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Modeling pancreatic cancer in mice for experimental therapeutics

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy that is characterized by early metastasis, low resectability, high recurrence, and therapy resistance. The experimental mouse models have played a central role in understanding the pathobiology of PDAC and in the preclinical evaluation of various therapeutic modalities. Different mouse models with targetable pathological hallmarks have been developed and employed to address the unique challenges associated with PDAC progression, metastasis, and stromal heterogeneity. Over the years, mouse models have evolved from simple cell line-based heterotopic and orthotopic xenografts in immunocompromised mice to more complex and realistic genetically engineered mouse models (GEMMs) involving multi-gene manipulations. The GEMMs, mostly driven by KRAS mutation(s), have been widely accepted for therapeutic optimization due to their high penetrance and ability to recapitulate the histological, molecular, and pathological hallmarks of human PDAC, including comparable precursor lesions, extensive metastasis, desmoplasia, perineural invasion, and immunosuppressive tumor microenvironment. Advanced GEMMs modified to express fluorescent proteins have allowed cell lineage tracing to provide novel insights and a new understanding about the origin and contribution of various cell types in PDAC pathobiology. The syngeneic mouse models, GEMMs, and target-specific transgenic mice have been extensively

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Author's contribution

MJ and SKG conceived the idea and designed the framework of the manuscript. KM completed the manuscript with the help of AA and SKG and helped in designing the figures and tables. SKB critically reviewed the manuscript and provided feedback.

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Conflict of interest

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used to evaluate immunotherapies and studying the therapy-induced immune modulation in PDAC yielding meaningful results to guide various clinical trials. The emerging mouse models for experimental parabiosis, hepatic metastasis, cachexia, and image-guided implantation, are increasingly appreciated for their high translational significance. In this article, we describe the contribution of various experimental mouse models to the current understanding of PDAC pathobiology and their utility in evaluating and optimizing therapeutic modalities for this lethal malignancy.

Keywords

Pancreatic cancer; mouse model; targeted therapy; immunotherapy; cachexia; parabiosis; metastasis; vaccine; antibody-targeted therapy

1.0. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal malignancy with a five-year survival of ~10% in the United States. Recent estimated new cases and deaths analysis in the US population suggest PDAC as the fourth leading cause of cancer-related deaths by 2021 [1]. Early detection correlates with better survival in PDAC, still the lack of biomarkers and the asymptomatic nature of disease lead to the diagnosis of PDAC at late stages, leaving the majority of patients' ineligible for resection [2, 3]. Other challenges associated with poor clinical outcomes in PDAC patients include early metastasis, high recurrence rate, and poor response to the existing therapeutic approaches [4]. The hypovascular, hypoxic, and desmoplastic pancreatic tumor microenvironment (TME) is the major determinant of therapy resistance, which is characterized by disrupted vascular transport [5-7]. The pancreatic TME is primarily comprised of cancer associated fibroblasts (CAFs) that secrete extracellular matrix (ECM) proteins to create a physical barrier that restricts the delivery of systemic therapies to the target tumor cells, leading to poor therapeutic response [8]. Together with CAFs, the tumor associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and other immune-regulatory cells secrete immune modulatory cytokines in the TME, leading to increased tumorigenesis, metastasis, and immunosuppression in pancreatic tumors [5, 9].

Considering the high lethality of PDAC, and challenges associated with poor therapeutic response towards existing treatment modalities, it is important to recapitulate the pathological hallmarks of human PDAC in preclinical models to evaluate therapeutic approaches more accurately for rationalizing the clinical trials. The experimental murine models have served as valuable tools for the development and optimization of targeted therapies for PDAC. These mouse PDAC models can be classified based on the manner of tumor induction, site of tumor implantation/growth, their histopathological characteristics, and clinicopathological relevance. We discuss here the evolution of various mouse models that have been employed for a mechanistic understanding of PDAC pathogenesis, biomarker development, and for evaluation and optimization of chemotherapy, radiation therapy, targeted therapies, and immunotherapy. We also describe the role of animal models in

developing novel therapies that have been translated into clinics and highlight the challenges that remain to be addressed to improve their relevance for clinical research.

2.1. Evolution of PDAC experimental mouse models

Based on the complexities and experimental requirements, the commonly used mouse models of PDAC can be classified into surgical and non-surgical implantation models, and spontaneous genetically engineered mouse models (GEMMs) (Figure 1). The early experimental mouse models of PDAC involved implanting human PDAC patient-derived cells in athymic nude rats to demonstrate tumor growth [10]. Subsequently, several studies were published to show the development of a reliable preclinical xenograft model of PDAC in different rodent species where human pancreatic tumor derivatives (tumor chunks and cells) were used for orthotopic implantation in the pancreas [11-13]. With the development of various human PDAC cell lines, implantation models remained the mainstay for *in vivo* studies for studying the gene functions and evaluating therapeutic modalities for several decades till the advent of genetically engineered mouse (GEM) models. The evolution of murine models of PDAC can be divided into pre- and post-GEM periods. Prior to the GEM models, the preclinical models employed for PDAC research were predominantly xenograft mouse models, or carcinogen-induced models in rats, and hamsters [11, 12, 14-16]. Various mouse models currently employed for PDAC research are depicted in Figure 1. Among the mouse models, immunodeficient mice, including the athymic nude mice and severe combined immunodeficiency (SCID) mice, have been extensively used as these models lack xenograft rejection and allow tumor development from the biological material (tissue and cell lines) derived from human PDAC tumors [12, 15, 17, 18]. The orthotopic implantation models where PDAC cells are surgically implanted in the pancreas of the mouse, (Figure 1A; **panel 1**), are considered superior to the subcutaneous xenograft model (Figure 1B; **panel 1**), because the tumors grow under the influence of the local organ-specific microenvironment. Since the late 1980s, the orthotopic models of PDAC have been widely used for the optimization of targeted therapies and continue to be used extensively for preclinical evaluation of therapeutic modalities [12, 19]. While implantation and propagation of human PDAC tumor fragments in mice have been practiced since the early 1990s, [20, 21], the use of such models, has increased exponentially in the last decade [22-24]. These models are now called patient-derived xenografts (PDXs) and can be initiated by implantation of tissue fragments or cells from surgical resections or biopsies without the need for *in vitro* expansion or derivatization. Similarly, the tumor fragments derived from GEMMs or carcinogen-induced murine tumors have been propagated as mouse-derived allografts (MDAs) or mouse derived homograft tumors to evaluate therapeutic modalities in PDAC [19]. In parallel, several models for metastatic PDAC were used to study the underlying mechanisms of metastasis and evaluate therapeutic agents [20, 25, 26]. Peritoneal dissemination model of metastatic PDAC was reported earlier in athymic nude mice and in hamsters with an intent to study the mechanism of peritoneal metastasis and to evaluate its preclinical significance (Figure 1B: **panel 3**) [16, 27]. Using these models, Yamamura et al. suggested that the peritoneal metastasis could either be fast by direct dissemination or slow via stomata in the diaphragm [16]. Similarly, the peritoneal dissemination model was used to

demonstrate that retroviral P53 vector targeted therapy could inhibit primary tumor growth as well as peritoneal metastasis [27].

Mutations in the KRAS gene are the most common early event in the progression of several epithelial malignancies including the cancers of the pancreas, lung, and colon [28, 29]. The development of genetically engineered mouse models harboring oncogenic mutant KRAS that could be conditionally expressed in a tissue-specific manner, ushered the development of gene-driven murine models of spontaneous tumorigenesis (Figure 1 C). The mutant KRAS-driven spontaneous mouse model was first described for non-small cell lung carcinoma [30] and later was modified to generate a model of spontaneous PDAC [31]. Subsequently, Hingorani and coworkers developed murine models harboring TP53^{R172H} mutation along with KRAS^{G12D} mutation that developed more aggressive tumors and faithfully recapitulated most of the pathological features of human PDAC [32]. This genetically engineered KRAS^{G12D}; TP53^{R172H}; Pdx-1-Cre (KPC) exhibits the early PanIN lesions from the age of 8-10 weeks of age and progresses to PDAC with high incidences of metastasis to liver, lung, diaphragm, and adrenals. The median age of KPC mice was reported to be 24 weeks. KPC tumors closely mimic complex TME of human PDAC, both pathologically and immunologically. Therefore, the KPC model has been widely used for the mechanistic understanding of PDAC progression and the optimization and preclinical evaluation of therapeutic approaches. The introduction of the KPC model revolutionized the progress in the field of biomarker development [33-36], targeted therapies [37-41], and immunotherapies [42-46] of PDAC. The advent of KC and KPC models has not only provided novel insights into the genetic, molecular, and immunological mechanisms driving PDAC, but also broadened the research in the preclinical experimental therapeutics for PDAC and facilitated rapid bench-to-bedside transition. Several KPC tumor-derived cell lines [47, 48], fibroblasts [47], organoids [49-51], and MDAs [52], have emerged as indispensable reagents for understanding pathogenesis and for evaluation of targeted therapies [37, 53-55]. The development of the syngeneic C57BL/6 KPC mouse models and reagents derived from GEMM-derived resources have rejuvenated the field of PDAC immunotherapy. The KPC mouse model has not only served as a valuable tool to understand mechanisms associated with immune activation and suppression, tumor immune microenvironment in PDAC, but also been utilized extensively for testing immunotherapy-based approaches, including therapeutic vaccines [56-58], immune checkpoint inhibition [59, 60], adoptive immune-cell therapy [58, 61], and CAR-T cell therapies [62, 63].

The emerging trend in PDAC clinical trials has been evaluate therapeutic modalities in conjunction with surgical resection either under adjuvant or neoadjuvant settings. While the implantation and spontaneous models of PDAC have been valuable tools for experimental therapeutics, these models are not amenable to surgery due to large size of orthotopic tumors or multifocal nature of spontaneous models. To circumvent these challenges several approaches have been devised to develop models with localized resectable tumors. These include orthotopic implantation of cells in the tail of the pancreas (Figure 1A; **panel 3**), or injection of plasmids encoding Cre (in the floxed mice) or oncogenes (Figure 1C) (detailed elsewhere in the article). The resultant tumors can be surgically removed, and animals can be followed for local recurrence and metastasis, to evaluate the efficacy of therapeutic agents.

Such models are highly relevant for the evaluation of therapeutic modalities in adjuvant and neoadjuvant settings and have been used in several preclinical studies.

2.2. Mouse models specific to different aspects of PDAC pathogenesis

The sequence of genetic and molecular events from early oncogenic mutations to PanINs and to PDAC have been thoroughly investigated to identify actionable targets in PDAC [64, 65]. In this regard, experimental models representing various PDAC progression stages have been used to evaluate targeted therapies (Figure 2). Experimental murine models can be classified based on the stage of progression, mode of tumor initiation, site of tumor development and their suitability of specific therapeutic modalities (Figure 2 A, B). In addition, murine models representing other risk factors for PDAC, such as pancreatitis, diabetes, alcohol, and smoking, have been studied to understand disease pathogenesis and used as experimental models for targeted therapies [66-72] (Figure 2C). Despite well-defined mechanistic cascades involved in PDAC pathogenesis, developing targeted therapies for PDAC remains challenging. Most of the genetic, molecular, and immunological targets reported as key determinants of PDAC pathogenesis failed during preclinical assessment due to multiple challenges associated with disease progression and lack of appropriate evaluation platforms [73]. However, the landscape for preclinical therapeutic evaluation of PDAC has gradually broadened with the emergence of novel experimental models and approaches [74]. Thus far, several murine models have been reported to recapitulate various aspects of PDAC initiation and progression (Figure 2A, 2C), including the experimental mouse models for pancreatitis, models for early and late PDAC, models for metastasis, and those representing the complexities of pancreatic TME.

2.2.1. Experimental models for inflammation and pancreatitis

Inflammation and pancreatitis are major risk factors in PDAC along with age, race, smoking, obesity, and diabetes [75-77]. Although direct role of pancreatitis, both acute and chronic, is obscure as a single factor, there is ample evidence suggesting that inflammation and pancreatitis could potentially trigger PDAC pathogenesis [78-80]. Both classical and genetically induced models have been used to study inflammation and pancreatitis [81-83]. Cerulein-based experimental models of inflammation and pancreatitis have been extensively employed for mechanistic understanding as well as for therapeutic evaluation. One of the earliest models to study acute pancreatitis was reported in 1977 by Lampel and Kern, which involved secretagogue hyperstimulation using cholecystokinin (CCK) in rat [84]. Since then, several studies have used cerulein, an orthologue of CCK, for induction of both acute and chronic pancreatitis (CP) in mice [75, 81]. We have summarized key mouse models that have been used to study pancreatitis in the context of pathophysiology, potentiating PDAC, biomarker discovery, or evaluating the therapeutic modalities (Figure 2C). Previously, it was demonstrated that acute pancreatitis (AP) enhances the aggressiveness of PDAC in K-Ras-dependent manner. In contrast, recent studies have shown that chronic inflammation independent of K-Ras mutations can induce PDAC in the presence of cyclooxygenase-2 or I κ B kinase-2 (IKK2) [85]. Further, combining alcohol with cerulein has been reported to induce CP [86]. Earlier, the cerulein-induced models for both AP and CP were used to demonstrate the role of CXCR2 and neutrophil recruitment in pancreatitis. Here,

inhibition of CXCR2 in *cxcr2*^{-/-} GEM or by pharmacological inhibitor significantly delayed pancreatitis [87]. Recently, ruxolitinib, an inhibitor of the JAK-STAT pathway was reported to reduce the severity of CP in the cerulean-induced murine model [88]. Thus, chemically induced and the genetically manipulated mouse models of pancreatitis serve as valuable platforms to evaluate targeted therapies.

2.2.2. Experimental models for pancreatic cancer

Experimental murine models to study therapeutic approaches in PDAC can be classified based on either primary source of tumor, site of implantation, and based on the experimental requirement (Figure 2A). For instance, all experimental studies using human pancreatic tumor as a source have been performed in immunodeficient mice such as athymic and severely combined immunodeficient (SCID) mice. Human PDAC cell lines, organoids, tumoroids, patient-derived xenografts (PDXs) have been used as a source material for the xenograft studies. Due to defined tumor growth kinetics and ease for genetic manipulation, PDAC cell lines have been extensively used for the optimization of therapeutic approaches in xenograft murine models [89, 90]. Depending on the source, the PDAC cell lines differ in genotype, morphology, doubling time, dose-response, invasiveness, tumor growth kinetics, and metastatic nature. Due to easier maintenance under defined culture conditions and their ability to yield consistent and reproducible data, the PDAC cell lines have been a preferred choice for the evaluation of therapeutic approaches for a long time. Moreover, due to their origin from the tumors of human PDAC patients, these cell lines were believed to recapitulate the genomic and molecular diversity of human cancers and their use for targeting cancer cell-intrinsic pathological mechanisms was viewed clinically more relevant [91]. In addition, cell lines are the easiest source for implantation in mice for assessment of therapeutic approaches in vivo. However, multiple in vitro passages and maintenance in a non-physiological environment frequently results in altered phenotypic and functional characteristics of cell lines, which may lead to variability in experimental outcomes [91]. There is a vast literature available for in vivo use of PDAC cell lines in tumorigenesis in mouse models and their use in the preclinical evaluation of various therapies [19, 92]. Of note, these cell lines-based xenograft mouse models are not obsolete yet, rather, are clinically relevant and continued to be used widely in PDAC research. For targets associated with genetic and epigenetic regulation of PDAC, cell line-based xenograft models are still being used for targeted therapies that include siRNA, shRNA, miRNA, and RNAi-based approaches [93-97]. Both genetic and epigenetic determinants of PDAC pathogenesis are conserved in cell lines and cell lines expressing the targets of interest can be selected for in vivo evaluation of targeted therapies in the xenograft mouse model. In contrast, the genetic and epigenetic regulations may differ in cell lines derived from murine and human pancreatic tumors, and therefore, a pre-validation of a target is required to select the right cell line model. Besides, it is important to analyze the cellular localization of a target to develop selective targeted therapy. Recent studies based on PDAC cell line based xenograft models have validated pharmacological inhibitors against targets associated with different oncogenic pathways, including EGFR-STAT3 signaling [98], YAP-TAZ pathway [96], KRAS signaling [99], CDK4/6 [100], SUMO pathway [101], and metabolic pathways [97, 102]. Mechanistically, anti-tumor efficacies of these pharmacological inhibitors have been reported either direct or through chemosensitization [103, 104]. Among other recent

developments, using PANC-1 xenograft tumors, it has been demonstrated that iExosomes loaded with siRNA or shRNA targeting KRAS^{G12D} suppressed the growth of orthotopic tumors of the pancreas [105]. All these studies strongly suggest that PDAC cell line-based xenograft models remain relevant and will continue to be integral for optimizing targeted therapies for PDAC.

The cell line-based xenograft models have several limitations. First, human pancreatic tumor-derived cells grow in immunodeficient mice and preclude the analysis of immunological parameters of the disease. Second, the PDAC cell lines alter their phenotype as well as tumorigenicity when cultured in vitro for longer passages. Therefore, care must be taken while using the PDAC cell line for xenograft studies. Third, the cell line-based xenograft tumors, particularly the SC implanted tumors, do not recapitulate the stromal complexities of human PDAC. Therefore, preclinical therapeutic assessment is now focusing more on co-implantation models using a combination of cancer-associated fibroblast and cancer cells, organoid models, and tumor fragment-derived xenograft and allograft models [8, 106, 107]. Recent studies have extensively used patient-derived xenografts (PDXs) and mouse derived allografts (MDAs) to evaluate targeted therapies in PDAC [8, 108-110]. Though the PDX and MDA models are of high clinical relevance in PDAC, obtaining fresh tumor tissues to propagate and maintain continuously is challenging. To determine the impact of tumor initiating material on tumor growth, histology, and survival, Tseng et al., compared orthotopic implantation of cell line derived from liver metastasis site of the KPC mouse and the tumor fragments derived from SC homograft implantation of the same cell line [111]. Interestingly, both models showed similar growth and survival, suggesting that the tumorigenic characteristics of the cell are conserved when implanted in the pancreas of the mouse. However, as compared to the cell lines derived from the primary KPC tumors, the liver metastasis derived cell lines and tumor fragments showed significantly higher liver metastases reflecting the preservation of metastatic traits between cell line and homograft implants. . Alternatively, the organoid models have been widely appreciated for drug screening and their therapeutic evaluation in vitro as well as in vivo [49, 50, 110]. More interestingly, organoid based models have been used to understand disease progression and subtyping of PDAC. A recent study reported a xenotransplantation model where the organoid neoplasm from PDAC patient was injected in the pancreatic duct of the mouse [112]. Interestingly, the analysis of global RNA-sequencing data of fast and slow growing organoids suggested that the fast-growing organoids showed “invasive” subtype and the MYC, E2F, mTORC, and KRAS signaling genes were significantly enriched in this set of organoids. In contrast, the slow-growing organoids were enriched in bile and fatty acid metabolism and showed gene signatures associated with “progenitor” and “classical” subtypes. This study suggested that the accurate modeling of molecular subtypes is achievable using organoids and is important for understanding the PDAC progression and for the evaluation of emerging therapies.

2.2.3. Subcutaneous vs. orthotopic implantation

Depending upon the experimental requirement, the subcutaneous (SC) and orthotopic (OT) impanation models have been preferably used to evaluate therapeutic approaches in PDAC [19, 92, 113]. The SC model is a non-surgical method of cancer cell implantation as

described in the figure (Figure 1B; **panel 1**), which is the most used method for the *in vivo* evaluation of therapeutic modalities because it is convenient to implant tumor cell line and tumor derived fragments without an extensive surgical procedure. In addition, it allows us to assess the tumor growth kinetics longitudinally. The periodic measurements of tumor volumes provide quantitative data of the growth kinetics from the SC tumor models. Most importantly, multiple tumors can be implanted in the same animal to evaluate tumor-specific target(s), e.g., both target positive and negative cells in the same mouse to investigate the effect of targeted therapy. In contrast, the OT tumors grow under the influence of the pancreatic microenvironment and therefore, are considered superior to the SC model in vascular development, stromal organization, and immune infiltration [15, 114]. Previous studies have reported that as compared to SC tumors, OT tumors due to their growth in natural microenvironment are more vascular with high stromal content, which histologically mimics the primary tumors in corresponding human malignancies [75, 114-116]. These vascular and histological differences in SC and OT tumor models cause a variable response to therapeutic agents. For example, the therapeutic response to FOLFIRINOX, a chemotherapeutic regimen for PDAC patients, was found to be variable in the mice bearing SC and OT tumors [114]. Interestingly, the difference in tumor growth, metastatic spread, blood vessel density, and the expression of Ki-67 and Caspase-3 suggested that the therapeutic response to FOLFIRINOX was better in the mice with OT tumors as compared to those with SC tumors. In another study, a comparative analysis of liver metastasis derived KPC cell line and its SC homograft tumor fragments was performed in OT implantation model [111]. The tumor growth, metastasis, histology, immune phenotype, and survival of the mice were not significantly different in the two groups implying that pancreas-specific microenvironment had greater influence on these parameters as compared to the implantation source. Further, the OT model of the pancreas is better for survival analysis as compared to the SC model because the increased tumor burden and associated pathological symptoms such as metastasis and ascites formation cause lethality in tumor-bearing mice. In contrast, the euthanasia in SC model is often dictated by humane endpoints (e. g, tumor volume, ulceration) defined by the local IACUC guidelines. Therefore, the survival data obtained from the SC model may not be truly an outcome of complications associated with tumor burden and metastasis. The OT models are relatively challenging to establish that require expertise in animal surgery to implant tumors. Recently, Erstad et al., have extensively optimized the surgical procedure to implant the PDAC cells in mouse pancreas and provided an excellent description of the techniques with all cautionary steps during the implantation [114]. The site of incision, cell number and volume, site of injection in the pancreas, angle of the needle during injection, and the gauge of the needle, are all important parameters to optimize for OT implantation. For instance, the most common incisions adopted for mouse pancreatic OT implantation are the midline incision, left subcostal incision, and left flank incision. However, the left flank incision with gentle pancreatic dissection provides better exposure of avascular regions of the pancreas to implant the cells [114]. Similarly, the angle of needle while injecting the cells is recommended parallel or at a minimum angle to the pancreas so that the bevel of the needle must not cross the thickness of the pancreas. Although the cell number in a volume of 50-100 μ l can vary depending on the aggressiveness of the cell type, the recommendation for KRAS^{G12D}; TP53^{R172H} mutant cell lines is in a range of $\sim 10^4$ cells/

mouse. Thus, considering these points during OT implantation in the mouse pancreas would help in more appropriate and non-erroneous assessment of therapeutic modalities. Recently, OT mouse model was used for mass screening of > 50 drugs and the study concluded that the combination of HSP70 and MEK inhibitors exerts synergistic effect against PDAC and could be taken for further evaluation [117].

To overcome the leakiness of injected cells during OT implantation, the solidifying gelatinous protein mixture such as Matrigel, which is a brand name of protein mixture secreted by mouse sarcoma cells and resembles the extracellular matrix environment present in different tissues, can be used to implant the cells. Several studies have used Matrigel based implantation method for testing various therapeutic modalities and reported that matrigel provides more consistent and proliferative tumors [118-121]. Alternative to surgical implantation method that is time-intensive and cause aberrant inflammation, a non-invasive method has been developed to implant the cells in the pancreas of the mouse with the help of USG imaging (Figure 1B; **panel 2**) [122]. Technically, as the transducer comes closer to the abdomen, pancreas can be visualized and the allowing a needle to be guided for the orthotopic implantation of the tumor cells. Like surgical implantation, bolus of cell suspension is the confirmation for accurate cell injection. Intrahepatic injections have also been performed for the orthotopic tumor implantation with the help of USG imaging [123], suggesting that the imaging-based approach could be an alternative to surgical procedures for OT implantation. Besides, there is heterogeneous tumor growth in different mice and requires careful animal randomization before therapy initiation, which could be achieved by bioluminescence imaging or by ultrasound guided (USG) imaging of tumors in appropriate settings. Additionally, if the cancer cells are unlabeled, it is difficult to follow the tumor growth in OT model longitudinally without the use of ultrasound or magnetic resonance imaging tools which are time-consuming and require additional expertise. Despite the challenges in implantation and monitoring, the OT models of the pancreas are clinically more relevant and considered superior to SC model for the evaluation of therapeutic approaches in PDAC.

2.2.4. Mouse models of metastasis and perineural invasion:

Early metastasis is a hallmark of PDAC and is one of the major causes of cancer-related deaths in PDAC patients [1]. Mechanisms regulating PDAC metastasis are not fully understood, but the genetic and molecular alterations have been reported to drive distant metastasis in PDAC, with the liver as the most favorable metastatic site [124, 125]. Therefore, several attempts have been made to generate experimental models of hepatic metastatic of PDAC. Earlier, studies indicated that orthotopic transplantation of tumor pieces from human pancreatic tumor specimen or after propagation of human PDAC cell lines in SC model resulted in metastatic spread of PDAC to several organs, including the liver [20, 126]. Similarly, the red fluorescent protein (RFP) labeled MIA-PaCa-2 cells were propagated in the SC flank of the mice and transplanted into the pancreas of nude mice [127]. In these transplantation models, metastases to multiple sites including liver, peritoneum, spleen, and lymph nodes, were observed. However, variability in metastatic burden and incidences make it challenging to evaluate targeted therapies specifically against metastases. Recently, OT implantation based model of PDAC metastasis showed that SUIT-2

is a more efficient cell line than Capan-1 for studying distant metastases with higher incidence and reliable dissemination to the liver, lung, and mesenterium [128]. As liver is the most common site for PDAC metastasis, several efforts have been directed to develop liver-specific models of PDAC metastasis. The most common and reliable models for liver metastasis are splenic and portal vein injection models that consistently form hepatic metastases and are considered suitable for evaluating targeted therapies and immunotherapy-based approaches (Figure 1A; **panel 2**) [129-131]. The highly consistent portal vein injection model has been used for liver metastasis in the multiple cancers including the colon, pancreas, breast, and uvea [132-135]. However, the portal vein injection model is surgically more cumbersome where a surgeon must flip all the abdominal organs to expose the portal vein. Soares et al., have described an alternative highly efficient and reliable model of hepatic metastasis that involves the injection of cancer cells into the hemispleen, as described in the figure (Figure 3) [129]. The hemispleen technique is highly reproducible in generating liver metastases and can be easily performed in xenograft and syngeneic mouse models. Recently, using hemispleen model, radiation-induced immunogenic “abscopal effect” in combination with immunotherapy was demonstrated to inhibit hepatic metastasis [136]. Overall, hemispleen model due to its ability to mimic pathophysiological aspects of hepatic metastasis is ideally suited to study the mechanisms of metastasis and evaluate targeted therapies, including immunotherapies.

Perineural invasion (PNI) is a mechanism of cancer cell metastasis through the nerves that is predominantly seen in the tumors lacking vascular and lymphatic invasion [137, 138]. Clinically, the PNI is considered to be a major cause of disease recurrence and pain in PDAC patients [139]. As the pancreatic tumors are hypovascular, lack lymphatic transport system, and located closer to the autonomic plexuses of the abdomen, the disseminating cancer cells tend to follow the perineural route to metastasize. Earlier, the histological analysis of resected pancreatic tumors and regional lymph nodes (N=14 patients) showed that pancreatic head is the primary site of nerve enrichment for the PNI and pattern of PNI is different than lymph node metastasis [140]. Later, Pour et al., demonstrated the evidence of PNI in Syrian hamster model where PNI was observed as most common route of metastasis (~89%) after intrapancreatic implantation of cells, but no vascular invasion [141]. The exact mechanism of PNI is still unknown; however, several oncogenic signals and growth factors such as MMPs, nerve growth factors, adhesion molecules, metabolic alterations, and cytokine signaling, have been reported to contribute in PNI in PDAC [142-151]. In an attempt to explain PNI and PDAC recurrence, Eibl and coworkers used a modified OT implantation model where the xenograft tumors developed after the implantation of MIA PaCa-2 and Capan-2 cells in the pancreas of athymic nude mice were completely resected and followed for disease recurrence [152]. Intriguingly, after 6 weeks of resection, recurrence was observed in the MIA PaCa-2 tumor group and histological evaluation suggested an extensive PNI of retroperitoneal nerve. Similarly, different PDAC cell lines (N=7) with high and low PNI properties were compared at RNA and protein level and used for the analysis of CD74 expression in xenograft mouse model [153]. Interestingly, the invariant chain CD74 was observed overexpressed in PNI high cell line group. In addition, the mouse PNI high cell lines Capan-1 and Capan-2 were found to invade mouse peripheral nerves, suggesting some overlapping human and mouse PNI pathways. Further,

the autochthonous KC mouse model has been used to delineate the molecular mechanisms and pathways that regulate PNI, and it was observed that ablation of sensory neuron significantly decreased the PNI [154]. Recently, axon guidance molecule SEMA3D was reported to activate receptor PLXND1 in OT model and knockout of PLXND1 in KPC model significantly decreased perineural invasion, which is highly regulated by dendritic root ganglion cells [155]. Overall, different xenograft and autochthonous mouse models have established the influence of PNI on various pathological aspects of PDAC including its role in local recurrence, metastasis, stromal modulation, and in the alteration of immune TME [138, 155, 156].

2.2.5. Mouse models for pancreatic tumor stroma

Tumor stroma is one of the critical determinants of therapy resistance in PDAC, which has gained greater appreciation with the advent of GEMMs. In fact, GEMMs were used to evaluate several therapeutic modalities targeting stromal components in combination with conventional chemo or radiation therapies [32, 157-159]. In addition to GEM models, recent studies have described several new *in vitro* and *in vivo* models to account for the contribution of stroma in evaluating therapeutic regimens [160-162]. Cancer-associated fibroblasts (CAFs) are the predominant stromal cells in pancreatic tumors that contribute to fibro-inflammatory responses within the TME, producing extracellular matrix (ECM) proteins and inflammatory and immunosuppressive cytokines [107, 163]. Therefore, experimental models based on co-implantation of cancer cells and fibroblasts are considered superior to cancer cells implantation alone and recapitulate tumor-stroma in experimental PDAC murine models [164]. Multiple reports suggest CAFs are key determinants of radiation therapy, immunotherapy, and other targeted therapies in PDAC [8, 106, 165]. Earlier, Nguyen and coworkers used the SC co-implantation model to investigate the role of fibroblast-expressed micro-RNAs (miRNAs) on tumor growth and demonstrated that miR 21 promotes tumorigenesis [166]. In contrast, in a recent study, the tumor-restraining role of fibroblasts associated with Meflin protein, a mesenchymal and stemness marker, was demonstrated using the co-implantation model [167]. Interestingly, Meflin knockdown in the fibroblasts rendered the straightening of collagen fibers and led to the tumor aggressiveness in the PDAC transplantation model, whereas the overexpression of Meflin caused the tumor growth suppression. Immunologically, CAFs play a significant role in immunosuppression and contribute to resistance to immunotherapies. Recent studies have used co-implantation models to determine the impact of targeted therapies and immunotherapy-based approaches on TME. For instance, targeting metabolic pathways by small-molecule glutamine analog in the co-implantation model not only reduced metastasis and stemness in cancer cells but also depleted ECM and sensitized tumors to immune checkpoint inhibition therapies [168, 169]. These findings strongly support the significance of including fibroblasts in tumor implantation models for evaluating targeted therapies in PDAC. The co-implantation model is further advanced by the introduction of organoids, PDXs, and MDAs because the tumors growing from these sources constitute high stroma and recapitulate human PDAC more accurately [49, 52, 170].

2.2.6. Mouse models for lineage tracing in PDAC progression

The lineage tracing models are highly relevant for identifying the cell of origin in spontaneous models and understanding the contribution of different cell types in cancer progression, TME constitution, therapy resistance, and immune cell repertoire. Previously, lineage tracing models have been employed to understand the development of pancreas, acinar to ductal transdifferentiation, and contribution of different cell types in the adult pancreas [171-173]. As the pancreatic tumors are highly heterogeneous in cellular composition with extensive phenotypic and functional diversity, lineage tracing approaches have provided a robust tool to understand the unique attributes of each cell lineage. Earlier, to explore the EMT and metastasis of epithelial cells in KPC mice, constructs were used to fluorescently tag the epithelial cells such that when spontaneous tumors develop, these cells could be tracked *in vivo* [174]. Based on this 'tag and track' KPC model, it was observed that yellow fluorescence protein (YFP) labeled tumor epithelial cells of the pancreas undergo EMT at early stage of tumor development as these fluorescently labeled cells were observed in circulation at very early stage, even before the histological confirmation of PDAC development. Similarly, with the help of lineage tracing approaches in KC and KPC models, role of stem cell populations was defined. For example, the stem cell subpopulation characterized by the expression of microtubule marker doublecortin like kinase-1 (DCLK-1) was found to be enriched in preinvasive lesions in mutant KRAS mouse model and play a significant role in pancreatic tumorigenesis [175, 176]. Further, using lineage tracing models of intraductal papillary neoplasm (IPMN) and PanIN lesions, a recent study demonstrated that DCLK-1+ stem cell subpopulation originates from Pdx-1+ progenitors [177]. In addition, the DCLK+ stem cells in IPMNs co-express the tuft cell markers tubulin and COX2, but not the tumor epithelial markers such as MUC5AC, suggesting distinct phenotypic characteristic of DCLK+ stem cells. Likewise, other molecular markers for specific cell lineages and their roles in PDAC progression and drug resistance have been explored using the lineage tracing models [178-181]. Besides, these lineage tracing models have opened avenues to explore the immune cell homing and origin during PDAC progression, which can be valuable tools in designing immunotherapy-based approaches against PDAC.

2.2.7. Mouse models of PDAC-induced cachexia

Cachexia is a complex metabolic and behavioral syndrome that is characterized by anorexia (loss of appetite), muscle wasting, and irreversible weight loss [182]. Up to 85% of PDAC patients exhibit features of oncogenic cachexia and ~30% of patients succumb to cachexia, not due to tumor burden [183-185]. The underlying mechanisms and factors that induce cachexia in PDAC patients remain poorly understood. Activation of proinflammatory cytokines, tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-6, and IL-8 in cachectic skeletal muscles lead to the activation of signaling pathways, e.g., JAK2/STAT3 and NF- κ B, which cause protein degradation and muscle wasting in PDAC patients [186-188]. Due to the lack of robust animal models and poorly understood mechanisms, cachexia management is highly challenging in PDAC patients. Previously, PDAC cell line xenograft and allograft murine models have been used to study cachexia [183]. Most of these studies analyzed weight loss, muscle wasting, and mRNA analysis of markers associated with cachexia. For instance, in a murine model of metastasis and cachexia, Greco et al. inoculated Panc02

and FC1242 cell lines in C57BL/6 mice and treated these mice with toll-like receptor-7/9 (TLR-7/9) inhibitor or anti-TGF- β antibody to investigate the role of TLR-7/9 or TGF- β mediated pathways [189]. Interestingly, treatment with anti-TGF- β antibody reverted the cachexia symptoms and impeded weight loss and improved survival. Further, several other molecular pathways, including MyD88, TLRs, CD25, and TGF- β , have been shown to regulate cachexia in PDAC [190-192]. In a comparative analysis, the KPC tumor cells were implanted at different sites (subcutaneously, intraperitoneally, and orthotopically) in C57BL/6 mice to study cachexia-related metabolic, phenotypic, and behavioral changes [193]. This study demonstrated that the implantation site is crucial while studying cachexia in mouse models. Both intraperitoneal and orthotopic implantation models developed more severe cachexia as compared to the subcutaneous implantation model. The KPC tumor-bearing mice showed a decrease in food intake and locomotive activity. Furthermore, the muscle catabolism (wasting) was observed in both the skeletal and cardiac muscles. In contrast to subcutaneous tumors, the orthotopic tumors are histologically closer and constitute a mimicking TME as present in human PDAC tumors; therefore, it is likely that factors associated with pancreatic TME play an important role in inducing cachexia. For example, recent studies suggest that tumor-associated macrophages play important role in muscle wasting and cachexia via activating STAT3 signaling [194, 195]. Hence, stroma intact murine PDAC models are more useful in understanding cachexia progression and for evaluating approaches for the management of cachexia.

2.2.8. Genetically engineered mouse models

The genetically engineered mouse models (GEMMs) of PDAC have served as useful tools for the preclinical evaluation of various therapeutic approaches and helped in understanding the disease progression [31, 32]. In fact, the current understanding of the early stages of PDAC progression is based on the observations made in the mutant KRAS mouse models of PDAC [196, 197]. The PDAC GEMMs can be classified based on three important categories: the target gene(s) driving oncogenesis; specificity of promotor used to regulate the Cre-recombinase expression, and the manner of oncogene induction (Figure 1C). In combination with the oncogenic mutant KRAS, several other genes have been targeted to enhance the pathological robustness of these murine models, which include P53, Smad4, Ikb1, Tgf- β , and Ink4 [19, 198, 199]. Similarly, the Cre-recombinase has been used under the control of different pancreas specific promoters such as Pdx-1, Elastase, P48, and Ptf-1- α [200-203] that are specific to distinct cell lineages in the pancreas. In another approach to rapidly produce the GEMMs, embryonic stem cells (ESCs) harboring the mutant KRAS, a homing cassette, and other genetic elements, were used at an early developmental stage to produce GEMMs with characteristics to develop tumors [204]. Due to similar histopathological features as human PDAC and rapid tumor progression, Pdx-1-Cre regulated KRAS^{G12D}; TP53^{R172H} (KPC) mouse model has been most widely used to understand PDAC biology and for the evaluation of targeted therapies [42, 55, 205-207], gene silencing, and pharmacological inhibition of selective targets [208-212]. There are several advantages of KPC mouse as an experimental model for the optimization of targeted therapies. First, the KPC mouse model recapitulates the pathobiology of human PDAC, including the progression, metastasis, stromal complexities, and immunological consequences of PDAC pathogenesis (Figure 1C), and therefore, it is considered suitable

to evaluate the effects of various risk factors and therapeutic agents on PDAC progression. For instance, recent studies have shown that smoking, which is a major risk factor for pancreatitis and PDAC, not only induces the stemness and endothelin-axis upregulation in KC mouse model but also promotes accumulation of myeloid-derived suppressor cells (MDSCs) in TME [71, 213, 214]. Second, PDAC progression is faster in KPC mice with a significant tumor burden by an average age of 20-24 weeks and metastases. Recently, tumors and cell lines derived from KPC mice have been used to demonstrate that selective metabolic reprogramming of stem cell populations promotes metastasis during PDAC progression [215]. Third, KPC mice exhibit high penetrance, with more than 90% of mice developing PDAC and succumbing to metastatic disease. Lastly, the resources generated from the KPC mouse models, including tumor cell lines, age-specific primary and metastatic tissue samples, serum samples, and intact tumor tissues for allograft implantation (MDAs) and tumoroid development, have emerged as valuable tools for PDAC research in immunocompetent animals. As generating ample number of KPC mice is challenging, the KPC cell lines and MDAs are useful alternatives that have been used for evaluating both targeted therapies and immunotherapy. Later, several other K-Ras mutations driven mouse models were introduced with other companion oncogenic and target specific mutations to study their effects in PDAC pathobiology [216, 217]. The KPC mouse model has been further utilized to develop gene specific knockout (KO) models to elucidate their role on PDAC pathobiology. For example, disruption of genes specific to stemness, metabolism, metastasis, glycosylation and immune pathways, have been demonstrated to alter the pathological features of gene specific KPC KO models [207, 210, 218, 219].

Despite several advantages, there is a variability in tumor initiation, progression, and incidences of metastasis in KPC mice, which can impact the experimental readouts during preclinical testing of therapeutic approaches. However, advanced imaging approaches have been employed to analyze tumor volumes, including USG imaging, MRI, and Computed Tomography (CT) in experimental murine models to follow the tumor growth and metastasis [220-222]. In addition, alternative approaches have been used to produce large batches of experimental mice that mimic the tumor progression and clinicopathological features of KPC mouse model. In this regard, tumor-derived homograft implantation and electroporation of oncogenic plasmids have been utilized to generate larger animal cohort and minimize the variability and challenges associated with KPC mice [210, 223, 224]. The electroporation to introduce plasmids encoding oncogenes or Cre recombinase have been used to efficiently produce focal disease in a more reproducible and predictable manner as compared to cumbersome breeding schemes where the yield of mice with the desired genotypes is low.

3.0. Therapeutic approach specific mouse models

Currently, preclinical evaluation of various therapeutic modalities for PDAC relies on sophisticated murine models to facilitate clinical translation. These models can either be immunodeficient to allow the growth of human PDAC cells, or immunocompetent where tumors are induced by carcinogen treatment, genetic manipulation or syngeneic cell implantation. The immune deficient models have been extensively used for evaluating chemotherapeutic agents, targeted therapies and radiation therapy, against tumors derived

from human cancer cell lines and patient derived xenografts. In contrast, immunocompetent models are ideally suited for evaluating immunotherapies or to examine the impact other therapeutic modalities on immune system components. Immunocompetent models are being used to evaluate the contribution of immune system in the response or resistance to non-immune therapies.

3.1.0. Murine models for chemotherapy and radiotherapy:

The treatment approach for PDAC patients is based on tumor stage and patient's response to therapies, which include surgery, chemotherapy, and radiotherapy [225, 226]. The optimization of chemotherapy and radiotherapy regimen in preclinical PDAC models is highly challenging because the small size animals differ in body weight, organ size, and basal metabolic rate (BMR), which are critical parameters for the chemotherapy dose optimization and for the delivery of radiotherapy. Despite that, major chemotherapies including gemcitabine, FOLFIRINOX, nab-paclitaxel, and capecitabine, have been optimized in mouse PDAC models [114, 227-231]. Earlier preclinical evaluations of chemotherapies and radiotherapy were intended to assess their effects on tumor growth, metastasis, and therapeutic resistance [18, 232-236]. However, most of these studies were usually performed in xenograft mouse models that were immune deficient and, it was difficult to make any immunological interpretation. For example, therapeutic agents' genistein and wortmannin that target DNA repair pathway and angiogenesis respectively, were reported to enhance the gemcitabine sensitivity in OT pancreatic tumors in SCID mice [234, 237]. Similarly, minnelide, a heat shock protein-70 (HSP70) inhibitor, was reported to overcome oxaliplatin resistance in OT PDAC xenografts derived from MiaPaCa cell line in athymic nude mice [236]. Similarly, preclinical assessment of radiolabeled monoclonal antibodies (mAbs) against the tumor specific targets employed xenograft mouse models [18, 233, 238-240]. SC and xenograft models have also been used to evaluate stereotactic body radiation therapy (SBRT) and chemotherapy-based combination treatment modalities [241-244]. However, the targeted delivery of SBRT to the mouse pancreatic tumors is challenging due to the small animal size and the anatomy of mouse pancreas, which hinders in focused delivery of radiation beam to the pancreatic tumors. However, a recent study showed that the radiation and chemotherapy-induced immunological alterations and their therapeutic effects in combination with immunotherapy-based approaches have been investigated thoroughly in immunocompetent syngeneic mouse models of PDAC [245-249]. Similarly, a combination of SBRT and anti-CD40 has been reported as effective in situ vaccination in PDAC mouse model, suggesting that the SBRT based combination therapies could be more effective in the treatment of PDAC [250]. The growing numbers of experimental mouse models for the preclinical evaluation of different therapeutic approaches are based on their ability to address the unique challenges associated with the pathological hallmarks of the disease and the therapeutic responses to the given treatment (Figure 2A-B). Besides chemo- and radiotherapy, other targeted therapies such as gene silencing approaches, pharmacological inhibitors, small peptides, and monoclonal antibodies, have been preferentially evaluated in SC and OT xenograft models using human PDAC cell lines, organoids, and PDXs.

Efforts have also been directed to generate experimental models to recapitulate clinical settings to optimize the therapeutic interventions in adjuvant and neoadjuvant settings. Several approaches have been employed to develop murine models amenable to surgical resection of primary tumors for evaluating the impact of therapeutic modalities on disease recurrence and survival (Figure 1A; **panel 3** & Figure 1C). A recent study demonstrated that there is high local recurrence and metastasis after the early resection of pancreatic tumors that were generated by the electroporation of oncogenic plasmid in the mouse pancreas. However, the treatment with gemcitabine (100 mg/kg) as adjuvant chemotherapy following the surgery significantly reduce the local recurrence and prolonged the survival of the treated mice [223]. Similarly, electroporation of cre-recombinase expressing oncogenic plasmids was performed in the pancreatic tail of the KRAS^{G12D}; P53^{fl/fl} B6 mice using the 5 mm diameter tweezer type electrode. A total of four pulses (35-ms/35 V) were given for sufficient plasmid electroporation, as depicted in the figure (Figure 1C). Once the focal tumor growth was observed, resection was performed following the standards commonly used in clinics [251]. For this, mice were laparotomized and surgical area was prepared. Next, the splenic blood vessel (BV) and blood supply to the tumor were stopped by applying the ligation clips. The important stages for this resection model are highlighted in panel 3 of Figure 1A. In this model, early resection coordinated with neoadjuvant and adjuvant therapies of gemcitabine, anti-PD-1 (T cell checkpoint) antagonist, and anti-CD96 (NK cell checkpoint) inhibitor provided long term survival benefit and significantly inhibited the local recurrence [251]. In another study, adjuvant radioimmunotherapy (RIT) was evaluated in a murine model following surgical resection of OT xenograft tumors. Intraperitoneal administration of 22.5 MBq dose of 64Cu-PCTA-retuximab after surgery significantly inhibited the local recurrence, liver metastasis, and peritoneal dissemination, as compared to the resected control group [252]. Recently, adjuvant co-therapy of acetylcholinesterase inhibitors with or without gemcitabine was investigated in orthotopic xenograft resection model [253]. However, it was observed that adjuvant co-therapy was not effective in enhancing the survival in this resection model. Thus, these xenograft and GEM resection models are useful tools for guiding the clinical trials for PDAC patients undergoing surgery with adjuvant or neoadjuvant therapies.

3.2.0. Experimental models for evaluating immunotherapies:

Immunotherapy-based approaches have been extensively evaluated for treating PDAC [254]. Immunotherapies such as immune checkpoint inhibition, cytokine therapy, therapeutic vaccines, and other immune-modulatory therapeutic approaches have been investigated primarily in syngeneic mouse models in C57BL/6 mice. Particularly, the cell lines and homograft's from KPC mice-derived tumors have served as valuable reagents for immunological studies in PDAC (Figure 2B). Immunotherapy-based approaches can be categorized into two subgroups: antibody-targeted therapies and vaccine-based therapies. While the antibody-targeted therapies may or may not require immunocompetent mice depending upon the target molecules, vaccine-based therapies are reliant on the intact host immune system. The third group of targeted immunotherapies is cell-based immunotherapies which involves adoptive immune cell transfer and chimeric antigen receptor T-cell (CAR T cell). Recently, progress has been made where cell based targeted

therapies have been evaluated in the xenograft, allograft, and GEM mouse models of PDAC [255-258].

3.2.1. Antibody-targeted therapies: Murine models of PDAC have been extensively used for testing the efficacy of various monoclonal antibodies (mAbs) targeting either tumor-associated targets, antigens associated with tumor vasculature, or targets within the tumor stroma. The latter class may include targets on stellate cells, fibroblasts, immune cells, or an acellular stroma component. Two kinds of models are generally used: the implantation models (subcutaneous and orthotopic) and genetically engineered mouse models, where tumors arise spontaneously in an immunocompetent mouse [115, 259, 260]. The SC and OT implantation models have been extensively used for evaluating monoclonal antibodies against PDAC. For example, anti-Her2 mAb Trastuzumab was shown to inhibit the growth of Capan-1 xenograft implanted subcutaneously in nude mice [261]. The antibody did not inhibit the growth of Capan-1 cells in in vitro assays, showing that the predominant mechanism of action of Trastuzumab in PDAC may involve the activation of immune effector mechanisms. Further, Trastuzumab administration exhibited synergy with gemcitabine in reducing the growth of Capan-1 xenografts. Similarly, dual inhibition of HER2 and EGFR exhibited synergy in reducing the growth of PDAC xenografts [262]. Antibodies like CS-1008, a humanized antihuman Death Receptor 5 (DR5) antibody and AMG 655 (a fully human anti-DR5 antibody) also synergized with gemcitabine in reducing the growth of SC and OT PDAC xenograft mouse model (MIA PaCa-2) [263, 264]. A human single chain antibody against DR5 again inhibited the growth of MIA PaCa-2 xenografts and synergized with gemcitabine [265]. Another such anti-MUC1 antibody called PankoMab-GEX was also shown to be highly tumor selective and was shown to induce potent ADCC towards its target cells [266]. Various other tumor-associated targets such as mesothelin, IGF-1R have been evaluated in similar xenograft models with varying degrees of success [267-269]. However, many agents that work very well in these models do not translate efficiently to the clinic with human patients. To circumvent these limitations, mAb-based therapies have also been evaluated in GEMMs. For example, in KPC mice model anti-CD40 mAb induced potent activation of macrophages in tumor tissue resulting in stromal depletion, improved delivery of chemotherapy, and enhanced tumor cell killing [270]. The KPC mouse model has also been extensively used to study the effects of anti-PD-L1 and anti-CTLA-4 immune checkpoint blockade and the poor response obtained in these models correlated well with the results obtained from human trials [271]. These observations further support that the KPC mouse model efficiently recapitulates the attributes of human PDAC in the sense that it is poorly immunogenic, excludes effector T cells, and provides an immunologically “privileged environment” for PDAC cells to thrive [260]. However, in the same study, a combination of anti-PD1 mAb and a CXCR4 inhibitor “AMD3100” induced tumor regressions in the KPC mice model. PD-L1 blockade also exhibited synergy with radiation therapy in both the KPC model and SC transplantation model using Panc02 cells [60]. KPC model was also used to test the efficacy of TME-associated targets such as a LOX-blocking antibody that demonstrated improved efficacy in combination with gemcitabine [189]. Similarly, a combination of an anti-MUC1 mAb called TAB004 with liposomal-MSA-IL2 provided therapeutic benefit in an immunocompetent MUC1 transgenic mice model [272]. This model exhibits the expression of human MUC1 in a spontaneously

arising tumor in the pancreas of the mouse, with similar apical luminal MUC1 expression in transformed ducts as seen in human PDAC tumors. KPC mouse model was also used to study the efficacy of targeting stroma as seen by targeting stromal matrix protein CTGF. In this study, it was shown that an antibody against CTGF could synergize with gemcitabine in reducing tumor growth [158].

3.2.2. Vaccine-based therapies: For immunological studies, the C57BL/6 strain of mice has been a model of choice as it predominantly elicits Th1-type immune response [273, 274], which is critical for anti-tumor immunity. However, the selection of immunogenic vaccine candidates and the development of relevant mouse models remains a challenge. In PDAC, three major types of therapeutic vaccines have reached clinical trials, including whole-cell vaccines, peptide vaccines, and dendritic cell (DC) based vaccines [275]. Preliminary studies are easier for DC-based vaccines because antigen-loaded DCs from the same mouse strain can be used to investigate the efficacy in an allograft mouse model or in a KPC mouse to analyze cytokine release, T cell activation, and anti-tumor effect [58, 276]. However, the anti-tumor immune response needs to be validated in human subjects. Thus far, whole-cell vaccine GVAX and multi-peptide vaccine Algenpantucel-L have advanced to phase II and phase III clinical trials, respectively [277]. Other vaccine candidates that are under investigation include MUC1, mutant K-Ras peptide, GV1001, and KIF20A. Recent studies suggest that other mucins, including MUC4 and MUC16, could be potential vaccine candidates against PDAC [278-280]. Most of the tumor antigens are self-antigens and demonstrate antigenic tolerance that led to their poor immunogenicity. However, recent studies suggested that tumor-specific mutations, splice variants, and post-translational modifications potentiate the immunogenicity of vaccine candidates. Besides, the lack of a reliable preclinical platform rendered slower development of vaccine-based immunotherapies in PDAC. Earlier, studies were performed using transfected murine cell line-based allograft models or using adoptive T-cell transfer methods to demonstrate the therapeutic efficacy of vaccine candidates in PDAC [281]. However, to investigate the therapeutic potential of candidate vaccines, antigen-specific transgenic (Tg) mouse models are required. For example, the development of the MUC1-Tg mouse significantly contributed towards understanding the immunogenic role of MUC1 and in the development of MUC1 specific vaccines [272, 282, 283]. Another approach to investigate the antigen-specific immune response of human PDAC antigens in murine models is by employing the human MHC transgenic mouse model. Human MHC-Tg mice models have demonstrated successful antigen presentation of human T-cell epitopes [284, 285], suggesting that the MHC-Tg mouse model could be used to investigate whether the selected antigen is efficiently presented on human MHC-I molecules to successfully prime T-cells for antitumor immune response. However, the generation of MHC transgenic needs an understanding of patient haplotype and therefore could be challenging to be use for personalized immunotherapy. Overall, vaccine development against PDAC is still an emerging field and it needs more robust and clinically acceptable mouse models for testing the vaccine based therapeutic approaches against PDAC.

3.2.3. Mouse models of parabiosis—Parabiosis is an experimental approach where two individual animals are joined anatomically to share blood and body fluids (Figure

4). Earlier use of parabiosis in biomedical research was described in the 19th century. In 1908, Sauerbruch and Heyde first coined the word “parabiosis”, and then several other groups showed the utility of parabiosis in experimental animal physiology [286, 287]. Pioneer work from Lambert and co-workers in the rat cancer model showed that the mouse tumor grew when transplanted in the rat in a parabiosis setting. Parabiosis models are of high relevance in studying cancer immunology and immunotherapy. For example, in an earlier attempt, Kross et al. showed that tumor cells when implanted in susceptible mice in immune and susceptible conjoint parabiosis system, exhibit reduced tumor growth in the susceptible mouse [288] suggesting the active exchange of immune components between the conjoined animals. Several recent studies have revived interest in parabiosis murine models for immunological studies [289-292]. For PDAC, a study published in 1964 showed in the rat parabiosis model that when one rat was irradiated while the other was shielded, it showed hormonal imbalance after irradiation but protected the supradiated mouse from lethal radiation dose. In addition, there was hardly any influence of parabiosis on tumor growth [293]. In another report, to decipher the role of host-derived MMP9 in angiogenesis and pancreatic tumor growth, MMP9 (–/–) and MMP9 (+/+) conjoint mice were used as parabiotic partners [294]. This study suggested that host derived MMP9 is crucial for tumor angiogenesis and growth. Recently, Zhu et al. used the parabiosis model by conjoining CD45.1⁺KPC mouse and CD45.2⁺ congenic wild-type mouse to investigate the contribution of circulating monocytes in tumor-associated macrophage population in PDAC [295]. Both the CD45.1⁺KPC and CD45.2⁺ wild-type mice were joined surgically at the age of ~14 weeks when KPC mice were pathologically at the PanIN stage and followed for next 6 weeks for chimerism in the partner KPC mouse. Interestingly, 28% of Ly6C^{hi} peripheral chimerism was observed in KPC mice in the first two weeks, which remained stable after 6 weeks of parabiosis. However, only 2.5% of chimerism was observed after 6 weeks of follow-up, suggesting that most TAMs are maintained independently of circulating monocytes. As PDAC is highly heterogeneous in the stroma and immune infiltration, the parabiosis models might help in deciphering the origins, trafficking and kinetics of infiltrating immune cells and fibroblasts.

4. Conclusion and future perspectives

Mouse models have played a crucial role in enhancing the current understanding of PDAC pathobiology and are central for the evaluation of novel therapeutic modalities. Beginning from the simplest cell line-based SC xenograft models to the complex GEMMs that are driven by oncogenic K-Ras mutation, each murine model has unique characteristics that provide flexibility to choose a specific model for the evaluation of targeted therapies against a given pathological hallmark. A catalog of existing PDAC mouse models describing their classification based on various parameters such as genetic background, tumor source and site for implantation, immunocompatibility, and targetable pathological hallmarks of the disease, can be a valuable resource for choosing the appropriate experimental model for evaluating emerging therapeutic approaches. While the cell line-based SC and OT implantation models have remained the mainstay of experimental therapeutics for PDAC, the greater appreciation of the complexities of disease progression, and the role of TME in determining the therapy response, fueled the development of new generation models involving fibroblast-PDAC

cell mixture co-implantation, PDXs, MDAs, and organoids. The advanced animal models recapitulate some of the stromal and vascular complexities of PDAC and are thus considered superior to the cell line-based models.

The mutant KRAS driven GEMMs have boosted the progress of immunotherapy and provided a deeper insight into PDAC initiation and progression. In the context of immunotherapy, the renewed interest in the parabiosis models to study immune cell trafficking, tumor-immune cell interactions, and associated immunomodulatory mechanisms, can pay rich dividends, particularly when mouse models for oncogenesis are combined with models engineered for altered immune/stromal cell functions. This can help circumvent challenges associated with simultaneous gene manipulations in multiple cellular compartments. Understanding molecular mechanisms associated with early metastasis and developing therapeutic approaches to prevent and treat PDAC metastasis have been challenging. In this regard, the mouse models of PDAC metastasis, including portal vein injection model, hemispleen injection model, perineural invasion model, and spontaneous models of metastasis, are poised to play a greater role in PDAC experimental therapeutics. To understand PDAC initiation and progression, the molecular cascade of early metastasis, and infiltrating immune cell populations, mouse models for cell lineage tracing have been recently used [296, 297]. These genetically engineered fluorescently labeled cell lineage tracing models will be highly useful in understanding PDAC initiation and for studying the origins and trafficking of metastatic cancer cells and infiltrating immune cells in PDAC. There is growing evidence suggesting that the targeting of pathological hallmarks in PDAC requires specific models. For example, the development of PDAC cachexia models has significantly expanded our understanding of its role in PDAC pathogenesis and therapeutic outcome.

Overall, to enrich the pipeline of novel targeted therapies and achieve high success rate in clinical trials, it is imperative to use robust and specific mouse models during preclinical assessment. Although recently evolved mouse models have provided clinically more relevant platforms for targeting various aspects of PDAC pathogenesis, there are still several unaddressed questions related to targeted therapies. For instance, experimental models required for targeted therapies against early PDAC, including precancerous lesions and PanINs, are not well defined. Another challenge in PDAC is to develop experimental models for targeting pre-metastatic phenotypes of PDAC cells, including EMT and stem cell phenotype. Similarly, mouse models for immunotherapy, particularly vaccine-based therapies, need to mimic the human immune system. Introducing humanized mouse models may benefit the development of therapeutic vaccines against PDAC. However, it is essential for a therapeutic vaccine to identify first the PDAC-specific immunogenic target antigen(s) and then develop antigen-specific transgenic mouse models to evaluate its therapeutic effects.

Emerging knowledge from various omics and single-cell sequencing-based studies has revealed extensive heterogeneity and functional dichotomy in tumor-associated fibroblasts, tumor-associated macrophage and immune cells. Some cellular subtypes in the pancreatic TME are tumor-promoting, while others exhibit anti-tumor effects. Further, genomic and transcriptomic, and immunological profiling have identified several subtypes of PDAC [298,

299]. This new knowledge on one hand forces us to rethink and recalibrate therapeutic modalities to combat PDAC, while on the other hand, it makes modeling such complexities in murine models a challenging endeavor. For example, choosing the right class of CAFs for co-implantation studies and predicting their behavior post-implantation will require careful planning and analysis. Similarly, the existing PDAC mouse models, to some extent, can address the cellular heterogeneity; however, designing the mouse models to elucidate the cellular plasticity in the pancreatic TME is still a challenge.

In summary, the new generation PDAC mouse models can address the challenges associated with PDAC progression, metastasis, stromal heterogeneity, and immunosuppression. However, future development of novel therapeutic approaches would need more improvisation in existing mouse models to specifically understand and target the pathological hallmarks of PDAC.

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Highlights

- Due to challenges in early detection, and limited efficacy of various treatment modalities, pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies and remains an active area of preclinical research employing animal models for improving diagnosis and therapy.
- The experimental mouse models have transformed PDAC research serving as indispensable tools for understanding disease progression and pathophysiology, and ideal platforms for evaluating therapeutic approaches.
- Both, the implantation models and genetically engineered mouse models (GEMMs), have been extensively used to optimize the targeted and untargeted therapeutic approaches against PC and facilitated their clinical translation.
- The introduction of mutant KRAS-driven syngeneic mouse model and its derivative tumor tissues, tumoroids, and cell lines have kindled the progress in developing the immunotherapy-based approaches for PC therapy.
- New generation models including patient derived xenograft (PDX), mouse derived allograft (MDA), organoids, and mouse models for liver metastasis, cachexia, parabiosis, lineage tracing, and perineural invasion, are set to dominate the preclinical research landscape for experimental therapeutics of PDAC.

Figure 1. Major PDAC experimental mouse models employed for preclinical research. Presentation of a surgical (A), non-surgical (B), and spontaneous models (C), for the experimental therapeutics of PDAC. (A) **Panel 1: Orthotopic implantation** of tumor source (cells/tumor derivatives) in the pancreas of the mouse. Lower panel depicts the approaches employed for implantation of cells and tumor growth in head of the pancreas. **Panel 2: Hepatic metastases models** are generated by hemispleen injection and portal vein injection. The lower panels indicate the injection site in the spleen (Left) and in the portal vein (Right). Details of the procedures are mentioned in the section 2.2.4. **Panel 3: Resection model** for the testing adjuvant and neoadjuvant therapies to evaluate their effect on local recurrence and metastasis. The lower panel depicts implantation of cells and tumor growth in the tail of the pancreas. Such models have allowed the R0 resections. Following resection mice can followed for local recurrence and metastasis .in the presence or absence of therapeutic modalities. (B) **Non-surgical procedures:** the SC implantation method (**panel 1**), non-invasive ultrasound guided (USG) injection model (**panel 2**), and peritoneal dissemination model (**panel 3**), are primarily the non-surgical methods to evaluate the therapeutics against primary tumor and peritoneal metastasis. (C) **Genetically modified mouse models (GEMMs):** Both endogenous and exogenous genetic modification can be performed to develop GEMMs of PDAC. Mouse models with mutant KRAS and/or TP53 genes driven by Cre-recombinases under the control of various cell-type specific promoters and described in the in the section 2.2.8. The spontaneous models recapitulate the pathological features of human PDAC with primary tumors and multiple metastases. Alternatively, exogenous induction of oncogenes can be achieved by the introduction of

plasmids via electroporation or injectable viral particles with different Cre recombinases to induce the tumor growth in the pancreas.

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Figure 2. Classification of murine models of PDAC based on various parameters.

Experimental models are classified based on different parameters including, progression stages, implantation source, implantation site, and therapeutic approaches. **(A)** Murine models based on progression stages include early and late PDAC and metastasis, including pancreatitis as a major risk factor (**Top; left panel**); models based on implantation material (**Top; right panel**), and site of implantation (**Bottom; right panel**). Lastly, therapy-specific models are described to highlight that based on therapy-specific requirements, models for immunotherapy, anti-stromal therapies, and other targeted therapies can be categorized (**Bottom; left panel**). **(B)** Illustration of implantation models based on tumor source and implantation site. Different PDAC sources including cell lines/organoids/PDXs/MDAs derived from tumors of the mouse or human pancreas are implanted surgically into the pancreas or injected subcutaneously. **(C) PDAC progression stage-specific experimental mouse models.** Experimental models are categorized based on different stages of pancreatic cancer progression. Following oncogenic mutations (Listed below the genetic mutations, which is the first event in PDAC progression), PDAC begins with PanIN lesions and progresses to PDAC with primary tumor and distant metastasis. Pancreatitis is included as a major risk factor for PDAC and based on some reports that suggest the development of PDAC from pancreatitis. In addition to selective models for targeted therapy, spontaneous

KC and KPC models are shown in all the stages of PDAC progression. **PanIN:** pancreatic intraepithelial neoplasia; **PDX:** Patient-derived xenograft; **MDA:** Mouse-derived allograft.
*Mutant KRAS is reported as driver mutation with others as complementary mutations.
**Pancreatitis is not an essential early event for PDAC but is considered as an important risk factor.

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Figure 3. Hemispleen injection technique for hepatic metastasis.

(A-B) Illustration of splenic hemispleen injection for hepatic metastasis in mouse mice.

(A) Left flank incision is followed by the splitting of spleen into proximal and distal parts after the ligation of the exposing ends with the help of clamps, as described in the main section. Injection of cells in the proximal hemispleen is followed by clamping the splenic blood vessel and removal of proximal hemispleen. (B) Diagram showing the development of liver metastases after 2-3 weeks of hemispleen injection. (C) Representative image showing liver metastases after the hemispleen injection of CD18-HPAF cell line in athymic nude mice with a Hematoxylin and Eosin (H&E) staining of the liver section to show the liver metastases. (D) Representative image showing multiple metastases after hemispleen injection of KPC tumor derived cell line with a representative H&E staining of liver section.

Sp-pro: spleen-proximal part; **Sp-dis:** spleen-distal part.

Figure 4. Depiction of mouse parabiosis model.

Either two male or female mice (**A&B**) with matching age, sex, weight, and genetic background are housed together for at least two weeks for adaptable cohabitation before the surgical union. For surgical union, the mice are placed together laterally and incisions are made longitudinally (**top; dotted lines**). The subcutaneous fascia is separated from the skin with the help of forceps, followed by suturing the olecranon with the help of non-absorbable sutures to connect the joints tightly. The surgical front is shown by the dotted back arrow (**middle**). In two weeks, mice start sharing blood vasculature and blood-borne components, as shown the circular arrows in the figure (**bottom**). An application of parabiosis models in PDAC research is depicted in boxes with blue dotted lines, where healthy mouse A is immunized with therapeutic vaccine to generate robust immune response and then conjoined to mouse B that has tumor in the pancreas and is can possibly exhibit localized and systemic immunosuppression and immune dysfunction (**Right side; Blue dotted panel**). Conjoining with immune activated mouse will help in immune reactivation in the tumor bearing mouse and can potentially restore anti-tumor immunity, as shown in the figure (**Left side, blue dotted panel**).