vitro* model systems on a chip, which do not require the introduction of perfusion loops between diverse organ compartments and ensure perfusion, vascularization, and high-throughput in a standard 384-well plate format (Beaurivage et al., 2019). Kramer and colleagues used S2-028 PDAC cells grown on a chip for gemcitabine treatment, which inhibits DNA synthesis and induces apoptosis. Therefore, the cells were cultivated in the 3-lane OrganoPlate® and the group observed a different effect of gemcitabine treatment in monolayer culture compared to Organs-on-a-Chip, thereby suggesting an overestimation of the actual drug efficiency in 2D (Kramer et al., 2019) (**Figure 3C**). Moreover, the HepaChip® with continuous perfusion of BxPC3, MiaPaCa2 and PANC1 lines was used by Beer et al. to show, that Organ-on-a-Chip platforms can be used for improved prognosis and drug testing, as their drug screenings initially proved, that *in vivo* drug responses are more closely mimicked with these platforms compared to normal 2D or 3D systems (Beer et al., 2017) (**Figure 3A**).

Also, vascularization can be studied in these platforms, as vascularization of the organoids is necessary to simulate *in vivo* conditions for waste removal and distribution of oxygen and nutrients (Huang et al., 2020). Another system called Integrated Vasculature for Assessing Dynamic Events (INVADE), based on a 96-well plate with inlet and outlet wells connected to a tissue chamber was used by Fook Lun Lai et al., 2020 in which human umbilical vein endothelial cells (HUVEC), human dermal fibroblasts and PDAC organoids were cultivated together. Subsequently, cytokine release, fluorescent dye distribution and viability after drug treatment for a better understanding of the TME in PDAC were studied. Fook Lun Lai et al., also observed a difference in organoid diameter and ECM remodeling between organoid monoculture and co-culture with fibroblasts. In addition, they also tested molecule transfer in monoculture and co-culture and concluded that the transfer from endothelial cell vessels is inhibited in the co-culture conditions, which might explain the role of the tumor stroma in insufficient chemotherapeutic treatment of PDAC (Fook Lun Lai et al., 2020) (**Figure 3D**). Ngyugen et al. used a similar setup and included a HUVEC vessel and primary mouse pancreatic cells in their model, to show a highly complex cellular interaction network, invasion of PDAC cells into the vasculature, thereby contributing to a better understanding of metastasis in PDAC with an Organ-on-a-Chip platform (Nguyen et al., 2019) (**Figure 3B**).

In addition, higher throughput can be achieved with these platforms as described by Drifka et al., as their tubing-free and easy-to-load microfluidic device opens the possibility for implementing automated liquid handling. Moreover, they also discuss the importance of the PDAC TME for incorporation of the *in vivo* complexities of 3D architecture and cell-cell interactions within a model containing primary PSCs and the PANC-1 human PDAC line (Drifka et al., 2013). Most Organ-on-a-Chip platforms reduce media and reagent consumption, are compatible with most laboratory equipment and the systems are often tube- and pump-free, allowing for the adoption of these platforms in many research labs. Thus Organ-on-a-chip platforms are revolutionizing research upon enabling tumor microenvironment studies in a high-throughput setting. Other advantages of Organ-on-a-Chip systems include a better mimicking of cell behavior and

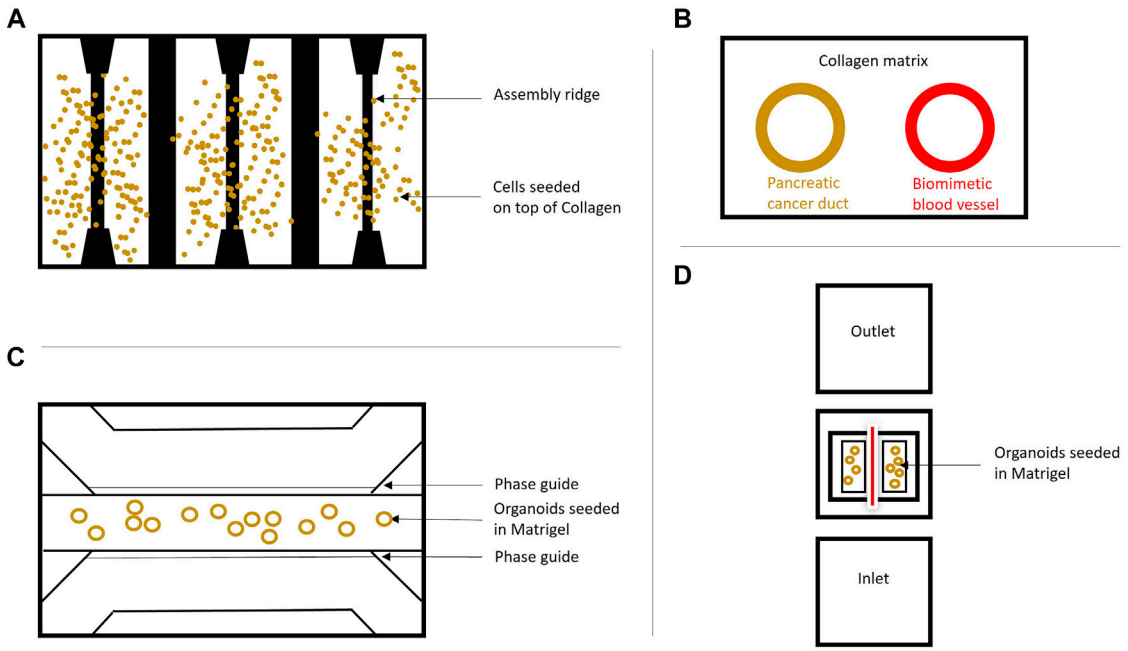


FIGURE 3 | Organ-on-a-Chip systems. **(A)** Schematic overview of the HepaChip used by Beer et al. (2017). The chip contains eight culture chambers, fluidic inlet and outlet and gold electrodes to simulate hydrodynamics. The surfaces were coated with collagen and three different cell types were seeded and compared to 2D and regular 3D cultures of these cells. This model shows that organs grown on a chip resemble the *in vivo* drug treatment behavior better than cells grown in 2D and 3D and supports the findings by Kramer et al. (2019). **(B)** Schematic cross-section of PDAC-on-a-chip containing pancreatic cancer cells forming a duct and endothelial cells forming a biomimetic blood vessel by Nguyen et al. (2019). This system was employed to study vasculature in PDAC. A similar system (dt-MOC) was developed by Bradney et al. (2020), where an epithelial cancer cell duct was created within a Collagen type I matrix to study intratumoral heterogeneity. **(C)** The microfluidic 3-lane OrganoPlate®, based on a 384-well plate containing 40 individual chips with three channels for perfusion and ECM separated by PhaseGuides™ can be used for growing Organoids on-a-Chip. The cells can be introduced into any of the three lanes depending on the purpose. Perfusion is ensured by the OrganoFlow®, which creates a height difference every few minutes at an adjusted angle to allow cell perfusion based on gravity leveling. This model was used by Kramer et al. (2019) to show a different treatment effect of chemotherapeutics in PDAC cells grown on a chip compared to monolayer culture. **(D)** Schematic InVADE platform used by Fook Lun Lai et al. (2020). Endothelial cells were suspended from the inlet and the outlet of the platform and unattached cells were removed upon perfusion. Attached HUVEC cells are represented in red, as they form a tube. The PDAC organoids were seeded in Matrigel within the middle chamber surrounding the endothelial cells. This model was established to show the importance of the tumor microenvironment in pancreatic cancer.

easier drug efficacy and toxicity screenings, which can in the long term enable treatment stratification and individualization. Moreover, this would also decrease the costs and duration of drug development. As research and development costs will decline upon fewer experiments needed in preclinical development due to better predictability, economic impacts will be lower during drug development studies with approximately 10–26% cost reduction. This would save several hundred million dollars per drug on the market (Franzen et al., 2019).

Although Organ-on-a-Chip platforms potentially provide an excellent tool to simulate the *in vivo* situation as closely as possible, there is still need for further development. A whole-body set-up as in animal models has not been modelled so far and thus physiological effects cannot be studied with current Organ-on-a-Chip platforms. Another limitation is that cultures cannot be maintained for a long time period which would be necessary to study long term treatment effects. In addition, there is still only a subset of cell types present, which does not fully recapitulate the cell-cell interaction and signaling within certain tissues. Moreover, high throughput of existing platform is not comparable to well-plates and thus this system will still find its main application in low throughput personalized patient treatment. Low throughput personalized treatment comprises patient cell sample isolation, expansion, Organ-on-a-Chip loading of cells and drug testing based on a patient's mutation status to quickly identify drug candidates (Esch et al., 2015). Thus, the selected treatment would be matched to a patient's disease status and thus treatment success can be guaranteed earlier as no other treatments have to be tested first in patients, thus potentially impacting patient survival. However, given the potential application of translational Organ-on-a-Chip based models, these platforms will likely in the long-term positively impact drug discovery and development programs making these more efficient and likely more cost effective.

As the technology matures and more scientists begin to use Organ-on-a-Chip platforms more data relevant for preclinical studies will become available and help improve the current platforms (Van den Berg et al., 2019).

DISCUSSION

PDAC remains one of the deadliest cancer types due to limited diagnosis at an early stage, multidrug resistance and its high metastatic potential into the liver and lung. Therefore, there is an urgent need for *in vitro* models that recapitulate tumor behavior more accurately and potentially also model patient specific responses. Organoids provide an outstanding tool for drug screening approaches in both academic proof-of-concept studies as well as in preclinical studies for replacement of 2D cell culture systems and animal testing. As numerous studies have suggested, morphological as well as functional (gene expression) differences occur between 2D and 3D models, better alternatives to 2D models are needed and organoids might in part overcome the gap between *in vitro* and *in vivo* responses. Also, for drug discovery programs as well as for personalized medicine it is necessary to be able to not only study intratumoral but also intertumoral heterogeneity, that exists between the diverse cell types and different patients.

The establishment of a "PDACoids" 3D system, which incorporates many different cell types and uses a synthetic matrix such as a hydrogel, thus eliminating batch-to-batch variation and potential pathogen transfer, is an important step to make personalized and preclinical research using organoids more robust (Dutta et al., 2017). Organoid experiments can be further expanded in Organ-on-a-Chip systems, which allow the incorporation of fluid flow and stromal cells for better tumor disease modelling. The major limitations of Organ-on-a-Chip platforms in personalized medicine approaches result from limited access to patient samples and corresponding clinical data as well as from relatively low-throughput and lacking automation of most Organ-on-a-Chip platforms.

Thus, PDAC-on-Chip models will likely evolve into low/medium-throughput platforms that will enable drug response studies and contribute to further development of the PDAC therapeutic field. Relevant PDAC models should include PDAC organoids that represent different patients in combination with a stromal cell compartment, represented by pancreatic stellate cells, cancer associated fibroblasts, and immune cells. The incorporation of lymphocytes into PDAC models is important to reveal potential reasons for failures of immunotherapeutic strategies and to model T cell function. The introduction of NK cells, DCs and macrophages might further allow to study receptor interactions and to provide a more complex *in vivo* like system. Also, the inclusion of endothelial cells for angiogenesis and lung and liver organoids for studying metastasis would favor a good simulation of the PDAC TME in conjunction with processes that impact tumor progression. Organ-on-a-Chip platforms could revolutionize PDAC research if improvements are made. High-throughput is still a limitation for most Organ-on-a-Chip platforms, whereas scalability of these assay units and automation are crucial for enabling larger drug screenings. Moreover, pump and tubing free methods whilst still assuring fluid flow will pioneer over their competitors, as these require lower maintenance, less time and are easier to handle. These platforms should also incorporate as many cell types as feasible to create a translational model and potentially replace animal models in the near future.

AUTHOR CONTRIBUTIONS

MG and KQ conceptualized and wrote the manuscript. MG created the figures.

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REVIEW
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Deconstructing Pancreatic Cancer Using Next Generation-Omic Technologies–From Discovery to Knowledge-Guided Platforms for Better Patient Management

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Comprehensive molecular landscaping studies reveal a potentially brighter future for pancreatic ductal adenocarcinoma (PDAC) patients. Blood-borne biomarkers obtained from minimally invasive "liquid biopsies" are now being trialled for early disease detection and to track responses to therapy. Integrated genomic and transcriptomic studies using resectable tumour material have defined intrinsic patient subtypes and actionable genomic segments that promise a shift towards genome-guided patient management. Multimodal mapping of PDAC using spatially resolved single cell transcriptomics and imaging techniques has identified new potentially therapeutically actionable cellular targets and is providing new insights into PDAC tumour heterogeneity. Despite these rapid advances, defining biomarkers for patient selection remain limited. This review examines the current PDAC cancer biomarker ecosystem (identified in tumour and blood) and explores how advances in single cell sequencing and spatially resolved imaging modalities are being used to uncover new targets for therapeutic intervention and are transforming our understanding of this difficult to treat disease.

Keywords: precision medicine, omic, pancreatic cancer, molecular profiling and subtyping, next-generation sequencing, single-cell 'omics, spatial transcriptomics, cancer biomarker

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INTRODUCTION

Large scale genomic sequencing of pancreatic ductal adenocarcinoma (PDAC) has revealed subgroups of patients that share clinically and biologically relevant molecular similarities (Jones et al., 2008; Biankin et al., 2012; Waddell et al., 2015; Bailey et al., 2016; Raphael et al., 2017; ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium 2020). An estimated 30–40% of PDAC tumours harbour an "actionable mutation" that can be matched to a known therapeutic regimen (Chantrill et al., 2015; Lowery et al., 2017; Aung et al., 2018; Singhi et al., 2019; Pishvaian et al., 2020). Precision oncology trials are now testing whether single biomarker drug combinations can be delivered in clinically relevant timeframes (Pishvaian et al., 2020). Beyond actionable mutations, "bulk" transcriptomic profiling has identified patient tumour subtypes with distinct biology that are associated with beneficial responses to standardised adjuvant chemotherapy (Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016; Raphael et al., 2017; Tiriac et al., 2018; O'Kane et al., 2019;

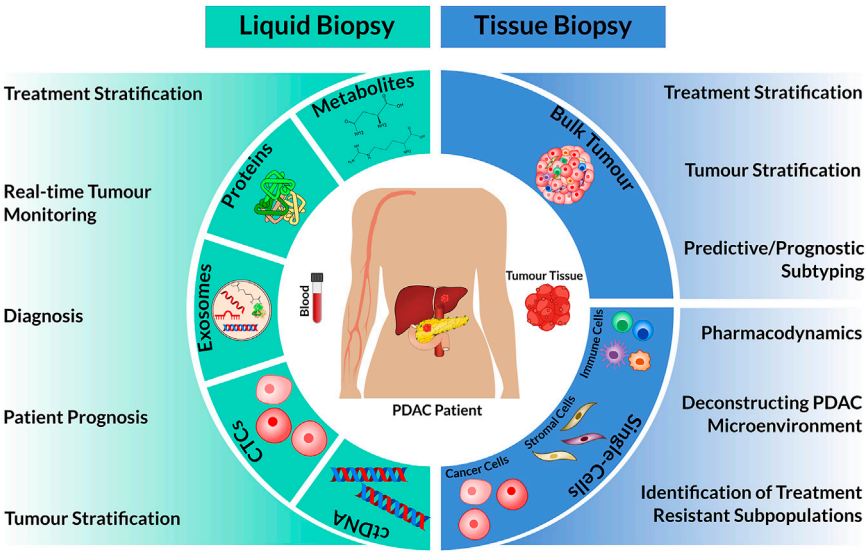

FIGURE 1 | Overview of patient materials isolated from liquid and tissue biopsies for omic analysis and their clinical utility. The circulating biomarkers: cell-free tumour DNA (ctDNA), circulating tumour cells (CTCs), exosomes, proteins or metabolites can be isolated and characterized from blood specimens. In comparison, tissue biopsies extract bulk tumour tissue, which can be used as a whole (Bulk Tumour) or dissociated into single cells. In combination with omic technologies, these biological materials can have diverse clinical use **(left and right)**.

Chan-Seng-Yue et al., 2020). Furthermore, transcriptomic signatures that are predictive of patient responses to standardised chemotherapy, e.g., gemcitabine, are now being tested in clinical trials to improve patient outcomes by identifying the optimal patient-specific chemotherapy.

Next generation single cell and single nucleus sequencing methods are augmenting "bulk" sequencing data to provide a comprehensive map of tumour cell communities and driver mutation dynamics. These methods are being deployed to investigate chemotherapy resistance and metastasis. In addition, advances in spatially resolved transcriptomics and multiplexed imaging modalities are providing fundamental information about neoplastic-immune cell interactions, with the potential to deliver precision immuno-oncology for PDAC.

Despite advances in surgical techniques and the optimisation of chemotherapy regimens, the overall 5-year survival for PDAC is only 10% (Siegel et al., 2021). PDAC is commonly diagnosed at advanced stages, at which time surgical resection is not an option (Mizrahi et al., 2020). As a result, there is considerable interest in the development of advanced genomic methodologies for the early detection of PDAC. The detection of somatic mutations and methylation signatures in circulating tumour DNA (ctDNA), which can be isolated from patient blood, provides an important first step in the development of minimally invasive blood tests for early PDAC diagnosis. In addition, the advancement of methods for the detection and characterisation of other blood-borne analytes will enable real-time monitoring of therapy response without the need for invasive biopsies.

The development and implementations of precision oncology for PDAC requires the appropriate deployment of cancer biomarkers into routine clinical care. Cancer biomarkers detected in tissue biopsies and/or blood or other fluids can help to diagnose early PDAC or its recurrence, be prognostic or predict a patient's response to specific drugs (**Figure 1**). Unfortunately, patient management for PDAC is currently hampered by a lack of defining biomarkers. This review examines the current PDAC cancer biomarker ecosystem and describes new "omic" technologies that promise to uncover new prognostic biomarkers by deconstructing patient tumours at single cell resolution.

GENOMIC PROFILING IDENTIFIES DRIVER EVENTS AND ACTIONABLE MUTATIONS IN PDAC

Large scale genomic sequencing studies have transformed our understanding of the genomic events that shape PDAC (Jones et al., 2008; Biankin et al., 2012; Waddell et al., 2015; Bailey et al., 2016; Raphael et al., 2017; Connor et al., 2019; ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium 2020). The PDAC mutational driver landscape is dominated by recurrent, predominantly overlapping mutations in KRAS, TP53, SMAD4, and CDKN2A (>50%) with a subset of additional genes including KDM6A, MLL3, ARID1A, TGFBR2, RBM10, and BCORL1 recurrently mutated in 5–10% of patient samples (Waddell et al., 2015; Bailey et al., 2016; Collisson et al., 2019). Most single gene biomarkers that can be matched to a drug such as ERBB2 amplification, BRAF gene fusions/mutations and mutations in DNA damage repair (DDR) genes BRCA1, BRCA2, or PALB2 are found in less than 4% of patients (Harada et al., 2008; Jones et al., 2008; Biankin et al., 2012; Witkiewicz et al., 2015).

Aggregation of genomic aberrations into common pathways and/or based on therapeutic intervention has helped to identify several "actionable molecular phenotypes" that are currently under clinical investigation (Collisson et al., 2019). A meta-analysis of 21,842 PDAC genomes has estimated that the pooled prevalence of germline and somatic mutations in DNA damage response genes (i.e., BRCA1, BRCA2, PALB2, ATM, ATR, CHEK2, RAD51, and FANC) that cause homologous recombination deficiency (HRD) is between 14.5 and 16.5% (Casolino et al., 2021). Recent evidence also suggests that surrogate biomarkers of HRD, such as unstable genomes (determined by SV analysis) and BRCA mutational signatures (BRCAness phenotype) may identify even greater numbers of HRD patients (24–44%) (Waddell et al., 2015; Casolino et al., 2021). HRD is a putative biomarker of therapeutic response to DNA damaging agents such as platinum and PARP inhibitors suggesting that a large segment of the PDAC patient population may benefit from these therapies (Collisson et al., 2019; Casolino et al., 2021).

Germline testing for the identification of known hereditary cancer syndromes, e.g., BRCA1/2 is increasingly recommended for patients with PDAC (Margaret A. Tempero et al., 2021). FOLFIRINOX (5-Fluorouracil, Irinotecan, and Oxaliplatin) has been shown to elicit significant responses in advanced PDAC patients who harbour germline BRCA1 and BCRA2 mutations (Golan et al., 2014; Wattenberg et al., 2020). Olaparib is FDA-approved for maintenance therapy in germline BRCA1/2 PDAC patients after platinum-based chemotherapy with response or stable disease (Golan et al., 2014). Maintenance trials with other PARP inhibitors are ongoing, with preliminary data showing improved responses in patients with advanced platinum sensitive BRCA or PALB2 mutated PDAC after treatment with Rucaparib (Rubraca) as a maintenance monotherapy (NCT 03140670).

Therapeutically targeting HRD may extend beyond germline carriers to patient groups harbouring somatic mutations in DNA damage repair genes or that exhibit a "BRCAness phenotype" (Casolino et al., 2021). Phase II precision oncology trials are now assessing whether single agent Olaparib therapy is effective in advanced PDAC patients that do not harbour germline BRCA1/2 mutations (NCT02677038). In addition, several early phase clinical trials are testing whether PARP inhibitors in combination with either standard chemotherapy (NCT03337087; NCT04228601) or other targeted therapies (NCT03842228; NCT04005690; NCT03682289) are effective in treating germline and/or somatic HRD in advanced PDAC (Singh et al., 2021).

Several alternative therapeutic strategies targeting HRD PDAC are also currently being explored. Patient-derived cell lines (PDCLs) harbouring germline and/or somatic mutations in BRCA1, BRCA2, PALB2 are sensitive to cell cycle checkpoint inhibitors specific for CHECK1, WEE1, ATR, and PLK4 (Dreyer et al., 2021). Synergies between PARP and ATR or DNA PKcs inhibitors can lead to synthetic lethality in ATM-deficient Genetically Engineered Mouse Models (GEMMs) and PDAC cell lines (Gout et al., 2021). Lessons from ovarian cancer also suggest that concurrent treatment of cell lines with PARP inhibitors followed by cell cycle checkpoint inhibitors can induce DNA damage and replication stress in both HRD and non-HRD tumour cells (Fang et al., 2019). High replication stress is commonly associated with greater sensitivity to cell cycle checkpoint inhibitors (Fang et al., 2019; Dreyer et al., 2021). Recent evidence in PDAC PDCLs demonstrates that cells having a Basal-like transcriptomic subtype (see below) may exhibit higher endogenous replication stress than those having a Classical subtype (Dreyer et al., 2021). Importantly, Basal-like PDCLs were found to exhibit greater sensitivity to cell cycle checkpoint inhibitors in a non-HRD dependent manner. These studies suggest that sequential treatment of PDAC with DNA damaging agents, either via standardised chemotherapy or PARP inhibitors, followed by cell cycle checkpoint inhibitors may be an effective treatment strategy for PDAC. In addition, stratification of patients by transcriptomic subtype may augment treatments that exploit replication stress.

TRANSCRIPTOMICS–IDENTIFICATION OF PROGNOSTIC AND PREDICTIVE OMIC SUBTYPES USING BULK AND LCM-DERIVED PATIENT MATERIAL

The recent identification of intrinsic transcriptomic subtypes of PDAC provides an alternative strategy for patient treatment selection–reviewed in depth by Xu Z et al. (2021). Transcriptomic profiling has identified 2 broad intrinsic PDAC classes, namely Classical and Basal-like with Basal-like tumours associated with significantly poorer outcomes and late-stage disease (Moffitt et al., 2015; Collisson et al., 2019). These classes are delineated by the differential expression of pancreatic specific transcription factors, such as GATA6, PDX1, and HNF1A, that act to specify and maintain Classical pancreatic identify and which are lost in Basal-like tumours (Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016; Raphael et al., 2017).

Findings from both COMPASS trial and pre-clinical models of PDAC demonstrate that Basal-like tumours are less likely to respond to standard-of-care chemotherapy than Classical tumours (Tiriac et al., 2018; Chan-Seng-Yue et al., 2020; Rashid et al., 2020). Transcriptional profiling of pancreatic cancer patient-derived organoids has identified treatment stratification signatures (TSS) that can identify best responders to gemcitabine-based therapy, or mFOLFIRINOX (Tiriac et al., 2018). When retrospectively applied to transcriptomic data from laser capture micro-dissected tumour tissues, obtained from either resected or advanced cases, these TSS were able to predict improved responses to mFOLFIRINOX or gemcitabine/nAb-Paclitaxel. Basal-like tumours were found to be significantly enriched in the mFOLFIRINOX non-sensitive group (Tiriac et al., 2018). As a corollary, it has recently been shown that GATA6 expression in tumours from patients with advanced disease (using RNA *in situ* hybridization) can discriminate Classical and Basal-like tumours. Importantly, the best progression-free survival in patients with advanced disease

was found in GATA6-positive Classical tumours given mFOLFIRINOX (O'Kane et al., 2020).

Detailed integrative analysis (combing both transcriptomic and genomic analyses) has demonstrated that while transcriptomic profiling can stratify PDAC into Classical and Basal-like subtypes the genomes of tumours falling within these same transcriptomic subtypes may exhibit considerable intra-tumoral heterogeneity (ITH) (Chan-Seng-Yue et al., 2020). Recent evidence demonstrates that both the Classical and Basal-like subtypes comprise at least 2 distinct subclusters that are driven by specific copy number (CN) gains in genes such as mutant KRAS and GATA6. Minor and major KRAS CN imbalances can further stratify the Basal-like subtype into 2 distinct subclusters, with minor KRAS CN imbalances associated with Primary disease (Stages I–III) and major KRAS CN imbalances linked to metastasis (Stage IV) and Squamous-like gene expression programmes. Importantly, Stage IV tumours with Major mutant KRAS CN gains were found to be significantly more resistant to FOLFIRINOX compared to those with minor mutant KRAS CN gains (Chan-Seng-Yue et al., 2020). These findings are incredibly informative in that they support a model of PDAC in which ongoing genomic instability during disease progression gives rise to different molecular phenotypes.

These findings also point to the potential utility of GATA6 and KRAS CN gains as biomarkers of disease progression, recurrence and/or opportunity for selective therapy (Miyabayashi et al., 2020).

EPIGENOMIC PROFILING FOR THE IDENTIFICATION OF THERAPEUTIC OPPORTUNITIES AND PATIENT PROGNOSIS

The 2 major transcriptomic subtypes of PDAC are defined by distinct epigenetic states. Bailey et al. were the first to demonstrate that distinct DNA methylation patterns define the Squamous and Classical subtypes. Squamous subtype tumours were found to exhibit a pattern of CpG DNA methylation significantly correlated with the downregulation of pancreatic specific transcription factors that control pancreatic cell fate determination (PDX1, GATA6, and HNF1A), and the activation of multigene programmes controlled by MYC and ΔNTP63 that drive Squamous-like differentiation (Bailey et al., 2016).

Lomberk et al., generated comprehensive chromatin state maps to characterise the epigenetic landscape of patient-derived PDAC tumour xenograft models (Lomberk et al., 2018). This analysis demonstrated that critical regulatory hubs, so called "super-enhancers", exhibit subtype specific activity. Mapping of super-enhancers to genes in the Classical subtype demonstrated that active super-enhancers regulate the expression of pancreatic specific TFs such as GATA6, HNF4A, FOS, FOXP1, FOXP4, KLF4, ELF3, and CUX1. In contrast, Basal-like specific super-enhancer regulation was associated with the hepatocyte growth factor receptor MET, MYC, and ΔNTP63. Interestingly, MET inhibition in Basal-like tumours resulted in a switch from Basal-like to Classical-like gene expression programmes. Subsequent mechanistic studies demonstrated that ΔNTP63 binds to the distal enhancer elements of genes important for Squamous-like differentiation (Somerville et al., 2018). Over-expression of ΔNTP63 in Classical cell lines was sufficient to reprogramme Classical cells towards a Squamous-like state. Together these studies implicate super-enhancers and the TFs that they regulate as critical determinants in PDAC subtype-specific gene expression. This data also suggests that phenotypic heterogeneity may be regulated by epigenetic elements and that these elements may be modulated by therapy (Lomberk et al., 2018). Therapies designed to modify the PDAC epigenome may, therefore, expose a therapeutic vulnerability for subsequent targeted intervention. Alternatively, therapies may reprogramme the epigenome and induce aggressive phenotypes or contribute to therapy resistance. Whether therapy induced epigenetic changes are hard wired or can be rewired to generate an original phenotype remains to be determined.

Mutations in chromatin modifiers, such as KDM6A, ARID1A, ARID1B, PBRM1, SMARCA2, PBRM1, SMARCA2, SMARCA4, or MLL2 are a feature of PDAC (Waddell et al., 2015). Mutations in KDM6A are enriched in the Squamous transcriptomic subtype, underpinning the importance of chromatin modifiers in shaping PDAC epigenetic states (Bailey et al., 2016). A Genetically Engineered Mouse Model of PDAC demonstrates that genetic inactivation of KDM6A rewires the epigenetic landscape of PDAC causing the aberrant activation of super-enhancers regulating the expression of ΔNTP63, MYC, and RUNX3. Activation of these TFs may subvert pancreatic identity and induce Squamous-like differentiation phenotypes (Andricovich et al., 2018). This work also demonstrates that KDM6A null tumours can be selectively targeted by the (Bromodomain and Extra Terminal) BET inhibitor JQ1. The actionability of mutations in other chromatin modifiers remains an open question. In other cancer types, cells harbouring mutations in ARID1A have been shown to exhibit defective DNA damage repair and increased sensitivity to ATR and PARP inhibitors (Williamson et al., 2016). Furthermore, ARID1A mutations have been demonstrated to induce immune phenotypes susceptible to immune checkpoint inhibitors in orthotopic and intraperitoneal ovarian cancer mouse models (Shen et al., 2018). Mutations in chromatin modifiers are found in up to 10% of all PDAC and represent a large yet underexplored therapeutic opportunity for PDAC.

Recent evidence also implicates the 5-methylcytosine hydroxylase TET2 as a critical modulator of PDAC epigenetic states (Eyres et al., 2021). Genome-wide epigenetic mapping of 5-hydroxymethylcytosine (5 hmc) DNA modifications in resected PDAC samples demonstrated that Squamous-like PDAC is associated with loss of 5 hmc at pancreatic specific gene loci, including GATA6. Loss of 5 hmc was directly associated with the concomitant downregulation of TET in Squamous PDAC samples. Moreover, TET2 expression was linked to SMAD4 mutational status, with SMAD4 inactivation associated with

loss of 5 hmc and downregulation of GATA6. Stabilising TET2 expression by treatment with metformin and vitamin C was sufficient to restore 5 hmc and GATA6 levels and increase biomarkers of Classical PDAC. These findings further highlight the importance of epigenetic mechanisms in governing PDAC transcriptional phenotypes and the ability of epigenetic states to be modulated by small molecules (Eyres et al., 2021).

Divergent epigenetic states not only discriminate PDAC subtypes, but also underpin tumour progression and metastases (McDonald et al., 2017; Lomberk et al., 2019). Combined genomic and epigenomic analyses using low passage cell lines derived from matched primary and metastatic PDAC demonstrated that cell lines derived from distant metastatic sites exhibit widespread epigenetic reprogramming when compared to cells of the primary tumour. Metastatic cell lines exhibited epigenetic programmes consistent with increased glycolytic dependency via the oxidative branch of the pentose phosphate pathway (oxPPP). Importantly, epigenetic reprogramming was observed in the absence of metastasis-specific driver mutations suggesting that epigenetic changes alone may drive adaption to metastatic niches (McDonald et al., 2017).

Brunton et al. used Assay for transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) to identify additional PDAC subgroups with potential therapeutic utility. This study demonstrated that differential chromatin accessibility, as assessed by ATAC-seq, can predict responsiveness and tolerance to GSK3β inhibitors in the Squamous subtype of PDAC (Brunton et al., 2020). ATAC-seq has also been applied to resected PDAC samples to identify a chromatin accessibility signature predictive of disease-free survival. Together these studies provide compelling evidence that epigenetic states play an important role in PDAC progression and response to therapy. The application of methodologies, such as ATACseq, and newly developed ATAC-array (Dhara et al., 2021), in different clinical settings, may uncover important new therapeutic opportunities for yet undefined patient subgroups.

DECONSTRUCTION OF PDAC AT SINGLE CELL RESOLUTION

Defining Actionable Segments Using scRNAseq and Spatial Transcriptomics

The modelling of PDAC subtypes from bulk tumour RNA profiling has been challenged by a complex admixture of expressed transcripts representing a diversity of normal and neoplastic cell types (Moffitt et al., 2015; Bailey et al., 2016; Collisson et al., 2019). PDAC classification schemes using bulk RNAseq have either attempted to define PDAC subtypes by including all expressed transcripts–and modelling tumour complexity as a whole or by enriching epithelial-derived cancer signals using informed informatic approaches or via laser capture microdissection (LCM) of the cancer-epithelium from abundant cancer stroma (Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016; Raphael et al., 2017; Puleo et al., 2018; Maurer et al., 2019; Chan-Seng-Yue et al., 2020).

The consensus classification of PDAC into 2 broad tumour cell intrinsic transcriptomic subtypes, namely Classical and Basal-like, is largely based on the later approach which favours the enrichment of gene transcripts that are representative of neoplastic gene programmes (Moffitt et al., 2015). Classification schemes that have defined additional subtypes such as aberrantly differentiated endocrine exocrine (ADEX), exocrine-like and/or immunogenic have been contested on the basis that these subtypes represent non-malignant stromal or normal cell contamination (Collisson et al., 2011; Bailey et al., 2016). Despite these criticisms, there is still some debate about the contribution of "normal" exocrine-like tumour tissue to PDAC. Further, the relationship between neoplastic cells and stromal cells remains unclear, although there is now a greater appreciation that diverse signals from epithelial-derived cancer cells shape the PDAC tumour microenvironment (Tape et al., 2016; Candido et al., 2018).

The 2-subtype consensus classification for PDAC, therefore, belies a complex tumour architecture made up of diverse cell types including "normal" adjacent, neoplastic, endothelial, Cancer-Associated Fibroblasts (CAFs) and immune cells. The contribution of these different cell types to PDAC, their inter-relationships and the importance of CAF and immune cells as mediators of standardised chemotherapy remain critical questions. single cell RNAseq (scRNAseq) is helping to address these questions by deconstructing tumours at single cell resolution (Peng et al., 2019; Hwang et al., 2020; N. G.; Steele et al., 2020; Hutton et al., 2021; Raghavan et al., 2021; Chen et al., 2021). Used in combination with spatially resolved transcriptomics and state-of-the-art multiplexed imaging platforms, new insights are emerging about neoplastic phenotypes (subtypes), the interplay between neoplastic and stromal cell populations and the evolution of resident tumour cell populations during disease progression, metastasis and in response to therapy (Raghavan et al., 2021; N. G.; Steele et al., 2020).

PDAC Subtypes at Single Cell Resolution

scRNAseq analysis of treatment naïve PDAC has demonstrated that Classical and Basal-like neoplastic cell phenotypes co-exist in the same tumour. Single cell profiling of biopsied liver metastatic lesions and primary PDAC has identified "hybrid" neoplastic cell populations that share common Classical and Basal-like gene expression profiles, corroborating some earlier bulk RNA expression studies (Chan-Seng-Yue et al., 2020; Hwang et al., 2020). Informatic approaches that infer the trajectories of single cell populations have also revealed that Classical and Basal-like neoplastic phenotypes may represent a dynamic disease continuum with the Basal-like state enriched for mesenchymal and stem-like programmes (Hwang et al., 2020; Raghavan et al., 2021). Recent findings have demonstrated that PDAC neoplastic cells may undergo dynamic phenotypic transitions after chemoradiation therapy (CRT) (Hwang et al., 2020). Single nucleus RNAseq analysis of treatment naïve resected and neoadjuvant CRT patient samples demonstrated a shift from a

Classical-like cell phenotype towards an "induced" Basal-like phenotype following CRT (Hwang et al., 2020). This finding suggests that therapeutic resistance may involve a transition towards more Basal-like states and is supported by earlier *ex vivo* findings demonstrating Basal-like phenotypic transitions following treatment of PDAC cell lines with FOLFIRINOX (Porter et al., 2019). Interestingly, CRT was also found to selectively enrich for a subset of neoplastic cells that exhibit acinar and neuroendocrine-classical like phenotypes. These cell phenotypes are reminiscent of the ADEX subtype, identified in bulk RNA studies, and are defined by overlapping sets of genes (Bailey et al., 2016; Hwang et al., 2020).

PDAC TME and Spatially Resolved Targetable Interactions

PDAC Immune Targets

The PDAC tumour microenvironment (TME) plays a fundamental role in disease progression and response to therapy (Candido et al., 2018; Sahai et al., 2020; N. G.; Steele et al., 2020; Hutton et al., 2021). The PDAC TME is generally considered "immunologically cold" with a scarcity of $CD8^+$ T cells and a highly immunosuppressive microenvironment rendering most tumours recalcitrant to immunotherapy (Neesse et al., 2015; Ho, Jaffee, and Zheng 2020).

The deconvolution of bulk RNA data using validated gene signatures, that define specific immune cell types and/or phenotype, has demonstrated that T cell, B cells, and myeloid cells contribute to complex patient immune profiles. Immunophenotyping of PDAC samples using multimodal single resolution methodologies, such as scRNAseq, spatial transcriptomics and multiplexed immunofluorescence, has revealed that Classical and Basal-like cell phenotypes are associated with distinct immune microenvironments. Principally, Basal-like cells are associated with increased macrophage infiltration and loss of cytotoxic T cell subsets in both primary and metastatic micro-niches (Raghavan et al., 2021). These findings suggest that Basal-like microenvironments may respond to immunotherapies that specifically target tumour-associated macrophages, such as CSF1R inhibitors (Zhu et al., 2014). Genetic context may be important here and the ability of DDR and MSI tumours to drive distinct immune profiles independent of an established neoplastic subtype remain unknown.

Tumour infiltrating T cells are associated with increased overall survival in PDAC and can potentially predict immunotherapy response (Carstens et al., 2017; Lohneis et al., 2017). Single cell analysis has demonstrated that $CD8^+$ T cell tumour infiltration is inversely correlated with myeloid cell enrichment (N. G. Steele et al., 2020). This analysis has also revealed that tumour infiltrating $CD8^+$ T cells exhibit exhausted phenotypes and that diminished $CD8^+$ T cell fitness increases with disease progression. Exhausted $CD8^+$ T cell signatures were associated with increased expression of the immune checkpoint TIGIT. The ligand for TIGIT, PVR was expressed in tumour, endocrine, endothelial cells and myeloid subsets, supporting the observation that myeloid cells promote immunosuppression in PDAC. Single cell data also demonstrates that immune checkpoint receptors are heterogeneously expressed between patients (N. G. Steele et al., 2020). In addition, recent studies have established that primary tumour and metastatic lesions support distinct immune infiltrates, which is largely heterogeneous between patients and also shaped by the metastatic niche (C. W. Steele et al., 2016; Lin et al., 2020; Raghavan et al., 2021; Ho et al., 2021). These data highlight the complexity of individual patient immune microenvironments and suggest that therapeutic approaches targeting immune checkpoints may need to be tailored to individual PDAC patients (N. G. Steele et al., 2020). It is also presently unclear how immune microenvironments change during patient treatment. Therefore, longitudinal single cell studies charting fluctuations in immune infiltrates and neoplastic-immune cell interactions will be an important next step in the development of new immunotherapies for PDAC.

Multiple studies have demonstrated that genomic drivers of PDAC, such as KRAS and MYC, can modulate the TME and support highly immunosuppressive microenvironments (Dey et al., 2020; Muthalagu et al., 2020; Ischenko et al., 2021). Biomarkers of immunotherapy response, however, are limited in PDAC. Hypermutated mismatch repair-deficient tumours which make up around 1% of PDAC are likely to respond to immune checkpoint inhibition (Le et al., 2017). While BRCA1 and BRCA2 deficient tumours are associated with increased immune infiltrates, rates of response to ICI are low. Recent evidence in mouse models of breast and colorectal cancer, suggest that BRCA2-deficient tumours are more susceptible to ICIs than BRCA1-deficient tumour (Samstein et al., 2020; Zhou and Li 2021). In addition, loss of CDKN2A, which is a feature of PDAC, has been identified as a biomarker of immune checkpoint therapy resistance in urothelial carcinoma (Nassar et al., 2021). These studies highlight a diversity of genomic events that may drive differential responses to ICIs and suggest that immunotherapy based on a matched genomic biomarker may be complex.

PDAC CAF Targets

CAFs are key components of the tumour microenvironment and are emerging as important therapeutic targets. CAFs are implicated in diverse functions such as matrix deposition and remodelling, reciprocal signalling with neoplastic cells and crosstalk with infiltrating T cells (Sahai et al., 2020). Reciprocal signalling between CAFs and PDAC cell lines grown in co-culture has been shown to promote epigenetic changes in PDAC cells and induce transcriptional and metabolic programmes that intersect with oncogenic KRAS signalling cascades (Tape et al., 2016). $KRAS^{G12D}$ PDAC cell lines grown in co-culture with heterotypic fibroblasts exhibit total proteomes and phospho-proteomes that are distinct from $KRAS^{G12D}$ PDAC cells grown autonomously. The reciprocal signalling between $KRAS^{G12D}$ PDAC cells and fibroblasts involves an IGF1R/AXL-AKT axis that mediates PDAC cell proliferation and apoptosis. The identification of IGF1R/AXL-AKT signalling cascades in reciprocally engaged tumour cells has uncovered additional therapeutic targets acting downstream of

oncogenic $KRAS^{G12D}$ and further reinforces the importance of understanding therapy in the context of complex heterocellular TMEs (Tape et al., 2016).

Single cell analyses of both murine and human PDAC have identified 3 distinct CAF cell populations, referred to as myofibroblastic CAFs (myCAFs); inflammatory CAFs (iCAFs); and antigen presenting CAFs (apCAFs) (Sahai et al., 2020). The complex roles attributed to these CAF populations are only just starting to be resolved, however, a link between myCAF phenotypes and tumour promotion is emerging. CAF myofibroblast programmes mediate immunosuppressive TMEs and may be enriched after CRT (Hwang et al., 2020). A limited clinical trial broadly targeting fibroblasts in PDAC was terminated due to disease acceleration (NCT01130142). Further studies are, therefore, required to understand CAF phenotypic heterogeneity and their role in inducing reciprocal signalling cascades in engaged tumour cells before stroma-targeting therapies are a reality in PDAC.

FUTURE -OMIC APPROACHES FOR UNDERSTANDING INTRA-TUMORAL HETEROGENEITY AND SPATIALLY RESOLVING PDAC TUMOURS

The characterisation of PDAC intra-tumoral heterogeneity (ITH) is in its nascent stages. ITH represents an important clinical challenge, as it provides a pool of tumour cell intrinsic variation that may drive cancer progression and lead to the emergence of drug resistance subclones (Gerstung et al., 2020; Dentro et al., 2021). Sub-clonal drug resistance and associated driver mutations are common and often emerge after therapy. Emerging studies in other cancer settings demonstrate that tumour ITH is complex and involves a combination of genomic, transcriptomic, and epigenetic programmes. To fully elucidate the scale and importance of ITH in PDAC integrated multi-omics approaches will be required to deliver a systems level view of tumour evolution and its vulnerabilities.

Profiling chromatin accessibility at single cell resolution using scATAC-seq provides a window into underlying epigenetic regulatory mechanisms that drive cell type differences. scATAC-seq can identify global changes that are not readily apparent at the transcriptome-level and has revealed rare subpopulations of tumour cells that result in therapeutic resistance and/or metastatic progression (Satpathy et al., 2019; Lim et al., 2020; Xu K et al., 2021). The integration of scRNA-seq and scATAC-seq data obtained from the same sample provides highly complementary information about tumour cell transcriptional programmes and the underlying epigenetic regulatory mechanisms that drive them. Recent studies integrating both scRNA-seq and scATAC-seq, have charted cell state changes between precursor lesions and primary disease and have defined complex cellular interactions along a continuum of neoplastic phenotypes (Satpathy et al., 2019). The application of these methodologies to PDAC will likely uncover new phenotypic states and help to determine whether Classical and Basal-like cells exist as a disease continuum. Moreover, complex regulatory information will provide new mechanistic insights into the evolution of PDAC and the selection of drug resistant cell populations.

Beyond spatially resolved transcriptomics new spatial genomic methodologies are emerging. Base Specific *In Situ* Sequencing (BaSISS) uses multiplexed highly sequence specific padlock probes to detect and visualise mutant and wild type alleles in fixed tissue specimens (Lomakin et al., 2021). Multiplexed with existing spatial transcriptomic and imaging modalities BaSiSS can generate large scale quantitative maps of cancer clones harbouring defined mutations. For the first time, specific cancer clones can be imaged and mapped to distinct histological features and associations between specific clones and stromal cell subsets followed over the course of a patient's treatment journey (Lomakin et al., 2021).

New Mass Spectrometry Imaging (MSI) methods are also now available that allow simultaneous visualisation of drugs, their metabolites, and endogenous compounds within tissue samples. MSI methods such as Matrix-assisted laser desorption ionization (MALDI) or Desorption Electrospray ionisation (DESI) can generate spatially resolved images of specific analytes using fresh frozen tissue sections (Cornett et al., 2007; Inglese et al., 2017; Vaysse et al., 2017). H&E staining, multiplexed immunofluorescence or imaging mass spectrometry can be applied to sequential tissue sections to develop a composite picture of the types of cells, histological features and drug metabolites associated with a defined region of interest. The integration of MSI with complementary imaging modalities will provide important insights into drug pharmacodynamics within complex tissue architectures and highlight distinct tumour regions associated with previously define biomarkers.

CIRCULATING BIOMARKERS IN PDAC

The rapid identification of circulating biomarkers, obtained from a liquid biopsy, e.g., routine venous phlebotomy, has the potential to facilitate real-time monitoring of a patient's treatment journey, from early diagnosis to response to therapy. The most extensively used blood-borne biomarker for PDAC is Carbohydrate Antigen 19-9 (CA19-9), which is primarily used to establish a primary diagnosis and to determine recurrence or progression of disease after therapy. CA19-9, however, is limited in its utility as an effective biomarker for PDAC for several reasons. Firstly, it is not specific for PDAC and is elevated in other cancers and benign conditions such as biliary obstructions. Secondly, it has no utility in approximately 10% of Caucasians and 22% of African Americans who are Lewis antigen negative (M. A. Tempero et al., 1987; Poruk et al., 2013; Goonetilleke and Siriwardena 2007).

Circulating blood-borne biomarkers including circulating tumour cells (CTCs), tumour DNA (ctDNA), and extra-cellular vesicles such as exosomes can all be identified in a blood sample. Rapid advancement in the detection and characterisation of these biomarkers, in PDAC and other cancers, point to their potential usefulness in early disease

detection and monitoring of therapy response (Crowley et al., 2013; J. Lee et al., 2019; Gall et al., 2019; Alix-Panabières and Pantel 2021; Li et al., 2021).

Circulating Tumour Cells

Local tissue and lymphatic invasion are common features of early PDAC. The dissemination of cancer cells from the site of primary lesion to surrounding tissues and ultimately distant organs is thought to involve circulating tumour cells (CTCs). CTCs are shed from the primary tumour into the bloodstream and are likely to be enriched for metastatic precursors (Lozar et al., 2019). CTCs are very rare, however, technical advances in methods for CTC isolation now allow for the molecular profiling of CTCs using diverse omic approaches (Yu et al., 2012; Ting et al., 2014; Jiang et al., 2015). The molecular profiling of CTCs, therefore, offers a non-invasive opportunity to identify novel biomarkers for early PDAC detection (J. Lee et al., 2019). In addition, as CTCs represent metastatic precursors molecular profiling may identify drivers of early recurrence and help to develop targeted therapies that inhibit metastatic disease (Gall et al., 2019).

CTCs have been found at all stages of PDAC. Pre-cancerous human pancreatic intraductal papillary mucinous neoplasms (IPMNs) release CTCs in comparable quantities to localised early PDAC (Court et al., 2018). Importantly, several studies have demonstrated that CTCs serve as prognostic biomarkers for PDAC. CTC positivity in the peripheral blood of PDAC patients is significantly correlated with shorter progression free survival and the development of metastases (Bidard et al., 2013; Court et al., 2018). The ability of CTCs to predict therapeutic response is unclear, although pharmacogenomic models of PDAC suggest that CTC profiling can identify patient responders for specific chemotherapy regimens.

Best estimates suggest that CTCs exist at a ratio of one to ten cancer cells per 10 billion normal blood cells in a mL of blood (Miller et al., 2010). CTCs are therefore typically enriched using phenotypic properties, such as size or density, or based on specific cell surface markers (Lianidou et al., 2014; Martini et al., 2019; J.; Lee et al., 2019). Enrichment of CTCs by flow cytometry or microfluidic systems provides sufficient material for omic analysis including WGS (Jiang et al., 2015), RNA-seq (Yu et al., 2012), or single-cell sequencing (Ting et al., 2014). The development of CTC-based biomarkers, therefore, may extend beyond simple CTC enumeration to mutational and expression profiling.

Transcriptomic profiling of CTCs isolated from Genetically Engineered Mouse Models (GEMM) of PDAC identified WNT signalling and aberrant extracellular matrix expression (ECM) as drivers of PDAC metastasis (Yu et al., 2012). Single cell sequencing of CTCs isolated from patients with locally advanced or metastatic pancreatic cancer corroborated findings in murine models showing specific upregulation of the ECM gene SPARC. SPARC expression was highly correlated with the expression of ZEB1 and vimentin, master regulators of Epithelial-to-Mesenchymal transition (EMT) (Lapin et al., 2017).

Franses et al. have recently performed RNA-sequencing of CTCs isolated from the blood of healthy donors, patients with either treatment naïve localised PDAC or metastatic PDAC (Franses et al., 2020). Differential gene expression analysis demonstrated that Mucin (MUC) genes, MUC3A, MUC4, MUC16, and MUC17 genes are significantly enriched in CTCs isolated from treatment naïve localised PDAC and metastatic PDAC versus normal controls. Importantly, correlation analysis identified 3 major subgroups of samples designated as LGALS3-high, WNT5A-high and LIN28B/KLF4-high. These genes are known as "stemness" markers and have been demonstrated to play a role in EMT. Of the "stemness" markers identified, LIN28B alone was found to be prognostic for poor survival. LIN28B plays an important role in modulating stem-like states by binding and blocking the maturation of let-7, a well-characterized tumour suppressor miRNA that targets multiple oncogenes. CRISPR-mediated silencing of LIN28B in cell lines and mouse model systems resulted in less aggressive metastatic phenotypes, primarily through the concomitant upregulation of let-7. CRISPR knock-out of let-7 target HGMA2 or chemical inhibition of LIN28B/let-7 binding mimicked the metastatic phenotypes observed in LIN28B CRISPR models. These results support the development of drugs targeting LIN28B/let-7 phenotypes for the control of metastatic disease.

Future strategies that utilise omic profiling of CTCs will provide important biomarkers and targets for therapeutic development, especially in the identification and control of early metastatic dissemination. The extension of these studies to longitudinal patient cohorts will likely expand our understanding of CTC evolution and identify additional biomarkers associated with therapeutic response. A significant drawback for the use of CTCs as an effective "liquid biopsy" is that they are exceedingly rare in standard blood draws.

Circulating Tumour DNA

Cell-free tumour DNA comprises a range of extracellular DNA including the fragments of circulating tumour DNA (ctDNAs). ctDNAs are released into the bloodstream by dying tumour cells and can be isolated from patient plasma providing a clinically relevant liquid biopsy analyte for tumour genotyping (Cohen et al., 2018; Jaworski et al., 2020). Somatic mutations and copy number alterations can be detected in ctDNAs by targeted gene sequencing or droplet digital PCR (Sausen et al., 2015; Botrus et al., 2021). These methodologies allow the detection of rare ctDNA mutations with MAFs as low as <0.2% (Le Calvez-Kelm et al., 2016). Whole-genome or whole-exome approaches for ctDNA analysis are complicated by low tumour cell fractions in ctDNA isolates and have not been widely applied in PDAC ctDNA analyses.

The mutational landscape of PDAC is well characterised, with oncogenic mutations in KRAS found in greater than 90% of PDAC (Biankin et al., 2012). The utility of KRAS ctDNA mutations as a prognostic marker has been demonstrated in several studies. Detection of KRAS mutant alleles in PDAC ctDNAs is associated with significantly poorer disease-free and overall survival (Hadano et al., 2016; Tjensvoll et al., 2016; Kruger et al., 2018; Perets et al., 2018; Bernard et al., 2019; Guo et al., 2020). Moreover, KRAS ctDNA mutations are correlated with tumour grade (Le Calvez-Kelm et al., 2016).

The profiling of ctDNAs also serves as a diagnostic tool for early PDAC. CancerSEEK a multi-analyte screening test that can simultaneously detect somatic ctDNA mutations in 16 genes and quantify 8 cancer-associated proteins (carbohydrate antigen 125 (CA-125), CA19-9, CEA, HGF, myeloperoxidase, prolactin, OPN, tissue inhibitor of metalloproteinases 1 (TIMP-1)) can identify cancer in approximately 70% of PDAC patients (Cohen et al., 2018).

Detection of 5-hydroxymethylcytosine (5hmC) in ctDNA may also provide a means for early PDAC diagnosis. Mapping of 5hmC changes in ctDNA between non-cancer and PDAC cohorts identified thousands of genes commonly deregulated in PDAC, including many genes involved in pancreas development (GATA6, GATA4) and pathogenesis (YAP1, PROX1, IGF1). 5hmC sites significantly enriched between PDAC and control groups were sufficient to discriminate PDAC from non-cancer patients (Guler et al., 2020).

Next generation sequencing has consistently found that 25% or more of PDAC tumours harbour actionable mutations (Aguirre et al., 2018; Pishvaian et al., 2020). Similarly, PDAC ctDNAs comprise high-confidence tumour mutations in actionable genes. Botrus et al. recently investigated actionable genetic alterations in ctDNA isolated from the blood of patients with locally advanced and metastatic PDAC. Several therapeutically actionable alterations were identified including mutations in the homologous recombination repair pathway genes BRCA1 (2.1%), BRCA2 (5%), and ATM (7%) (Botrus et al., 2021). The identification of actionable mutations in ctDNAs using a liquid biopsy may significantly improve the time between first detection and therapeutic intervention.

PDAC Blood Metabolomes

Metabolomics is defined as the quantitative analysis of metabolites (endogenous low molecular weight components <1 kDa) in a biological specimen. Several studies have explored the utility of blood borne metabolites for PDAC early diagnosis, patient stratification and patient monitoring. These studies, while providing some prospects, have failed to identify a single metabolite biomarker that can accurately discriminate PDAC from chronic pancreatitis (CP) (Mayerle et al., 2018).

The development of multi-analyte signatures comprising CA19-9 and several metabolite biomarkers may provide increased specificity for PDAC diagnosis. Mayerle et al., investigated 477 metabolites from patients with CP or PDAC and demonstrated that a panel of nine metabolites (Proline, Sphingomyelin (d18:2,C17:0), Phosphatidylcholine (C18:0,C22:6), Isocitrate, Sphinganine-1-phosphate (d18:0), Histidine, Pyruvate, Ceramide (d18:1,C24:0), Sphingomyelin (d17:1,C18:0)) in conjunction with CA19-9 levels can distinguish PDAC from CP (Mayerle et al., 2018).

The effectiveness of metabolomic profiling for patient stratification has also recently been investigated. Metabolomic profiling of 361 PDAC blood plasma samples identified three subtypes, with sphingolipid metabolism exhibiting differential enrichment between subtypes. Integrative analysis revealed that the 3 identified subtypes do not overlap with previously defined transcriptomic subtyping schemes and are not associated with clinical outcome (Mahajan et al., 2021). While identifying sphingolipid metabolites as potentially important biomarkers in PDAC, the utility of serum based metabolic profiling for patient stratification remains unclear.

PDAC Exosomes

Exosomes are membrane-bound extracellular vesicles ranging in size between 40 and 160 nm in diameter. Derived from the endosomal compartment, exosomes are secreted from cells and can be readily detected in blood and other bodily fluids (Kalluri 2016; Kalluri and LeBleu 2020). Importantly, exosomes have been demonstrated to contain a multitude of analytes, including nucleic acids (DNA, mRNA, microRNA, long non-coding RNA) (Valadi et al., 2007; Kahlert et al., 2014; Hinger et al., 2018), proteins (Hurwitz et al., 2016; Kowal et al., 2016), lipids (Haraszti et al., 2016), and metabolites (Altadill et al., 2016). The biological role for exosomes as mediators of cell-cell communication now seems established, with exosomes delivering functional cargoes to, or binding membrane bound receptors of recipient cells (Alexander et al., 2015; Hoshino et al., 2015; Muller et al., 2017). The diverse role of exosomes in tumorigenesis, metastasis, immune regulation, and TME remodelling coupled with their long half-life in circulation make exosomes and their cargoes attractive biomarkers for patient diagnosis, prognosis, and biological discovery (Capello et al., 2019; Sun et al., 2020). Moreover, given that exosomes are programmable via genomic and/or proteomic modification exosomes are now being used as therapeutic vehicles (Zitvogel et al., 1998; Kamerkar et al., 2017; Usman et al., 2018; Kugeratski and Kalluri 2021).

Several recent studies suggest that the profiling of exosome cargoes may have clinical utility for PDAC diagnosis. miRNA profiling of exosomes isolated from the plasma of PDAC patients has identified miR-10b, miR-196a, miR451a, miR-21, and miR-17-5p as biomarkers for the diagnosis of early PDAC (Que et al., 2013; Joshi et al., 2015; Y.-F. Xu et al., 2017; Takahasi et al., 2018). In addition, miRNA profiling of exosomes isolated from the plasma of patients with either CP, benign pancreatic tumours (BPTs), or PDAC has demonstrated that specific exosomal miRNAs (miR-21, miR-17-5p) can be used to discriminate PDAC from CP and/or BPTs (Que et al., 2013). Furthermore, analysis of exosomes isolated from the pancreatic juice of PDAC patients or subjects with CP identified exosomal miR-21 and miR-155 as biomarkers capable of differentiating PDAC from CP (Nakamura et al., 2019).

Exosome proteins may also serve as important biomarkers for PDAC diagnosis. Quantitative proteomics has identified core exosomal proteins that are highly expressed in PDAC and other cancer cell types. Glypican-1 (GPC1), a cell surface proteoglycan, is enriched in PDAC-derived exosomes. GPC1 positive exosomes isolated from patient sera were shown to discriminate PDAC patients from healthy volunteers and patients with benign pancreatic disease (Melo et al., 2015). Macrophage migration inhibitory factor (MIF) is also highly expressed in PDAC-derived exosomes. MIF expression is higher in exosomes derived from stage I PDAC patients who later develop liver metastasis

(Costa-Silva et al., 2015). Despite the potential of diagnostic specific exosome protein biomarkers in PDAC, future analyses will be required to both validate and extend these findings.

KNOWLEDGE-GUIDED PLATFORMS FOR BETTER PATIENT MANAGEMENT: CHALLENGES AND OPPORTUNITIES

The early detection of cancer is now a major focus of health care systems around the world. Galleri™, a multi-cancer early detection (MCED) test that can detect over 50 types of cancer from a blood draw has been hailed as a "game changer" for cancer care with the potential to reduce deaths and decrease healthcare costs by detecting cancers early before they metastasise (https://www.galleri.com/). The Galleri™ test, now being trialled by the National Health Service (NHS) in the United Kingdom, employs a methylation-based ctDNA classifier to detect cancer and its location (Braunstein and Ofman 2021; Klein et al., 2021; Nadauld et al., 2021). Galleri™ has been demonstrated to detect Stage I, II and III PDAC with sensitivities ranging from 61.9 to 85.7% (Klein et al., 2021).

The deployment of MCED ctDNA-based tests for the routine early detection of PDAC remain challenging. Firstly, PDAC is rare in the general population and false positive screening may increase costs and result in patient harm from unnecessary procedures (Hruban and Lillemoe 2019; Lennon et al., 2019). Secondly, even though detected at early stages PDAC may still be extremely difficult to treat. For example, several studies demonstrate that venous invasion is a common attribute of PDAC, and as veins within the pancreas empty into the liver, it has been suggested that early liver metastases may be a frequent feature of early stage PDAC (Noe et al., 2018; Hruban et al., 2019; Hong et al., 2020). Beyond early detection, ctDNAs might serve as minimally invasive blood-borne biomarkers for monitoring patient treatment response. Comprehensive longitudinal studies charting ctDNA changes over the course of standardised therapy will be an important first step in making this a reality.

The identification of actionable mutations in an estimated 30–40% of PDAC has amplified calls for the routine genomic profiling of patient tumours. The "Know Your Tumour" trial has performed targeted sequencing on 1856 patient tumours with only 46 patients (2.5%) receiving a selected second line therapy matched to an actionable mutation (Pishvaian et al., 2020). This study points to survival gains for patients who receive a matched therapy based on genomic profiling, however, there is some debate as to whether these gains represent a substantial effect (Pishvaian et al., 2020). A significant difficulty in implementing precision oncology for PDAC is the length of time needed to acquire a patient sample, perform genomic profiling, and guide a patient to a molecularly matched therapy. Precision oncology trials in PDAC have largely focused on advanced disease with genome-matched therapies offered in the second line after standardised chemotherapy. The short survival times of patients with advanced PDAC means that timelines for meaningful intervention are significantly reduced. In addition, the current paradigm of guiding therapy based on single agent matched therapies often results in recurrence as resistant subclones emerge after therapy with repeat biopsies and genomic profiling leading to diminishing returns.

Several other clinical trials, including EPPIC (Enhanced Pancreatic Cancer Profiling for Individualised Care Study; https://www.tfri.ca), Precision Promise (https://www.pancan.org/research/precision-promise/) and ESPAC6, are pursuing multi-omic strategies to better define patient selection for standardised chemotherapy and to accelerate drug development for PDAC. The ESPAC6 clinical trial will assess whether a treatment specific transcriptomic signature can inform patient selection for either gemcitabine or FOLFIRINOX adjuvant therapy. In addition, ESPAC6 will generate a comprehensive cohort of patient-derived organoids. Organotypic models derived from patient samples with matched primary resectable material and clinical response data may represent a more clinically relevant approach for the testing of new drugs and/or biomarker hypotheses. Collectively, these studies will be highly informative and will likely define broader-based multianalyte selection strategies for patient management.

The next phase of omic driven discovery science has arrived. Single cell and single nucleus sequencing are providing new insights into PDAC ITH. Spatial transcriptomic and multiplexed imaging modalities are revealing critical cell-cell interactions, receptor-ligand interactions, and complex tumour architectures that will likely identify a next generation of cancer biomarkers and therapeutic opportunities for PDAC. The utility of single cell transcriptomics is limited for now to discovery; however, single cell data can be obtained from real-world PDAC biopsies (J. J. Lee et al., 2021). Multiplexed imaging modalities, such as MSI, that can generate composite maps of different cell types and drug metabolites may represent a more informative approach to the analysis of clinical samples in the future. Importantly, integrative systems and machine learning approaches are unlocking the potential of these new omic techniques to identify new cancer biomarkers and transition towards next generation knowledge-guided platforms for clinical decision making.

AUTHOR CONTRIBUTIONS

DS, SB and PB designed and drafted the manuscript. DS, JN, SB, and PB discussed the topics of the manuscript and approved the final version.

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REVIEW
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Modulation of Type I Interferon Responses to Influence Tumor-Immune Cross Talk in PDAC

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Immunotherapy has revolutionized the treatment of many cancer types. However, pancreatic ductal adenocarcinomas (PDACs) exhibit poor responses to immune checkpoint inhibitors with immunotherapy-based trials not generating convincing clinical activity. PDAC tumors often have low infiltration of tumor $CD8^+$ T cells and a highly immunosuppressive microenvironment. These features classify PDAC as immunologically "cold." However, the presence of tumor T cells is a favorable prognostic feature in PDAC. Intrinsic tumor cell properties govern interactions with the immune system. Alterations in tumor DNA such as genomic instability, high tumor mutation burden, and/or defects in DNA damage repair are associated with responses to both immunotherapy and chemotherapy. Cytotoxic or metabolic stress produced by radiation and/or chemotherapy can act as potent immune triggers and prime immune responses. Damage- or stress-mediated activation of nucleic acid-sensing pathways triggers type I interferon (IFN-I) responses that activate innate immune cells and natural killer cells, promote maturation of dendritic cells, and stimulate adaptive immunity. While PDAC exhibits intrinsic features that have the potential to engage immune cells, particularly following chemotherapy, these immune-sensing mechanisms are ineffective. Understanding where defects in innate immune triggers render the PDAC tumor–immune interface less effective, or how T-cell function is suppressed will help develop more effective treatments and harness the immune system for durable outcomes. This review will focus on the pivotal role played by IFN-I in promoting tumor cell–immune cell cross talk in PDAC. We will discuss how PDAC tumor cells bypass IFN-I signaling pathways and explore how these pathways can be co-opted or re-engaged to enhance the therapeutic outcome.

Keywords: innate immunity, PDAC, IFN-I, nucleic acid sensing, immunotherapy, tumor microenvironment

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INTRODUCTION

PDAC accounts for more than 90% of pancreatic malignancies and is currently the third leading cause of cancer-related death in Western countries (Luchini et al., 2016; Pishvaian and Brody, 2017; McGuigan et al., 2018). High mortality rates are mainly due to late detection, a high level of tumor heterogeneity, and a desmoplastic immunosuppressive microenvironment (Ryan et al., 2014). Although advances in treatment have significantly increased 5-year survival rates for many other

cancer types (Brahmer et al., 2012; Nixon et al., 2018), the overall 5-year survival rate for PDAC is only 10% (Siegel et al., 2021). Mono- and multi-agent chemotherapy regimens are the standard treatments. Conventional cytotoxic therapies in PDAC include the nucleoside analog gemcitabine which may be combined with the microtubule poison nab-paclitaxel, and FOLFIRINOX (folinic acid, fluorouracil, irinotecan, and oxaliplatin) (Mohammad, 2018). Both treatment regimens improve the overall survival for patients with localized disease but are less effective for the majority of patients who are diagnosed with advanced or metastatic disease (Bliss et al., 2014). Improvements in progression-free survival have been achieved for patients with BRCA-mutated tumors when PARP inhibitors are used as maintenance treatment following chemotherapy (Golan et al., 2019). Therefore, despite incremental advances, there is an urgent need for new therapeutic approaches.

Immune checkpoint inhibitors (ICIs) are revolutionizing the treatment of cancer, mainly when used in combination with chemotherapy or radiotherapy. In ICI-sensitive diseases such as non-small-cell lung cancer (Lim et al., 2020), head and neck squamous cell carcinoma (Burtness et al., 2018), and triple-negative breast cancer (Schmid et al., 2018), combining chemotherapy and immunotherapy generates durable responses in a subset of patients (Reck, 2018). Combining standard-of-care chemotherapy with ICIs has been largely unsuccessful in PDACs [reviewed in Henriksen et al. (2019)], albeit in trials where patients were not selected based on any favorable biomarker.

PDAC is generally considered immunologically cold with a low incidence of tumor-infiltrating lymphocytes (TILs) (Stromnes et al., 2017; Blando et al., 2019; Gorchs et al., 2019; Seo et al., 2019), thought to be a result of poor T-priming by the tumor. Poor T-cell priming or activation in PDAC is commonly attributed to low neo-antigen content, although PDAC tumors do have potentially actionable neo-epitopes. Indeed PDAC tumors with good quality neo-antigens are associated with better outcomes (Bailey et al., 2016a; Balachandran et al., 2017). How PDAC tumor cells evade the immune system is unclear. Tumor cell intrinsic mechanisms play important roles in regulating both intrinsic and therapy-induced engagement or avoidance of the innate or adaptive immune systems (reviewed in Wellenstein and de Visser (2018)). Tumor cells can evade the immune system by reducing antigen processing, cell surface antigen presentation, or expression of cell surface proteins that engage innate immune cells. They also secrete growth factors, chemokines, and cytokines that shape an immunosuppressive microenvironment (Grivennikov et al., 2010; Gonzalez et al., 2018). In addition to high tumor T-cell infiltration, "immunologically hot" tumors that respond well to immunotherapy-based treatment typically express IFN-I (IFN-α, -β), type II interferons (IFN-II) (IFN-γ), and high levels of interferon-stimulated genes (ISGs) that sustain antitumor immune responses (Gajewski et al., 2013; Corrales et al., 2015; Wang and Wang, 2017). PDAC does not commonly show high expression of interferons or ISG signatures, but if tumor-targeted therapies such as chemotherapy or targeted agents induce robust IFN-I and/or innate responses, immune system engagement is more likely to occur. Understanding why this fails in PDAC could guide new therapeutic or patient selection approaches that will ultimately harness the immune system to deliver more durable clinical responses.

IFN CASCADE IN TUMORS

The IFN cascade in tumors is complex, as shown in **Figure 1**. Type I interferons (IFN-α and -β) can be expressed as a result of stress damage to tumor cells or stromal cells such as macrophages (Medrano et al., 2017). Type I interferons stimulate many cells in the TME driving both tumor cell and immune cell responses (Zitvogel et al., 2015). The secretion of type I interferons can kill or senesce tumor cells and directly or indirectly stimulate T cells, natural killer (NK) cells (Fenton et al., 2021), and potentially macrophages or dendritic cells to secrete the type II interferon (IFN-γ). The type II interferon is one marker of cytotoxic immune effector cells in the TME (Bhat et al., 2017). The IFN system can be self-enhancing. For example, chemotherapy-mediated damage may induce the secretion of IFNs from both tumor cells and cells in the TME amplifying immune cell activation throughout the tumor (Budhwani et al., 2018). Understanding the cause and effect in this pivotal cascade is challenging as both type I and -II interferons drive common signaling cascades. IFN responses in tumors are commonly monitored using bulk RNA gene expression signatures which, in the absence of single-cell sequencing approaches, are unable to distinguish which cells initiate, or which cells respond to IFN-driven signals. This complex interplay between cells in tumors presents different opportunities to drive IFN induction and activation of antitumor immune cell activity, even when normal pathways are dysfunctional.

CHEMOTHERAPY AND IMMUNOMODULATION IN PDAC

Chemotherapeutic agents are first-line standard-of-care treatments for PDAC. Despite that the combination of gemcitabine with nab-paclitaxel and FOLFIRINOX increases efficacy compared to gemcitabine alone, patients' overall survival rates remain particularly low, and toxicity limits their use (Conroy et al., 2011; Von Hoff et al., 2013; Javed et al., 2019). PDAC patients rapidly develop resistance to chemotherapy through both tumor cell intrinsic and tumor microenvironmental factors (Chand et al., 2016). Despite the limited benefit, there is evidence of chemosensitivity in subsets of PDAC patients. For instance, several clinical trials report increased sensitivity to platinum-based chemotherapy in DNA damage repair (DDR)-deficient patients (Pishvaian et al., 2019; Golan et al., 2014; O'Reilly et al., 2020; Blair et al., 2018).

In addition to tumor cell cytotoxic activity, chemotherapies may also potentiate immunomodulatory responses (Bailly et al., 2020; Piadel et al., 2020). Treatment of PDAC with gemcitabine has been associated with enhanced T-cell-mediated responses through increased naive T-cell activation (Plate et al., 2005) and $CD8^+$ T-cell function, which is in part dependent on dendritic cell (DC) activity (Plate and Harris, 2004). In addition, following

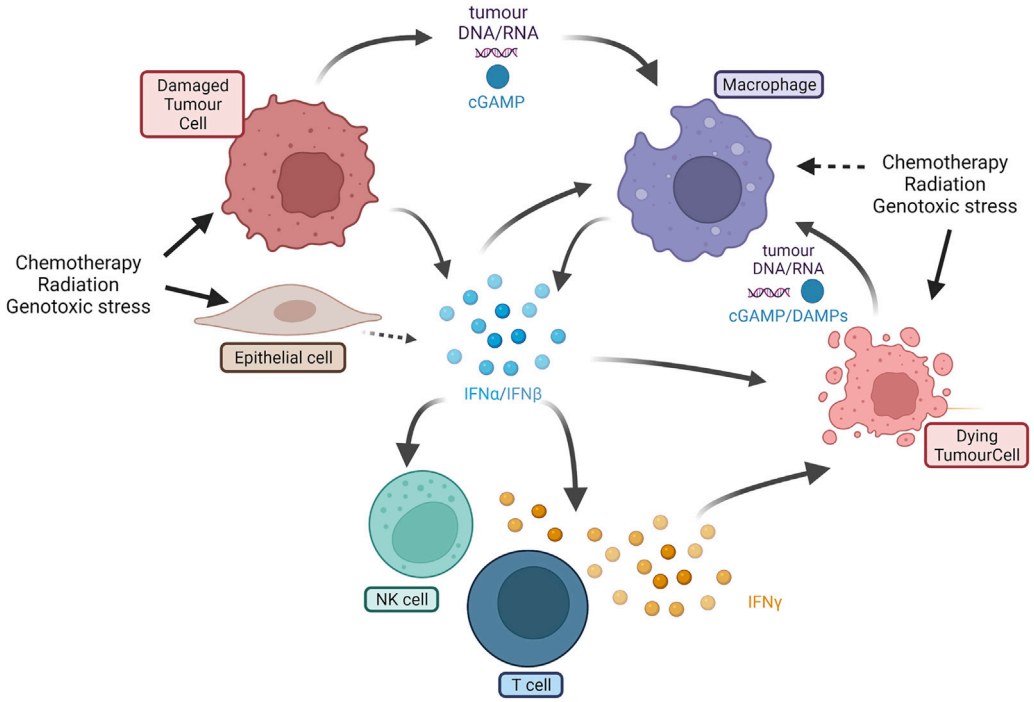

FIGURE 1 | Overview of IFN-I and IFN-II cascade in tumors. IFN-I (interferons α and β) can be expressed by damaged tumor cells, epithelial and stromal cells (e.g., macrophages) as a result of cell damage or stimulation. Secreted IFN-I activates macrophages, NK cells, and T cells, and kills other tumor cells. Cross talk can also be mediated by DNA or RNA fragments or DAMPs such as cGAMP. T cells produce IFN-II (IFN-γ) that is cytotoxic to tumor cells. Dying tumor cells can release further DAMPs or cell fragments that further stimulate new macrophages or other innate immune cells (e.g., dendritic cells not shown). IFN = interferon; NK = natural killer; cGAMP = cyclic guanosine monophosphate; DAMP= damage-associated molecular patterns (Created using BioRender®).

gemcitabine or FOLFIRINOX treatment, suppressive immune populations such as regulatory T cells (Tregs) (Kan et al., 2012; Homma et al., 2014; Liu et al., 2017; Hui et al., 2021; Sams et al., 2021) and myeloid-derived suppressor cells (MDSCs) (Suzuki et al., 2005; Vincent et al., 2010; Eriksson et al., 2016; Wu et al., 2020a) are downregulated in patients and in preclinical models. In mice, gemcitabine also increases the immunogenicity of tumor cells (Liu et al., 2010). Therefore, global changes in the immune profile of tumors following treatment can be both direct and indirect. Consistent with these findings, an *in vitro* analysis of the proteasome and immunopeptidome of human PDAC cell lines showed that gemcitabine induces overexpression of MHC-I molecules (Gravett et al., 2018) with novel peptides. Similarly, in KPC tumor-bearing mice, prolonged treatment with gemcitabine leads to increased MHC-I along with increased secretion of CCL/CXCL chemokines (Principe et al., 2020). Gemcitabine and oxaliplatin (the platinum-based element of the FOLFIRINOX regimen) also induce the expression of immunogenic cell death-associated damage-associated molecular patterns (DAMPs) including ATP and HMGB1 (Hayashi et al., 2020; Smith et al., 2021), which can signal NK cells and potentiate the innate immune system. Finally, in PDAC patients, chemotherapy has been shown to stimulate the immune response by increasing T-cell response to tumor-associated antigens (Mandili et al., 2020).

The immunostimulatory effects of these chemotherapeutics highlight their potential use in combination with immuno-oncology therapies. Especially with ICIs, *in vitro* and *in vivo* studies suggest that gemcitabine increases the expression of immune checkpoint molecules such as PD-L1 (Principe et al., 2020; Smith et al., 2021). While favorable changes in the immune profile of tumors can be seen following treatment, why these changes do not translate into more sustained benefit alone or in combination with other therapies remains unclear. This may be because the impact of chemotherapy on tumor cells is rapidly reversed or because the TME remodels quickly restraining the potential benefits. Understanding the kinetics of induction and recovery in the tumor cells versus TME following chemotherapy treatment would give useful insights.

TREATMENTS TARGETING IMMUNE CELLS ALONE ARE NOT ACTIVE IN PDAC

Clinically, antitumor immune responses are stimulated by therapeutic antibodies that target T-cell checkpoints such as PD-1/PD-L1 and, to a lesser extent, CTLA-4/B7H4, which act to inhibit T-cell function. In PDAC, both PD-L1 and CTLA-4 are upregulated and associated with poor prognosis (Nomi et al., 2007; Cancer Genome Atlas Resea, 2017). Early trials showed no beneficial response to ipilimumab (anti-CTLA-4 monoclonal

antibody) in patients with metastatic and locally advanced PDAC (NCT00112580), although treatment was associated with advanced toxicity (Royal et al., 2010). These results were mirrored with another anti-CTLA-4 monoclonal antibody (NCT02527434), tremelimumab (Sharma et al., 2018). Anti-PD-L1 monoclonal antibodies showed no objective response in the 14 PDAC patients in a phase I clinical trial (NCT00729664) (Brahmer et al., 2012), while disease control (partial response or stable disease) was observed in 21% of the 29 PDAC patients in another phase I trial with durvalumab (NCT01693562) (Segal et al., 2014). Interestingly, PD-1 blockade with pembrolizumab showed a 62% objective response in a phase II clinical trial in 8 PDAC patients harboring mismatch repair (MMR) deficiency (NCT01876511) (Le et al., 2015). Pembrolizumab has now been approved by the Food and Drug Administration (FDA) for solid tumors harboring MMR defects including PDAC (Le et al., 2017); however, these only represent about 1% of PDAC patients (Hu et al., 2018). In the other patients, very limited responses to ICIs have been observed. Some studies are still ongoing such as a phase II trial of atezolizumab (NCT03829501), as well as combinations of antibodies targeting CTLA-4 and PD1/PD-L1 (NCT01928394). Consistent with findings in other tumor types, it is probable that combination approaches, for example, with chemotherapy, will be required.

Cell-based vaccines alone or combined with chemotherapy and ICI present alternative strategies to achieve tumor-targeted immune stimulation. The granulocyte–macrophage colony-stimulating factor (GM-CSF)-secreting allogeneic pancreatic tumor cell vaccine (GVAX) consists of irradiated human allogeneic pancreatic tumor cells that secrete GM-CSF (Lutz et al., 2011). It is given as a cellular vaccine to present pancreatic cancer cell epitopes and also improve antigen presentation by inducing GM-CSF-mediated maturation of dendritic cells. Despite showing positive changes in the immune microenvironment and potential efficacy in early clinical trials (Lutz et al., 2014), a more extensive phase II trial in combination with the CTLA4 targeting ICI ipilimumab failed to show increased clinical benefit (Wu et al., 2020b). Studies like this which assess immune biomarker changes suggest that while the activation of immune cells can be achieved in PDAC, they do not translate into a durable effect. The use of GM-CSF in GVAX is interesting as it has both positive and negative effects. In addition to promoting dendritic cell recruitment, increased GM-CSF can promote pancreatic tumor development (Pylayeva-Gupta et al., 2012) and drive recruitment of immunosuppressive immune cells (Bayne et al., 2012).

CHEMOTHERAPY AND IMMUNOTHERAPY COMBINATIONS IN PDAC

Greatest response to ICI treatment is observed in combination with chemotherapy. Both chemotherapy and radiotherapy can increase antitumor immunity by inducing immunogenic tumor cell death and cell stress signaling pathways, and depleting immunosuppressive cells or stimulation of T cells to complement the effects of ICI treatment (Burtness et al., 2018; Galluzzi et al., 2020; Salas-Benito et al., 2021). In PDAC, the combination of ICI with chemotherapy is marginally more effective than monotherapy ICI treatment; however, benefits remain limited. Combining ipilimumab with gemcitabine in a phase Ib trial (NCT01473940) resulted in 2 partial responses and five stable diseases out of 11 patients (Kamath et al., 2020). In a larger phase I clinical trial that combined tremelimumab and gemcitabine (NCT00556023), 2 out of 28 PDAC patients showed a partial response and seven stable diseases (Aglietta et al., 2014). Chemotherapy in combinations with ICIs compared to chemotherapy alone conferred longer overall survival in a clinical trial comprising 58 patients with advanced PDAC (Ma et al., 2020). Other phase I and Ib trials have combined nivolumab or pembrolizumab with nab-paclitaxel and gemcitabine. In the first of these trials (NCT02309177), nearly half of the 50 PDAC patients had stable disease, 8 partially responded and one completely responded (Wainberg et al., 2020). In the second trial (NCT02331251), all patients achieved disease control, of which 3 showed partial response (Weiss et al., 2018). In a large phase II clinical study looking at the combination of gemcitabine and nab-paclitaxel with or without durvalumab and tremelimumab (NCT02879318), no benefits in terms of progression-free and overall survival were observed in the 191 metastatic PDAC patients; however, disease response rates were improved (Renouf et al., 2020).

Overall, chemoimmunotherapy improves response rates in PDAC compared to ICI or chemo monotherapies; however, these benefits remain limited and do not impact survival. Hence, the combination of ICI and chemotherapy is relevant in PDAC, but further potentiation of the immune system is needed to obtain significant benefit.

PDAC IMMUNE MICROENVIRONMENT AND IFN-I

PDAC is characterized by a dense tumor stroma mainly composed of the extracellular matrix, fibroblasts, and vasculature (Hosein et al., 2020). It also contains immunosuppressive cell types such as MDSCs and Tregs, which accumulate during disease progression to suppress $CD4^+$ and $CD8^+$ T-cell function, contributing to poor prognosis (Clark et al., 2007; Bayne et al., 2012; Thyagarajan et al., 2019; Huber et al., 2020).

Transcriptomic profiling of PDAC has identified 2 broad consensus subtypes, namely, classical and basal-like, which largely represent predominant neoplastic gene programs (Collisson et al., 2011; Moffitt et al., 2015; Bailey et al., 2016b). Additional transcriptomic subtypes, such as ADEX/exocrine-like and immunogenic, exhibit significant overlap with the classical subtype (Bailey et al., 2016b). Deconvolution of bulk transcriptomic RNAseq data using validated immune cell type signatures has demonstrated that the 2 consensus subtypes of PDAC are associated with distinct immune profiles (Bailey et al., 2016b; Karamitopoulou, 2019; Santiago et al., 2019). The basal-like subtype is usually associated with an "immune escaping" phenotype, with high Tregs and suppressive macrophages (M2 or

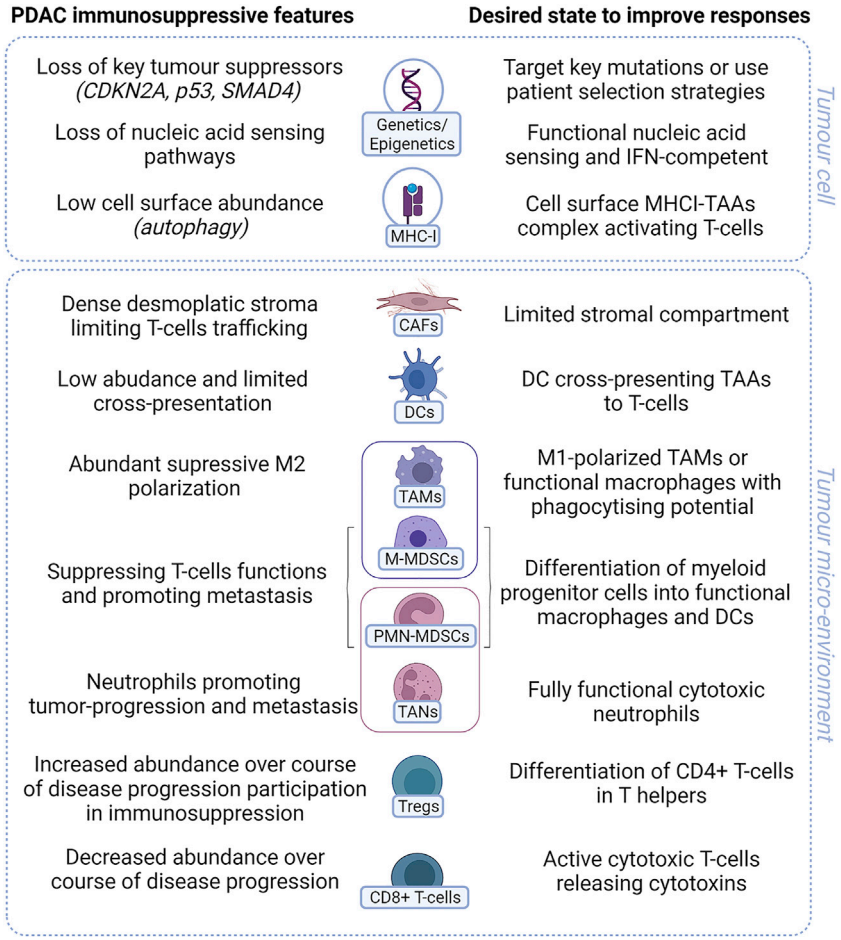

FIGURE 2 | Key immunosuppressive features of PDAC. PDAC has many features that prevent activation of T cells in tumor. Both tumor cell intrinsic characteristics (genetic or changes in gene expression) along with tumor microenvironmental factors including suppressive or dysfunction cells all contribute to render PDAC immunologically "cold" with poor response to treatment. IFN= interferon. MHC-I= major histocompatibility complex class I; TAA= tumor-associated antigen; CAF= cancer-associated fibroblast; DC= dendritic cell; TAM= tumor-associated macrophage; M-MDSC= monocytic myeloid-derived suppressor cell; PMN-MDSC= polymorphonuclear myeloid-derived suppressor cell; TAN= tumor-associated neutrophil; Treg= regulatory T cells (Created using BioRender®).

m-MDSC) and low cytotoxic T cells (Wartenberg et al., 2018; Karamitopoulou, 2019). The classical subtype appears relatively "immune rich," with lower Tregs and M2 macrophages and a higher percentage of $CD4^+$ and $CD8^+$ T cells as well as inflammatory (M1) macrophages (Wartenberg et al., 2018). IFN-I plays a pivotal role in regulating immunomodulatory chemokines and cytokines that in turn reshape the tumor immune microenvironment. In addition, IFN-I upregulates the expression of MHC-I and antigen presentation (Yang et al., 2004; Wan et al., 2012), increasing tumor cell recognition by the immune system. However, the presentation of neoantigens at the cell surface by MHC-I may be reduced by the targeted degradation of MHC-I in PDAC cells (Yamamoto et al., 2020). With respect to immune cells, IFN-I also enhances antigen presentation and cross-priming of the immune system as it stimulates DC activation and maturation (Lorenzi et al., 2011; Binnewies et al., 2019). In PDAC, DCs are rare and decrease upon disease progression, further limiting antigen cross-presentation and T-cell activity (Hiraoka et al., 2011). Similarly, IFN-I mediated activation of NK cell cytotoxicity (Swann et al., 2007; Bergamaschi et al., 2020) is impaired in PDAC (Marcon et al., 2020). Tumor-associated macrophages (TAMs) represent a dominant immune cell population in PDAC. High levels of the M2 or suppressive macrophage phenotype are associated with poor prognosis and disease progression (Kurahara et al., 2011; Candido et al., 2018). IFN-I can promote the antitumor M1 or inflammatory macrophage phenotype and enhance phagocytic functions (U'Ren et al., 2010; Sampson et al., 1991).

The PDAC TME is accepted as being highly immunosuppressive (**Figure 2**). Many preclinical studies have shown the potential for TME modulators to enhance immunotherapy. Few have yet to translate to the clinic, but a number of agents targeting myeloid cells or stromal cells are being tested clinically with ICI or in chemotherapy/ICI combinations (Liu et al., 2019; Roma-Rodrigues et al., 2019; Ho et al., 2020;

Zhong et al., 2020). $CD4^+$ and $CD8^+$ T cells are abundant at the initial stages of PDAC tumor development, and their presence and activation decrease during disease progression due to an immunosuppressive microenvironment (Knudsen et al., 2017). The dense fibroblastic stroma limits T-cell trafficking and access to the TME. MDSCs and Tregs suppress T-cell activation, which is already limited by poor antigen cross-presentation. Type I IFNs induce the secretion of cytokines such as CCL5, CXCL9, and CXCL10, which can attract T cells (Lorenzi et al., 2011) along with factors that negatively modulate immunosuppressive regulatory T cells (Gangaplara et al., 2018), facilitating effective cytotoxic T-cell functions. However, targeting different elements of the TME by modulation of myeloid cells with CXCR2, CCR2, or CSF1R (Steele et al., 2016; Candido et al., 2018; Nywening et al., 2018; Siolas et al., 2021) or CXCR4 antagonists (Feig et al., 2013; Biasci et al., 2020) can regulate the TME through changes in myeloid cells and stroma, improving preclinical responses to immune checkpoint inhibitors. Myeloid cells and the stroma can initiate IFN-I responses or be triggered as a result of tumor cell damage. The suppressive cell phenotypes in the TME may have limited capacity to enhance IFN-I or other innate signals. Targeting stromal cells in addition to tumor cells and T cells with chemotherapy and ICI may yield better clinical responses.

GENETIC FEATURES OF PDAC MAY INFLUENCE IMMUNE CELL FUNCTION

PDAC tumors exhibit genetic characteristics that may underpin the lack of observed intrinsic responses to immune checkpoint therapy. Mutational heterogeneity is associated with a better response to ICI (Reuben et al., 2017; Iyer et al., 2021; Vitale et al., 2021). While PDAC tumors have many low-abundance mutations, there are four dominant driver mutations, namely, oncogenic activation of KRAS and inactivation of the tumor suppressors TP53, CDKN2A, and SMAD4 (Jones et al., 2008). CDKN2A loss correlates with reduced immune infiltrates in several tumor types, including PDAC (Siemers et al., 2017). Retrospective analysis of clinical data in different tumor types revealed an association of CDKN2A loss with worse overall and progression-free survival following ICI treatment in combination with chemotherapy (Horn et al., 2018; Gutiontov et al., 2021). Preclinically, specific loss of CDKN2A has also induced resistance to immunotherapy (Gutiontov et al., 2021; Han et al., 2021). CDKN2A is located on the human chromosome 9p21 locus (Sasaki et al., 2003), in close proximity to MTAP, JAK2, and a large cluster region coding for 16 IFN-I genes (Diaz, 1995). Co-deletion of these genes is common, for example, concomitant mutation of CDKN2A and MTAP in PDAC is estimated at 26% according to The Cancer Genome Atlas (TCGA) dataset (Mavrakis et al., 2016) and may also contribute to resistance to ICI-based therapy. In melanoma, CDKN2A-associated JAK2 mutation leads to loss of functional IFN-γ signaling which also limits response to immunotherapy (Horn et al., 2018). CDKN2A loss results in the activation of CDK4/6 kinases. In preclinical models, treatment with CDK4/6 inhibitors re-sensitized melanoma tumors (Jerby-Arnon et al., 2018) and syngeneic models (Deng et al., 2018) to treatment. In preclinical pancreatic cancer models, CDK4/6 inhibitors can influence tumor growth (Chou et al., 2018) and combine to sustain antitumor effects following chemotherapy (Salvador-Barbero et al., 2020). Combining the CDK4/6 inhibitor palbociclib and the MEK inhibitor trametinib not only reduced growth of patient-derived xenograft models but also enhanced response to PD-1 inhibition in a transplantable tumor model derived from the KPC model (Knudsen et al., 2021). This suggests that appropriate combinations with CDK4/6 inhibitors in PDAC may enhance immune response in tumors with loss of CDKN2A through tumor-centric and possibly TME mechanisms. In addition, it is not known whether loss of SMAD4 and p53 and mutation of KRAS also cooperate with CDKN2A/B loss to further increase the tumor immune-resistant status. For example, in lung cancer, loss of the tumor suppressor STK11 is regarded as driving resistance to both chemotherapy and immunotherapy (Skoulidis et al., 2018). Interestingly, it is the co-occurrence of a RAS mutation that renders these tumors most resistant to ICI-based treatments (Ricciuti et al., 2021). Therefore, given the dominant mutations in pancreatic cancer, it may be challenging to achieve strong immune activation.

The IFN-I genes are also expressed on Chr9 (close to CDKN2A and MTAP) and can sometimes be lost. It is also possible that even when not deleted, the disruption of other genes in the locus may reduce gene expression. Tumor cells with reduced IFN-I expression may evade immune cells due to a lack of IFN-α and -*ß* cross talk to the immune system. Loss or reduction of this central coordinator mechanism for the tumor–immune interaction would contribute to immune escape and ICI resistance (Grard et al., 2021), especially in the context of tumor-targeted combination approaches.

Less than 4% of PDAC tumors comprise mutations in DNA damage repair genes, such as BRCA1, BRCA2, or PALB2 (Waddell et al., 2015; Bailey et al., 2016b). BRCA1- and BRCA2-deficient tumors are associated with increased immune infiltrates in some patients; however, rates of response to ICI are low (Sønderstrup et al., 2019; Wen and Leong, 2019; Mei et al., 2020). Recent evidence in mouse models of breast and colorectal cancers suggests that BRCA2-deficient tumors are more susceptible to ICI than BRCA1-deficient tumors (Samstein et al., 2020; Zhou and Li, 2021). ARID1A alteration is found in about 6% of PDAC patients (Cancer Genome Atlas Resea, 2017) and has been observed to modulate responses to immunotherapy. In fact, ARID1A deficiency has been shown to lead to the inactivation of MMR genes, increase in PD-L1 expression, increase in tumor mutation burden through DDR dysfunction *via* ATR inhibition, and TIL recruitment and inflammatory response by IL-6 production (Hu et al., 2020; Wang et al., 2020). Consistently, ARID1A-mutated patients showed better response to ICI with prolonged progression-free and overall survival observed in various solid cancer types, suggesting that ARID1A deficiency may be a patient selection biomarker for ICI combinations (Jiang et al., 2020; Okamura et al., 2020). It is unlikely that mutation status alone will be sufficient to drive patient segmentation in PDAC; therefore, new

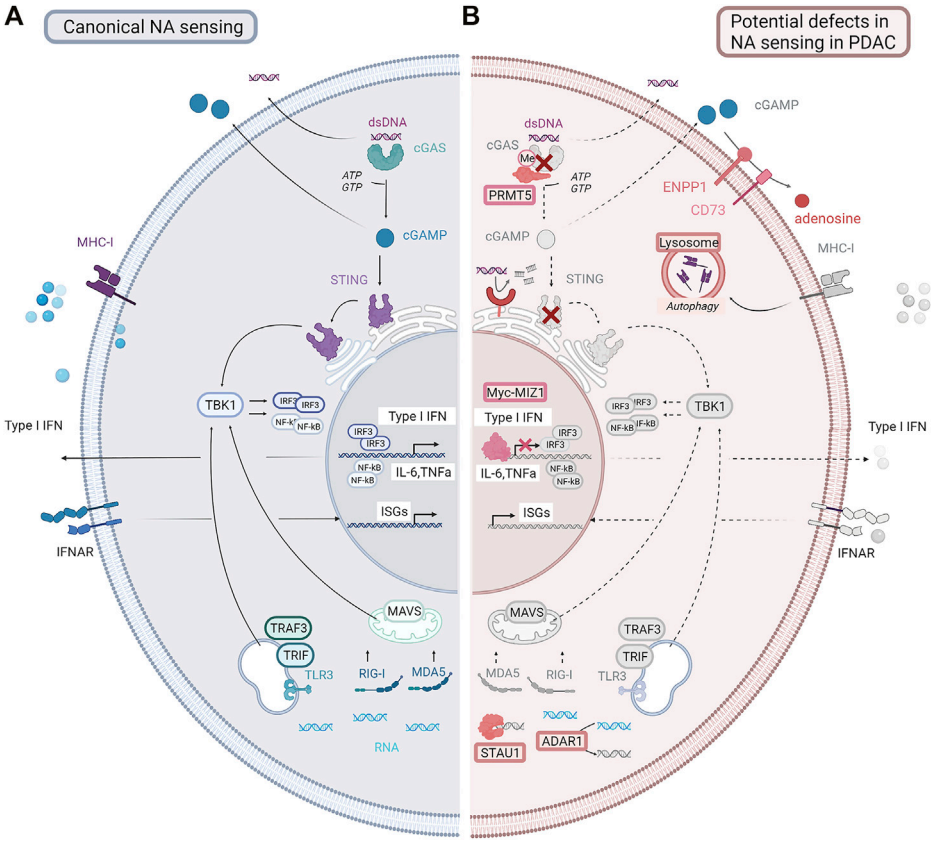

FIGURE 3 | Canonical nucleic acid-sensing pathways and potential defects in PDAC. **(A)** Canonical nucleic acid-sensing pathways detect dsDNA or dsRNA fragments (DAMPs) generated as a result of stress, or following drug treatment, which accumulate in the cytoplasm. dsDNA fragments activate the cGAS-STING pathway. dsRNA fragments activate the MAVS/RIG-I/MDA5 or other sensing pathways, for example, TRAF3/TRIF. These complexes drive TBK1, resulting in endogenous type I interferon production and interferon-stimulated gene expression. Nucleic acids along with signaling molecules such as cGAMP can be secreted by tumor cells and activate nucleic acid-sensing or innate damage-sensing pathways in normal immune or stromal cells. Secreted IFN-I can activate IFN signaling in other tumor cells. Antigen presentation is upregulated with increased MHC-I expression. **(B)** In PDAC, these pathways are disrupted. The cGAS-STING pathway can be lost by deletion or downregulation. RNA sensing can be inhibited through the upregulation of STAU and ADAR1. cGAMP can be degraded by the upregulation of ENPP1. IFNAR receptor can be downregulated. Antigen presentation can be inhibited by the downregulation of MHC1 expression. *Pathways that are lost or downregulated in PDAC are shown in gray*. NA= nucleic acid; dsDNA= double-stranded DNA; cGAMP = cyclic guanosine monophosphate; IFN = interferon; ISGs = interferon stimulated genes; IFNAR: interferon α/β receptor; MHC-I = major histocompatibility complex class I (Created using BioRender®).

strategies to define patient subsets for specific combination treatments will be important.

TRIGGERING INNATE IMMUNE RESPONSES IN PDAC

To maintain immunological homeostasis, an organism must discriminate self from non-self. Upon infection, viral nucleic acids stimulate antiviral responses, but the same innate pathways can also recognize damaged self-DNA and/or RNA (Iurescia et al., 2018). Activation or dysfunctional regulation of proteins involved in these pathways causes disorders known as interferonopathies, which are associated with increased interferon production. For example, the Mendelian autoinflammatory disorder Aicardi–Goutieres syndrome is a type I interferonopathy caused by mutations in genes such as TREX1, which plays a pivotal role in endogenous nucleic acid sensing (Tao et al., 2019; Baris et al., 2021).

Chromosome instability (CIN) is a hallmark of cancer (Pikor et al., 2013), and damaged DNA within micronuclei has been demonstrated to induce innate immune responses (Mackenzie et al., 2017). Although nuclear DNA is shielded from cytoplasmic nucleic acid sensors by the nuclear membrane, membrane rupture during mitosis and/or cytotoxic stress can expose nucleic acids to pattern recognition receptors (PRRs). PRR stimulation activates the immune system through the modulation of the tumor cell surface and secreted proteins (Maciejowski and Hatch, 2020). PDAC is characterized by DDR deficiency, CIN, and metabolic stress (Bailey et al., 2016b; Aguirre et al., 2018). These tumor cell intrinsic properties should trigger canonical innate immune pathways. However, responses are limited in PDAC because of the activation of anti-autoimmune regulatory mechanisms that

facilitate immune evasion and disease progression (Moskovitz et al., 2003; Waddell et al., 2015; Bailey et al., 2016b).

DNA- AND RNA-SENSING SIGNALING PATHWAYS IN PDAC

Chemotherapy, radiation, and therapeutics that damage DNA can prime immune responses through the stimulation of nucleic acid-sensing pathways. DNA or RNA fragments stimulate innate immune pathways in the tumor, including the secretion of IFN-I, which cross talks to immune cells (**Figure 3**). Double-stranded DNA (dsDNA) is generally located in the nucleus. However, in cells with CIN, due to DNA damage and/or defects in DNA damage repair machinery, cell cycle regulation (Fenech et al., 2011; Crasta et al., 2012; Sahin et al., 2016), or drug treatment dsDNA is found in the cytoplasm. Canonically, cytoplasmic dsDNA is sensed by cGAS (cyclic GMP-AMP synthase), a DNA-binding protein that catalyzes the production of the second messenger cGAMP (2′-3′ cyclic GMP-AMP) (Li et al., 2013). cGAMP interacts with the adaptor protein stimulator of interferon genes (STING) (Ablasser et al., 2013; Kato et al., 2017), causing dimerization and translocation from the endoplasmic reticulum to the Golgi (Ishikawa and Barber, 2008; Ishikawa et al., 2009). STING activates the kinase TBK1, driving translocation of the transcription factor IRF3 to the nucleus and inducing the expression of IFN-I (Liu et al., 2015). This sensing pathway can also be triggered in non-tumor cells with build-up of cytoplasmic dsDNA in the TME, with a transfer of dsDNA and/or cGAMP from tumor to host cells (Schadt et al., 2019; Zou et al., 2021). STING and TBK1 activation trigger the recruitment of IκB kinases (IKK), which results in NF-κB pathway activation by phosphorylation of the inhibitory IκB, thus enabling the translocation of NF-κB transcription factors to the nucleus (Abe and Barber, 2014; Yum et al., 2021). Similarly, the NF-κB and IFN-I pathways can be activated by sensing DNA through endosomal TLR3 and TLR9 (Kawai and Akira, 2007). The STING-TBK1 signaling axis has shown functionality in several PDAC models upon stimulation with STING agonists or modulators (Jing et al., 2019; Ren et al., 2020; Liang et al., 2021; Miller et al., 2021). However, it is not clear whether STING responses are functional in the majority of PDAC tumors, or whether the pathway is attenuated as tumors progress. Interestingly, cGAS-STING pathway agonists show efficacy in preclinical PDAC tumors by targeting macrophages in the TME (Ager et al., 2021), suggesting that tumor cells or cells within the TME may respond to activation of this pathway.

RNA sensing allows cells to detect cytoplasmic double-stranded RNAs and specific single-stranded RNAs, which are usually signs of viral infection. However, following treatment (Ranoa et al., 2016), cytoplasmic dsRNAs may accumulate in treated cells and be recognized by RNA-sensing pathways. Cytoplasmic dsRNAs are sensed, according to their size and location, by toll-like receptors such as TLR3 located in endosomes (Alexopoulou et al., 2001) or cytosolic ubiquitously expressed RIG-I-like receptors (RLRs) such as RIG-I and melanoma differentiation associated gene-5 (MDA5) (Hornung et al., 2006; Kato et al., 2006). The former signals through the adaptor protein TRIF, while the latter induces the activation of mitochondrial antiviral signaling protein (MAVS). Following a cascade of downstream events, both converged on TBK1, and subsequent IRF3 activation results in IFN-I and NF-kB responses (Seth et al., 2005; Sun et al., 2006; Kawai and Akira, 2018). These pathways are functional in preclinical models of PDAC following stimulation by PRR agonists (Duewell et al., 2014; Metzger et al., 2019). It is therefore possible that triggering these pathways with radiation or chemotherapy, or directly with pathway agonists to target STING or RIG-I in both tumor and the TME could add benefit as part of a combination strategy with ICI.

MECHANISMS REGULATING DNA- AND RNA-INDUCED IFN RESPONSES IN PDAC

As cancer cells are altered-self cells, recognition by the immune system can be facilitated by damage-associated molecular patterns (DAMPs). The term DAMP encompasses a group of molecules that can signal in a cell-intrinsic manner or trigger cross talk to the innate immune system after being released from damaged cells (e.g., cGAMP, DNA or RNA fragments, or intracellular proteins such as actin, HMGB1, and histones) (reviewed in depth in Gong et al. (2020)). DAMPs commonly activate a range of PRRs and trigger IFN-I responses (Teijaro, 2016; Musella et al., 2017) as a result of genetic changes or drug treatments (Stetson and Medzhitov, 2006; Reikine et al., 2014). Tumor cells commonly undergo changes that abrogate or make these sensing pathways less effective or reduce the secretion of DAMPs.

TREX1 and ENPP1 can antagonize signals resulting from damaged DNA (**Figure 3**). Both enzymes are highly expressed in PDAC (Carozza et al., 2020), and overexpression reduces the activation of the cGAS-STING pathway. TREX1 is an ER-associated exonuclease that degrades cytosolic dsDNA and therefore restrains cGAS-STING activation (Mohr et al., 2021). ENPP1 is a transmembrane protein with a hydrolase extracellular domain capable of cleaving a variety of substrates including cGAMP preventing activation of the cGAS-STING pathway in surrounding cells (Li et al., 2021). In preclinical studies, cGAMP plays a role in driving cross talk to the TME following radiation treatment (Vijayan et al., 2017). Moreover, cGAMP hydrolysis by ENPP1 results in the formation of AMP, a substrate of NT5E, a transmembrane hydrolase that catalyzes the formation of adenosine (Allard et al., 2019) which also drives immunosuppression in the TME (Stagg et al., 2010; Vijayan et al., 2017). Whether cGAMP and ENPP1 play a similar role in PDAC has not been explored in detail.

Epigenetic regulation is another strategy cancer cells use to modulate response to DAMPs and attenuate PRR activation or downstream signaling. cGAS and STING are epigenetically silenced by the methylation of their respective promoter regions in PDAC (Konno et al., 2018). KRAS and MYC aberrations, which are among the most common in PDAC, suppress IFN-I response by inducing binding of the Myc–MIZ1 complex to IFN regulator promoters (IRF5, IRF7,

STAT1, and STAT2) (Muthalagu et al., 2020). Posttranslational modifications such as methylation, phosphorylation, sumoylation, acetylation, and ubiquitination also modulate constituents of the cGAS-STING pathway in different cancer types (Brown et al., 2018; Tan et al., 2018; Wu et al., 2019; Zhang et al., 2020; Ma et al., 2021). Interestingly, the epigenetic factor PRMT5, which is upregulated in PDAC and correlates with poorer survival (Qin et al., 2019), directly methylates cGAS and limits binding to dsDNA (Muthalagu et al., 2020). It is possible that inhibiting TREX1 or ENPP1 function could result in more effective activation of DNA-sensing pathways in specific subsets of PDAC (e.g., subsets with defects in DNA repair pathways), or following treatment with chemotherapy or radiation.

RNA-sensing pathways can also initiate damage signals, and proteins that prevent sensing of dsRNA can be upregulated in tumors (**Figure 3**). For example, the protein STAU1 stabilizes dsRNA, preventing recognition by RNA-sensing proteins (Chengjin et al., 2018), while ADAR1 targets modified RNA fragments, assisting evasion from triggering of RNA-sensing complexes (Mehdipour et al., 2020). ADAR1 destabilizes specific inverted Alu repeats in dsRNA motifs by catalyzing adenosine-to-inosine editing. Alu repeats are a major source of drug-induced dsRNA, and their destabilization by ADAR1 limits activation of MDA5 and RIG-I (Pichlmair and Reis e Sousa, 2007; Liddicoat et al., 2015; Wang et al., 2017; Herbert, 2019). While the regulation of the RNA-sensing machinery has not been widely explored in PDAC, ADAR1 is upregulated in PDAC and is associated with poor prognosis (Sun et al., 2020). Interestingly, IFN-I also induces tumor cell apoptosis and DAMP release in cell lines (Kimura et al., 2003; Gómez-Benito et al., 2007; Kazaana et al., 2019), including PDAC cells (Vitale et al., 2007) (**Figure 1**).

The potential to harness these pathways to initiate an immune trigger in PDAC is underexplored. Chemotherapy may trigger responses in the tumor cells or the TME, while fragments released from the tumor may stimulate the TME. Given the high density of macrophage-like cells found in PDAC and the high stromal content, agonists of DNA- or RNA-sensing pathways could also play an important role in enhancing more sustained immune responses in the appropriate treatment regimens.

THERAPEUTIC STRATEGIES TO RESTORE TUMOR IFN-I OR INNATE RESPONSES IN PDAC TO MAXIMIZE IMMUNE CELL ENGAGEMENT

There are a variety of pathways that can be activated or inhibited to elicit an interferon response. Reducing the presence of DAMPs is a strategy for cancer cells to evade NA sensing; therefore, increasing DAMP expression could initiate an effective IFN-I response. This could be achieved by targeting the DNA damage response. Conventional treatments such as chemo- and radiotherapy act as potent DNA-damaging agents that can induce IFN-I and recruit antigen-presenting cells (Lugade et al., 2005). PARP inhibition leads to the accumulation of cytosolic DNA by blocking DNA repair. Recently, the PARP inhibitor (PARPi) olaparib has been approved by the FDA for the treatment of BRCA-mutated metastatic PDAC after having shown promising results in the POLO clinical trial (Golan et al., 2019). Interestingly, phase II and III clinical trials have shown PARPi efficacy in patients with non-mutated BRCA1/2 ovarian cancer, suggesting a potential use in a broader spectrum of patients (Gelmon et al., 2011; Mirza et al., 2016). In subcutaneous mouse syngeneic tumor models, the combination of PARPi and anti-PD-L1 treatment increases therapeutic efficacy in BRCA-deficient tumors (Shen et al., 2019). In PDAC tumors with BRCA mutation, inhibiting PARP in combination with ICI may give further enhanced benefits. This same principle could apply to other PDAC subtypes with deficient DNA repair pathways, perhaps giving similar but more sustained IFN-I and innate stimulation than that achieved with chemotherapy.

Inhibiting the function of ATM which repairs double-stranded breaks also increases cytoplasmic DNA (Zhang et al., 2019), TBK1 phosphorylation, and IFN-I expression in preclinical PDAC models. Interestingly, ATM loss of function (which renders cells dependent on the alternative DNA repair enzyme ATR) has been associated with an immune-rich phenotype in PDAC (Wartenberg et al., 2018). Preclinically inhibiting ATR alone or in combination with PARP inhibition has shown therapeutic benefit (Dunlop et al., 2020; Dreyer et al., 2021; Gout et al., 2021; Parsels et al., 2021). Finally, inhibiting DNA-PK, which facilitates non-homologous end joining in combination with radiotherapy increases micronuclei formation and DNA damage, activates IFN-I and increases $CD8^+$ T-cell response (Ciszewski et al., 2014). Therefore, in specific patient subgroups, PARP, ATM, ATR, or DNA-PK inhibitors given chronically (alone or in combination) may sustain DNA damage to trigger immune engagement. Indeed targeting these enzymes with more chronic treatment may be more effective at driving IFN-I and other damage responses than chemotherapy, which only achieve short transient stimulation of these pathways (Reisländer et al., 2020).

There are other ways to increase nucleic acid-mediated activation of DNA damage or stress pathways. Although not investigated in PDAC, the nucleoside analog 6-thio-DG, which induces DNA damage and telomerase stress, activates STING, IFN-I signaling, and $CD8^+$ T cells in colorectal cancer models (Mender et al., 2020). The DNA-sensing cGAS-STING pathway can also be targeted directly with agonists. Intratumoral injection of the STING agonist ADU-S100 combined with radiotherapy increased tumor interferon-stimulated gene expression and T-cell infiltration, causing a reduction in local and distal tumor burden in PDAC murine models (Vonderhaar et al., 2021). Combining STING agonists with anti-PD-1 also showed promising antitumor activity in preclinical mouse syngeneic models (Ghaffari et al., 2018; Kim et al., 2019; Lemos et al., 2020). STING activation in PDAC can occur within either tumor or macrophages to drive a response. Despite these promising preclinical studies, selectively triggering STING also presents challenges. Overactivation of STING may also drive systemic toxicity through the release of TNFα and other cytokines and may

limit the activation that can be achieved, or the duration of treatment. Preclinical tool compounds capable of stimulating the RIG-I RNA-sensing pathway have also shown interesting proof of concept in PDAC models (Ellermeier et al., 2013; Bhoopathi et al., 2014; Das et al., 2019) and may provide an alternate approach in tumors where the cGAS-STING pathway is not functional.

Activating PRRs or reducing suppression by inhibiting negative regulators will also drive IFN-I induction. Although not explored extensively in PDAC, administration of TLR7/8 agonists such as R848 has shown promising results in preclinical models (Michaelis et al., 2019). However, as with STING agonists, these agents can be limited by toxicity, requiring careful scheduling of treatment or the requirement for intratumoral injection to mitigate toxicity (Mullins et al., 2019). TLR9 agonists SD-10-1 and CMP0001 sensitized ICI-resistant mouse models of the colon (Wang et al., 2016) and head and neck squamous cell carcinoma (Sato-Kaneko et al., 2017) to PD-1 blockade. Response was linked to IFN-I stimulation, increase in interferon-stimulated gene expression, and subsequently expansion of $CD8^+$ T cells. Approaches to stimulate these pathways may be worth considering for PDAC to enhance the triggering of immune responses.

Modulators of the DNA methylation state can cause DNA or RNA stress within cells. DNA methyltransferase inhibitors (DNMTi) reduce methylation of endogenous retroelements (Roulois et al., 2015) and increase levels of cytosolic dsRNA (Goel et al., 2017). 5-Azacytidine (a DNMTi) induced re-expression of silenced genes leading to increased T-cell-stimulating chemokines *in vitro* and increased T-cell infiltration in PDAC *in vivo* models (Ebelt et al., 2020). Effects of epigenetic modulators can however be context-dependent. The inhibition of PRMT5 with EPZ015666 increased IFN-I and cGAMP production upon stimulation with DNA fragments *in vitro* (Ma et al., 2021). However, in another study, EPZ015666 decreased IFN-I expression and impaired interferon-stimulated gene expression upon stimulation with poly(I:C) (Cui et al., 2020). Last, consistent with ADAR1 being associated with dampening RNA-sensing pathways, knockout in tumor cells conferred vulnerability to immune checkpoint inhibitors (Ishizuka et al., 2019).

Vaccines or oncolytic viruses can stimulate IFN-I responses, while delivery of recombinant IFN-α or -β to tumors agonizes the pathway directly. Early trials with IFN-I conjugates were not successful, largely due to toxicity issues; however, next-generation approaches that deliver IFN-I conjugates more safely are being developed. Tumor vaccines will stimulate broad innate immune responses. Both cell-based tumor vaccine approaches such as GVAX (Wu et al., 2020b) and GVAX, and a *Listeria*-based vaccine expressing mesothelin (an antigen upregulated in pancreatic cancer) have been trialed (Le et al., 2019). Unfortunately, these vaccine-based approaches have not yielded positive clinical signals. Oncolytic viruses can stimulate significant IFN-I induction (reviewed in Harrington et al. (2019); Evgin et al. (2020); Cao et al. (2021); Rosewell Shaw et al. (2021)) and drive sustained T-cell activation. Novel strategies combining oncolytic viruses with CAR-T therapy are being developed to drive targeted sustained responses in preclinical pancreatic cancer tumor models.

There are many different strategies to increase tumor immune cross talk through IFN-I responses. However, these approaches are challenging, and therapeutic index is likely to be an issue requiring careful dose selection clinically and the ability to focus on specific patient subsets to induce an effective innate immune response (Konno et al., 2018) (Salvador-Barbero et al., 2020) (Muthalagu et al., 2020). Moreover, IFN-I may act differently when induced acutely or with sustained chronic upregulation. How the IFN-I response is modulated and for how long needs to be considered as complicated regulatory mechanisms can have both activating and suppressive effects, depending on the context.

CONCLUSION

PDAC has limited sensitivity to chemotherapy and resistance to most current immunotherapy approaches. While chemoimmunotherapy shows better overall responses than chemotherapy or immunotherapy alone, this benefit is marginal for most patients. In PDAC, many mechanisms may prevent the activation of the immune response, from failure to sustain tumor damage- and stress-related immune triggers (e.g., IFN-I release, antigen presentation, or DAMP release) to indirect resistance in the TME. IFN responses can however be stimulated with tumor-targeted treatment (e.g., chemotherapy and DDR inhibitors) or induced in the TME. Improved clinical responses could be achieved by enhancing the cross talk between the tumor and TME through the induction of IFN-I signaling, and there are promising approaches to achieve this in the right setting. A number of clinical translational programs such as Pan Can, ESPAC, and Precision Panc are using multi-omic assessments of human clinical trial samples to look at features that influence response to treatment. Studies such as these can provide new ways to look at where and how IFN response may be suppressed in patients as well as identify tumors that may respond well to specific treatment strategies. This work will contribute to our understanding of the intrinsic tumor cell-initiated immunomodulatory pathways in PDAC. Moreover, focusing on treating subsets of tumors in a biomarker and mechanism linked way will hopefully enable the field to identify and build on efficacy signals with more confidence. Ultimately, this will guide the development of improved combination strategies that potentiate immune response in this challenging disease.

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