Chapter 4
Mouse Models of Pancreatic Cancer

Katherine T. Ostapoff, Michael T. Dellinger, Niranjan Awasthi, Rolf A. Brekken and Roderich E. Schwarz

Abstract Pancreatic ductal adenocarcinoma (PDAC) is a disease characterized by aggressive tumor biology, desmoplasia and chemoresistance. Given the insidious nature of its onset, multiple models have been developed to study progression from in situ lesions (PanIN) to PDAC in transgenic mouse models. These have been developed using known mutations that are present in human tumors including *K-ras*, *p53*, *DPC4*, *CDNK2a*, *p16* and *Brca2*. The metastatic character of each of these models is variable and described here. Metastasis to the lymph nodes, liver and peritoneum are also prominent features of PDAC. Syngeneic models and xenograft models (i.e. orthotopic, direct xenograft and metastatic models) are also used to study primary tumor development and metastatic disease and are described.This chapter seeks to describe murine models of experimental PDAC that are currently used to investigate mechanisms of carcinogenesis and metastatic progression, individual risk factors, tumor biology aspects, mechanisms of in vivo chemoresistance, analysis of therapeutic targets and experimental therapies.

R. E. Schwarz (✉)
IU Health Goshen Center for Cancer Care,
Indiana University School of Medicine, Goshen, USA
e-mail: RSchwarz@iuhealth.org

K. T. Ostapoff · N. Awasthi · R. A. Brekken
Department of Surgery, Division of Surgical Oncology,
Hamon Center for Therapeutic Oncology Research, University of Texas,
Southwestern, Dallas, USA

M. T. Dellinger
Hamon Center for Therapeutic Oncology Research,
University of Texas, Southwestern, Dallas, USA

R. A. Brekken
Department of Pharmacology, Simmons Comprehensive Cancer Center,
University of Texas, Southwestern, Dallas, USA

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Abbreviations

CEA	Carcinoembryonic antigen
CK-19	Cytokeratin 19
DDRs	Discoid domain receptors
EGFR	Epidermal growth factor receptor
EL	Elastase
Fbln5	Fibulin 5
GEMM	Genetically engineered mouse model
GFP	Green floursecent protein
HCC	Hepatocellular cancer
IPMN	Intraductal papillary mucinous neoplasm
KPC	$Kras^{G12D}Trp53^{R172H}Pdx1^{Cre}$
MRI	Magnetic resonance imaging
PanIN	Pancreatic intraepithelial neoplasm
PAS	Periodic acid-Schiff
PDAC	Pancreatic ductal adenocarcinoma
PET/CT	Positron emission tomograph/computed tomography
RFP	Red flouresecent protein
RTKi	Receptor tyrosine kinase inhibitor
SCID/NOD	Severe combined immunodeficiency/non-obese diabetic mice
Shh	Sonic hedgehog
shRNA	Short hairpin RNA
SPARC	Secreted protein acidic and rich in cysteine
TGF-α, TGF-β	Transforming growth factor -alpha, -beta
VEGF-C, VEGF-D	Vascular endothelial growth factor -C, -D
VEGFR-3	Vascular endothelial growth factor receptor 3

4.1 Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer death in the United States with a five year survival that remains at less than 5% [1]. While surgical resection remains the only option for cure, the majority of patients present with advanced metastatic disease unsuitable for surgical resection, and the majority of resected patients experience subsequent recurrence and death. The current standard of care in terms of systemic therapies includes gemcitabine, or for patients with high performance status FOLFIRINOX (5-Fluorouracil, Irinotecan and Oxaliplatinin) [2]. Radiotherapy is usually considered for non-resection candidates, and may be applied in an adjuvant setting. Despite these options, few patients have tumors that are sensitive to gemcitabine (26%) [3] or are able to tolerate the high side effect profile of FOLFIRINOX [2].

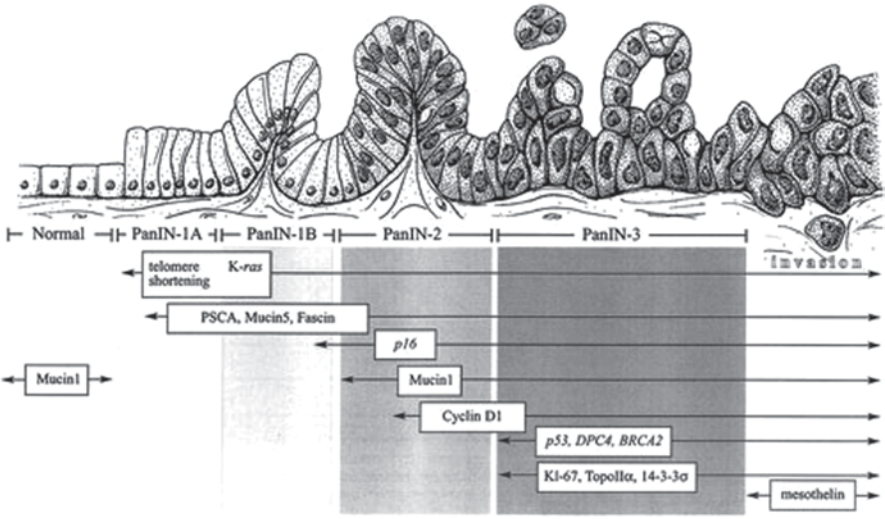


Fig. 4.1 Multiple mutations are required for the development of PDAC. Activating mutations in KRAS have been implicated as an early driver of neoplasia, which progresses through pre-neoplastic stages including pancreatic intraepithelial neoplasia (PanIN) lesions illustrated above. Subsequent mutations in CDKN2A, Mucin1, and Cyclin D1 drive additional changes in ductal epithelia. Additional mutations in p53, DPC4 and BRCA2 and others foster progression towards fully developed PDAC. Adapted with permission from Maitra A et al., Mod Pathology 2003 [137]

Pancreatic ductal adenocarcinoma (PDAC) accounts for over 90 % of pancreatic malignancies, with the remaining 10 % consisting primarily of acinar carcinomas, papillary or cystic mucinous adenocarcinomas (including intraductal papillary mucinous neoplasms, IPMNs) and malignant neuroendocrine tumors. The remarkably poor survival characteristic of PDAC is multifactorial due to advanced stage upon presentation, rapid tumor progression with early metastatic mechanisms, and biologic factors that lead to poor treatment susceptibility. PDAC often has an insidious onset with patients generally remaining asymptomatic until they have near total ductal obstruction (jaundice, exocrine insufficiency), pain (through neural invasion mechanisms) or impairment of endocrine function (diabetes) through peripheral insulin resistance. Risk factors include diabetes [4], chronic pancreatitis, family history, smoking [5, 6] and obesity [7]. Given the increasing prevalence of these conditions and the persisting challenges regarding early diagnosis and effective therapy, the study of carcinogenesis and the progression spectrum of PDAC carry great clinical relevance.

Mutations that are known to frequently contribute to PDAC development include KRAS, DPC4, p53 and CDKN2A (p16) in 90, 60, 75 and 95 % of cases respectively (Fig. 4.1) [8]. In addition, BRCA2 is mutated in 10 % of sporadic cases and 19 % of familial PDAC. Patient survival correlates with these mutations, as a worse prognosis is linked to mutations in Kras [9], or functional deletions of SMAD4 (encoded by DPC4) [10, 11], p53 [12] or p16 [13]. The identification of these mutations and

their inverse correlation with survival provide a potential opportunity for the individualization of treatment based on mutation analysis [14], although currently this remains an area of research without established clinical applications.

Clinical progression of PDAC frequently appears rapid but its development is thought to occur over many years [15]. In fact, it has been shown in patient samples that the development of distant metastasis due to mutations arising in the primary lesion can take up to ten years to develop [16]. Genetic mutations that occur early in carcinogenesis and the prolonged nature of tumor development are consistent with the PanIN model [17]. Furthermore, the high incidence of metastasis at disease diagnosis emphasizes the importance of studying carcinogenesis and metastatic mechanisms, including the progression from micro- to macrometastasis. It has been hypothesized that the desmoplasia of the tumor microenvironment contributes to intrinsic tumor cell resistance to current cytotoxic chemotherapy regimens that typically accompanies PDAC. Understanding the contribution of tumor cell autonomous and non-autonomous factors in the response of PDAC to therapy remains a key challenge to developing therapeutic strategies to treat localized and metastatic disease.

This chapter seeks to describe murine models of experimental PDAC that are currently used to investigate mechanisms of carcinogenesis and metastatic progression, individual risk factors, tumor biology aspects, mechanisms of in vivo chemoresistance, analysis of therapeutic targets and experimental therapies.

4.2 Genetically Engineered/Transgenic Models

Multiple research groups have targeted known mutations in human PDAC tumors for the development of mouse models to study early and late carcinogenesis [18] as well as therapeutics [19]. The spectrum of available models ranges from those that identified molecular mechanisms for pre-invasive lesions for the study of chemoprevention to those utilizing the introduction of specific mutations to follow tumor development from in situ lesions (PanIN 1-3) to invasive PDAC and subsequent metastasis. Herein we describe several of the more commonly utilized models of pancreatic adenocarcinoma and focus on their in vivo disease characteristics as summarized in Table 4.1. The earliest models failed to consistently achieve the development of PDAC as in those employing TGF-α and Pdx-1Cre $Kras^{G12D}$, which has limited the utility of these models.

4.2.1 TGF-a

Early transgenic models failed to develop PDAC but provided important information with respect to other subtypes of pancreatic cancer. Discovered in the 1990s, *TGF-α* has been used as a driver of tumorigenesis when combined with various promoters (Elastase, Simian Virus 40 T antigen or c-myc oncogene) to form acinar carcinoma. These mice have a median survival of less than 15 weeks [20]. Large

Table 4.1 Transgenic models of pancreatic adenocarcinoma development

Model	Median survival (mo)	PDAC	Predominant histology	Metastasis(y/n)	Liver	Ascites	Extra pancreatic malignancy	Reference
EL-TGFα	15	Yes	Acinar	Not described			Primary hepatic tumors	Sandgren EP et al. 1993 [20]
EL-TGFα $P53^{-/-}$	3–8	Yes	Acinar	Yes	Yes	No	Thymic lymphoma	Wagner M et al. 2001 [21]
p48-Cre $Kras^{G12D}$		Rare (2/29)		No (in 2/29 that developed PDAC)	No	No	No	Hingorani SR et al. 2003 [25]
p48 Cre $Kras^{G12D}$ $Cdnk2^{lox/lox}$	2.5	Yes	Sarcomatoid	Yes, rarely	Yes	Yes	CNS tumors	Aguirre AJ et al. 2003 [26], Hingorani SR et al. 2003 [25]
$Kras^{G12D}$ Pdx1-Cre $Smad^{lox/lox}$	8	Yes	Mucinous	Rare	Rare	No	Pdx1 cre-gastric tumors	Bardeesy N et al. 2006 [27], Kojima K et al. 2007 [28]
$Kras^{G12D}$ p48Cre $Smad4^{lox/lox}$	8	Yes	Invasive and mucinous	Yes 53% Micro > Macro	Yes	Yes	MCN	Izeradjene K et al. 2007 [29]
$Kras^{G12D}$ p48Cre Smad4 $^{lox/wt}$	15	Yes	Invasive and mucinous	Yes 35% Micro > Macro	Yes	Yes	MCN	Izeradjene K el al. 2007 [29]
Mist1$Kras^{G12D}$	10.8	Yes	Acinar	Occasional gross	Rare	Yes	HCC	Tuveson DA et al. 2006 [30]
Mist1$Kras^{G12D}$ $Trp53^{R12D}$	6.4	Yes	Pleomorphic	Yes	Yes	Yes	HCC 1/12 mice	Tuveson DA et al. 2006 [30]
$Kras^{G12D}$ p48Cre $Trp53^{R12D}$	5	Yes	Glandular	Yes	Yes	Yes	Rare thymic lymphomas or teratocaricinomas	Hingorani SR et al. 2005 [31]
$Kras^{G12D}$ p48Cre Trp53 $Brca2^{Tr/\Delta 11}$	2.8	Yes	Tubular 100%, sarcomatoid (50%)acinar 18%	Yes, liver and lymph node	Yes	Unk	1 report of neuroendocrine tumor	Skoulidis F et al. 2010 [32]
Pdx1-Cre $Trp53^{R12D}$ $Brca2^{f/f}$	12.3	Yes	Glandular and sarcomatoid	Yes (rare), in longer survivors	Yes	No	Early deaths without tumors	Feldman G et al. 2011 [33]

All transgenic models listed above develop PanIN lesions

h head, *t* tail, *b* body predominant location of tumor, *CNS* central nervous system tumors, *MCN* mucinous cystic neoplasms, *HCC* hepatocellular carcinoma

(>2 cm) hepatic tumors (hepatocellular and cholangiocarcinomas) were found in over 50% of the mice [20].

Subsequent work resulted in *EL-TGF-α/ p53*$^{-/+}$ and *EL-TGF-α/ p53*$^{-/-}$ mice [21]. In the heterozygotes (p53$^{+/-}$) 3% of mice developed epithelial tumors or sarcomas. Mice in each group (*EL-TGF-α/ p53*$^{-/+}$ and *EL-TGF-α/ p53*$^{-/-}$) however developed pancreatic tumors after extended periods of time that expressed multiple epithelial markers and had limited fibrosis [22]. Unique to this model, additional mutations were acquired during tumor development. Although they did not occur in 100% of tumor bearing mice, new loss of heterozygosity occurring in the *Ink4a/Arf (Cdkn2a)*, *Rb* and *Smad4* loci were identified [21]. These have since been attributed to mutations on chromosome 11 and 15 [23]. Furthermore, a tumor specific immune response was identified in this model showing increased intratumoral levels of TNF-α, IFN-γ, IL-6, and MCP-1 [24].

Although mice with TGF-α mutations do not uniformly develop PDAC, this early model provided evidence that fibrosis is a key feature in PDAC development. This model continues to be used to study tumor immunology [24]. With the development of new mutations during tumor development, this model also has the potential to be used for studying the mechanisms of acquired mutations in metastasis.

4.2.2 *Kras^{G12D} p48-Cre*

This model relies on expression of an activating *Kras* mutation predominately in the pancreas. Identified by Kawaguchi et al., p48/Pft-1 is a transcription factor during embryological development expressed by pancreatic precursor cells both of endocrine and exocrine lineages [25]. Under the effect of this pancreas-specific promoter, cells within the pancreas under model conditions express mutations in Kras^{G12D} and subsequently generate a more nodular pancreas [26]. These mice were found to have predominantly normal pancreata at 9 weeks, but subsequently developed PanIN 1-3 lesions by 9 months, with only few mice developing invasive PDAC [26]. In 29 mice, only 3 developed invasive PDAC with hallmark features of hemorrhagic ascites and liver metastasis [26]. Other groups using this model also found minimal development of invasive PDAC, with significant PanIN lesions seen within 30 weeks [27]. Despite the lower incidence of invasive lesions, a proteomic signature was developed that identified preinvasive lesions with a sensitivity of 90.5% and specificity of 97.7%. Further work with this model has shown that TGF-β and BMP4 are highly expressed; this has propelled further investigations on the contribution of TGF-β to tumor progression and metastasis [28].

4.2.3 *LSL-Kras^{G12D}; Cdkn2a$^{lox/lox}$; Pdx1^{Cre}*

Given the slow disease progression of animals engineered to express *Kras^{G12D}* in the pancreas, additional mutations have been added to force more consistent PDAC

disease promotion. Mutations in p16/p19 are commonly identified in clinical specimens. These mutations result in the deletion of the *Cdkn2a* (Ink4a/Arf) locus product [29] and in concert with activated Kras promote PDAC tumorigenesis [30]. By combining an activating mutation in Kras (*LSL-Kras^{G12D}*) and deletion of Cdkn2a (*Cdkn2a$^{lox/lox}$*) with pancreas-specific Cre expression (*Pdx1^{Cre}* or *p48^{Cre}*) mice developed solid lesions that can progress rather rapidly; animals can become moribund with evidence of ascites, weight loss or jaundice between 7 and 11 weeks [27]. These mice at early time points (as early as 3 weeks) were shown to have precursor PanIN lesions and early stage invasive PDAC (as early as 4 weeks).

On necropsy, mice in general did not show grossly evident liver metastases but showed histologic evidence of lymph node metastasis and liver micrometastasis. Development of jaundice due to bile duct obstruction aside from ascites in end stage disease is common. In one survival experiment, 23 of 24 mice had local invasion into adjacent organs by the primary tumor [27]. Primary tumors had glandular features with abundant stroma with strong collagen (trichrome) staining and abundant mucin (PAS positive).

These mice have been shown to retain p53 function throughout tumor progression, a deviation from the common clinical tumor characteristics [31]. However, these models retain a dense stromal component, which is consistent with the human disease. This model has been used to evaluate therapeutic efficacy using a variety of strategies including sonic hedgehog inhibition [32], and combination of a smac mimetic and cytotoxic chemotherapy [33]. *Kras^{G12D} Cdkn2a* tumors are sensitive to gemcitabine in vivo as well, rendering the model a tool for the examination of combination therapy approaches.

4.2.4 Kras^{G12D} Pdx1-Cre Trp53^{R172H} (KPC mice)

The relatively low incidence of gross metastasis in murine models utilizing active Kras and loss of Cdkn2a promoted further work directed towards identifying a highly metastatic murine PDAC model. Trp53^{R172H} was originally identified as a driver of Li-Fraumeni Syndrome [34]. Hingorani et al. discovered that heterozygous mutation of p53(*Trp53^{R172H}*) combined with Pdx1-Cre Kras^{G12D} (KPC) resulted in an aggressive PDAC model with a median survival of 5 months and 100% mortality by 1 year [35]. These animals typically present with pancreatic head lesions with biliary obstruction. Furthermore, this model of PDAC generally progresses with tumor sequelae that are consistent with the human disease, including ascites and macroscopically identifiable liver, peritoneal and lung metastases (Fig. 4.2).

In this model lesions expressed high levels of ductal markers, including CK-19 and mucin, in well differentiated areas. Across tumor samples, investigators found heterogeneity of EGFR and Her2 expression between PanIN and PDAC lesions. However, uniform expression of Shh was found to be elevated in preinvasive and invasive lesions throughout the pancreas. Interestingly, during the course of disease progression, mice were found to develop homozygous loss of p53 in all primary

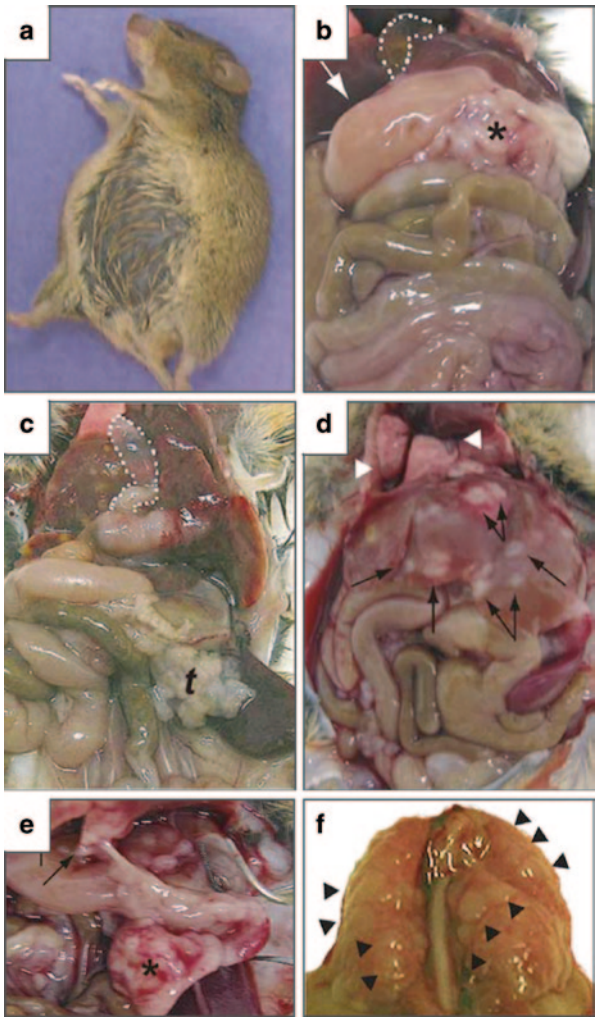


Fig. 4.2 KPC mice develop significant metastatic burden. Once moribund, mice develop significant ascites **a**. Primary tumors are typically in the pancreas head location (*) and cause biliary and duodenal obstruction (*white arrow*, **b** and gallbladder distension **c**. Multiple liver **d**, diaphragm **e** and lung metastases (*black arrows*) are seen **f**. (Adapted with permission from [35])

tumors and metastases. In addition, cells lines from tumors at different time points were found to display chromosome instability. Throughout tumor progression, pancreatic lesions were found to maintain p16Ink4a, Smad4, Rb, Akt and Myc protein expression without development of mutations or other alterations [35].

This model has been also used to evaluate various therapeutic strategies. For instance, the use of a Shh inhibitor enhanced delivery of chemotherapy and improved tumor control in KPC mice [36]. Additionally, dasatinib, a receptor tyrosine kinase inhibitor (RTKi) that inhibits Src, BCR-Abl, and DDRs among others, was found to inhibit metastasis but not primary tumor growth, within this model [37]. This model has also been used for evaluating chemoprevention. Use of enalapril and aspirin resulted in decreased development of invasive PDAC lesions compared to untreated

mice (17 vs. 60%) [38]. Synthetic triterpenoids also prolonged survival when added to the chow of these mice [39].

The KPC model is useful to study tumor biology as well as for therapeutic investigation. With a prolonged time course and high metastatic incidence, it represents human PDAC disease characteristics well. This model does not acquire new mutations during development, making it a good choice for examining the biology of metastasis without the spontaneous development and impact of new mutations. Although many transgenic mouse models lead to development of liver metastases, this model generates frequent lung metastases. KPC mice are also good for studying chemoprevention given the slower time course of disease development. Perhaps one of the most desirable features of this model is that KPC tumors are resistant to gemcitabine as a single agent in vivo, similar to the relative insensitivity of patients to gemcitabine.

4.2.5 *Kras^{G12D} Smad4$^{lox/lox}$*

Dpc4 is the gene that encodes Smad4, the signaling intermediate critical for canonical TGF-β signaling. Its activation drives transcription of genes that counteract or prevent mechanisms of tumor proliferation, migration, survival and epithelial to mesenchymal transition. As a major regulator of TGF-β, Smad4 has been shown to be mutated in aggressive tumor phenotypes. Furthermore, patients with PDAC mutations in Smad4 have a decreased survival [11]. The role of this mutation has been studied in many transgenic models. Although loss of Smad4 alone was insufficient for tumor formation, homozygous loss of *Smad4* combined with *Pdx1* or *p48* driven *Kras^{G12D}* activation resulted in tumor formation at 7-12 weeks of age and a median survival of 8 months [28]. In addition to invasive PDAC, *Kras^{G12D} Smad4$^{lox/lox}$ Pdx1-Cre* animals also developed IMPNs and squamous and adenosquamous gastric cancers. PDAC tumors in this model showed elevated stromal components [28], although metastasis was infrequent and mostly associated with a sarcomatoid histology compared to *Kras^{G12D} Pdx1-Cre* mice [40]. Additionally, these tumors had areas of inflammatory cells and evidence of chronic pancreatitis in 6 of 16 mice [40].

Median survival for *Kras^{G12D} Smad4$^{lox/wt}$ p48Cre* (haploinsufficiency) mice was 15 months with low grade PanIN lesions seen at 7-8 months [41]. Tumors were identified in major and minor ducts, a feature not represented in the majority of genetic models of PDAC [41]. Tumors from these animals were found to have elevated EGFR, ErbB2, Hedgehog and Hes1 expression [41].

Although this model has not been used for therapy studies to date, its prolonged time course and modeling of an important mutation in PDAC make it a valuable model to study early therapeutic strategies. An unusual but important feature of *Kras^{G12D} Smad4$^{lox/lox}$ p48Cre* mice is that they develop both PanIN and IPMN lesions. By having both types of precursor lesions, the mechanisms of invasive tumor development and metastasis from these convergent pathways can be studied.

4.2.6 *Mist1-Kras^{G12D}*

Mist1 is a protein that is expressed highly in mature pancreatic acinar cells and is required for normal acinar architecture [42]. The Tuveson group proposed that mutations in *Mist1* may participate in PDAC development. Unlike previous models described, in *Mist1Kras^{G12D}* mice a mutation in *Kras^{G12D}* is knocked into the *Mist1* locus. *Mist1Kras^{G12D}* mice have a median survival of 10.8 months and typically develop ascites when 3 months old. Addition of a targeted p53 mutation *(LSL-p53^{R172H})* reduced median survival to 6.8 months [43]. Mice without p53 mutations had cystic and papillary features with few cases of glandular differentiation. A minority of *Mist1Kras^{G12D}* mice developed liver metastasis but 25 of 44 mice developed hepatocellular cancer. Conversely, in *Mist1Kras^{G12D} Trp53^{R172H}* animals, liver metastasis occurred in roughly 50% of mice, and rare (1 of 12) hepatocellular cancers were noted. These mice had pleomorphic carcinomas. *Mist1Kras^{G12D}* mice were found to have increased protein expression of Akt and Ras and mRNA expression of *Hes1, Hey1* and *Hey2* but decreased levels of *Mist1* [43]. When comparing an elastase inducible *Pdx1-Cre Kras^{G12D}* to *Mist1Kras^{G12D}* mice, Habbe et al., found similar patterns of PanIN lesions between the models [44].

This model provides important additional evidence for the mechanisms of pancreato-hepatobiliary carcinoma. The high incidence of other hepatobiliary tumors (HCC and cholangiocarcinomas) make this a difficult model to use for PDAC therapy studies, but there is the potential to provide insight into the common causes of chemoresistance and metastasis between these tumor types.

4.2.7 *Models of Familial Pancreatic Cancer with Brca2 mutations*

Familial pancreatic cancer represents a small percentage of patients who develop PDAC. The most common mutations found in familial cases are BRCA2 [45, 46] and PALB2 [47]. Skoulidis et al describe *Kras^{G12D} Brca2$^{flox/wt}$* animals in which a heterozygosity for *Brca2* results in murine PDAC [48]. They also found that homozygous deletion of *Brca2* in the *KPC* model resulted in a high penetrance of adenocarcinoma and reduced median survival (86 vs. 168 days) compared to *KPC* mice. Interestingly, heterozygous loss of *Brca2* also resulted in decreased median survival of 143 days. These mice frequently developed liver and lymph node metastases. In mice with wild-type p53, i.e. *Pdx1-Cre Kras^{G12D} Brca2$^{flox/wt}$*, the mice frequently developed pancreatic insufficiency. This suggests that mutation of one copy of *Brca2* is not sufficient, even in the context of *Kras* activation, to drive tumor formation and highlights the redundancy of DNA repair mechanisms. The predominant histology of the *Kras^{G12D} Pdx1-Cre Trp53^{R172H} Brca2$^{lox/wt}$* model includes tubular (~100% of mice), sarcomatoid (~50%) and acinar cell histology, which mirrors those found in patients with familial *BRCA2^{999del5}* PDAC. In the het-

erozygous model, all of the mice were found to retain one copy of wild type *BRCA2* expression within their tumors.

An additional Brca2-based model has been developed to mimic familial PDAC. This author group found that pancreas-specific homozygous mutations of *BRCA2* with or without *p53* mutations also results in PDAC development. In *Pdx1-Cre* $Brca2^{lox/lox}$ and *Pdx1-Cre* $Trp53^{R172H}$ $Brca2^{flox/flox}$ mice, median survival is 454 and 375 days respectively [49]. In the cohort of mice that died early, histological analysis revealed pancreata replaced with adipose tissue and depletion of acinar cells. In mice that lived > 1 year, preinvasive lesions were identified. Tumor histology was sarcomatoid and glandular. Mutations in *p53* resulted in increased frequency of invasive lesions and metastatic disease at 15-17 months. In mice with only *Brca2* mutations, metastatic events were infrequent. There were no acquired mutations in *Kras* found in either tumor type. Increased expression of *Shh* was found only in neoplastic glands and not in non-neoplastic cystic epithelium [49].

Despite the low incidence of familial PDAC, these models provide evidence for an alternative mechanism for tumor development in susceptible individuals. With these models, (particularly the *KPC* $Brca2^{lox/lox}$ mice) the potential to study chemoprevention aimed at this population is apparent. Other known genetic alterations linked to familial PDAC have not been modeled in animals, such as the mutation and overexpression of the actin associate palladin [50]. Interestingly, isoform overexpression of palladin in murine PDAC tumors has been identified primarily within fibroblasts but not epithelial cells indicating scenarios of specific mechanisms of tumor invasion and metastasis that deserve recognition for future disease modeling efforts [51].

4.2.8 Additional Models

Additional genes have been targeted in an attempt to develop PDAC models that mirror human disease. Although $Kras^{G12D}$*-Nestin* lesions developed PanIN lesions and did not progress to PDAC, evidence suggests *Nestin* positive cells are progenitors to PDAC [52]. *CK-19* driven $Kras^{V12}$, resulted in mice that developed ductal hyperplasia in the pancreas and stomach but no PanIN lesions [53].

Genetically engineered mouse models (GEMM) certainly have many advantages for studying carcinogenesis and early metastasis. They recapitulate many of the progressive genetic events that are believed to account for the development of pancreatic cancer. Depending on the model, they have various time courses with median survivals ranging from 2 months to > 1 year [27, 41]. An additional benefit of these mice is that they are bred on immunocompetent backgrounds and allow for the evaluation of innate and adaptive immunity in tumor biology.

Conversely, disadvantages include the lengthy time period for development of new models or use of existing models. Regardless of the model, all mice that are competent to develop PDAC will eventually develop tumors throughout the entire pancreas and various stages in a manner often unlike the course of disease in human

patients. As areas within the pancreas develop tumors at different rates, it can be challenging to conduct efficient therapeutic experiments, or to study targeted pathways such as TGF-β, which can inhibit tumor progression early and drive progression at later stages. The genetic background of the model is also a critical feature that can be altered by crossing different strains of mice; naturally, the comparison of median survival across strains is challenging. Furthermore, genetically engineered mouse models are costly and require significant resources to maintain. However, despite these challenges, they are a valuable resource for understanding tumor progression and metastasis in a heterogeneous tumor cell population.

4.3 Syngeneic Models

Although the isolation of cell lines from transgenic mice is occurring more frequently [35], this is rather labor intensive, and not all cell lines isolated will form tumors once implanted in vivo [24]. As the role of the immune system is important in tumor progression and metastasis, specific models are needed in immunocompetent systems. These models are useful for studying potential immunotherapy and vaccine therapy in PDAC [54].

Currently two cell lines are commercially available that can be grown in immunocompetent C57BL/6 mice. These cell lines, Pan02 (Panc02) and Pan03 (Panc03), (DTP, NCI) were isolated from C57Bl/6 mice that developed a chemically induced pancreatic adenocarcinoma. To establish these cell lines, a suture impregnated with the carcinogen 3-methyl-cholanthrene (3-MCA) was sutured into the pancreas of a C57BL/6 mouse [55]. Two of the 13 PDAC tumors established survived explant culture and re-passage (Pan02 and Pan03). Pan02 was found to be high grade (grade 3 but became undifferentiated with additional passages) and highly metastatic with 80% of mice developing lung metastasis in the original report of its use [55]. Availability of murine syngeneic pancreatic cancer cell lines has facilitated investigation of function and contribution of the innate and adaptive immune system in pancreatic cancer progression and metastasis [56, 57]. Additionally, the function of T cells in pancreatic tumors has been studied in Pan02 tumors [58]. Pan02 tumors have high levels of T regulatory cells and macrophages. Inhibition of TGF-β mediated T cell differentiation into T regulatory cells can result in a reduction in tumor growth and metastasis [59]; induction of Th17 cells results in prolonged survival [60]. Further description of this model with respect to metastatic incidence (Table 4.2) and lymphatic metastasis are discussed in later sections of this chapter.

The availability of C57BL/6 animals with genetic ablation of target genes has enabled the evaluation of the function of target proteins in the development and progression of Pan02 tumors. For example, orthotopic Pan02 implantation in *fibulin* $5^{-/-}$ mice, resulted in smaller and less invasive tumors than tumors grown in *wild-type* animals. *Fibulin-5* (*Fbln5*) is a matricellular protein implicated in regulation of angiogenesis [61] and elastic fiber formation [62]. Importantly, Pan02 tumors grown in the absence of *Fbln5* displayed reduced microvessel density. Investiga-

Table 4.2 Orthotopic tumor establishment and metastatic potential of cell lines

Cell line	Number cells injected	Time length of experiment	Location of metastasis	Reference
AsPC-1	1×10^6	6 weeks	Peritoneum, Liver	Fujioka SF et al. 2003 [78]
BxPC-3	2 mm^3 sc tumor	8 weeks	LN, minimal metastasis noted	Matsuo Y et al. 2009 [79]
Capan-1	1 mm^3 sc tumor	14 weeks	Liver, LN	Bhargava S et al. 2007 [80]
Capan-2	1×10^6	12 weeks	LN 60%, Liver 50% Spleen, GI	Bailey JM et al. 2009 [81]
CFPAC-1	1×10^6	8 weeks	Not described	Yao J et al. 2010 [82]
Colo357 (L3.6pl)	1×10^6	5 weeks	Liver, LN, occasional peritoneal	Bondar VM et al. 2002 [83]
HPAC	1×10^6	3-4 weeks	None	Mohammed RM et al. 1998 [77]
HPAF-II	2×10^6	5 weeks	LN only	Fujisawa T et al. 2009 [84]
Hs766T	2×10^6	5 weeks	LN and 50% Liver	Fujisawa T et al. 2009 [84]
MiaPaca-2	1×10^6	8-10 weeks	Liver	Dineen SP et al. 2010 [39]
MPanc-96	1×10^6	Not described	Lung 100% Liver100%	Ramachandran V et al. 2008 [85]
Panc-1	1×10^6	12 weeks	Minimal	Awasthi N et al. 2011 [86]
SW1990	1 mm^3 sc tumor	8 weeks	80–100% Metastasis, Liver	Jia L et al. 2005 [87]
Pan02*	5×10^5	7 weeks	Liver, peritoneum, LN	Dineen SP et al. 2008 [88]

The following lines have only been done in subcutaneous models: Panc03.27, Su 86.86, PL45, Panc 10.05. According to literature search the following ATCC lines have not be performed in vivo models: Panc 08.13, Panc02.03, Panc02.13, Panc04.03, Panc05.04

sc primary tumor implanted from subcutaneous tumor, *LN* lymph node metastasis, *GI* gastrointestinal metastasis

*denotes mouse cell line

tion of the mechanisms underlying reduced tumor growth in the absence of Fbln5 resulted in the identification of a new function for this protein. It was discovered that Fbln5 competes with fibronectin for binding to integrin α5β1. Elevated ligation of the integrin resulted in increased production of reactive oxygen species, which resulted in endothelial cell apoptosis, reduced angiogenesis and poor tumor growth [63]. Similarly, studies of Pan02 tumor growth in the absence of the matricellular protein "secreted protein acidic and rich in cysteine" (SPARC) documented that SPARC is critical for the appropriate stromal response to pancreatic cancer formation [64]. A defining feature of tumors grown in *SPARC*$^{-/-}$ animals was reduced collagen deposition and an increase in vascular perfusion [65]. These features resulted in an increase in local invasion and distant metastases, an effect that was dependent in part on TGFβ activity [66].

Recently, groups that work with the KPC transgenic model have developed clones from primary tumors that they have implanted either subcutaneously or orthotopically [67]. These tumor cell lines are implanted in histocompatible synegenic mice depending on the background of the mouse from which the original cell line was derived. Differences between the parental cell lines vs. subcutaneous tumors vs. orthotopic tumors were however noted on CGH array.

The syngeneic model provides a tumor microenvironment with innate and adaptive immunity, but it also has some limitations. Limited variability in in vivo modeling can be achieved given that there are only two cell lines available. Furthermore, these two cell lines have not been fully characterized beyond reports of Pan02 cells expressing wild type Kras [68] and a Smad4 mutation (Arnold SA, unpublished data) and mutation in p16 (Ostapoff KT, unpublished data). Finally, Pan02 cells express a highly mesenchymal phenotype. Although this makes them highly metastatic, their lack of epithelial marker expression in vitro and in vivo may limit their utilization for some experiments.

4.4 Xenograft Models

Despite the increasing utilization of genetic models of PDAC, xenograft modeling has been and remains the mainstay of in vivo pancreatic cancer research. A finite number of PDAC cell lines have been widely used for in vivo experimentation throughout the years. An early such effort resulted in the development of Panc-1 cells that Lieber et al. found to be a stable cell line after 2 years in culture [69]. Subsequently, multiple cell lines have been developed from human primary tumors at all stages of disease, including isolates from primary tumors, lymph node metastases, liver metastases and malignant ascites. Currently, the ATCC (American Type Culture Collection) has 21 human pancreatic cancer cell lines available for purchase. Additionally, groups at MD Anderson and Johns Hopkins University have continued to develop cell lines from patients before and after preoperative chemotherapy. Baseline information on mutational status of many of these cell lines have been established and can be used as a guide for experimental testing (Table 4.3).

The development of cell lines has allowed the investigation of a variety of aspects of PDAC biology in vitro as well as in vivo, which has encompassed subcutaneous, orthotopic and intraperitoneal injection models.

4.4.1 Subcutaneous Models

In immunodeficient mice, tumors are established on the flank after subcutaneous injection of tumor cells. Tumor growth is easily followed with calipers allowing for rapid real time responses to drug treatments. Direct effects of therapeutic

Table 4.3 Genetic alterations in human pancreatic cancer cell lines

Cell line	Origin	Kras	p53	CDKN2A/p16	DPC4
AsPC-1	Ascites	12 Asp mutation	Frameshift mutation, 135 Δ1 bp, Intro 4 Δ200 bp splice site, HD exon 5	Δ2 bp frameshift mutation, HD, wild type	Wild type
CFPAC-1	Liver metastasis	12 Val mutation	242 Arg mutation	Methylated, deletion, wild type	HD mutation
HPAF-II	Ascites	12 Val mutation	151 Ser mutation	29–34 in frame deletion	Wild type
MDAPanc-3	Liver Metastasis	12 Ala mutation	273 Cys mutation	−36 to (+5) C deletion	Wild type
MiaPaca-2	Liver metastasis	12 Cys mutation	248 Trp mutation	HD mutation	Wild type
Panc-1	Primary tumor	12 Asp mutation	273 His , 273 Cys mutation	HD mutation	wild type
PancTu-I	Primary tumor	12 Val mutation	176 Ser mutation	Methylated, deletion	Wild type
Suit-2	Liver metastasis	12 Asp mutation	273 His mutation	69 Glu to Stop mutation	Wild type
Capan-1	Liver metastasis	12 Val mutation	153 Val mutation	HD mutation	577 Leu, 343 STOP mutation
Hs 766T	Lymph node metastasis	Wild type	225-282 deletion, Δexons 2-4 mutation, wild type	Intron 2 splice site mutation, , wild type	HD mutation
BxPC-3	Primary tumor	Wild type	220 Cys Mutation	HD mutation, wild type	Wild type
Capan-2	Primary tumor	12 Val mutation	wild type,Intro 4 Δ200 bp splice site	6 bp ins, 7 bp ins mutations, wild type	Low protein expression, wild type
Colo357	Lymph node metastasis	12 Asp mutation	Wild type	HD mutation, wild type,	HD mutation or wild type
SU86.86	Liver metastasis	12 Asp mutation	245 Ser mutation	HD mutation	Wild type

Modified and Adapted from Moore PS et al., Virch Arch 2001 [68], Deer EL et al., Pancreas 2010 [69] and Sipos B et al., Virch Arch 2003 [70]
HD homozygous deletion, Δ- deletion, *ins*- insertion, *bp*-base pair

interventions can be followed and regressions are in fact best seen with this model. Typically, analysis of these subcutaneous tumors allows for evaluation of tumor vasculature [70], drug delivery, apoptosis and proliferation [71]. For instance, this model has frequently been used in shRNA experiments to demonstrate the impact of specific targets on tumor growth. It is also useful for injecting a mixed cell popula-

tions. For example, the contribution of fibroblasts to the tumor microenvironment has been studied in this model by injecting mice with genetically modified fibroblasts with tumor cells [72] and by injecting tumor cells with pre-treated fibroblasts [73].

The ease and accessibility of the tumor cell injection site has made subcutaneous models the most frequently used. The model is suitable for investigation of therapeutic efficacy and tumor biology. However, primary subcutaneous pancreatic tumors typically do not metastasize and often incorporate less stroma than transgenic tumors or tumors grown in the orthotopic setting. Other possible putative differences between pancreas-based spontaneous and skin-based injected tumors are not proven, but may exist.

Subcutaneous models are certainly not ideal for analysis of metastatic parameters. Furthermore, given that these experiments are performed in immunodeficient animals, there is also a limited ability to study the role of innate immunity in tumor progression. Despite these shortcomings, advocates of this model emphasize its ease of use and application, aside from the limited training needed for performing these experiments in a quick and reliable fashion. In addition, multiple different, well-characterized cell lines can be assessed in vivo in this fashion.

4.4.2 Orthotopic Model

More recently orthotopic modeling gained more popularity since the subcutaneous model is critiqued for its altered tumor architecture and its presence of a distinct capsule. Tumor vasculature is derived from skin which may be different from the network that is provided by the pancreas. Most importantly, the subcutaneous model does not metastasize, a distinction from a common and important clinical feature of PDAC.

The orthotopic model approach attempts to recapitulate the tumor microenvironment by directly implanting human tumor cells into the pancreas of immunodeficient mice. Such developing tumors are infiltrated with murine stromal components that resemble the stroma of PDAC from patients. Critical factors that influence growth of tumors in the orthotopic site are: (1) cell line selected for implantation; (2) volume and number of cells injected; and (3) the operative technique used for tumor cell injection (Table 4.2).

Tumor cell implantation occurs at either the head or tail equivalent of the pancreas. Mice with tumors in the pancreatic head often succumb to local invasion and develop biliary obstruction prior to extensive liver metastasis. Although these may develop fewer metastases, this clinical presentation is consistent with human disease. Alternatively, tumor injection can occur at the tail of the pancreas. Tumors in the tail are able to grow for longer periods of time, typically develop more frequent metastases and provide more tissue for mechanistic analysis. Various experiences with orthotopic injection, cell numbers and metastatic incidences are included in Table 4.2. Several techniques have been developed to establish orthotopic tumors.

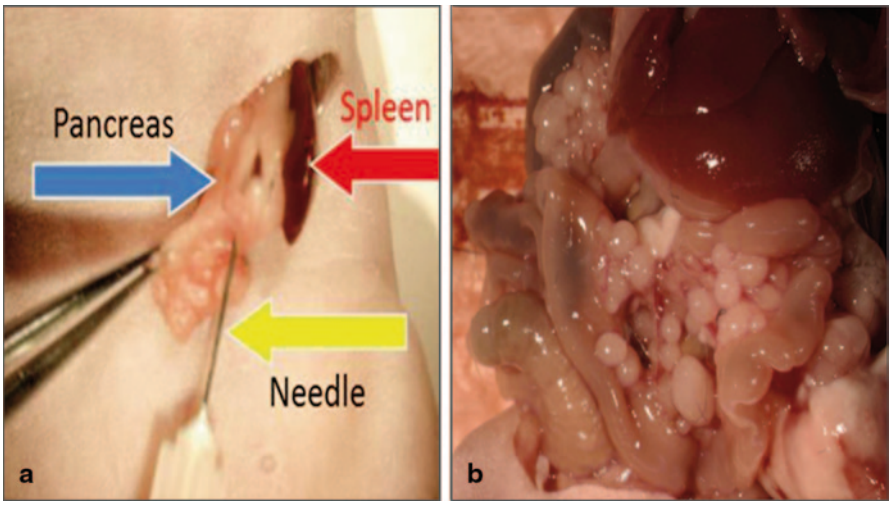


Fig. 4.3 Orthotopic injection of pancreatic cells

This includes the initial placement of subcutaneous tumor. Once established, subcutaneous tumors are harvested and cut into 1–2 mm^3 pieces and implanted with suture fixation into the pancreas [74]. Alternatively, subcutaneous tumors are minced, processed into a cell suspension [75], injected into the pancreas and followed for tumor growth [76]. Cells can also be directly injected into the pancreas from cell culture (Fig. 4.3). Prior to injection, cells are grown in culture, counted and suspended in sterile PBS or serum free medium in a desired cell concentration. After anesthetizing the recipient mouse, a left upper quadrant incision is made and the distal pancreas is introduced into the wound. Tumor cells are injected under sterile conditions into the pancreas, and the incision is closed. These two methods have been compared in several cell lines, including MiaPaca-2, Capan-1, AsPC-1 and HPAF-II. Tumors established by tumor piece implantation had an increased overall survival but decreased metastatic incidence compared to tumors established by direct tumor cell inoculation from culture [74]. Given the increased incidence of metastasis in the injection model, we use this method for orthotopic implantation. The difference in metastatic rates between these two methods is intriguing and warrants further study to further characterize the mechanisms of metastasis involved.

After anesthesia induction, a left lateral incision is made and the spleen and distal pancreas are externalized. A subcapsular injection is made forming a bubble as shown and then returned to abdomen and skin is closed. Tumor growth is then monitored (Fig. 4.3a). Many cells ultimately metastasize. Three months after MiaPaca-2 tumor cell injection, gastrointestinal, liver and lymph node metastasis are seen throughout the abdomen of the mouse (Fig. 4.3b)

The orthotopic model has been widely represented in the literature. Although gemcitabine has been used frequently as the standard of care in in vivo experiments, the dose and frequency of administration have not been similar between

Table 4.4 Gemcitabine sensitivity in vivo

Cell line	Tumor location	Gemcitabine dose	Sensitivity in vivo	Reference
AsPC-1	Subcutaneous	100 mg/kg twice weekly	Yes	Awasthi A et al. 2011 [96]
BxPC-3	Orthotopic	300 mg/kg weekly 150 mg/kg weekly	Yes 48 %No 51.8 %	Sun FX et al. 2003 [97]
Capan-1	Subcutaneous	100 mg/kg twice weekly	Yes T/C=~20 %	Kimura K et al. 2006 [98]
CFPAC-1	Subcutaneous	150 mg/kg every 3 days	Yes T/C = 5.6	Mercalli A et al. 2007 [99]
L3.6pl (Colo357)	Orthotopic subcutaneous	250 mg/kg twice weekly 62.5 mg/kg twice weekly	Yes T/C 28 % No T/C=68.9	Bruns CJ et al. 1999 [100] Bondar VM et al. 2002 [83]
HPAC	Orthotopic	2.5 mg/kg daily	No T/C=63 %	Mohammad RM et al. 1998 [77]
HPAF-II	Orthotopic	120 mg/kg	Yes T/C=25 %	Hotz B et al. 2010 [101]
MiaPaca-2	Orthotopic	25 mg/kg twice weekly	Yes T/C= 50 %	Dineen SP et al 2010 [39]
MPanc-96	Orthotopic	50 mg/kg100 mg/kg weekly	No No T/C >100 %	Pan X et al. 2008 [102] Ramachandran et al. 2008 [85]
Panc-1	Orthotopic subcutaneous	25 mg/kg twice weekly100 mg/kg	No T/C=77 % Yes T/C ~25 %	Awasthi N et al 2010 [86] Du JH et al. 2010 [103]
SU 86.86	Subcutaneous	100 mg/kg every 3 days	Yes T/C= 31%	Feng N et al. 2003 [104]
SW1990	Orthotopic subcutaneous	100 mg/kg weekly 50 mg/kg weekly 50 mg/kg every 3 days	Yes T/C =30 %, No T/C= 77 % No T/C 93 %	Jia L et al. 2005 [87] Xie Q et al. 2009 [105]
Pan02*	Orthotopic	3.5 mg/animal weekly	Yes T/C =40 %	Dineen SP et al. 2008 [88]

*murine pancreatic adenocarcinoma cell line T/C ratio is the weight of the gemcitabine treated tumor/ weight of the control treated tumor. Cell lines are considered sensitive to gemcitabine if tumor growth is reduced by 50 % or less compared to control tumor weights. T/C ratio as reported by reference or approximated from representative figures.

Literature search reveals no in vivo sensitivity results for the following ATCC lines: Capan-2, PL45 & Panc 10.05 (derived from same human tumor), Panc03.27, Panc 08.13, Panc02.03, Panc02.13, Panc04.03 and Panc05.04

experiments. Table 4.4 provides a summary of in vivo models using human PDAC cell lines and the gemcitabine dose used and the tumor response (gem tumor weight/ control tumor weight = T/C ratio). It is important to note that despite being gemcitabine-resistant in vitro, many of the human cell lines are somewhat gemcitabine-sensitive in vivo such as Panc-1 and HPAF-II (KT Ostapoff, unpublished observations). Interestingly, the converse is also true. MiaPaca-2 cells are sensitive to

Gemcitabine in vitro but are relatively resistant in vivo (KT Ostapoff unpublished observations). The role of gemcitabine effects on metastasis has not been fully characterized in these models.

4.4.3 Direct Xenografting

Direct xenografts are the newest method for studying tumor growth and metastasis in vivo. This technique uses patient samples to establish tumors. The exact method of placement varies. Small**,** usually 1 mm^3 pieces from fresh surgical specimens can be isolated and then directly implanted with suture onto the head/portal area of the pancreas [77], middle [78] or tail [79]. For tumor samples that are too small to immediately implant, tumor pieces are implanted subcutaneously and later harvested in a manner similar to that described for the orthotopic model [80]. Tumors can alternatively be isolated into single cell suspensions and then directly injected orthotopically or maintained in culture prior to injection. Groups that routinely perform these types of injections find that tumor growth improves when implantation occurs within 1 h of resection [81]. Tumors typically establish at faster rates with each additional passage.

There are several advantages to the direct xenograft model. Tumor specimens contain stromal elements from the primary tumor. Although these elements may not persist with additional passages, they contribute to the tumor microenvironment and metastasis. These stromal elements may hold a key to the increased rate of metastasis compared to cell suspensions in other models [80]. Early reports have suggested that metastasis isolated from these tumors develop mutations after transplantation in the mouse [79]. The cause of these new mutations has not been identified as of yet. It is unclear whether these new mutations are caused by interactions with mouse cells in the tumor microenvironment or if they reflect the natural progression of these cells as they find the metastatic niche (i.e. these same mutations are present in metastasis subsequently found in the patient).

A significant advantage to this approach is the potential to improve drug selection and identify individual tumor susceptibility in patients. Analysis of patient samples in vivo, have allowed researchers to study potential mutations in surgical specimens that may predict response to therapy [82]. Additionally, tumors isolated from patients after preoperative chemotherapy have been used to study drug resistance within the viable tumor and/or used to identify additional drug sensitivity of remaining tumor cells post-operatively [81].

Several disadvantages also exist for this model. Not all tumor specimens contain viable tumor for implantation. The tumor take rate (or establishment rate) is not consistent between different samples, and may in fact be problematically low (i.e. around 20%). The same sample placed in different locations (i.e. liver, pancreas or colon) within the mouse interacts differently with its host environment [83]. This method also requires specialized training and a well-organized system in place to assure rapid delivery of tumor from operating room to bench and implantation. Dif-

ferent samples reflect the clinical heterogeneity of tumors, rendering generalizable investigations more difficult due to the absence of detailed knowledge of molecular and biologic alterations involved. Regardless of these potential caveats, this model has great potential for improving patient care and chemotherapeutic agent delivery, as samples used are as much reflective of clinical tumor specimens as feasible. It also has the potential to allow researchers to study mechanisms of chemo- and radiation resistance within previously treated tumors. It provides a platform for studying the additional genetic events that occur when tumor cells develop metastases.

Xenograft models have provided excellent advances in the knowledge of disease progression and metastasis. Most cell lines invade into local tissues and metastasize to lymph nodes and liver in an orthotopic model. This allows for the study of molecular pathways of invasion, tumor to stromal interactions and metastasis as well as the study of targets in these pathways [84]. Many of these cell lines have been fully characterized in terms of their known mutations (Table 4.3) and may allow for studying specific responses to targeted therapy.

4.4.4 Monitoring Growth in PDAC Models

Several methods aid in the understanding of mechanisms of PDAC progression and metastasis that can be used both in vivo and ex vivo. Subcutaneous tumor growth is easily measured with calipers. Orthotopic models, however, present a unique challenge given intraperitoneal tumor location. Several methods have been developed for noninvasive imaging of orthotopic tumors, allowing for size monitoring in the same animal over time. If cell lines are labeled with a non-invasive probe (e.g. fluorescent protein), primary tumors and metastases can be visualized using fluorescence stereo microscopes [85]. Visualization for this technique is optimized by creating a skin flap over the primary tumor which improves sensitivity of detection of fluorescence (Fig. 4.4) [86]. Furthermore, Bouvet et al., have shown that there is strong correlation between metastatic disease using GFP or RFP labeled cells by imaging between fluorescence optical imaging (FLU), magnetic resonance imaging (MRI) and ultrasound without the use of additional contrast agents [87]. Recently, this group has shown using GFP and RFP labeled cells that micrometastasis can be identified using in vivo laparoscopy in mice [88]. In fact, using fluorescently labeled CEA (carcinoembryonic antigen), this group was also able to identify micrometastasis throughout the peritoneum after orthotopic tumor establishment with laparoscopy [89].

Bioluminescent imaging (BLI) can be used for luciferase labeled cells. Mice are injected with a luciferase substrate, anesthetized and subsequently imaged (Fig. 4.5) [90].

This technique can adequately demonstrate the development of metastatic disease during tumor progression and can be quantified. However, in our experience, luciferase labeled cells grow slower in vivo than their parental cell line and often inconsistently develop metastases; however disseminated lesions that do develop

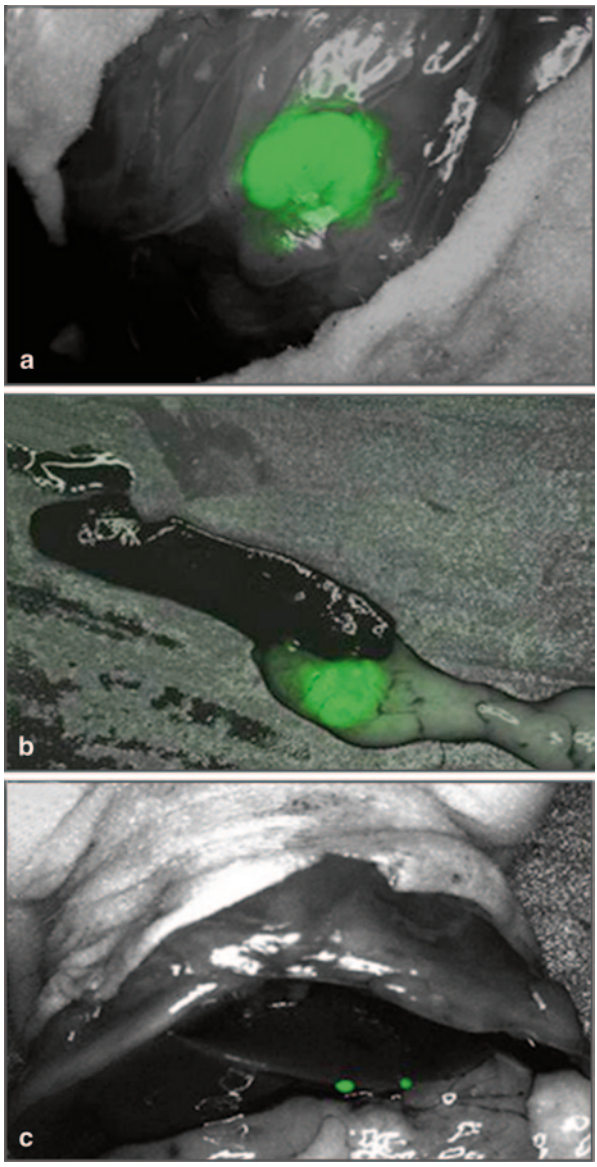


Fig. 4.4 Panc-1-GFP cell lines metastasize. After injection of GFP-Panc-1 cells orthotopically in the pancreas, primary tumors are established as shown **a** in vivo and **b** ex vivo. Liver metastases are established on liver as shown by green areas **c**

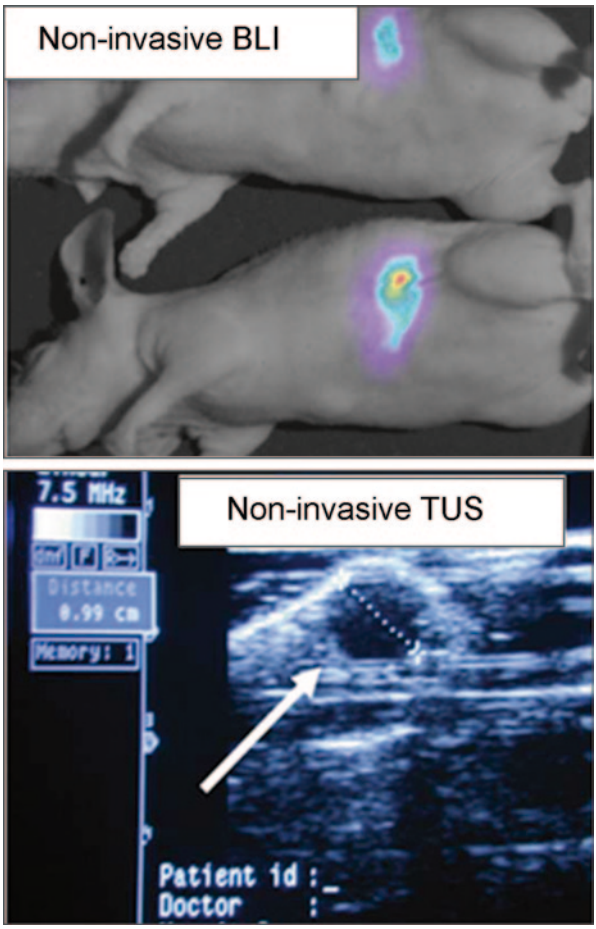


Fig. 4.5 Noninvasive imaging of tumor and metastatic growth. Using luciferase labeled cells; tumor growth after orthotopic implantation can be followed using BLI (*upper panel*). The tumor intensity is measured and can be followed over time. Without labeled cells, simple ultrasound imaging can provide evidence of tumor growth that is measureable (*lower panel*, *dotted line* is tumor measurement)

can be followed easily. Additionally, several options also exist for monitoring unlabeled cells including ultrasound with or without microbubbles [91], MRI [92] and PET/CT [93](Fig. 4.5).

4.5 Modeling Metastatic Disease

Orthotopic models facilitate studying tumor to stromal interactions and some other mechanisms of tumor progression. However, direct metastatic models are beneficial for the evaluation of the ability of tumor cells to colonize and grow at distant sites. Not all circulating tumor cells result in metastasis, and the processes under which some arrive in the metastatic niche and go on to form demonstrable metastases are complex. Specific models to disrupt or study these mechanisms are required to un-

derstand this complex relationship. It has also been suggested that specific human cell lines have predilections for metastasis from an intrapancreatic location to specific target sites (i.e. BxPC-3 tends metastasizes to lymph nodes while MiaPaca-2 tends to metastasize to the liver) [94]. This may be due to mechanisms inherent to the cell line, or due to specific evolution of tumor to stromal interactions. Several methods of developing metastatic only models allow one to study the development of micro- to macrometastasis and invasion from the vasculature into the site of metastasis.

In addition, for patients who are able to undergo pancreatic resection, survival is governed by the pre-therapeutic development of metastasis. Murine models that allow evaluation of strategies to inhibit circulating tumor cells within the metastatic niche in order to prevent subsequent metastasis would therefore have important clinical implications. This section will describe models of perineural invasion, lymphatic metastasis, liver metastasis and peritoneal spread of pancreatic cancer.

4.5.1 Perineural Invasion Models

Perineural invasion is a significant feature of human PDAC which is associated with a worse survival and an increased risk of metastasis. It also correlates with an increased risk of local recurrence after resection. The specific mechanism and its role in the evolution of metastasis remains incompletely understood.

Early reports indicated a lack of perineural invasion in orthotopic models [77, 95]. Recently however, perineural invasion has been documented in an orthotopic model. MiaPaca-2 and Capan-2 orthotopic tumors were established and then resected at 4, 6 and 8 weeks. After resection, mice with MiaPaca-2 tumors that were 6 weeks or older developed tumor recurrence that showed extensive retroperitoneal perineural invasion on histological analysis [96]. There are also two models which use nerve grafts to establish perineural invasion. In the human model, celiac or superior mesenteric artery nerve plexus were taken from recent human autopsy specimens and implanted subcutaneously in SCID/NOD mice. After 4 weeks of nerve engraftment, human cell lines were then injected in an adjacent area subcutaneously and allowed to grow [97]. Although all cell lines used (Capan-1, Capan-2, CFPAC and MPanc96) had some perineural invasion, they frequently did not invade the nerve itself. Alternatively, human pancreatic cancer cell lines were injected subcutaneously on the midline of the mouse back and allowed to grow towards the spine. Both Capan-1 and Capan2 had significant perineural invasion (in 55% and 69% of mice respectively) compared to none in HPAF-II, AsPC-1 and Panc-1 cell lines used. All lines had epineural invasion and nerve involvement [97]. Additionally, use of the mouse perineural model has been used to analyze potential transcription factors that contribute to perineural invasion and metastasis [98, 99].

With the additions of both of these animal models of perineural invasion, in vivo models are available to study mechanisms of perineural invasion that can lead to local recurrence and metastasis after a successful pancreatic resection.

In order to investigate the mechanism of perineural tumor invasion, Gil et al. used dorsal root ganglia extracted from 2–4 week old Balb/c mice and allowed MiaPaca-2 and Panc-1 tumor cells to migrate through a matrigel [100]. They found that some cell lines were able to grow towards neural cells and in fact those that did formed a spindle-shape morphology. Tumor cells also migrated towards neural cells in Boyden chambers and this was blocked by anti-GDNF antibodies[100]. The use of myenteric plexus cells isolated from Sprague-Dawley rats, plated with T3M4 pancreatic cancer cells, also demonstrates a proclivity of tumor cells to migrate towards nerve cells and change their morphology [101].

4.5.2 Lymphangitic Metastasis Models

PDAC cells exhibit a predilection to colonize regional lymph nodes. This clinical observation suggests that lymphatic vessels carry an integral function in the metastatic process of PDAC; in fact, expression of the primary lymphatic growth factor vascular endothelial growth factor-C (VEGF-C) tends to correlate with lymph node metastasis in patient specimens [102–105]. The presence of VEGF-C expression in cancer cells is associated with increased incidence of lymph node metastasis but does not correlate with decreased patient survival [102, 106]. It has also been shown that the density of lymphatic vessels is lower in intratumoral regions than normal regions of the pancreas, and intratumoral lymphatic vessels are collapsed whereas peritumoral lymphatics are enlarged in human PDAC specimens [106]. Analysis of human PDAC cell lines shows that T3M4, MiaPaca-2, Panc-1, Colo357 and BxPC-3 all express VEGF-C but do not express VEGFR-3 (vascular endothelial growth factor receptor-3) [106].

VEGF-D also plays an important role in lymph node metastasis. VEGF-D null mice after orthotopic implantation with Pan02 cells have significantly increased mesenteric lymph node metastasis and a reduction in lymph vessel diameter compared to control mice [107]. However, there is no discernable difference in primary tumor weight. As a result, this provides evidence that lymphatic vascular function is specifically and abnormally regulated in the PDAC environment. As lymphatic metastasis may play a critical role in PDAC progression, specific models may aid in understanding and studying this relationship.

The first study of the normal pancreatic lymphatic network dates back to 1881, with the work of Hoggan and Hoggan [108]. Since that time, injection methods, basic histological staining, and electron microscopy have been employed to characterize the lymphatic network of the pancreas [109]. Tumor lymphangiogenesis is seen in both subcutaneous and orthotopic tumors. Lymph node metastases are common in orthotopically implanted MiaPaca-2, BxPC-3, HPAF-II, PancTu-1, aPt45P1 and Colo357 [110–112]. Using these models, the mechanisms of lymphatic metastasis have been studied. Inhibition of TGFβRI enhances intratumoral lymphatics in subcutaneous MiaPaca-2 and BxPC-3 tumors [113]. Using MiaPaca-2 orthotopic tumors, Schultz et al. found that induction of p16 expression in tumors resulted in

an inhibition of lymph node metastasis and reduced the tumor burden in the rare affected lymph nodes [114]. Furthermore, overexpression of αVβ3 that results in c-Src activation in FG cells (a clonal line from parental Colo357 cells) was found to promote lymphatic metastasis specifically to the bowel mesentery and hepatic hilar lymph nodes [115]. This was further confirmed with the use of dasatinib (a Src inhibitor) which resulted in reduced lymph node metastasis, overall LN mass and tumor burden within the lymph node [115].

For certain lymphatic intervention approaches such as nanoparticle delivery a lymphatic metastasis model can be used, in which the mouse hindfoot is injected with tumor cells known to metastasize to lymph nodes (BxPC-3) [116]. After several weeks, lymph nodes from the popliteal, inguinal and iliac regions are harvested for analysis.

One of the limitations of xenograft models is the lack of a fully functioning immune system which quite possibly impacts the biology of lymphatic metastasis. In contrast, Pan02 cells are injected into immunocompetent mice. Furthermore, orthotopic Pan02 tumors are highly metastatic towards mesenteric lymph nodes, even though they do not exhibit profound intratumoral lymphatic vessels [117]. Lymph node metastasis can be easily calculated in this model with control mice often having 20–30 lymph node metastases. Due to this observation, multiple studies have used this model to look at effects of different therapies on lymphatic metastasis [111]. Using the Pan02 model, macrophages have been shown to play an important role in regulating peritumoral lymphangiogenesis and lymph node metastasis [117]. Furthermore, lymphatic vessel density is decreased in the presence of macrophage depletion with clodrolip or with the use of anti-PlGF (placental like growth factor) in vivo [117]. In the pancreas, there is growing evidence that lymphatic vessels facilitate the lymphatic spread of PDAC. However, the underlying mechanisms remain poorly defined. Future *in vitro* and *in vivo* studies will shed light on the pathways controlling the lymphovascular regulation and the resulting lymphatic spread of PDAC. This may help elicit novel biomarkers to identify patients at risk for disease recurrence and also identify potential therapeutic targets.

4.5.3 Liver Metastasis Models (discussed in Chapter 7 in detail)

The most common site of metastasis for PDAC is in the liver. The two most common models used to establish metastasis are portal vein injection and splenic injection models (Fig. 7.2).

4.5.3.1 Portal Vein Injection Model

In the portal vein injection model, cells are injected directly into the portal vein and subsequently form liver lesions with high frequency. Initial studies used India ink to stain tumor cells within the liver, and confirmed that cells left untreated after portal

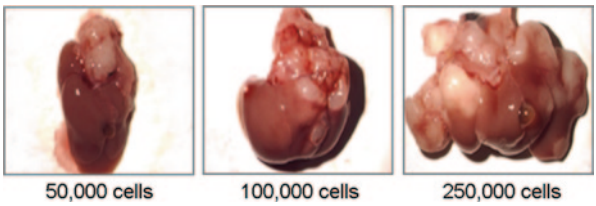


Fig. 4.6 Splenic injection results in large metastasis

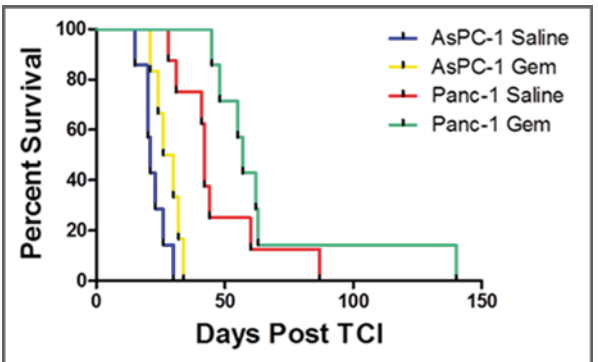
injection can reliably form hepatic parenchymal metastases [118]. As early as 72 h post injection, I^{125}labeled tumors cells can be identified within the liver in the form of micrometastases [119]. In one study portal vein injection resulted in higher incidence of metastasis (71% vs. 51%) compared to orthotopic implantation of the same cell line [120]. Portal vein injection is technically challenging and bypasses many hurdles that metastatic tumor cells must overcome to establish lesions in the liver. It should however be noted that tumor cells injected into the portal vein do reach the systemic circulation and can form metastases in other organs, too (e.g., lung).

4.5.3.2 Splenic Injection Model

In addition to portal vein injection, the splenic injection method offers a reliable route to establish liver metastases. In this method, tumor cells are injected into the lower pole of the spleen and then pass through the splenic vein to the liver and systemically. Investigators using this method will often remove the spleen shortly after injection prior to closing the incision [121], although some allow the spleen to remain in place, especially if an immunotherapeutic component may be affected by a splenectomy [122]. An approach of hemisplenectomy with spleen splitting, removal of the hemispleen through which tumor cells had been injected, and preservation of the unaffected hemispleen has been described [123–125]. Tumor burden can be measured by liver weights or by gross inspection. This model typically leads to micrometastases as early as 7–14 days after injection, and may lead to near complete liver replacement with tumor typically between 5–8 weeks (Fig. 4.6).

Pan02 cells were injected into the spleen of C57bl/6 mice. After 5 weeks mice were sacrificed and evaluated for metastatic deposits. Representative livers are shown above. Metastatic burden can grossly be appreciated between different cell numbers injected.

One group comparing different cell lines found that while some lines did not form metastases even when injecting 10^6 cells (Capan-2 and PL45), other more aggressive lines could form metastases after injection with less than 10^4 cells (MiaPaca-2, AsPC-1, Panc1, Capan-1 and BxPC-3 in NOD/SCID or NOG/SCID mice [126]. This method has been effective for studying some mechanistic aspects of metastatic tumor implantation, such as by either pretreating cells prior to implantation or by treating mice after tumor cell injection with specific inhibitors of the TGF-β pathway [121].



	Median Survival (Days)
AsPC-1 Saline	21
AsPC-1 Gemcitabine	28
Panc-1 Saline	42
Panc-1 Gemcitabine	57

Fig. 4.7 Survival outcomes after intraperitoneal PDAC cell injection, and potential for therapeutic testing. Control mice in both AsPC-1 and Panc-1 groups have short survival (21 and 42 days respectively). Addition of standard chemotherapy in form of gemcitabine extends survival minimally for ASPC-1 cells (gemcitabine resistant in vitro), and moderately for Panc-1 cells (gemcitabine sensitive in vitro)

The splenic injection model may still not prevent cells from circulating systemically and ultimately forming extrahepatic tumors. In our experience, after intrasplenic injection of Pan02 cells in C57bl/6 mice, the animals do not develop lung metastases (KT Ostapoff, unpublished observations). The hepatic metastases that form in this model are sufficiently large (as demonstrated in Fig. 4.6) to retrieve tissue for immunohistochemistry, protein isolates for Western blots or proteomic studies, and RNA or DNA samples for genomic arrays.

4.5.4 Intraperitoneal Injection Model

While liver metastases are common in patients, peritoneal spread of disease is also a hallmark feature of PDAC. Mechanisms of intraperitoneal recurrence and the development of intraperitoneal disease are important to understand the progression to end stage disease. Models that address the spontaneous intraperitoneal progression of PDAC have not been described. However, disease progression after intraperitoneal injection can be followed for various cell lines in xenograft or syngeneic injection models. Mice are injected with cells directly into the peritoneal cavity and develop peritoneal implants as soon as 48 h after injection. In cases of injection of AsPC-1 and Panc-1 cell lines into SCID or nude mice, spontaneous homing of the cells to the pancreas occurs [127]. After establishment of tumors, very few animals

develop ascites, but obstructive jaundice, lymph node metastases and liver metastases are common; animals must be sacrificed due to tumor associated morbidity soon after [71, 127, 128]. This model therefore can serve as a simple, reproducible and reliable survival model for experimental therapy approaches.

It has been shown to be highly replicable with respect to median survival of control and gemcitabine treated animals in both Panc-1 and AsPC-1 models and used in studying therapeutic interventions (Fig. 4.7) [71, 127–131].

Using overall survival as its primary endpoint, the intraperitoneal model is analogous to a human clinical trial. Multiple agents in combination can be tested at once, with toxicities to certain combinations identified early in the in vivo evaluation. A downside to this approach is that model outcomes will depend on the specific cell line characteristics, and therefore carry some shortcomings compared to the complexity and heterogeneity of spontaneous human PDAC tumors.

4.6 Conclusions

Due to the complexity of PDAC biology, no single animal model will provide complete understanding of the mechanisms of this disease. In fact, we can comfortably go as far as to assume that no model will completely be able to represent this disease complexity. However, we now can resort to numerous in vivo models that are able to address specific factors with relevance to PDAC development and therapy.

Genetically engineered mouse models provide improved understanding of carcinogenesis and tumor initiation; they are likely continuing to evolve through the introduction of new mutations into existing transgenic mice. Syngeneic (and transgenic) models provide an opportunity to investigate the importance of immune cells for the regulation of tumor progression, and to evaluate immunotherapy approaches in vivo. Work with established human cell lines has multiple benefits of evaluating drug response and tumor to stromal interactions. More recently, direct human-derived xenografts provide some opportunity to study chemoresistance and sensitivity in human samples as well as potentially direct therapy.

Despite the assets of these known models, multiple challenges face the field as we move towards better understanding the mechanisms of metastasis. The role of pancreatic stem cells, while still controversial, opens a new opportunity of research. Recent work suggests that cells from the pancreas and bone marrow work in conjunction in a carcinogen-induced model of pancreatic cancer [132]. There is also evidence that pancreatic stellate cells, which are known to participate in the desmoplastic reaction within the primary tumor also facilitate metastasis [133, 134]. Similarly work with mesenchymal stem cells shows promise to understand the function of host-stroma interactions and has been proposed as a potential target for treatment [135, 136].

In the era of emerging individualized care, multiple in vivo models give testament to the fact that progress is being made in the study of this challenging disease. Future work will need to address the added complexities of both the tumor microenvironment and pluripotent nature of PDAC cells. We therefore

anticipate that additional PDAC models will aid the process of ever improving insight into the molecular and genetic mechanisms and therapeutic strategies for pancreatic cancer.

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