



Review

Pancreatic ductal adenocarcinoma: From genetics to biology to radiobiology to oncoimmunology and all the way back to the clinic



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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death. Despite improvements in the clinical management, the prognosis of PDAC remains dismal. In the present comprehensive review, we will examine the knowledge of PDAC genetics and the new insights into human genome sequencing and clonal evolution. Additionally, the biology and the role of the stroma in tumour progression and response to treatment will be presented. Furthermore, we will describe the evidence on tumour chemoresistance and radioresistance and will provide an overview on the recent advances in PDAC metabolism and circulating tumour cells. Next, we will explore the characteristics and merits of the different mouse models of PDAC. The inflammatory milieu and the immunosuppressive microenvironment mediate tumour initiation and treatment failure. Hence, we will also review the inflammatory and immune escaping mechanisms and the new immunotherapies tested in PDAC. A better understanding of the different mechanisms of tumour formation and progression will help us to identify the best targets for testing in future clinical studies of PDAC.

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1. Introduction

Pancreatic cancer constitutes the fourth leading cause of cancer mortality. Pancreatic ductal adenocarcinoma (PDAC) is the most frequent histological type and is characterised by a dismal prognosis with a 5-year survival rate of 5% [1,2]. The American Cancer Society has reported 43,920 new cases of PDAC in the US in 2012. The majority of patients are diagnosed at a late stage due to the lack of diagnostic symptoms in the early stage disease [1]. Despite the advent of new agents, the clinical outcome of patients with PDAC remains poor. Hence, there is an urgent need for a better understanding of the biological phenotype of this deadly disease. In the present article, we comprehensively review the current literature on genetics, biology, radiobiology, mouse models, metabolism, circulating tumour cells and oncoimmunology as well as the implications of the findings for the management of patients with PDAC.

2. Genetics of pancreatic ductal adenocarcinoma

PDAC is characterised by genetic alterations that induce tumorigenesis that determine the disease phenotype. Genetic mutations occur commonly in patients with PDAC and preinvasive lesions, such as pancreatic intraepithelial neoplasia (PanIN), exhibit mutations that are similar to those found in advanced disease [3]. Also, some pancreatic cancers present familial inheritance [4]. Importantly, the disease phenotype can be closely recapitulated using genetically-engineered mouse models (GEMMs) with conditional modification of the expression of genes involved in PDAC formation and progression, such as Kras and p53 [5]. These data support the notion that PDAC has a genetic aetiology.

2.1. Preinvasive lesions

Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystadenomas (MCNs) are the three main preinvasive lesions that have been identified in the pancreas [3]. PanIN likely emerges from the Her1-positive stem cell-like cells found at the acini–ductal epithelium junction or the mature acinar cells [6]. PanIN has three different stages of progression before it gives rise to PDAC [7,8]. These are PanIN-1, PanIN-2 and PanIN-3, depending on the degree of cytological atypia of the duct lining cells. PanIN-1 presents minimal atypia with mucinous differentiation of the ductal cells, whereas PanIN-3 has marked atypia and essentially

represents a carcinoma in-situ [7,8]. IPMNs stem from the mucin-producing main pancreatic duct or its branches and have cystic morphology. MCNs present an ovarian-like stromal content and have a mucinous epithelial lining. The majority of PDACs arise from PanINs, whereas IPMN- or MCN-induced PDAC only occurs sporadically.

2.2. Telomeres in pancreatic ductal adenocarcinoma

Telomeres are DNA–protein complexes that cap the chromosome ends and are made of tandem repeats of the 5′-TTAGGG-3′ sequence [9]. They have evolved by eukaryotic organisms to solve the problem of DNA end-protection. Shortening of the telomeric DNA at chromosome ends limits the lifespan of human cells and short telomeres activate the DNA damage response (DDR).

Activation of telomerase that is responsible for telomeric DNA synthesis is essential in cancer cell immortalization and cancer progression [9]. Patients with PanIN have abnormally shortened telomeres compared to the ductal epithelium of normal pancreas [10]. Also, patients with PDAC often have activation of telomerase expression and telomere dysfunction [11,12].

2.3. Pancreatic development and genetic alterations in pancreatic ductal adenocarcinoma

Early pancreatic development is mediated by Pdx1 and Ptf1a genes in conjunction with the Sonic Hedgehog (Shh) and Notch signalling pathways. The pancreatic homeobox transcription factor Pdx1 is mediated by the Shh repression and is expressed in the nascent pancreatic bud. The Pdx1 promoter has been used in GEMMs to direct transgene expression at the bud stage [13]. Ptf1a is a basic helix–loop–helix (bHLH) transcription factor present in acinar cell nuclei that is required for the development of the pancreas. Ptf1 is involved in the activation of the gene promoters that encode the secretory digestive enzymes and in pancreatic tissue development [14]. In addition, nestin, an exocrine pancreas progenitor marker and Mist1 are involved in early organ formation [15–19]. All four genes have been used as promoter in the development of GEMMs of PDAC. Two common pathways involved in pancreatic organogenesis and development are the Shh and Notch signalling pathways [6,20]. Preclinical evidence has suggested involvement of both pathways in initiation of PanIN and tumour growth. Shh is not expressed in normal adult pancreas but aberrant signalling has

been reported in more invasive stages. Also, Shh contributes to the formation of desmoplasia. Similarly, Notch promotes the formation of early PanIN lesions via TGF- α signalling [6,20].

PDAC is a genetic disease induced by mutations in oncogenes and tumour suppressor genes that lead to tumour formation and disease formation. Ductal reprogramming of the acinar and insulin-positive endocrine cells occurs before transformation of normal tissue to pancreatic intraepithelial neoplasia (PanIN). Together with aberrant expression of master switch genes, such as ZIP4, these genetic alterations gradually accumulate throughout the stages of PDAC development starting from PanIN [21].

Kras (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) oncogene mutation occurs in 95% of patients with PDAC [22]. It is an early initiation event in PDAC tumorigenesis leading to cell proliferation, differentiation and survival. Kras encodes a ~21 kDa small GTPase, which cycles between GTP-bound active and GDP-bound inactive states [22]. Mutations of KRAS found in human PDAC include point mutations at codon G12, G13 and Q61 that impair intrinsic GTPase activity of the KRAS protein. This leads to activation of Kras downstream pathway that drives tumour progression. Mutations in Kras gene are already encountered at the PanIN lesions, being the earliest genetic alteration that promotes formation of PDAC [23].

Tumour suppressor genes such as cyclin-dependent kinase inhibitor 2A (CDKN2A/p16^{INK4A}), tumour protein p53 (TP53), mothers against decapentaplegic homolog 4 (SMAD4/DPC4), serine/threonine-protein kinase 11 (LKB1/STK11) and the breast cancer 2 early onset gene (BRCA2) are involved in PDAC formation [24]. CDKN2A, also known as p16 or INK4A, is a tumour suppressor gene that inhibits cell cycle progression through the G1-S checkpoint. CDKN2A is inactivated in 90% of patients with PDAC and is found in PanIN-2 precursor lesions [25]. Loss of function occurs by either homozygous deletion, intragenic mutation or epigenetic silencing by promoter methylation [26]. Germline mutation in CDKN2A is associated with both Familial Atypical Mole-Malignant Melanoma (FAMMM) syndrome and PDAC [27].

The p53 protein is a master regulator of genome integrity and plays an important role in regulation of cell cycle, such as G1-S checkpoint, G2-M arrest and induction of apoptosis [28]. p19ARF leads to p53 stabilization by preventing MDM2-dependent proteolysis. In PDAC, defects in p53 tumour suppressor function occur commonly due to p19ARF loss and missense mutation within the DNA-binding domain of [29]. Loss of p53 function is found on chromosome 17p and occurs via an intragenic mutation with loss of the second allele [28]. Approximately 50%–75% of patients with PDAC have p53 loss, whereas gene mutations are found as early as PanIN-3 lesions [30]. Sansom and colleagues have shown that Trp53 loss or mutation permits growth of Kras-mutated cells and promotes progression of premalignant lesions to PDAC through failed growth arrest and senescence [31]. Of note, mutant p53(R172H) correlated with increased metastases, both in a mouse model of PDAC and in human specimens.

SMAD4, also known as the deleted in pancreatic carcinoma 4 gene (DPC4), is found on chromosome 18q21 [32]. SMAD4 deficiency can inhibit TGF- β -induced cell cycle arrest and migration, contributing to tumour formation [33]. SMAD4 inactivation occurs by either homozygous deletion (30%) or by intragenic mutations and loss of the second allele (25%) and it is found as early as PanIN-3 lesions [34]. The John Hopkins group conducted a rapid autopsy in 76 patients with PDAC [35]. From those, 30% had a locally destructive disease and 70% distant metastases. Loss or mutation of SMAD4 correlated with distant metastases ($p = 0.007$), indicating that this marker could be potentially used to stratify patients for more aggressive local or systemic treatment [35]. The same group examined 79 matched primary and metastatic samples for mutations in KRAS, CDKN2A, and TP53 and expression of CDKN2A, TP53, and SMAD4. Interestingly, only 39% of patients presented alterations in all 4 genes. The majority of patients with SMAD4 loss in primary tumours also had coexistent TP53 alterations. In contrast, TP53 alterations occurred in patients with both SMAD4-wild type and loss (approximately 1:1). Multivariate analysis showed that the number of

driver gene alterations was an independent prognostic factor for overall survival ($p = 0.046$) [35]. Finally, BRCA2 tumour suppressor gene mutation is associated with familial breast and ovarian cancer but it only occurs in 1–2% of patients with PDAC [36].

2.4. Sequencing of human samples of pancreatic ductal adenocarcinoma

An important multicentre study performed exome sequencing to investigate the genetic and molecular alterations in 24 human PDAC [37]. In that work, 20,661 protein coding genes that represented 99.6% of the coding genome were studied. Copy number gains or deletion were also assessed by copy number chips. In total, 31 of 69 gene sets were identified and were grouped into 12 core signalling pathways with distinct functions in tumour formation and growth [37]. These included apoptosis, DNA damage control, G1/S phase transition, Shh, homophilic cell adhesion, signalling of integrins, c-Jun, Wnt/Notch, TGF- β , Kras and other GTPases. Although the genes that showed alteration in any given PDAC varied widely within the individual pathways, notably only one gene member of each pathway was altered in any given tumour. This is an important finding indicating that using the core pathways instead of large number of genes might aid to the better understanding of the mechanisms of tumour formation in PDAC [37]. Recently, Biankin et al. conducted exome sequencing in 142 patients with Stage I and II PDAC [38]. In total, 2016 non-silent mutations and 1628 copy-number variations were detected. Also, 16 known and novel mutated genes and somatic aberrations in SLIT/ROBO axon guidance signalling were described [38].

2.5. Clonal evolution in pancreatic ductal adenocarcinoma

There is a progressively increasing interest in investigating the clonal evolution in PDAC. The cancer genome data of seven patients with PDAC and their matched metastases were examined [39]. The genetic profile of the surgically resected tumours and their matched metastases were compared with each other. Intriguingly, analysis of the genetic features showed that the total number of genetic alterations and the spectrum of the mutations were similar. These tumours had approximately 61 mutations, the majority of which were missense mutations that lacked predictive value with regard to tumorigenesis [39]. This study indicated that the genetic alterations observed in patients with advanced disease are comparable to those seen in the primary tumour resection samples.

To gain further understanding, a comparative lesion sequencing was conducted using the samples of these seven patients [39]. Two types of mutations were identified. The first group included mutations that were commonly shared in both the primary tumour and their matched metastatic samples and were called “founder” mutations. The authors hypothesized that founder mutations must have occurred already at the PanIN precursor lesions and must be present in the majority of the cells within the tumour mass, mediating tumour formation. Indeed, these included mutations in driver genes, including Kras, CDKN2A, p53 and SMAD4 but also other candidate cancer genes (CAN) according to their mutation frequencies and types. The second group included mutations that were only found in a subset of tumour samples from each patient. These “progressor” mutations likely represent genetic alterations that occurred after founder mutations due to clonal evolution of the parental clone [39].

Altogether, there are three main differences between founder and progressor mutations. First, there is quantitative difference i.e. significantly more founder mutations were detected compared to progressor mutations and were contained within the parental cancer clone. Second, the majority of key mutations that highly determine tumour aggressiveness, such as Kras, were included in the founder mutations. Third, most of founder mutations were actually homozygous mutations and hence the majority of alterations that lead to genomic instability and drive cancer growth are harboured by the parental clones. Campbell et al. conducted parallel paired-end sequencing in 13 patients with pancreatic cancer to explore clonal relationships among metastases [40].

They found that the majority of genetic alterations and rearrangements occurred early, long before the development of metastatic dissemination. Indeed, fold-back inversions occurred early in tumourigenesis and likely constitute crucial drivers of tumour progression.

Computational analyses of genome sequencing data estimated the time between tumour initiation and formation of macroscopic metastases [39]. This mathematical model included published data on the rate of cellular proliferation of both normal and malignant pancreatic cell lines as well as passenger mutation rates. Remarkably, the time from tumour initiation to the birth of the parental cancer clone was estimated to be almost 12 years. According to this model, the parental clone is not sufficient for the development of metastatic spread and additional subclones develop over an additional period of approximately 7 years. Finally, the time from metastatic dissemination of the subclone(s) until the patient's death is approximately 2.7 years. This is a crucial finding and indicates that developing methods of early tumour detection might provide a window of opportunity for medical intervention before the development of metastatic disease. A similar model has been generated to predict the optimal therapeutic window for administration of anticancer therapies in PDAC and showed that chemotherapeutic agents that efficiently reduced the growth rate of cells earlier in the course of treatment result in superior treatment outcome [41].

2.6. Genetic subtypes of pancreatic ductal adenocarcinoma

Hanahan and colleagues examined the transcriptional profile of PDAC samples and also human and murine PDAC cell lines. The array datasets revealed three molecular subtypes of PDAC, based on the gene signatures: classical, quasimesenchymal and exocrine-like [42]. The classical subtype mainly expressed genes associated with cell adhesion and also epithelial genes, whereas the quasimesenchymal subtype demonstrated expression of genes encountered in mesenchymal cells. Finally, the exocrine-like subgroup exhibited expression of genes involved in the regulation of digestive enzymes. Of note, the classical subtype was associated with significantly better survival than the quasimesenchymal or exocrine-like group in patients that underwent surgical resection in multivariate analysis ($p = 0.038$). Notably, quasimesenchymal cells showed better response to gemcitabine as compared with the classical phenotype. The latter was more sensitive to erlotinib, indicating that the survival of Kras-dependent PDAC cells depends on EGFR, at least in part [42]. These data provided important insight as they could help stratifying patients to different therapies according to their genetic signature.

3. Mouse models in pancreatic ductal adenocarcinoma

Several models of PDAC, including xenograft, orthotopic and GEMMs, have been used to study the biology of the disease. Below we discuss the different mouse models, their main characteristics and the differences between the human and mouse phenotype.

3.1. Subcutaneous mouse model

The subcutaneous tumour model has been used extensively in PDAC research [43]. It is relatively inexpensive and permits direct tumour growth assessment. However, highly relevant to PDAC, it fails to recapitulate the histopathological features of the human pancreatic tumour. Additionally, immunodeficient mice do not allow the study of the tumour cell-host immune response interaction that appears to play a key role in tumour growth and progression in patients with PDAC. Last but not least, in contrast to the orthotopic or GEMM, xenograft tumour models rarely develop distant metastases that commonly occur in patients with PDAC [43].

3.2. Orthotopic xenograft model

The orthotopic model has been increasingly used during the last decade. It resembles more closely the human PDAC pathophysiology and in contrast to the xenograft mode, it results in the frequent development of distant metastases. Nevertheless, the majority of the models have used immunodeficient mice that lack immune cells, a critical component in tumour development and metastatic spread [44,45].

Several methods have been tested to develop orthotopic PDAC mouse models. Some groups have employed direct injection of PDAC cells into the pancreas, either body or tail following surgical operation. Although relatively simple, it can be associated with increased risk of complications, such as infection, abdominal bleeding, disruption of pancreatic capsule and intraabdominal tumour cell spillage and peritoneal spread [44,45]. Additionally, injection into the main pancreatic duct has been reported using green or red fluorescent protein (GFP/RFP)-tagged cells into the pancreas to enable visualization and monitoring of tumour growth and metastatic spread [46]. An alternative method, ultrasound-guided injection into the pancreas, appears to be feasible and safe, allowing faster injection times and shorter recovery time [47]. A fluorescence-guided orthotopic tumour implantation using a tumour fragment from subcutaneous xenograft has been demonstrated [48]. Even though more traumatic, the tumour take and growth rates in nude mice were excellent using this technique and tumours closely resemble the histological features of the human disease. An alternative, less invasive option to surgical tumour implantation directly into the pancreas is wrapping the pancreas around the tumour xenograft but its validity remains to be defined [49]. The orthotopic model has been previously used to examine tumour recurrence, perineural invasion and the role of pancreatic cancer stem cells [45].

3.3. Genetically engineered mouse models

GEMMs including transgenic mouse models, gene knock-in and knock-out models, and conditional/inducible models have been used extensively during the last decade to study the biology of PDAC.

3.4. Transgenic mouse models

Transgenic mice enable probing of gene function and allow monitoring the effects of genetic alterations experimentally. Pancreas constituted one of the first organs to be studied using transgenic tumour induction through the recognition of tissue-specific promoter elements in the elastase I locus [50]. The elastase promoter was used to examine the impact of Kras oncogene activation in *Ela-KrasG12D* mice that presented precancerous morphological changes that failed to develop to PDAC [50]. More advanced models demonstrated the key role of Kras in tumour initiation and progression. *Ela-1-myc* was created following insertion of the c-Myc gene via SV40-Tag under the regulation of the elastase promoter and resulted in the formation of acinar and acinar-ductal carcinomas with a high degree of ductal metaplasia [51]. In a different model, the elastase-1-TGF- α construct exhibited a high amount of fibrosis associated with tubular complexes formed by cells with mixed ductal-acinar phenotype [52]. Interestingly, mice older than 1 year of age demonstrated loss of the acinar phenotype and preservation of the ductal-like morphology. Lewis et al. used a different approach by delivering the oncogene-bearing avian retroviruses to the mouse model that expressed the receptor TVA (receptor for avian leukosis sarcoma virus subgroup A (ALSV-A)) [53]. Insertion of either the mouse polyoma virus middle Y antigen (PyMT) encoding ALSV-A-based RCAS vectors or C-MYC to elastase-tv-a transgenic *INK4a/ARF* null mice resulted into pancreatic tumour formation [53].

PDX1 is a marker for pancreatic progenitor cells that has been implicated in the formation of PDAC and its loss is associated with pancreatic agenesis [16]. PDX1 and its promoter has been explored for genetic manipulation in transgenic mice to prevent expression of transforming

oncogenes in the embryonic stage and target specific cell groups within the pancreas [54]. The PDX-Shh mice have been used to study the effect of Shh pathway [17]. This model develops tubular structures of both PanIN-1 and PanIN-2 and exhibits mutations of both Kras and HER-2/neu oncogenes. The main disadvantage of the transgenic mouse model is the relatively low efficiency when inserting foreign DNA into the host genome using microinjection. In addition, in transgenic mice, two wild-type alleles are positioned at the transgene integration site, whereas the human tumour genome contains one wild-type and one mutant allele [55].

3.5. Knock-in and knock-out mouse models

To create the knock-in and knock-out mouse models, the endogenous genomic sequence is modified by either inserting or deleting the gene at a specific locus. Embryonic stem cell-based techniques are used to insert the transgene in its native chromosomal location. To study the role of Kras, Tuveson et al. created the *Mist1-KrasG12D/+* mice by targeting the expression of KrasG12D to the *MIST1* locus [56]. *MIST1* is expressed at early stages of pancreatic exocrine development and these mice exhibited acinar–ductal metaplasia and hyperplasia. Insertion of a concomitant *Trp53* $+/-$ mutation led to the formation of PDAC and liver metastases [56].

3.6. Conditional knock-in and knock-out models

For conditional knock-in and knock-out mouse models, a silencing transcriptional element is inserted between the promoter and the gene of interest to avoid universal expression during embryogenesis and early postnatal development. The gene of interest is activated in a tissue-specific manner and the most popular method is Cre–Lox recombination [57]. This permits temporal and spatial control of Kras expression, enabling transcriptional control of the mutant Kras allele by the endogenous Kras promoter. Crossing mice expressing a Cre-activated KrasG12D allele with mice expressing Cre recombinase in pancreatic tissue resulted in the formation of the endogenous KrasG12D model that presents PanIN lesions during the first weeks of life [5]. Deletions of *INK4a/ARF* or *TP53* alone did not show tumorigenesis, but mice formed aggressive PDAC by 7–12 weeks with shorter latency and metastasis formation following combination with Kras [5]. In a different model, Carriere and colleagues used the Nestin-Cre; LSL-KrasG12D to better elucidate the origin of PDAC [19]. This model exhibited PanIN that was similar to the Pdx1-Cre; LSL-KrasG12D model, indicating that PDAC might derive from Nestin-expressing cells.

The KrasG12D; *Tgfr2* knockout model has been created to examine the effect of the suppressor TGF- β [58]. *Tgfr2* knockout led to blockade of the TGF- β signalling pathway and the pancreas demonstrated PanIN-like lesions and extensive desmoplasia that almost completely substituted pancreatic tissue by 6–7 weeks of age. The morphology following blockade of TGF- β by knocking out SMAD4 closely resembled intraductal papillary mucinous neoplasm (IPMN) or mucinous cystic neoplasm (MCN) pre-cancer lesions that are considered to be less aggressive than PanIN [58].

Crossing CLEG2 transgenic mice with Pdx1-Cre mice has been used to create the Pdx1-Cre; CLEG2 conditional knockin mouse model to investigate the role of the SHH signalling pathway [59]. This model develops pancreatic tumours that fail to resemble PDAC histological characteristics. Intriguingly, crossing Pdx1-Cre; CLEG2 mice with the LSL-KrasG12D mouse model led to PanIN-2 and PanIN-3 lesions that failed to progress to PDAC. In this model, Hedgehog ligand expression was aberrant [59]. A similar model has been developed to assess the role of the Notch signalling pathway in PDAC [60]. Crossing Notch-1 transgene, *Rosa26NIC*, along into Kras and Pdx1-CreERT transgenes was used to generate the Pdx1-CreERT; LSL-KrasG12D; *R26-Notch* mouse model. The latter was characterised by the rapid development PanIN of acinar origin. Ling and colleagues studied the NF- κ B pathway

by generating the Pdx1-Cre; KrasLSL-G12D; IKK2/ β F/F and Pdx1-Cre; KrasLSL-G12D; *Ink4a/Arff/F*; IKK2/ β F/F mouse models. These exhibited inactivation of the pancreas-target IKK2/ β that inhibits NF- κ B signalling. IKK2/ β /NF- κ B was activated by KrasG12D through the IL-1 α /p62 axis, whereas mice failed to develop PDAC, highlighting the importance of NF- κ B in tumorigenesis [61].

3.7. Inducible transgenic mouse models

A major challenge when using transgenic method is that the transgenic rather than the native promoter regulates the transgene expression. To avoid early expression, an inducible promoter is inserted to enable activation of the transgene when needed. For that purpose, two systems, the tamoxifen-inducible Cre-ER and the tetracycline-inducible Tet-ON/OFF have been employed [62,63]. Inactivation of the studied gene occurs via binding of the chimeric Cre protein to HSP90. The Pdx1-CreERT; LSL-Kras mouse models only PanIN without PDAC formation following tamoxifen binding [2]. In a recent study, Ji et al. created a novel PDAC mouse model by inserting KrasG12V after the CAG promoter, and after a loxp-GFP-loxp cassette (cLGL-KrasG12V) [64]. Kras upregulation in acinar cells led to PanIN, cystic papillary carcinoma and PDAC. Interestingly, higher level of Kras activity was associated with chronic pancreatitis-like features, such as inflammation and fibrosis, which can promote genetic instability and tumour progression. This work showed the impact of high level of Kras activation in promoting transformation of PanIN to PDAC [64].

3.8. Patient-derived xenograft mouse model

There is an increasingly accumulated interest in developing patient-derived xenografts (PDXs) of PDAC [65]. Following surgery of human PDAC, a resected tumour specimen is implanted in immunodeficient mice, usually the non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mouse. This model lacks functional T and B cells and also harbours additional deficiencies in natural killer cells (NKs) that permit tumour establishment from a very low number of tumour cells. PDXs recapitulate closely human tumour histopathology, including the formation of stroma, and preserve tumour heterogeneity, providing a more reliable platform for the study of human PDAC compared to other xenograft models [45,65]. Hidalgo and colleagues implanted 94 resected specimens of PDAC of which 61% presented successful engraftment [66]. Interestingly, SMAD4 inactivation correlated with better engraftment rate, faster growth and higher metastatic gene expression signature that predicted for worse response of patients to chemotherapy and unfavourable outcome [66].

4. The stroma of pancreatic ductal adenocarcinoma

Desmoplasia is a prominent characteristic of PDAC characterised by extensive stroma that constitutes approximately 80% to 90% of the tumour volume [67,68]. Desmoplasia results from the significant overproduction of extracellular matrix (ECM) proteins and extensive proliferation of myofibroblast-like cells called pancreatic stellate cells (PSCs) [67,69–71]. The stellate cell-mediated alterations in the stromal cell proliferation and the deposition of ECM components result in increased stiffness, solid stress and interstitial fluid pressure. These common features contribute to the overall tissue heterogeneity, impaired delivery of oxygen and nutrients, resulting in chemo- and radioresistance and tumour progression. The presence of extensive matrix component in stroma cells has been associated with poor outcome in PDAC [72,73]. Genetic alterations, including oncogene and tumour suppressor gene mutations have been reported in both the tumour epithelial and stromal compartments [74]. Interestingly, normal stroma appears to prevent or even delay tumour growth, whereas abnormal stromal components facilitate tumour progression [75].

4.1. Components of the stroma

The desmoplastic stroma is composed of pancreatic stellate cells (PSCs), ECM, fibroblasts, macrophages, blood vessels, pericytes, lymphatic vessels, bone marrow-derived cells (BMDCs) and stem cell-like cells and also inflammatory cells [76–79]. The mechanisms of stroma formation remain unclear. Pancreatic cancer cells produce several modulating growth factors that can alter the adjacent stroma to facilitate tumour progression. These factors can act in a paracrine manner to induce an inflammatory response and activation of PSCs, fibroblasts smooth muscle cells and adipocytes [76,80]. These lead to the release of cytokines, pro-tumourigenic growth factors and proteases that form a positive feedback interaction loop to activate both cancer cells and the surrounding stroma to enhance tumour growth.

Currently, there is a poor understanding of the precise origin of stromal cells. Although stromal cells might stem from the normal pancreatic tissue stroma, cancer cells might be an alternative source of stroma following epithelial–mesenchymal transition (EMT) [81]. In addition, stroma might derive from BMDCs. Interestingly, in a model of chronic pancreatitis, BMDCs contributed to the formation of PSCs [82,83]. Moreover, BMDCs and stem-like cells have the ability to differentiate into different stroma cell subtypes [82]. Proinflammatory and immune cells enable immune escape that, in conjunction with proangiogenic and profibrotic factors, contribute to increased desmoplasia [84]. Chronic pancreatitis constitutes a major risk factor for the development of PDAC and it is associated with a 7.2 fold increase in the incidence of the disease [85]. Additionally, it is characterised by extensive organ fibrosis and shares many histopathological and molecular similarities with PDAC, including identical stromal composition [85,86]. Chronic pancreatic inflammation, as induced by smoking and diabetes can lead to fibrosis and scarring of pancreatic acini [87]. Exposure of tumour and stromal cells to high insulin levels is correlated with increased mitogenesis and stromal proliferation.

4.2. Growth factors

Several growth factors secreted by cancer cells regulate the proliferation and survival of stromal cells. These include TGF- β , Shh, Wnt signalling, secreted protein acidic and rich in cysteine (SPARC), PDGF, FGF, IGF-1, EGF, NGFs, HGF/Met, COX-2, MMPs, neurotrophins, and EGF. This crosstalk between cancer cells and stromal cells contributes to tumour angiogenesis, invasion and metastasis.

4.3. TGF- β

TGF- β mediates epithelial cell growth and formation of ECM proteins and is commonly upregulated in patients with PDAC, being expressed predominantly in the stroma [88]. TGF- β promotes survival of cancer cells and contributes to resistance to chemotherapy and radiotherapy [89,90]. It exerts a paracrine effect in modulating the stroma via stimulation of angiogenesis, expression of ECM proteins and immunosuppression [91,92]. Transfection of pancreatic cancer cells with TGF- β induced a rich stromal reaction following orthotopic pancreatic tumour cell implantation in a nude mouse model [91]. Furthermore, TGF- β promoted EMT resulting in increased fibrogenesis in TGF- β -producing human PDAC cell lines [93].

4.4. The extracellular matrix

The ECM provides mechanical support and adherence of cells and facilitates cell–cell contact and communication. Collagens, glycosaminoglycans, glycoproteins, growth factors and proteoglycans, SPARC, tenascin C, periostin, and thrombospondin are components of the ECM. Alterations in the expression of ECM components modulate tumour cell invasiveness and metastatic potential [76–79]. Fibroblast activation protein (FAP)- α can alter the morphology of fibronectin to

enhance the invasive potential of PDAC cells [94]. Collagens I, III and fibronectin constitute important components of PDAC stroma and alterations in collagen expression can increase tumour cell proliferation, migration and survival [95]. Importantly, adherence of PDAC cells to ECM components, such as collagen, laminin and fibronectin decreases response to chemotherapy in PDAC [96]. Glycosaminoglycans, like hyaluronic acid, contribute to vascular collapse and decreased drug penetrability in PDAC [97]. Recent studies have attempted to deplete hyaluronic acid using the enzyme hyaluronidase. The addition of this enzyme to gemcitabine enhanced its intratumoural concentration and improved survival in GEMMs bearing PDAC [98]. This study supported the crucial role of stroma in mediating chemoresistance in PDAC. Also, the nerve fibres and release nerve growth factors (NGFs) enhance tumour growth and spread [99,100].

4.5. Pancreatic stellate cells

PSCs resemble fibroblast cells and express various adhesion molecules, such as α -smooth muscle actin (α -SMA), vimentin, and desmin [101]. They contain vitamin-A-storing lipid droplets in the cytoplasm, a unique characteristic that is not found in either normal smooth muscle cells or fibroblasts [102]. Under normal conditions, PSCs comprise only 3–4% of the normal pancreas volume [102]. PSCs are the master regulators of desmoplasia in PDAC and the main source of ECM in pancreatic fibrosis. Several studies have shown that PSCs enhance tumour invasiveness and aggressiveness [78,103]. Intriguingly, PSCs have been found in distant metastases, indicating that PSCs might migrate simultaneously or even in association with pancreatic cancer cells to form a favourable microenvironment and enable distant metastatic spread [104].

Culturing PDAC cells using conditioned media from PSCs led to upregulation of the β -galactoside-binding protein galectin-3 in PSCs [105]. PSCs produce several factors including transforming growth factor β (TGF- β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumour necrosis factor α (TNF- α), interleukin (IL)-1 and IL-6, activin A, matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs) and cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGFA) and collagen I and reactive oxygen species [106–112]. These profibrogenic molecules augment PSC activation and enhance secretion of ECM components via activation of various signalling pathways, such as the mitogen-activated protein kinase [111, 112]. In consequence, these autocrine loops increase deposition of fibrotic tissue, resulting in excessive desmoplasia in PDAC [113].

4.6. Fibroblasts

Fibroblasts play a key role in the pancreatic cancer formation and growth [114]. They are involved in epithelial differentiation, production of ECM proteins as well as inflammation and wound healing [115]. Fibroblasts express α -SMA, vimentin, desmin and fibroblast activation protein (FAP) [116]. Accumulating evidences suggests that cancer-associated fibroblasts (CAFs) interact closely with tumour cells in PDAC through various factors, such as TGF- β and PDGF [116]. Furthermore, CAFs secrete stromal-cell-derived factor 1 (SDF-1) that results in the release of serine proteases and MMPs and increased tumour invasiveness [117,118]. Similarly, production of hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF1) by CAFs promotes tumour cell proliferation and migration [116,119]. Co-culture of CAFs with human PDAC cells enhanced their invasiveness [120]. Moreover, comparison of gene expression between human pancreatic CAFs and normal pancreatic fibroblasts revealed upregulation of the Hedgehog receptor *Smoothened* only in CAFs, whereas co-culture of human PDAC cells that express *Hedgehog* with fibroblasts resulted in Gli activation in fibroblasts [121,122]. Also, co-culture of CAFs with human PDAC cells augmented tumour cell survival via upregulation of COX-2 in both cell

types [120]. These data support the role of CAFs in promoting tumour cell survival and metastatic spread in PDAC.

4.7. Developmental pathway aberrations

4.7.1. Sonic Hedgehog pathway

The Shh signalling pathway is involved in the development of normal pancreatic tissue [20]. The Shh ligands and Smo are expressed in up to 70% of human PDAC tumours but not in normal pancreas [123]. Shh contributes to early and late tumourigenesis and promotes cell migration and invasion [17,124]. It also increases desmoplasia by modifying the differentiation of PSCs and fibroblasts [125,126]. Indeed, treatment with Shh enhanced α -SMA expression in fibroblasts and up-regulated collagen I and fibronectin in a xenograft mouse model [125, 126]. Moreover, Shh enhances tumour growth via a paracrine mechanism, whereby tumour cell-secreted Shh induces hedgehog-target genes in the surrounding stromal tissue [124].

4.7.2. Wnt pathway

Wnt constitutes one of the major signal transduction pathways involved in pancreas organogenesis [127]. It utilises β -catenin dependent (canonical) and β -catenin independent (noncanonical) signals to regulate cell phenotype differentiation, proliferation and migration. Aberrant Wnt signalling has been implicated in the development of various cancers but its role in PDAC remains ambiguous [127]. Pancreas-specific Wnt/ β -catenin activation via CTNNB1 mutations led to the formation of pseudopapillary tumours but not PanIN or PDAC in a transgenic mouse model [128]. Similarly, the p48-Cre; Ctnnb1 exon3/+; LSL-KrasG12D mouse model that carries both a Kras G12D mutation and a β -catenin stabilizing mutation developed an intraductal tubular-like tumour but not PanIN or PDAC, indicating that Wnt can, under certain circumstances, impair Kras-mediated oncogenesis [129]. This possibly occurs via acinar cell regeneration in response to the Kras-mediated acinar-to-ductal metaplasia [129].

However, the seminal genomic sequencing study by Jones et al. revealed that one of the 12 core pathways that present genetic alterations in PDAC is the Wnt pathway (Fig. 1) [37]. Also, epigenomic studies have shown that Wnt pathway genes, such as WNT5A, WNT7A, and WNT9A, FZD9, SOX1, SOX7, SOX14, and SOX17 are targets of hypermethylation in PDAC [130]. Additionally, a recent gene microarray work demonstrated increased expression of the Wnt transcriptional activation target AXIN2 in 15 out of 26 PDAC samples as compared to normal pancreatic tissue [131].

In contrast to early-stage PanIN lesions, late stage PanIN exhibit positive nuclear β -catenin expression in the LSL-Kras mouse model and nuclear and/or cytoplasmic β -catenin is expressed in up to 65% of human PDAC samples [132–136]. Blockade of Wnt/signalling by β -catenin shRNA or dominant-negative LEF1 impaired PDAC growth in-vitro [136]. Furthermore, β -catenin expression increased upon co-culture of PDAC cells with immortalized pancreatic stellate cells [135]. Kocher and colleagues showed that treatment with all-trans retinoic acid (ATRA) induced pancreatic stellate cell quiescence and attenuated paracrine-mediated Wnt signalling activity through increased secretion of frizzled related protein in the KPC mouse model of PDAC [137]. ATRA increased apoptosis and inhibited tumour growth in the KPC mouse model [137].

Cumulatively, the above findings indicate that aberrant Wnt signalling occurs in PDAC. However, in contrast to other tumour types, such as colorectal cancer, the role of the pathway in PDAC initiation and progression is more diverse. Indeed, the impact of Wnt signalling deregulation on PDAC pathogenesis appears to be time-dependent. Indeed, aberration of the pathway at early stages appears to oppose Kras-mediate tumourigenesis, whereas aberrant signalling in already-developed PDAC appears to promote tumour progression.

4.8. Depletion of stroma: recent unexpected findings

Although the protumourigenic role of the stroma has been described in numerous previous studies, until recently there were only limited options for depleting stromal elements. The Fearon group has previously identified FAP as a key stroma subset [138]. Elimination of FAP-expressing cells in a PDAC xenograft model using a KPC cell line enhanced immunological control of tumour growth [138]. In that study, mice had been previously immunized using non-Tg and diphtheria toxin receptor (DTR) Tg recipients with a mesothelin peptide and were later treated with the diphtheria toxin (DTX) to deplete FAP-positive cells. The Fearon group also used the same method to assess the impact of depletion of FAP-expressing cells in normal tissues [139]. Intriguingly, experimental ablation of these cells caused extensive loss of muscle mass and reduction of haematopoiesis, resulting in cachexia and anaemia. Notably, elimination of FAP-expressing cells strengthened response to T-cell checkpoint therapy [140]. Indeed, FAP-expressing CAF were the main source of the chemokine CXCL12 that was mainly located in pancreatic cancer cells. Blockade of the CXCL12/CXCR4 axis using AMD3100 resulted in intratumoural T-cell infiltration and remarkable tumour regression when combined with an anti-PD-L1 inhibitor [140]. These data underline the crucial role of FAP in promoting immune evasion and tumour progression in PDAC.

Hingorani and colleagues identified hyaluronan as a key primary determinant of the stromal barrier in PDAC [141]. Enzymatic depletion of hyaluronan using the drug PEGPH20 decreased interstitial fluid pressure, improved vascular functionality and gemcitabine delivery and increased survival in a GEMM of PDAC. Although the findings of this novel approach of stromal targeting were encouraging, two recent studies examined in detail the impact of depleting the stroma using different strategies and reported unexpected findings in GEMMs of PDAC [142,143]. In the first study, the authors used transgenic mice of PDAC to deplete aSMA + myofibroblasts [142]. Surprisingly, depletion of these cells resulted in more aggressive, undifferentiated tumours that were characterised by increase in hypoxia, EMT and cancer stem cell numbers. These tumours had increased infiltration by FOXP3 + Tregs indicating an immunosuppressive environment. Immunohistochemical analysis of resected human PDAC samples showed that patients with high number of aSMA-expressing myofibroblasts had significantly better survival compared to patients with low amount of stroma. Intriguingly, depletion of aSMA + myofibroblasts resulted in enhanced tumour response to anti-CTLA4 but not gemcitabine [142]. In a similar study, transgenic mice were generated to target the Shh [143]. Knock-out of Shh decreased the stromal content and also resulted in more aggressive, undifferentiated PDAC tumours with increased vessel density. Interestingly, targeting VEGFR increased survival in these mice [143]. Altogether, these two studies reported completely unexpected findings and provided novel insight on the protective role of the stroma against tumour progression. The phenotypic alterations observed after stromal depletion highlighted the remarkable plasticity of the PDAC cells.

4.9. Special drug delivery system

PDAC constitutes a characteristic example of abnormal tumour microenvironment characterised by a chaotic vascular morphology with low vessel density, immature, leaky, collapsed vessels that result in hypoxia and poor delivery of chemotherapy agents [144,145]. Vascular endothelial growth factor (VEGF), the master regulator of tumour angiogenesis, is commonly upregulated in PDAC and is correlated with poor disease outcome [146]. Preclinical work has demonstrated that inhibition of VEGF receptor reduced growth and angiogenesis in PDAC [147]. In addition, blockade of VEGF receptor enhanced the cytotoxic effect of gemcitabine resulting in decreased tumour growth and metastasis in-vivo [148].

However, targeting the VEGF pathway using novel agents have failed to produce a survival benefit in the clinical setting [149]. Huber

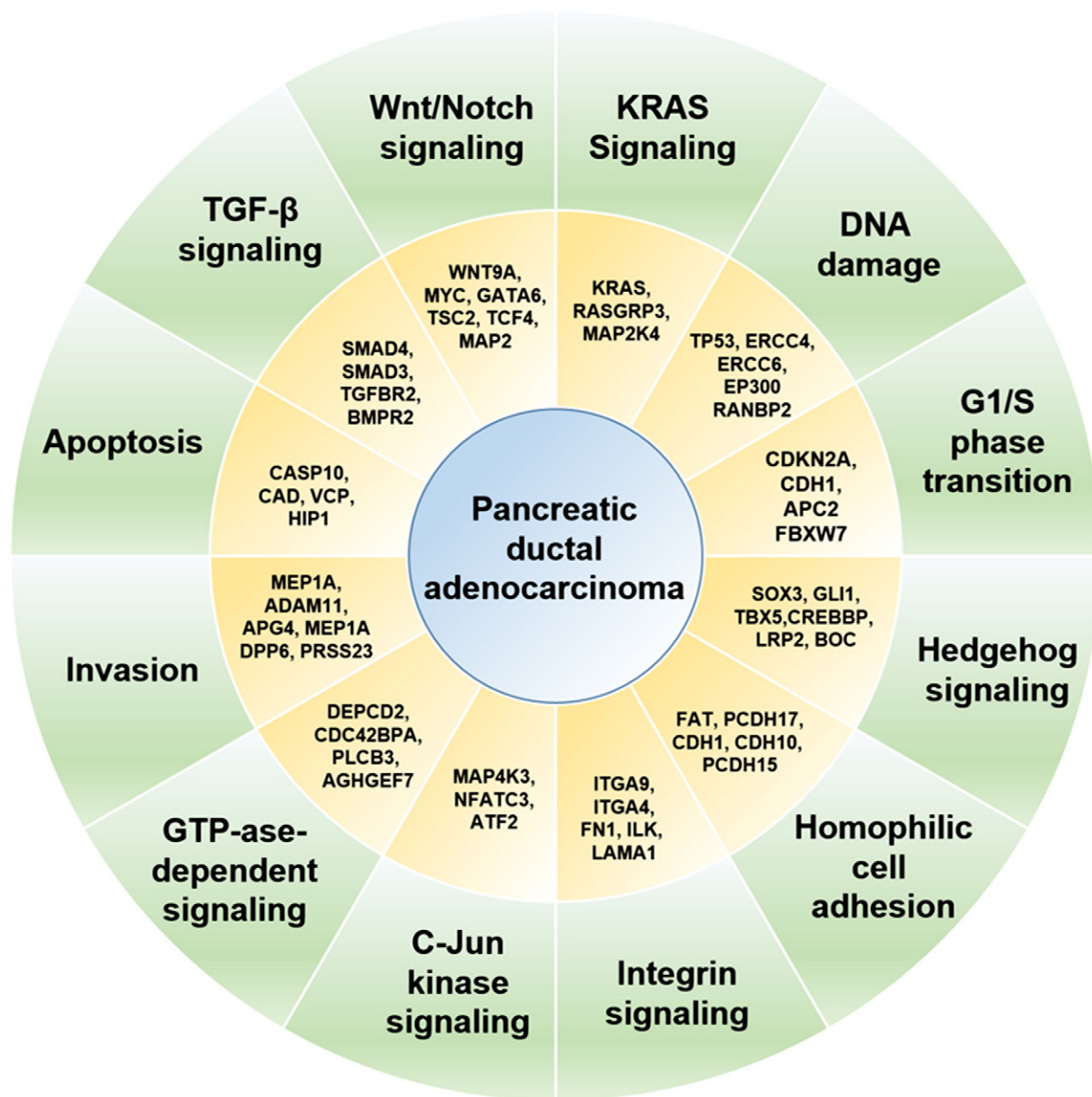


Fig. 1. A human pancreatic ductal adenocarcinoma (PDAC) contains an average of 63 genetic alterations. These have been assigned to a core set of 12 major signalling pathways as revealed by global genomic analyses of 20,735 genes in 24 human PDAC samples [37]. The incidence of alterations in these 12 pathways is as follows: Kras – 100%, TGF- β – 100%, Sonic Hedgehog signalling – 100%, Wnt/Notch signalling – 100%, G1/S-phase transition regulation – 100%, apoptosis – 100%, c-Jun N-terminal kinase signalling – 96%, invasion – 92%, DNA damage control – 83%, homophilic cell adhesion – 79%, small GTPase Ras-independent signalling – 79% and integrin signalling – 67%.

and colleagues examined the angiogenic switch in PDAC using gene microarrays [150]. By comparing gene expression between PDAC with chronic pancreatitis samples, they discovered that the peroxisome proliferative-activated receptor delta (PPAR δ) played a key role in promoting aberrant angiogenesis. PPAR δ overexpression was associated with a more aggressive malignant phenotype characterised by increased recurrence and metastatic spread [150]. Special drug delivery systems, such as nab-paclitaxel, have been created to facilitate better drug penetrability into the tumour mass [151,152]. Nab-paclitaxel is a drug designed to bind to paclitaxel to albumin nano-particles to improve tumour penetrability. In a phase I/II trial, Von Hoff and colleagues demonstrated improved overall survival in patients with metastatic PDAC following addition of nab-paclitaxel to gemcitabine, with a response rate of 48%. Importantly, SPARC expression in the surrounding stroma, but not in the intraepithelial compartment, predicted for improved outcome [151,152]. A preclinical study demonstrated the

stroma-depleting ability of nab-paclitaxel [153]. In that work, addition of nab-paclitaxel significantly decreased the amount of desmoplasia and increased gemcitabine penetrability by 2.8-fold [153]. Tuveson and colleagues had previously demonstrated that nab-paclitaxel increases the intratumoural concentration of gemcitabine in GEMMs of PDAC by blocking cytidine deaminase, a key gemcitabine metabolizing enzyme [154]. This effect was induced through the enhanced production of reactive oxygen species by nab-paclitaxel. Interestingly, SPARC-deficiency reduced intratumoural concentrations of nab-paclitaxel in a SPARC-deficient GEMM of PDAC [155].

5. Chemoresistance and radioresistance

Patients with PDAC commonly receive chemotherapy, either in a curative or palliative intent [156–158]. However, despite the advent of new agents, such as nab-paclitaxel and the small improvement using

multiagent regimens, such as FOLFIRINOX, the majority of patients with PDAC respond poorly to chemotherapy [156]. Several reasons could be responsible for this outcome. First, patients with PDAC often have a deficient vascular network that, in conjunction to the extensive desmoplasia, impairs effective drug delivery to tumour cells [72,80]. In that context, Tuveson and colleagues have recently demonstrated that paracrine hedgehog signalling is directly involved in the interplay between tumour and stromal cells, contributing to treatment failure [126]. Indeed, the addition of a Shh inhibitor resulted in increased vascular density and increased the penetrability of gemcitabine that led to tumour regression. However, this effect was only temporary and tumours shortly after presented increased stromal amount and hypovascularity, indicating that PDAC can evade inhibition of this pathway [126]. Second, cancer cells that developed resistance to gemcitabine had acquired an EMT phenotype, characterised by growth of pseudopodia, spindle-like shape, decreased E-cadherin and increased vimentin expression in association with upregulation of Notch-2 and its ligand, Jagged-1 [159,160]. These data are consistent with the notion that Notch signalling pathway directly mediates the acquisition of the EMT and cancer stem-like-cell phenotype. Notch signalling suppression resulted in partial reversal of the EMT phenotype with mesenchymal-epithelial transition (MET) and downregulation of vimentin, Slug, Snail and ZEB1 [160].

Third, antiapoptotic mechanisms can decrease chemotherapy efficacy. In an elegant study by Neese et al., blockade of cytidine deaminase resulted in increased levels of gemcitabine in murine PDAC tumours but, unexpectedly, this increase failed to delay tumour regrowth [161]. On the other side, inhibition of the connective tissue growth factor by FG-30190 enhanced cell killing without increasing intratumoural penetrability by gemcitabine. This effect was associated with a downregulation of the X-linked inhibitor of apoptosis protein (XIAP), a factor known to mediate chemotherapy resistance [161]. Notably, a recent study indicated that YAP was upregulated in human PDAC, whereas YAP deletion prevented the progression of preneoplastic lesions to PDAC [162]. YAP functioned downstream of the Kras-MAPK pathway and was necessary for Kras-mediated proliferation of pancreatic cancer [162].

Fourth, PDAC cells often have deregulated cellular transport proteins that can compromise uptake of chemotherapy agents by tumour cells. The human equilibrative nucleoside transporter 1 (hENT1) is the primary transport protein that facilitates bidirectional transport of pyrimidine nucleosides, including gemcitabine, into cancer cells [163]. Several clinical reports have indicated that hENT1 is a predictive marker for pancreatic cancer patients treated with gemcitabine [164]. The expression of hENT-1 protein varies widely in human PDAC and patients with strong hENT-1 intratumoural expression presented better outcome, most likely due to differential gemcitabine uptake. Greenhalf et al. recently assessed the predictive value of hENT-1 in patients treated as part of the European Study Group for Pancreatic Cancer (ESPAC)-3 phase 3, randomized controlled trial that compared the outcome between adjuvant gemcitabine and 5-FU after resection of PDAC [165]. In line to previous findings, patients with low/absent hENT-1 expression had worse outcome after gemcitabine chemotherapy and the authors suggested that these patients should be treated with 5-FU or other agents [165].

Additionally, the SCALOP phase II trial compared capecitabine with gemcitabine-based radiotherapy after 12 weeks of induction chemotherapy in patients with locally advanced PDAC. The median overall survival favoured capecitabine over gemcitabine (15.2 months vs 13.4 months; $p = 0.012$) and the gemcitabine group was associated with significantly higher toxicity [166]. Although this important study suggested that capecitabine might be a better option for treating PDAC with radiotherapy, the reasons for this outcome remain unclear [158]. Jodrell and colleagues examined the effect of capecitabine and gemcitabine in preclinical models of PDAC. Capecitabine-induced tumour growth delay was comparable to gemcitabine but their

combination was associated with increased gastrointestinal toxicity. Intriguingly, in contrast to gemcitabine that is characterised by impaired delivery due to the desmoplastic stroma in *in situ* KPC tumours [167], the concentrations of 5-FU were equivalent in both the KPC allograft and the *in situ* tumours after treatment with capecitabine [168]. This important observation indicated that oral dosing might, through extended systemic exposure, result in more sustained delivery and saturation in tissue compartments.

Although there is controversy with regard to the role of radiotherapy in PDAC, it becomes increasingly recognised that even patients with borderline resectable or primarily resectable tumours need multimodal treatment including chemotherapy and/or radiotherapy to enhance resectability and decrease local recurrence [169–171]. Functional imaging and modern planning methods have the potential to further improve the accuracy of radiotherapy and facilitate dose escalation to improve local control [172–174].

Regarding the biological response to radiotherapy, co-culture of cancer cells with irradiated pancreatic fibroblasts increased the invasiveness and aggressiveness of PDAC cells [175]. In that work, culturing of tumour cells using conditioned media from irradiated fibroblasts led to phosphorylation and activation of c-Met and MAPK. In a different study by Brunner and colleagues, PSCs conferred radioprotection to tumour cells and increased their growth both *in vitro* and *in vivo* [176]. The same group has previously demonstrated that patients with non-resectable PDAC and high stromal SPARC expression had significantly worse survival after chemoradiation compared to patients with low SPARC expression [177]. This occurred in a $\beta 1$ -integrin-dependent manner as targeting integrin significantly improved response to radiotherapy. In a similar study, PSCs conferred a cancer stem cell-like phenotype to PDAC cells, upregulated the expression of several EMT markers and increased their radioresistance that was reversed by a TGF β neutralizing antibody [90]. A recent work showed that the ataxia-telangiectasia group D-associated gene (ATDC) protein is commonly expressed in human samples of PDAC and confers radioresistance following ATM-mediated phosphorylation by the MAPKAP kinase 2 [178]. In addition, Li et al. examined the role of CD44, a surface marker for tumour-initiating cells, in PDAC. CD44 overexpression predicted for worse survival in patients with PDAC, whereas blockade of CD44 using an antibody reduced tumour growth, metastatic spread and recurrence following irradiation in a mouse model of PDAC [179]. This effect was mediated by stem cell genes, such as Nanog, Sox-2 and Rex-1, and the signal transducer and activator of transcription 3 (STAT3) [179]. Wei et al. performed an siRNA library screen to discover novel radiosensitizers in PDAC cells [180]. Inhibition of PPP2R1A, a scaffolding subunit of protein phosphatase 2A (PP2A) resulted in radiosensitisation of PDAC cells *in-vitro* and *in-vivo* by altering CDC25C/CDK1 and inhibiting homologous recombination repair [180]. Our group recently examined the potential of ATM-Rad3-related (ATR) inhibition in sensitising PDAC cells to radiation. Blockade of ATR using two novel inhibitors, VE-821 and VE-822, resulted in profound radio- and chemoradiosensitisation of PDAC cells with minimal toxicity in normal cells and a murine abdominal irradiation model [181,182]. Similar radiosensitisation studies have been reported by Morgan and colleagues following combination of radiation with Chk1 inhibitors in PDAC cells and xenograft models [183,184].

Moreover, immunohistochemical analysis of hypoxia inducible factor-1 α (HIF-1 α) expression in $n = 48$ samples of human PDAC revealed nuclear and stromal HIF-1 α expression in 88% and 43%, respectively [185]. Blockade of HIF-1 α by the novel agent PX-478 reduced vascular volume fraction and permeability and enhanced the response of PDAC xenografts to radiation and chemoradiation [185]. Furthermore, targeting EGFR using the inhibitors cetuximab and erlotinib blocked phosphorylation of Akt and EGFR and enhanced gemcitabine-based chemosensitisation in a xenograft mouse model of PDAC [186]. The human immunodeficiency virus protease inhibitors can sensitize tumours to irradiation by blocking Akt signalling [187]. A

phase I trial testing the combination of nelfinavir and gemcitabine/cisplatin-based chemoradiotherapy in $n = 12$ patients with locally advanced PDAC showed promising results as 5 of the 9 patients that received PET-CT presented complete response, whereas the toxicity was acceptable [188].

An elegant study by Klug et al. showed that low-dose irradiation (LDI) can improve vascular morphology and facilitate, either directly, or following ex-vivo transfer, a CD8⁺ T-cell-mediated rejection of pancreatic cancer in different mouse models [189]. In that report, LDI-mediated reprogramming of macrophage differentiation was mediated by the inducible nitrogen oxide synthase (iNOS) and led to endothelial activation and expression of cytotoxic chemokines as well as suppression of proangiogenic and immunosuppressive factors. Examination of human samples of PDAC from patients that received preoperative chemoradiotherapy revealed increased numbers of iNOS⁺ macrophages and CD8⁺ T cells, and improved vascular morphology [189]. Altogether, this important work highlighted the potential of radiotherapy in enhancing the effect of immunotherapies.

6. Pancreatic ductal adenocarcinoma metabolism

PDAC is characterised by altered metabolism that facilitates survival of malignant cells in conditions of nutrient and oxygen deprivation. Tumours present a close link between oncogenic addiction and alterations in metabolic cascades [190], whereby Kras activation appears to orchestrate the metabolic reprogramming in PDAC. Otto Warburg observed that, even under normoxia, cancer cells predominantly utilize aerobic glycolysis rather than mitochondrial oxidative phosphorylation of pyruvate as an energy source [191–193]. Several enzymes of the glycolytic pathway are overexpressed in PDAC, including lactate dehydrogenase A and B (LDHA, LDHB), hexokinase 2 (HK2), pyruvate dehydrogenase kinase isozyme 1 (PDK1), pyruvate kinase muscle (PKM), glucose transporter 1 (GLUT1) and monocarboxylate transporters 1 and 4 (MCT1 and MCT4) [194–198]. Hypoxia led to the glycolytic switch of PDAC cells to lactate production and the activation of hexosamine biosynthesis to promote tumour growth [194]. Blockade of LDHA by siRNA or the novel inhibitor FX11 impaired glycolysis, reduced ATP production, induced oxidative stress and suppressed tumour growth in a mouse model of PDAC [199]. Goldberg et al. combined farnesylthiosalicylic acid (FTS), an inhibitor of Ras, with the glucose analogue 2-deoxy-D-glucose (2-DG) that blocks glycolysis, in a xenograft model of PDAC. The two drugs additively blocked proliferation, whereas a synergistic apoptotic effect and tumour growth delay was observed [200].

In a recent elegant study, DePinho and colleagues conducted transcriptomic and metabolomic analysis using a Kras mouse model of PDAC [201]. Importantly, Kras activation promoted uptake of glucose and shunting into glycolysis, hexosamine and pentose phosphate pathway (PPP) biosynthesis. Kras augmented ribose biogenesis through the nonoxidative arm of PPP, whereas its inactivation led to rapid tumour regression. Interestingly, inactivation of Kras did not alter the glucose flux into the oxidative arm of the PPP and the TCA cycle. The results of that study were unexpected as, in contrast to the current notion that generation of non-oxidative mediators is coupled with the generation of oxidative mediators, it showed that Kras bypasses the oxidative NADPH biosynthesis and is connected to the nonoxidative PPP phase and DNA biosynthesis [201].

Glutamine is an important source of carbon for the TCA and also provides nitrogen for the generation of amino acids, nucleotides and nicotinamide. Glutamine is converted to α -ketoglutarate, either via oxidative deamination that is catalyzed by the glutamate dehydrogenase (GLUD1), or through transamination by transaminases [193]. Son et al. revealed that PDAC cells use a non-canonical glutamine (Gln) metabolism pathway to convert glutamine-derived aspartate into oxaloacetate [202]. In turn, oxaloacetate can generate malate and pyruvate to increase NADPH levels and maintain the cellular redox state via generation of reduced glutathione. This metabolic reprogramming was

regulated by Kras, whereas blockade of the genes along this metabolic pathway resulted in tumour regression [202]. Viale et al. recently demonstrated that Kras inactivation does not eliminate all cells in PDAC [203]. Indeed, a subpopulation of dormant PDAC cells survived and were characterised by more active mitochondrial respiration, impaired glycolysis, activated autophagy and decreased dependence on glycolysis. Importantly, blockade of oxidative phosphorylation efficiently blocked the spherogenic and tumorigenic potential of PDAC cells, highlighting the combination of Kras and oxidative phosphorylation inhibition as a promising method to avoid tumour recurrence following regrowth of these dormant cell subpopulation [203].

Autophagy (otherwise known as macroautophagy) is a catabolic process of cytoplasmic organelles and macromolecule degradation. Paradoxically, autophagy can have both pro- and antitumorigenic role in cancer progression [204]. In PDAC, Kimmelman and colleagues have shown that autophagy is required for the growth of xenografts and GEMMs, as genetic (ATG5 depletion) or pharmacologic (chloroquine) blockade of autophagy suppressed tumour growth via decreased oxidative phosphorylation, and reactive oxygen species production that enhanced DNA damage [205]. However, Ryan and colleagues recently raised concerns regarding the role of autophagy in PDAC [206]. In that work, deletion of the key autophagy mediators Atg5 and Atg7 demonstrated accumulation of low-grade PanIN, whereas tumour progression to PDAC was suppressed. Remarkably, Kras-driven tumours with p53 deficiency presented accelerated tumour growth following genetic autophagy loss and targeting of autophagy using hydroxychloroquine [206]. Due to the findings on autophagy inhibition in mice with homozygous Trp53 deletion, the Kimmelman group employed a Trp53 LOH mouse model and patient-derived xenografts with either p53 wild type or loss. Autophagy blockade resulted in tumour growth suppression, independently of the p53 status [207]. These contradictory reports could be attributed to the different mouse models used and hence more research is needed to better elucidate the impact of autophagy inhibition in PDAC.

7. Circulating tumour cells in pancreatic ductal adenocarcinoma

PDAC is characterised by frequent and early dissemination of the disease. PDAC commonly develops metastases to the liver, lung and skeletal system, indicating that PDAC cells have the ability to intravasate and spread through the circulation to distant organs. Circulating tumour cells (CTCs) are cells that have detached from the primary tumour and entered the blood circulation [208,209]. CTCs have been described in several tumour types. CTCs enter the circulation either by direct cancer cell shedding from the primary tumour or via the active process of EMT. Interestingly, disparities between CTCs, primary tumours cells and cells from distant metastases have been described and heterogeneity has been observed among the CTCs' population [208,209]. This could be attributed to the mode of CTCs' dissemination (passive vs active). In addition, CTCs can originate from both the primary tumour and distant metastases that could also contribute to the heterogeneity of the CTCs' population. Remarkably, it has been estimated that as few as 0.01% of CTCs survive to give rise to distant metastases, as most of CTCs die.

Although several methods have been developed for the detection of CTCs, the most common technique involves the use of immunomagnetic enrichment of CTCs expressing EpCAM in combination with immunocytochemical labelling of CD45⁺ leukocytes and epithelial cells, such as cytokeratins 8, 18 and 19 (CK 8, 18 and 19) [208,209]. The first study on CTCs in PDAC was reported by Chausovsky and colleagues [210]. In that work, cytokeratin 20 mRNA was used to detect CTCs in 28 patients with PDAC. Nested RT-PCR revealed detection of CK20 in 79% of patients. Since then, several groups have investigated the presence of CTCs in peripheral blood of patients with PDAC and correlated the findings with the clinical outcome. In a large study, Soeth et al. examined the prognostic role of CK20-positive CTCs in bone marrow and blood from 172 patients with PDAC that received resection at various stages of the

disease (Stages I–IV) [211]. CTCs were detected in 33% of patients with CK20-positive cells in blood samples and correlated with significantly worse overall survival (17.9 months vs 26.1 months; $p = 0.05$). In a cohort of 20 patients that received curative resection for pancreatic cancer, Matakis et al. examined the prognostic value of CTCs by obtaining blood at the baseline and then every 3 months post-operatively [212]. Nested RT-PCR for carcinoembryonic antigen (CEA) mRNA expression revealed CEA mRNA-positivity in six (30%) patients during follow-up analysis, of which five presented early disease recurrence ($p = 0.007$). By using the CellSearch system to study CTCs in 26 patients with PDAC, Kurihara et al. showed that eleven (42%) patients had CTCs in peripheral blood that correlated with significantly reduced overall survival ($p < 0.001$) [213]. In a similar work, Albuquerque et al. used qRT-PCR for several markers, including CK19, MUC1, EpCAM, CEACAM5 and BIRC5 to detect CTCs in 34 patients with PDAC [214]. In total, 16 (47.1%) patients showed positivity for at least one marker that was correlated with significantly worse progression-free survival ($p = 0.01$). The recent LAP07 multicenter randomized study assessed the outcome of patients after treatment using induction chemotherapy followed by chemoradiotherapy [215]. Analysis of a subgroup of patients ($n = 79$) revealed CTCs in 5% and 9% before and 2 months after treatment, respectively. CTCs-positivity correlated with worse overall survival in multivariate analysis ($p = 0.01$) [215].

Interestingly, some studies have failed to detect a prognostic role for CTCs in PDAC. In a large study in 105 patients with PDAC, CTCs were found in 3 patients with resectable tumours (9%) and in 24 patients with unresectable tumours (33%) [216]. However, CTCs failed to demonstrate prognostic significance for disease progression ($p = 0.08$). Sergeant et al. used qRT-PCR to analyse EpCAM in blood and peritoneal cavity samples from 40 and 8 patients with resectable and unresectable PDAC, respectively [217]. Interestingly, surgery resulted in increased EpCAM-positive CTCs in peripheral blood samples that decreased 1 day after surgical resection. EpCAM was positive in 10 (25%) and 27 (67.5%) patients preoperatively and immediate after surgery, respectively ($p < 0.0001$), whereas CTCs were found in only 23.5% six weeks postoperatively. However, CTCs failed to predict for either cancer-specific survival ($p = 0.17$) or disease-free survival ($p = 0.28$).

Several issues need to be addressed before routine implementation of CTCs in diagnosis and management of patients with PDAC. First, the heterogeneity observed in CTCs poses a major challenge [218]. It currently remains unclear whether and at which stage of the disease CTCs stem from the primary tumour and/or overt metastases. Second, the potential of CTCs in detecting early disease in PDAC remains to be validated. In an elegant study, Rhim et al. showed that pancreatic cancer cells with a mesenchymal phenotype and stem cell properties had spread into circulation and the liver already before the formation of the primary pancreatic tumour but clinical evidence is currently lacking [219]. Third, the potential of CTCs to guide therapy in PDAC remains to be confirmed [220]. CTC genotyping and phenotyping and analysis of circulating DNA have been recently explored in blood samples from patients with PDAC [221] but there is currently lack of information on the CTCs' genetic alterations following conventional chemotherapy and radiotherapy.

8. Inflammation in pancreatic ductal adenocarcinoma

Tumour progression from PanIN to invasive PDAC is associated with changes in the inflammatory microenvironment. Tumour-associated macrophage (TAMs) infiltration increases early in PDAC formation, whereas neutrophils are abundant in PanIN lesions, being sporadic in invasive PDAC. TAMs have also high phenotype plasticity and can be either anti-tumourigenic (M1) or pro-tumourigenic (M2) TAMs that enhance angiogenesis and suppress adaptive immunity [222]. The switch from the M1 to the M2-phenotype is achieved by polarization in response to various signals, such as the cytokines IL-10 and TGF- β that stem from tumour and immune cells [222]. Varying quantities of T cells

are present within both PanINs and PDAC [223,224]. Clark et al. showed that human PDAC is increasingly infiltrated by T regulatory cells (Tregs) and Th2 cells, over the course of disease progression from preinvasive lesions to PDAC, whereas cytotoxic T lymphocytes (CTL) decreased over time [224]. MDSCs constitute immature myeloid cells that suppress both innate and adaptive immunity by promoting cysteine sequestration, arginase overexpression, increased formation of reactive oxygen species and secretion of TGF- β [225]. These result in blocking of T and NK cell function. In preinvasive lesions, effector T cells were scarce and MDSCs were abundant. This close correlation between the presence of MDSCs and the absence of tumour-infiltrating effector T cells highlights the progressive accumulation of immunosuppressive cells in PDAC [223,224].

Neutrophils are recruited into tissues by CXCR2-mediated chemokine signalling and have the ability to remodel the tumour microenvironment. Fridlender et al. demonstrated that neutrophils show plasticity in PDAC [226]. Indeed, inhibition of TGF- β was associated with an anti-tumour neutrophil phenotype called N1-neutrophils [226]. PDAC exhibited strong infiltration by immunosuppressive MDSCs that can differentiate into macrophages and neutrophils. Interestingly, there is evidence that MDSCs of splenic origin can differentiate into a pro-tumourigenic neutrophil phenotype called N2-neutrophils. Kras oncogene activation stimulated granulocyte macrophage-colony stimulating factor (GM-CSF) that attracted MDSCs and suppressed CTL functionality, promoting tumour growth. Blockade of GM-CSF prevented MDSCs recruitment to the tumour microenvironment and facilitated inhibition of tumour growth by infiltrating CTL cells. Conversely, CTL depletion rescued tumour growth [227,228]. In addition, neutrophils activated, via production of reactive oxygen species, the NF- κ B signalling pathway to induce an inflammatory response and enhance PDAC growth [229].

Improved outcome has been described in patients with high infiltration of CD8 + cytotoxic T cells. In line with this finding, strong tumour infiltration by CD8 + T cells predicted for better outcome in patients with PDAC, whereas CD8 + T cells could be detected both in the circulation and bone marrow of these patients [230]. Conversely, progression of precursor lesions to PDAC was correlated with decrease in the number of CD8 + effector cells, impaired function of circulating CD8 + T cells and increase in Tregs tumour infiltration [231,232].

Paradoxically, blockade of the Toll-like receptor 4 (TLR4) signalling promoted dendritic cell-mediated recruitment of T helper 2 (Th2) CD4 + cells that was associated with increased pancreatic inflammation and tumour progression [233]. PDAC presented increased expression of the Toll-like receptor 7 (TLR7) compared to normal pancreas. TLR7 ligation led to activation of the STAT3 and NF- κ B pathways that increased tumour growth in a murine PDAC model [234]. Mice deficient in TLR7 in their inflammatory cells did not show tumourigenesis, whereas TLR7 inhibition protected against tumourigenesis [234]. Furthermore, STAT3 often upregulated in PDAC and experimental studies in GEMMs with Kras activation showed STAT3 activation after pancreatitis-induced damage to the pancreatic acini [235,236]. STAT3 deletion resulted in decreased infiltration by CD45 + cells and down-regulation of cytokines, including IL-6, and decreased inflammation-induced formation of PanIN. Notably, IL-6 was mainly expressed in infiltrating macrophages and serum IL-6 levels were significantly increased in patients with PDAC [235,236].

A recent elegant work revealed that Kras activation upregulated IL-17 receptors on PanIN cells and promoted immune cell infiltration and acceleration of PanIN to PDAC formation [237]. Blockade of the IL-17 signalling prevented PanIN formation and progression to PDAC. Furthermore, a large PDAC cell line screening study revealed increased sensitivity of STAT3-expressing cells to a JAK2 inhibitor [238].

NF- κ B contributes to PDAC formation by inducing a chronic inflammatory reaction. Stimulation of NF- κ B by TNF- α resulted in activation of several molecules, including IL-6, cyclin D1 and c-Myc. NF- κ B promoted the release of Shh by TAMs [239]. Activation of NF- κ B occurred

in response to IL-1 α release from tumour cells to promote invasion and formation of liver metastases [240]. This phenomenon was mediated by the IKK2/ β and was reversed after NF- κ B blockade in an orthotopic Kras mutated PDAC model. Similarly, genetic inactivation of NF- κ B in a GEMM model prevented formation of PDAC over a period of 1 year [61]. This was associated with lack of the inflammatory infiltrate typically seen in NF- κ B activated GEMM (Pdx1-Cre; KrasG12D/+; IKK2/Bfl/−) of PDAC, including infiltration by macrophages and neutrophils.

CXC chemokines display pleiotropic effects in mediating immune response, angiogenesis, and metastases and deregulation of CXC chemokines occurs in late-stage PDAC [241,242]. The CXCR2 ligand, CXCL5, is overexpressed in patients with PDAC and is significantly correlated with advanced disease stage and poor outcome [241,242]. CXCR2-knockout mice harbouring PDAC presented impaired recruitment of endothelial progenitor cells from bone marrow that resulted in decreased angiogenesis and tumour growth. Hiraoka et al. showed that the chemokine (C-X-C motif) ligand 17 (CXCL17) mediated intratumoural infiltration of immature dendritic cells, while the intercellular adhesion molecule (ICAM2) facilitated CD8+ cell-mediated cytotoxicity and tumour cell killing [231]. Interestingly, during tumourigenesis progression from precursor lesions to PDAC, downregulation of CXCL17 and ICAM2 tempered immune response that led to immune tolerance [231]. Furthermore, blockade of TGF- β receptor II in a mutant Kras mouse model resulted in an aggressive disease phenotype with increased secretion of CXCL1 and CXCL5 ligands [241]. Of note, CXCR2 expression was higher in stromal fibroblasts compared to epithelial cells, whereas signalling attenuation using a CXCR2 inhibitor decreased vessel density and improved survival in tumour-bearing mice.

9. Immune therapy in pancreatic ductal adenocarcinoma

During the last decade, there has been a progressively increased interest in studying the therapeutic potential of immune therapy in PDAC [243]. The concept of cancer immunoediting has added to the better understanding of how the host immune system determines tumour fate in three distinct phases, by sculpting the immunogenicity of cancers [244]. During the first phase, elimination, transformed cells are destroyed by a competent immune system. Both the innate and the adaptive immune system protect against tumourigenesis and identify and kill cancer cells using cancer immunosurveillance. For that purpose, the orchestration of the antitumour immune response by various types of immune cells, such as cytotoxic CD8 T cells, T helper-1 (Th1) cells, NK cells, mature dendritic cells (DCs) and activated pro-inflammatory macrophages (M1) is crucial. In the second phase, sporadic cancer cells that have managed to evade immune destruction enter equilibrium where editing occurs. Finally, in the third phase of escape, malignancies present a highly immunosuppressive microenvironment that promotes tumour progression, whereby tumour cells high-jack the immune system [244]. Indeed, the interference with MHC class I peptide presentation in conjunction with the activation of tolerance-inducing molecules and cytokines, including PD-L1, IL-10, TGF- β , VEGF, to name a few, result in recruitment of Tregs, MDSC, TAMs and fibroblasts as well as acquirement of a T helper cell type 2 phenotype (Th2) [245]. These lead to immune suppression and enable growth of PDAC. Below we discuss the function of the different immune cells that mediate tumour progression in PDAC and the evidence on the different immune therapies used in PDAC.

9.1. Tregs

T-regulatory cells (Tregs) are FOXP3+ CD4+ CD25+ cells [246]. Under normal circumstances, Tregs are essential for the prevention of auto-immune disease, via expression of CTLA-4 and secretion of TGF- β and IL-10. In malignancies, Tregs lead infiltrate tumours at increased numbers and lead to immunosuppression [246]. In human PDAC,

increased numbers of Tregs in the tumour and the circulation are associated with increased risk of disease recurrence after surgery [232,247].

9.2. Dendritic cells

Dendritic cells (DCs) are key regulators of the anti-tumour immune response [248]. Similarly to macrophages, they are member of the antigen-presenting cell (APC) family and express MHC class II. Under normal conditions, this enables presentation of antigenic peptides to CD4+ T cells. Through internalization and degradation of tumour antigens, they facilitate their presentation, via MHC class I and II molecules, to CD4+ and CD8+ T cells, respectively [248]. In malignancies, including PDAC, DC maturation and survival are compromised [249]. Also, the presence of DCs in the tumour mass or in the blood circulation predicts for improved survival in patients with PDAC [250]. However, other studies have suggested that DCs might have a protumourigenic effect. For example, Ochi et al. recently demonstrated that DCs mediated the protumourigenic impact of MyD88 blockade to activate pancreatic antigen-restricted Th2-deviated CD4+ T cells and accelerated the transition from pancreatitis to PDAC [233].

9.3. Natural killer cells

Natural killer (NK) cells and natural killer T (NKT) cells constitute subsets of lymphocytes that present functional and phenotypical similarities [243,251]. Through the secretion of cytokines, such as interferon- γ (IFN- γ), they participate in adaptive antitumour immune response. In PDAC, NK cell activity is decreased and the levels of the NK cell activating receptor NKG2D are reduced, whereas increased levels of NK cells in blood circulation is correlated with better outcome in the clinical setting [252,253].

9.4. T cell activation

Activation of T cells involves a series of complex immune ligand-receptor interactions [254]. Stimulation of CD8+ and CD4+ T cells occurs via APC-mediated presentation of antigenic peptides on MHC class I and II molecules, respectively, to the T cell receptor (TCR). Activation of T cells requires the presence of co-stimulatory receptors, including CD40, CD28, OX40 and 4-1BB. This activation is inhibited by receptors such as CTLA-4 and programmed death 1 (PD-1) that are expressed on the surface of T cells. This inhibition of T cells occurs via binding to their corresponding ligands CD80/CD86 and PD-L1/PD-L2, respectively [254].

PD-1 is often overexpressed in PDAC and impairs CTL infiltration, leading to tumour growth and poor outcome [255,256]. In contrast, high expression of CD40 and CD40L in tumour cells predicts for good prognosis in patients with PDAC [257]. Similarly, the presence of the immune inhibitor ligand B7-H33 is associated with improved prognosis in PDAC. CD40 is a member of the tumour necrosis factor (TNF) receptor superfamily member and plays a key role in T-cell-dependent antitumour immunity [258]. In a seminal study by Vonderheide and colleagues, addition of a CD40 agonist antibody to gemcitabine resulted in tumour regression in some patients with surgically incurable PDAC [259]. Preclinical work by the same group surprisingly showed that tumour regression required macrophages but not T-cells or gemcitabine to induce regression in a GEMM of PDAC. Tumours exhibited rapid infiltration by CD40-activated tumouricidal macrophages that led to stromal depletion [259]. A recent phase I trial in $n = 22$ patients with advanced PDAC using the CD40 agonist CP-870,893 in combination with gemcitabine showed good tolerance and led to antitumour activity [260].

9.5. Blockade of immune checkpoints

Tumours resist immune attack by expressing ligands that engage inhibitory receptors and compromise T-cell functions. Several preclinical

and clinical studies have indicated that blockade of immune checkpoints can significantly augment antitumour immunity. Strategies targeting these checkpoints include inhibitors of the receptor–ligand interaction on T-cells and tumours, such as blocking monoclonal antibodies to cytotoxic T-lymphocyte associated protein-4 (CTLA-4) and the programmed cell death 1/programmed cell death 1 ligand 1 (PD-1/PD-L1) receptor/ligand pair [261]. Treatment of 14 patients with PDAC with a novel anti-PD-L1 antibody showed no response in any of the patients [262]. Similarly, in a phase II trial in 27 patients with locally advanced or metastatic PDAC, the anti-CTLA-4 agent ipilimumab was ineffective as no response was observed [263].

The DeNardo group has shown that targeting TAMs and monocytes by blocking the myeloid cell receptors colony-stimulating factor-1 receptor (CSF1R) or chemokine (C-C motif) receptor 2 (CCR2) reduced tumour-initiating cells (TIC) in pancreatic tumours, inhibited metastatic spread and increased tumour infiltration by cytotoxic CD8 + T-cells that enhanced response to chemotherapy in different mouse models of PDAC [264,265]. The same group demonstrated that CSF1R blockade upregulated PD-L1 and CTLA4 that can limit its therapeutic potential [266]. Notably, combination of CSF1R blockade with PD1 and CTLA4 antagonists led to tumour regression, even in large tumours in a mouse model of PDAC, indicating the potential of combining inhibitors of myeloid cell recruitment with T cell checkpoint inhibitors.

9.6. Soluble immunosuppressive factors

Indoleamine 2,3-dioxygenase (IDO) catabolizes tryptophan into kynurenin and is upregulated in PDAC [267,268]. Tryptophan is necessary for T cell activation and its depletion led to impaired T cell function that was paralleled by stimulation of Tregs development. Another group of soluble immunosuppressors, galectins, were found to be upregulated in PDAC [269]. They induced IL-10 production in Tregs and promoted a Th2 cytokine profile. Although Galectin-1 is expressed in both tumour and stellate cells in PDAC and has been correlated with advanced disease stage, its predictive value remains unclear as increased expression of Galectin-1 has been reported in PDAC patients with long-term survival [269,270].

9.7. Anticancer vaccines in pancreatic ductal adenocarcinoma

During the last decade, there has been an intensive effort towards the development of novel vaccine therapies for cancer (Table 1) [271–273]. Vaccine therapy includes the administration of compounds that contain a tumour-expressing antigen. The purpose is to activate and multiply dormant tumour-specific T cells and/or to strengthen the natural ability of immune cells to recognize and destroy malignant cells. Several tumour antigens have been proposed for the development of vaccines in PDAC. These include mutated proteins that are only present in PDAC cells, such as the mutated Kras oncogene, or proteins that are over-expressed in tumour tissues, including mesothelin, MUC-1, CEA, Wilms tumour gene (WT-1) and VEGF-R. Methods of administration include peptide fragments, whole proteins, DNA or RNA.

The first peptide-based vaccine tested in clinical studies used mutated Kras as a target [274]. Kras is a promising target as its mutant form is only expressed in tumour but not normal cells that offers tumour selectivity to the vaccine therapy. In a phase I/II trial, vaccination using mutated Kras led to immune response in 2 out of 5 patients [275]. In a similar phase I/II trial using Kras-derived synthetic peptides in combination with GM-CSF in 48 patients with advanced PDAC, peptide-specific immunoresponse was observed in 58% of cases, whereas responders to vaccination showed a significantly increased median survival compared to non-responders (148 vs 61 days; $p = 0.0002$) [276]. In a different study, a 21-mer epitope that encompassed the patient-specific Kras mutation was administered monthly for in total 3 months in patients with PDAC following surgical resection [277]. Intradermal GM-CSF

was administered as well. The median survival was 20.3 months and the vaccine was well-tolerated.

Weden et al. reported the 10-year follow-up data of post-operative vaccination with long synthetic mutant Kras peptides (CTN-95002 and CTN-98010) in 23 patients with PDAC treated as part of two Phase I/II trials [278]. The data of twenty patients were available and 85% of those showed immunologic response to the vaccine, with a median survival of 28 months. Importantly, the 10-year survival was 20% versus 0% in patients that did not receive the vaccine, highlighting the therapeutic potential of Kras vaccination as a promising adjuvant therapy in patients with PDAC.

MUC-1 is a glycoprotein located in cell membrane and contributes to PDAC growth and metastasis by promoting EMT. Interestingly, MUC-1 can induce CD8 + T cell responses and production of anti-MUC antibodies to result in improved survival [279]. In a large phase III trial in 255 patients with metastatic PDAC, vaccination consisting of a vaccinia virus expressing CEA and MUC1 (PANVAC-V) showed no survival benefit compared to gemcitabine-based chemotherapy [280].

Gilliam et al. tested the antagatrin immunogen G17DT in 79 patients with advanced PDAC that were unsuitable for chemotherapy [281]. Seventy five patients received a placebo. Immune responders showed better median survival compared to non-responders or patients on placebo (median survival, 176 vs 63 vs 83 days, $p = 0.003$) and the vaccine was well-tolerated [281].

In a phase 2 trial, Hardacre et al. examined the efficacy of algenpantucel-L in combination with chemoradiotherapy in $n = 70$ patients with PDAC in the adjuvant setting [282]. Algenpantucel-L is an allogeneic PDAC vaccine generated by genetic engineering. It is composed of two human PDAC cell lines (HAPa-1 and HAPa-2) that have been designed to express α Gal via a retroviral transfer of the murine α GT gene. The vaccine had a safe toxicity profile, whereas the 12-month disease-free survival and overall survival were 62% and 86%, respectively [282]. A phase III study is currently underway.

Although less well explored, Wilms tumour gene 1 (WT-1) is expressed in 75% of patients with PDAC, whereas it is undetectable in normal pancreatic tissue [283]. Currently, a phase II trial using a WT-1-based vaccination is ongoing in patients with advanced PDAC. The oral DNA vaccine VXM01 that uses *Salmonella typhi* Ty21 as a vector targets VEGFR-2 and has also entered clinical trial in patients with locally advanced and metastatic PDAC [284].

The telomerase-targeting vaccine (GV1001) is 16-aa peptide derived from the human telomerase reverse transcriptase (hTERT), a potent immunogenic antigen that binds to multiple major histocompatibility complex (MHC) molecules [285]. In a phase I/II trial conducted in 48 patients with non-resectable PDAC, GV101 in combination with GM-CSF for 10 weeks produced detectable T cell response in 63% of patients and improved survival [285]. However, two recent phase III studies using GV101 in combination with gemcitabine (Primovax and TeloVac trials) failed to improved survival in patients with metastatic PDAC [272,286].

Whole cancer cell vaccines are a promising alternative because they are patient-specific and do not require targeting a selected tumour associated antigen (TAA), as they rely on previously irradiated cancer cells that express the whole repertoire of TAAs [287]. Because autologous vaccines need isolation of a relatively large amount of malignant tissue and take time to process the cancer cells, allogenic tumour cell lines have been developed to be used as vaccines. In a phase I clinical study, Jaffee et al. administered a novel allogeneic GM-CSF-secreting vaccine in 14 patients with surgically resected PDAC followed by adjuvant chemoradiotherapy [288]. Three of the patients presented delayed-type hypersensitivity responses that led to prolonged disease-free survival. The same group from John Hopkins subsequently examined the efficacy of this vaccine in a phase II trial. Disease-free survival and overall survival were 17 and 24 months, respectively [289]. These data underline the promising therapeutic potential of immunotherapy in PDAC.

Listeria monocytogenes stimulate robust, cell-mediated adaptive immune responses by targeting DCs in vivo [290,291]. A live-attenuated

Table 1
Clinical trials of vaccine therapy in pancreas cancer.

Study	Type of vaccine	Phase	Patients	Treatment	Clinical outcome
Jaffee et al. 2001 [288]	GM-CSF vaccine	I	n = 14, resected PDAC	GM-CSF vaccine combined with chemoradiotherapy	3 patients disease-free at 25 months
Gjertsen et al. 1995 [275]	Kras-based vaccines	I/II	n = 5, locally advanced PDAC	Mutated Kras peptide	2 immune responders with longer survival
Gjertsen et al. 2001 [276]		I/II	n = 48 (10: resected PDAC, 38: locally advanced PDAC)	Mutated Kras peptide combined with GM-CSF	Median OS: Responders vs non-responders: 148 vs. 61 days (p = 0.0002)
Abou-Alfa et al. 2011 [277]		–	n = 24, resected PDAC	Mutated K-ras peptide	Median RFS: 8.6 months, Median OS: 20.3 months
Gilliam et al. 2012 [281]	Antigrastrin vaccine	–	n = 154, locally advanced PDAC, unsuitable for chemotherapy	G17DT gastrin peptide vaccine or placebo	G17DT vs placebo: 151 vs 82 days (p = 0.03)
Laheru et al. 2008 [273]	Kras-targeting vaccines	II	n = 50, locally advanced PDAC	GM-CSF vaccine or Cy/GM-CSF vaccine	Median OS: 2.3 vs 4.3 months
Lutz et al. 2011 [289]		II	n = 60, resected PDAC	GM-CSF vaccine with 5-FU-based chemoradiotherapy	Median OS: 24.8 months
Le et al. 2013 [290]		II	n = 60, metastatic PDAC	Cy/ GM-CSF vaccine followed by CRS-207 vs Cy/GM-CSF vaccine	Median OS: 6 vs 3.4 months (p = 0.0114)
Weden et al. 2011 [278]		I/II	n = 23, resected PDAC	6–10 doses of Ras oncogene peptide vaccine after surgery	10-year OS: 20%
Le et al. 2012 [293]	Live-attenuated <i>Listeria</i> vaccine	I	n = 28, mixed histology (pancreatic, lung, ovarian cancer, mesothelioma)	Live attenuated <i>Listeria</i> vaccine (ANZ-100) vs Live attenuated mesothelin expressing <i>Listeria</i> vaccine (CRS-207)	15-month OS: 37% in CRS-207 arm
Hardacre et al. 2010 [282]	Algenpantucel-L	II	n = 62, resected PDAC	Algenpantucel-L with gemcitabine-/5 FU-based chemoradiotherapy	1-year DFS: 62% 1-year OS: 86%
Lepisto et al. 2008 [295]	MUC-1 pulsed autologous DC vaccine	I/II	n = 12, resected PDAC	MUC-1 pulsed autologous DC vaccine	Median OS: 26 months
Le et al. 2012 [294]	Ipilimumab plus Whole cell vaccines	Ib	n = 30, locally advanced/ treatment-refractory/ metastatic PDAC	Ipilimumab-only vs Ipilimumab plus Cy/GM-CSF vaccine	Median OS: 3.6 vs 5.7 month (p = 0.072)
Bernhardt et al. 2006 [285]	Telomerase-targeting vaccines	I/II	n = 48, locally advanced PDAC	GV1001 telomerase peptide with GM-CSF	Median OS: Responders vs non-responders: 216 vs 88 days (p = 0.0001)

DFS, disease-free survival; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; GM-CSF, granulocyte-macrophage colony stimulating factor; LAK, lymphokine-activated killer cells; DC, dendritic cell, MUC-1, mucin 1.

Listeria monocytogenes strain (Δ actA/ Δ inlB) has been generated following deletion of the genes encoding 2 virulence determinants to produce a cancer vaccine. A recent preclinical study by Jaffee and colleagues assessed the efficacy of such an attenuated intracellular *Listeria monocytogenes* vaccine engineered to express Kras [292]. When used in combination with an anti-CD25 and cyclophosphamide to deplete Tregs, the vaccine depleted Treg cells, reduced progression of early but not late PanIN and increased infiltration by inflammatory cells [292]. A phase I study revealed good tolerance and sufficient immune activation [293]. In consequence, a phase II trial of a mesothelin-coding listerial vaccine (ANZ-100) has been initiated in patients with metastatic PDAC.

In a phase Ib trial, Le et al. examined the outcome of patients with either advanced or treatment-refractory PDAC after treatment with the anti-CTLA-4 agent ipilimumab alone or in combination with GVAX [294]. The combination treatment showed a trend towards better overall survival (3.6 vs 5.7 months; p = 0.072). Both arms showed grades 3–4 immune reactions in approximately 20% of patients [294].

Antigen pulsed DCs' vaccine is consisted of patient DCs that have been previously isolated, pulsed with peptides, autologous, or allogeneic tumour lysate, or transfected with RNA [295]. Once developed, these DCs are re-infused in patients. The first phase I/II trial examined the efficacy of MUC-1-pulsed DCs in 12 patients with PDAC and biliary cancer following tumour resection. Four of those 12 patients were alive and recurrence-free at 4 years [295]. In a recent study, a DC vaccine plus lymphokine-activated killer cell therapy was administered in combination with gemcitabine and/or S-1 to 49 patients with inoperable pancreatic cancer. The treatment was well-tolerated and the median survival was approximately 1 year, whereas 2 patients showed complete remission [296].

9.8. Adoptive cell transfer and chimeric antigen receptors

Adoptive cell transfer (ACT) immunotherapy is a promising approach for the treatment of cancer [254]. In brief, T cells are harvested from the tumour tissue by apheresis followed by ex-vivo culture, genetic engineering and expansion of purified cells and re-infusion in patients. ACT enables cell priming to tumour antigens, or recombinant DNA transfection to T cell receptors that directly targets tumour-specific antigens. During the process of cell engineering and expansion, the patient is receiving low-dose cyclophosphamide and/or fludarabine to induce lymphodepletion [297]. Furthermore, genetic engineering of lymphocytes has been explored to carry chimeric antigen receptors (CARs). The latter has an extracellular domain with a Fab fragment of an antibody that has been specifically constructed to recognize a tumour-associated antigen, whereas its intracellular domain is responsible for signal transduction of the TCR. T cell binding to specific tumour antigens results in their activation. Proliferation of T cells is enhanced via co-stimulatory molecule domains integrated into the intercellular domain of CARs [298].

Kondo et al. used dendritic cells pulsed with MUC1 peptide (MUC1-DC) and CTL sensitized in 20 patients with unresectable (n = 8) or recurrent PDAC (n = 12) [299]. From the 8 patients with unresectable tumours, only 1 developed hepatic metastasis. In a preclinical study, Ablate-Kaga et al. used prostate stem cell antigen (PSCA), a glycoprotein that is upregulated in PDAC, to design a CAR. For that purpose, the authors tested the human monoclonal antibody Ha1-4.117-based CAR for adoptive T cell therapy in a xenograft model of PDAC. The treatment resulted in significant tumour regression and lack of toxicity [300].

10. Future perspectives and conclusion

There is now strong evidence that PDAC is a genetic disease (Fig. 2). Common genetic alterations that promote progress of tumour from PanIN to PDAC have now been discovered. The advent of new technologies, such as exome and whole genome sequencing, has provided novel insight. Indeed, recent genomic studies incorporating mathematical modelling have indicated that there is a large time gap between the development of parental and metastatic subclones, which comes in contrast to the notion that the PDAC life span is only few years. Inevitably, the interrogation of PDAC's clonal evolution has raised questions. For example, it currently remains unclear to which extent subclones contribute to metastatic spread in PDAC as most metastasis suppressor genes present haploinsufficiency. Second, it remains to be demonstrated whether genome sequencing accurately describes the genetic identity of PDAC independently of intratumoural heterogeneity. This is a key point as there is currently an increased interest in designing studies based on genome sequencing to guide personalized treatment with novel agents. Third, if the time window between the development of parental clones until the appearance of macrometastases is indeed longer than a decade, how do we explore this finding in the clinical setting? Such observations have important implications for the role of future population screening to facilitate early diagnosis. Surgery is the cornerstone in PDAC management but the majority of patients are diagnosed with locally-advanced, inoperable disease. Hence, early detection of PDAC, for instance during the PanIN stage can, in theory, improve the clinical outcome. However, in contrast to other tumour entities, there is still a lack of a valid screening and early detection method in PDAC and hence the design of new technologies will be crucial. Detection of Kras mutations in patient's blood and/or stool might be a promising way towards this direction but several issues, including public awareness and developments of strategies to avoid overtreatment of patients, need to be addressed first.

Desmoplasia is one of the hallmarks of PDAC. Traditionally, desmoplasia had been associated with biological aggressiveness, treatment failure and adverse prognosis in PDAC. However, recent studies demonstrated that stroma depletion results in even more aggressive, undifferentiated tumours, whereas patients with higher amount of stroma had longer overall survival. Currently, it is difficult to envisage the best way to interpret these novel observations and cross-validation by other groups should be encouraged. Even though it will be challenging to completely change the current paradigm, these unexpected findings highlight the plasticity of PDAC cells and emphasize that caution is needed when targeting the tumour stroma. A careful reassessment of the novel targeted agents and their impact on tumour microenvironment is needed. Importantly, these observations support a protective role for the desmoplastic stroma. In older studies, Stoker observed that the proliferation of transformed hamster kidney cells is suppressed by normal fibroblasts [75]. Also, although fibrosis manifests at the cost of organ function loss, it is commonly encountered in many different non-malignant inflammatory diseases in an attempt of the body to develop a protective barrier to prevent spread of the noxious stimuli [301]. Hence, it is plausible that the fibrotic reaction encountered in PDAC might represent a defence mechanism of the host to prevent progression of preneoplastic to neoplastic lesions and/or mechanically restrain the tumour from growing and spreading.

Although it remains unclear why PDAC has higher stromal amount and less vasculature compared to other tumour types, targeting the tumour cells without modifying the stroma to allow better oxygenation and drug penetrability is unlikely to improve the outcome to conventional radiotherapy and chemotherapy. On the other hand, although it rendered the tumour more aggressive, stromal depletion “unmasked” the immunosuppressive microenvironment and tumours responded better to immune checkpoint blockade and antiangiogenic treatments. Hence, it will be interesting to analyse the potential of a combining

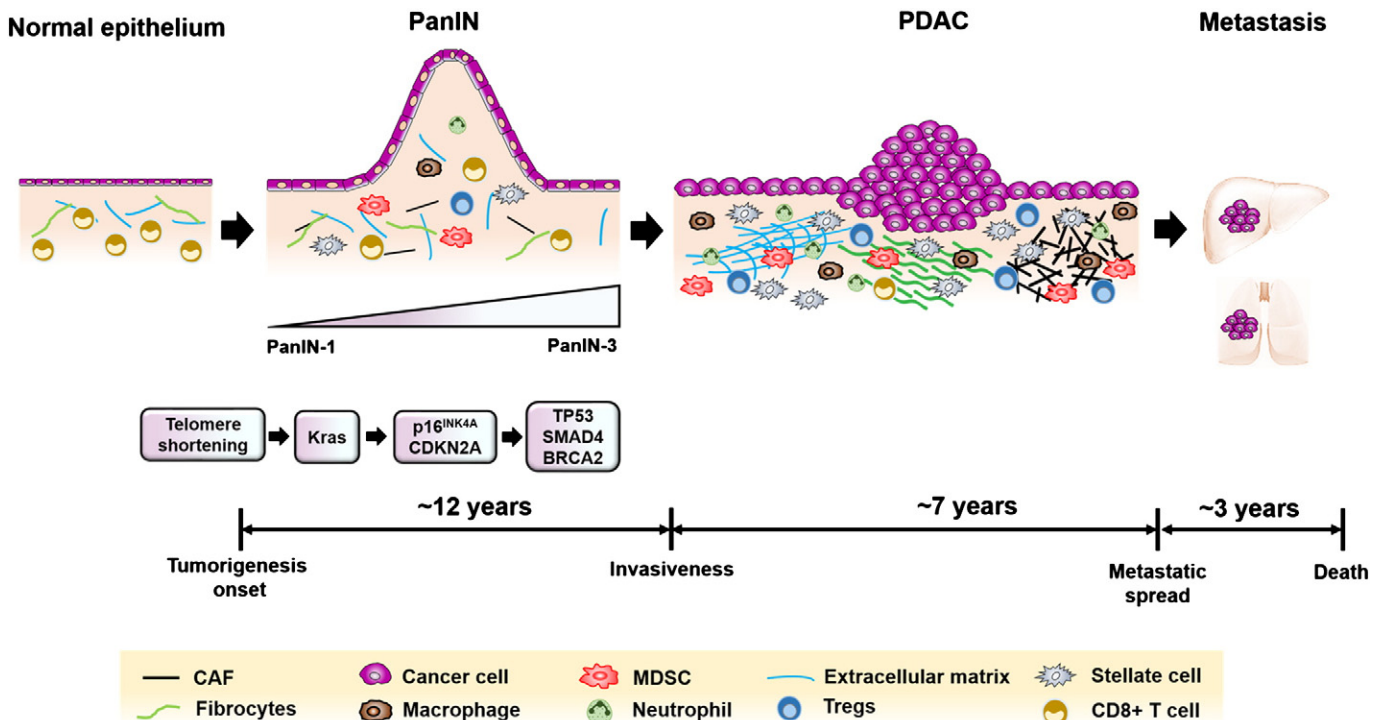


Fig. 2. Tumour microenvironment and progression in pancreatic cancer. Pancreatic ductal adenocarcinoma (PDAC) develops from pancreatic intraepithelial neoplasia (PanIN), a well-defined precursor lesion. Tumour progression is associated with genetic and biological alterations. Telomere shortening and Kras mutations occur early during pancreatic tumorigenesis (PanIN-1) followed by inactivating mutations of CDKN2A (PanIN-2) and late mutations in TP53, SMAD4 and BRCA2 (PanIN-3). In a similar fashion, progression of PanIN to PDAC is characterised by accumulation of the desmoplastic stroma that drives immunosuppression, tumour growth, invasiveness and metastatic spread, leading to patient death. Genetic studies have shown that the average time from early tumorigenesis to patient death from metastatic disease is approximately 22 years. CAF, cancer-associated fibroblast; Treg, regulatory T cell; MDSC; myeloid-derived suppressor cell.

conventional anticancer therapies with stromal depletion and novel immune checkpoint inhibitors and/or antiangiogenic agents.

Accumulating evidence has underlined the key role of Kras in regulating the metabolism of PDAC. Several metabolic pathways, such as glycolysis, glutamine and lipid metabolism and autophagy facilitate the adaptation of PDAC cells in conditions that lack nutrients and oxygen and contribute to the aggressive behaviour of PDAC. Exploitation of these metabolic niches has shown promising results in preclinical models of PDAC but clinical evidence for their efficacy is currently lacking.

Analysis of CTCs might potentially have clinical applicability in diagnosis and management of PDAC. Although some reports have demonstrated a prognostic role for CTCs, other studies have failed to show a prognostic significance. This discrepancy could be attributed to several factors, including the different methods and technologies used to detect and analyse CTCs, the low patient number and the retrospective nature of some studies. Thus, further investigation is needed before CTCs are used as a routine diagnostic and prognostic marker in patients with PDAC.

In the light of the crucial role of the inflammatory milieu and immunosuppression in tumour initiation and progression, testing anti-inflammatory strategies and novel immunotherapeutics might further improve tumour response to chemotherapy and radiotherapy. Some experimental vaccines have yielded promising findings. However, the immunosuppressive microenvironment often impairs the cytotoxic effect by immune effector cells and prevent vaccines from eliciting an effective tumour response. Hence, more trials are needed towards this direction.

Also, there is uncertainty on whether we should combine these novel immunotherapeutics with chemotherapy and radiotherapy, or administer them as single agents. Radiation and chemotherapy can either enhance or impair the therapeutic potential of immunotherapies in pancreatic cancer [302,303]. This effect possibly depends on timing, dose and administration schedule. Indeed, application of radiation and/or chemotherapy might impair an already triggered antitumour immune response. For example, if adoptive T cell or vaccine therapy is used, administration of radiation after T cell transfer could disrupt the T cell response. Conversely, an immunoadjuvant could enhance the cytotoxic impact of radiation and chemotherapy. In theory, administration of radiation and chemotherapy prior or simultaneously to immune checkpoint inhibitors might be a better approach as it could enhance de novo generation of antigens.

Altogether, PDAC continues to pose a major therapeutic challenge. So far, only small progress has been performed in the diagnosis and management of patients with PDAC. The recent discoveries in the field of PDAC genetics, biology, metabolism and oncoimmunology have created new opportunities to develop novel approaches for earlier diagnosis and more effective treatment. More research is clearly needed to improve our biological understanding. It remains to be demonstrated whether therapeutic targeting of the different components of the tumour microenvironment, either alone or in combination with conventional treatments, will improve the outcome of patients with PDAC in the near future. Intense efforts should aim to bring this knowledge all the way back to the clinic.

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