

**BRIDGING THE GAP BETWEEN PRECLINICAL MODELS AND CLINICAL TRIALS
USING A NOVEL KRAS- AND MYC-DRIVEN MOUSE MODEL OF PANCREATIC
CANCER AND 3D-BIOPRINTING**

By

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LIST OF ABBREVIATIONS

15919-T	Primary patient-derived PDAc cell line from study participant ST-00015919
18136-T	Primary patient-derived PDAc cell line from study participant ST-00015919
2D	Two-Dimensional
3D	Three-Dimensional
5-FU	5-Fluorouracil
ABC	Avidin-Biotin Complex kit
Abraxane	Albumin-bound Paclitaxel (also known as nAb-paclitaxel or nAb-pac)
ADM	Acinar-to-ductal metaplasia
AEC	Aminoethyl carbazole
AKT	AKT Serine/Threonine Kinase
ALK	Anaplastic Lymphoma Kinase
AMG 510	Amgen (Biopharmaceutical company) 510 (KRAS ^{G12C} inhibitor)
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3-related
α SMA	Alpha-Smooth Muscle Actin
BAT	Brown Adipose Tissue
BCCPC	Brenden Colson Center for Pancreatic Care
BET	Bromodomain and Extra-Terminal motif proteins
BRAF	B-Rapidly Accelerated Fibrosarcoma (a proto-oncogene)
BRD4	Bromodomain Containing 4
BSA	Bovine Serum Albumin
c-MYC	Cellular MYC 'myelocytomatosis' proto-oncogene, BHLH transcription factor
CAF	Cancer-Associated Fibroblast
CD11c	Cluster of Differentiation 11c
CD3	Cluster of Differentiation 3
CD31	Cluster of Differentiation 31
CD4	Cluster of Differentiation 4
CD45	Cluster of Differentiation 45
CD8	Cluster of Differentiation 8
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
clF	Cyclic Immunofluorescence
CN	Copy Number
CNA	Copy Number Alteration
CODA	CODA (acronym does not stand for anything), code algorithm

COMBAT	ComBat (Empirical Bayes method for batch effect correction in genomics data)
CRC	Conditionally Reprogrammed Cell
CreERT2	Cre recombinase with modified estrogen receptor (ERT2)
CST	Cell Signalling Technologies, Inc.
CXCL12	C-X-C Motif Chemokine Ligand 12
DAB	Diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindole
DC	Dendritic Cell
DE	Differential Expression
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E2F	E2F Transcription Factors
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EIC	Early Invasive Carcinoma
EMT	Epithelial-Mesenchymal Transition
EPI	Exocrine Pancreatic Insufficiency
ERK	Extracellular Signal-Regulated Kinase
ERK1/2	Extracellular signal-regulated kinases 1 and 2
F-12	Ham's F-12 Nutrient Mixture
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FDR	False Discovery Rate
FFPE	Formalin-Fixed Paraffin-Embedded
FIJI	Fiji Is Just ImageJ
FINDS	Filters for Next Generation Sequencing, code algorithm
FOLFIRINOX	Leucovorin (folinic acid, FOL), 5-fluorouracil (F), irinotecan (IRIN), and oxaliplatin (OX)
G2M	Gap 2/Mitosis phase of the cell cycle
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
Gem	Gemcitabine
Gem/nAb-pac	Gemcitabine/nanoparticle Albumin-Bound Paclitaxel (also known as Gem/Ab)
GEMM	Genetically Engineered Mouse Model
GFP	Gonadal fat pad
GM	Gastrocnemius muscle
GO	Gene Ontology

ABBREVIATIONS

GSEA	Gene Set Enrichment Analysis
GSK	GlaxoSmithKline (Pharmaceutical Company)
GSVA	Gene Set Variation Analysis
H&E	Hematoxylin and Eosin Staining
HA	Hyaluronic Acid
HeLa	Human cervical cancer cell line, derived from cells from Henrietta Lacks
HG	High grade
HLA	Human Leukocyte Antigen
HPAF-II	Human Pancreatic Adenocarcinoma cell line II
hPSC	Human Pancreatic Stellate Cell
HR	Hazard ratio
HRP	Horseradish Peroxidase
HSR	Histopathology Shared Resource at OHSU, core facility
HUVEC	Human Umbilical Vein Endothelial Cell
I.P.	Intraperitoneal
IACUC	Institutional Animal Care and Use Committee
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-6	Interleukin 6
Ink4a	Inhibitor of Cyclin-Dependent Kinase 4a (also known as p16, or specifically as p16INK4A in humans)
IPMN	Intraductal Papillary Mucinous Neoplasm
IRB	Institutional Review Board
JQ1	BET bromodomain inhibitor JQ1
KC	GEM: LSL-Kras ^{G12D/+} ; Pdx1-Cre
KC ^{ERT2}	GEM: LSL-Kras ^{G12D/+} ; Ptf1a-Cre ^{ERT2}
KMC ^{ERT2}	GEM: LSL-Kras ^{G12D/+} ; LSL-ROSA-Myc ; Ptf1a-Cre ^{ERT2}
KPC	GEM: LSL-Kras ^{G12D/+} ; Trp53 ^{R172H/+} ; p48-Cre
KRAS	KRAS Proto-Oncogene, GTPase, Kirsten Rat Sarcoma Viral Oncogene Homolog
KRAS ^{G12C}	KRAS Gene Mutation (G12C)
KRAS ^{G12D}	KRAS Gene Mutation (G12D)
LAZR	Label-free and Automated Zonal Reactivity
LG	Low grade
LSL	Lox-Stop-Lox
Ly6G	Lymphocyte Antigen 6 Complex, Locus G
MAPK	Mitogen-Activated Protein Kinase
MCERT2	GEM: LSL-ROSA-MYC ; Ptf1a-Cre ^{ERT2}
MDSCs	Myeloid-Derived Suppressor Cells
MEK1/2	Mitogen-activated protein kinase kinase 1 and 2
mFOLFIRINOX	Modified FOLFIRINOX (also known as mFXX)
miHC	Multiplex Immunohistochemistry

MPSSR	Massively Parallel Shared Sequencing Resource at OHSU (core facility)
mRNA	Messenger RNA
MRTX1133	Small molecule inhibitor of KrasG12D mutation
MSC	Mesenchymal Stem Cell
MYC	MYC 'myelocytomatosis' proto-oncogene, BHLH transcription factor
nAb	Nanoparticle Albumin-bound
nAb-paclitaxel	Nanoparticle Albumin-bound Paclitaxel (also known as Abraxane, nAb-paclitaxel, or nAb-pac)
NCBI	National Center for Biotechnology Information
NE	Neuroendocrine
NES	Normalized Enrichment Score
NGF	Nerve Growth Factor
NGF/TrkA	Nerve Growth Factor/Tropomyosin Receptor Kinase A
NGS	Next-Generation Sequencing
NOTCH	Notch Signaling Pathway
O.G.	Oral Gavage
OHSU	Oregon Health & Science University
OMO	Omomyc
OMO-103	Anti-MYC miniprotein therapeutic
OPTR	Oregon Pancreas Tissue Registry at the BCCPC at OHSU
OX	Oxaliplatin
p48	Protein 48 (also known as Ptf1a)
p48-Cre	Protein 48-Cre
p53	Protein 53
PanCK	Pan-Cytokeratin
PanIN	Pancreatic Intraepithelial Neoplasia
PANTHER	Protein ANALysis THrough Evolutionary Relationships
PARP	Poly (ADP-Ribose) Polymerase
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
PD-L1	Programmed Death-Ligand 1
PDAc	Pancreatic ductal adenocarcinoma
PDCL	Patient-Derived Cell Line
PDMC	Patient-Derived Model of Cancer
PDX	Patient-Derived Xenograft
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PI3K/AKT/GSK	Phosphoinositide 3-Kinase / AKT / Glycogen Synthase Kinase

ABBREVIATIONS

PIN1	Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1
PK	Pharmacokinetics
PLL	Poly-L-Lysine
PP	Pancreatic Polypeptide
PP2A	Protein Phosphatase 2A
pS62	Phospho-Serine 62; phosphorylated Serine 62 locus on MYC gene
pS62-MYC	MYC, phosphorylated at Serine 62; indicative of an active and stable state
PSC	Pancreatic Stellate Cell
Ptf1a	Pancreas Transcription Factor 1a (also known as p48)
Ptf1a-Cre	Pancreas Transcription Factor 1a-Cre
Ptf1a-Cre ^{ERT2}	Pancreas Transcription Factor 1a-Cre ^{ERT2}
PVDF	Polyvinylidene Fluoride
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat Sarcoma Viral Oncogene Homolog
RGB	Red Green Blue (color palette)
RMC-6236	RAS Multi-targeting Compound 6236
RMC-6291	RAS Mutant Compound 6291
RNA	Ribonucleic Acid
RNAseq	RNA sequencing
ROCK	Rho-Associated Protein Kinase
ROI	Region of Interest
RPM	Revolutions per Minute
RRID	Research Resource Identifier
RT	Room temperature
RTK	Receptor Tyrosine Kinase
S62	Serine 62
SD	Standard Deviation
shPIN1	shRNA to PIN1
shRNA	Short Hairpin RNA
SMAD4	SMAD Family Member 4
SNV	Single Nucleotide Variant
SoC	Standard of Care
STR	Short Tandem Repeat (sequencing)
T58	Threonine 58
TBST	Tris-Buffered Saline with Tween
TCGA	The Cancer Genome Atlas
TEMPUS	Tempus (Genomic and clinical data technology company)
TGF	Transforming Growth Factor
TGF β	Transforming Growth Factor Beta
TiME	Tumor immune microenvironment

TMA	Tissue Microarray
TME	Tumor microenvironment
TP53	Tumor Protein 53
TPM	Transcripts per million
UTSW	University of Texas Southwestern
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VST	Variance stabilizing transformation
WES	Whole Exome Sequencing
WGS	(Low pass) Whole Genome Sequencing
Y-27632	ROCK inhibitor

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ABSTRACT

Pancreatic Ductal Adenocarcinoma (PDAC) ranks among the top three most aggressive cancers in the United States and is projected to increase in incidence over the next few years. Standard-of-care (SoC) treatment for PDAC consists of a cocktail of harsh chemotherapies, which have improved overall survival by only a few percentage points—to a 5-year survival rate of 13%. This dismal prognosis is a result of its primarily late-stage diagnosis and lack of durable response to treatment, the latter of which is partially due to PDAC's desmoplastic and immunosuppressive microenvironment. One commonly deregulated pathway in PDAC is *c-MYC* (*MYC*). *MYC* is a transcription factor that plays an important role in tumor progression and whose overexpression has been linked with tumor aggressiveness, immune suppression, increased metastatic potential, and therapeutic resistance in cancers including PDAC. In creating a novel genetically engineered mouse model (GEMM) of *MYC*-driven PDAC and bioprinted PDAC models, the aim of my dissertation work has been to better understand and recapitulate the human disease's microenvironment and biologic properties of the tumor cells in an effort to improve clinical translation of preclinical research.

In this dissertation, I will describe the generation and characterization of our novel PDAC GEMM, the KMC^{ERT2} (LSL-*Kras*^{G12D};Rosa-LSL-*Myc*;Ptf1a-Cre^{ERT2}) mouse. This model has inducible, Cre-driven expression of both mutant *Kras* and low-deregulated *Myc* in the pancreas. I will show that while this level of deregulated *Myc* is not tumorigenic on its own, it cooperates with *Kras*^{G12D} in the adult pancreas to drive PDAC. I will also describe how the KMC^{ERT2} model nicely recapitulates histologic and transcriptomic features seen within clinical PDAC samples. Human PDAC is often resistant to SoC therapies such as gemcitabine/nAb-paclitaxel and FOLFIRINOX. Orthotopic therapeutic studies using cell lines derived from our KMC^{ERT2} tumors demonstrate a similar resistance to these

therapies, allowing us to use this model to better understand the mechanisms leading to therapeutic resistance and to test new therapies. In addition, we find consistent metastasis to the liver in both spontaneous and orthotopic transplant settings. Together, this work investigates the role of deregulated *MYC* expression in metastatic behavior, immune phenotypes, and therapeutic response in murine PDAC. It also provides both spontaneous and orthotopic mouse models of PDAC that recapitulate the heterogeneous and highly metastatic nature of the human disease, allowing for important therapeutic testing opportunities.

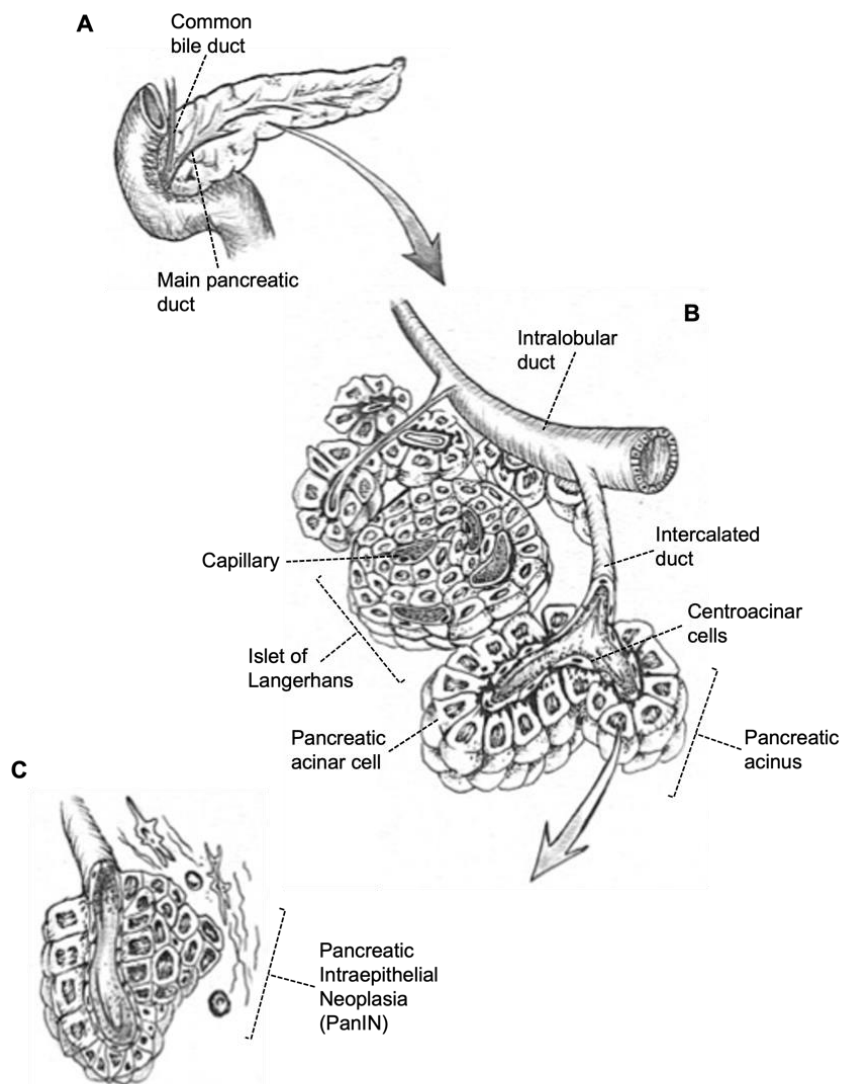
While current preclinical mouse models do facilitate important advances in scientific knowledge, as I will show when discussing our KMC^{ERT2} model, fewer than 11% of ensuing clinical trials produce actionable results in the form of FDA-approved therapeutic regimens. I will further explore non-murine preclinical models of disease. I will investigate the use of extrusion-based 3D-bioprinting technology to create disease- and, eventually, patient-specific tumor models, allowing for additional drug screening and personalized treatment approaches. This dissertation will demonstrate the potential of both the novel KMC^{ERT2} mouse model and 3D-bioprinting to improve our understanding of pancreatic cancer progression and treatment response, ultimately leading to more effective therapies for patients.

CHAPTER 1: INTRODUCTION

THE PANCREAS IN HEALTH AND DISEASE

Function

The pancreas is a vital organ in the human body that plays a crucial role in maintaining homeostasis. It is located behind the stomach and is responsible for producing both endocrine and exocrine secretions that are essential for regulating blood glucose levels, digesting food, and maintaining overall health [1, 2].



McKinna Tillotson

Figure 1.01 Illustrated representation of the pancreas and its main components.

Particular focus is placed on **A)** position of pancreas in the body, **B)** pancreatic lobule comprised of an Islet of Langerhans and multiple pancreatic acini, and **C)** progression from normal acinus to pancreatic intraepithelial neoplasia (PanIN). This illustration was drawn by Dr. McKinna Tillotson with the express intent of publication in this dissertation.

The exocrine compartment of the pancreas makes up 80-90% of the gland and is comprised of acinar cells, which are responsible for producing and releasing digestive enzymes which are then carried through ducts into the small intestine (**Fig. 1.01**) [1]. These enzymes help break down proteins, fats, and carbohydrates into smaller molecules that can be absorbed by the body. The exocrine compartment also includes duct cells, which secrete bicarbonate ions to neutralize stomach acid and create an optimal environment for enzyme activity. Together, these components of the exocrine compartment play a crucial role in the digestion and absorption of nutrients in the body.

The endocrine compartment of the pancreas is comprised of five cell types that make up the Islet of Langerhans. These cell types include alpha cells, beta cells, delta cells, gamma cells, and epsilon cells. Each cell type plays a crucial role in regulating blood sugar levels and maintaining overall metabolic balance in the body [3]. Alpha cells are responsible for producing and secreting glucagon, a hormone that raises blood sugar levels. Beta cells, on the other hand, produce and release insulin, which helps lower blood sugar levels. Delta cells secrete somatostatin, a hormone that inhibits the release of both glucagon and insulin. Gamma cells, also known as pancreatic polypeptide (PP) cells, produce pancreatic polypeptide, which helps regulate digestion and nutrient absorption. Epsilon cells help stimulate appetite by secreting ghrelin, but their function(s) are still being elucidated [4].

Dysfunction

The dysfunction of each type of pancreatic cell can lead to varied pathogenic states [1], such as hyperglycemia, somatostatin deficiency, exocrine pancreatic insufficiency (EPI), and diabetes mellitus. Diabetes mellitus, or diabetes, is a chronic metabolic disorder characterized by high blood sugar levels that is the result of beta cell dysfunction [5, 6]. It

occurs when the body either does not produce enough insulin or is unable to effectively use the insulin it produces. This dysfunction can be caused by a variety of factors, including genetic predisposition, obesity, and an unhealthy lifestyle, leading to damage to the insulin producing pancreatic beta cells; in turn resulting in an imbalance in blood sugar regulation [7–9]. If left untreated, diabetes can lead to serious complications such as heart disease, kidney damage, or nerve damage, and has been shown to predispose patients to PDAC [9, 10]. Interestingly, diabetes can also be secondary to pancreatic cancer and pancreatitis [5, 7, 11].

Pancreatitis is a serious pancreatic dysfunction condition characterized by inflammation of the pancreas. The most common causes of pancreatitis are gallstones (40% acute cases) and excessive alcohol consumption, but others include cigarette smoking, certain medications (e.g. estrogens, steroids, thiazide diuretics), diabetes, and pancreatic tumors [12–16]. Pancreatitis is either acute—characterized by sudden and severe abdominal pain, nausea, and vomiting—or chronic. Acute pancreatitis can be a life-threatening condition if not treated promptly, but it often resolves with proper medical intervention [12, 14, 17–19]. Chronic pancreatitis, on the other hand, is a long-term condition that can lead to permanent damage to the pancreas and that impairs its ability to produce digestive enzymes and insulin [13, 15, 20]. It is typically characterized by recurring episodes of abdominal pain, weight loss, and malabsorption of nutrients. Both conditions require medical intervention and lifestyle changes to manage symptoms and prevent further complications. When left unchecked, acute pancreatitis can lead to chronic pancreatitis, which significantly increases a patient's chance of developing pancreatic cancer [21–23].

PANCREATIC DUCTAL ADENOCARCINOMA

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer. Representing only 3% of annual cancer diagnoses in the United States but 8% of cancer-

related deaths, estimated at 66,440 new cases and 51,750 deaths in 2024 [24], PDac is arguably the most aggressive cancer. PDac is currently the third leading cause of cancer-related deaths in the United States, with a five-year survival rate of only 13%, and it is projected only to increase in incidence over the coming years [24]. The pancreas sits deep in the abdomen, making it difficult to palpate and, therefore, without additional specific symptoms, pancreatic cancer is difficult to detect at early stages (**Fig. 1.02**). Most patients present with late-stage disease after seeking medical attention for jaundice, chronic pain, weight loss, fatigue, or change in digestion. This late stage detection and diagnosis is one of the main drivers of the dismal prognosis of PDac [25–30]. Studies of RNA expression in PDac across patients have shown the disease to have two main molecular subtypes: the classical/ductal/glandular subtype and the basal-like/quasi-mesenchymal/squamoid subtype [31–36]. While patients with classical-subtype tumors generally survive longer than those with basal-like-subtype tumors, both subtypes nonetheless result in unfavorable outcomes for patients [37–39]. Ongoing work is being done to determine whether subtyping of pancreatic cancer has clinical utility, for example, in the decision making around treatment.

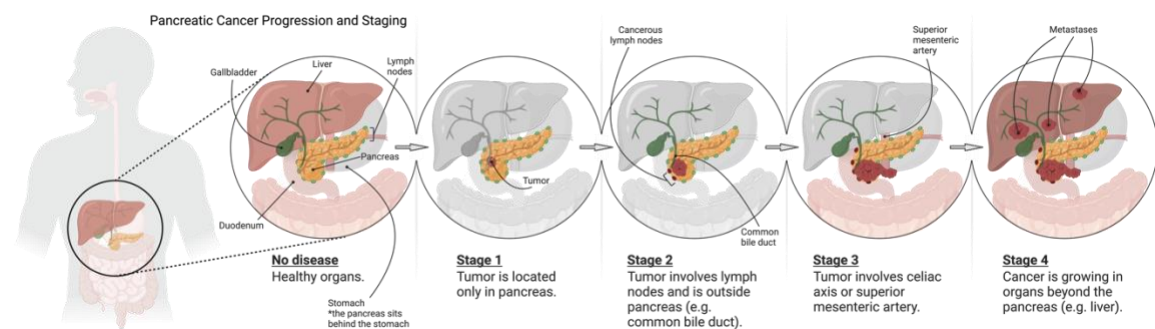


Figure 1.02 Schematic representation of the pancreatic cancer disease progression and staging.
Figure created by author with BioRender.com

Pancreatic cancer development

While genetic predisposition to PDAC accounts for approximately 10% of cases [40], researchers have also identified a growing list of modifiable risk factors that contribute to a majority of new diagnoses [41]. In addition to chronic pancreatitis (hazard ratio (HR) = 16.16), smoking (HR = 1.4-3.38), and excessive alcohol consumption (HR = 1.2), which have been understood as risk factors for many years, causative risk factors now include: a lack of physical activity, diabetes (HR = 1.85), obesity (HR = 3.85), diet, viral hepatitis, microbial dysbiosis, and environmental exposures to chemicals included in pesticides, asbestos, benzene, and chlorinated hydrocarbons [41–44]. It is important to note that for a majority of PDAC patients, more than one of these risk factors applies and they often correlate synergistically with an increased risk of PDAC development. For example, one study performed in Italy showed that while many pancreatic cancers could be attributed to excessive drinking (13.0%) or tobacco smoking (13.6%), this proportion nearly doubled when the two risk factors were considered together (25.7%) [44].

One of the challenges of studying PDAC development is that it often develops slowly, with a premalignant disease that does not cause symptoms. Research has shown that pre-malignant lesions can exist for many years before progressing to invasive carcinoma. Pancreatic intraepithelial neoplasms (PanINs) are the most common pre-malignant lesions of the pancreas, and are classified into two grades based on the level of dysplasia (**Fig. 1.03**). Low-grade PanINs are characterized by mild cellular atypia, while high-grade PanINs show more severe dysplasia and are more likely to progress to invasive carcinoma. By the time a patient is diagnosed, the cancer has often spread beyond the pancreas, making treatment more difficult.

A number of genetic and transcriptomic studies generating robust datasets from human PDAC have identified key molecular drivers of the disease. The most prevalent driver is that of the Kirsten Rat Sarcoma viral oncogene homologue (*KRAS*), present in

approximately 84-95% of human PDAC cases [45–47]. Through the use of genetically engineered mouse models, acinar cells have been shown to be extremely susceptible to activating mutations in *Kras*, which are sufficient to initiate a progression from normal acini towards a ductal phenotype [48, 49]. This transdifferentiation is known as acinar to ductal metaplasia (ADM). While mutant activation of *KRAS* is an early and near-universal event in PDAC [50, 51], it is not sufficient to drive disease progression past PanIN stages, and the acquisition of additional genetic mutations is required during the evolution to invasive ductal adenocarcinoma (**Fig. 1.03**) [52]. These additional mutations often occur in tumor suppressor genes *TP53* (56-72%), *CDKN2A* (30-50%), and *SMAD4* (32-50%) [45–47, 53–64]. The loss of *CDKN2A* expression initiates the transition from ADM into early PanIN stages [65, 66]. The subsequent loss of *TP53* and/or *SMAD4* expression further drives the progression into high-grade PanIN stages and eventually fully developed PDAC [67–70].

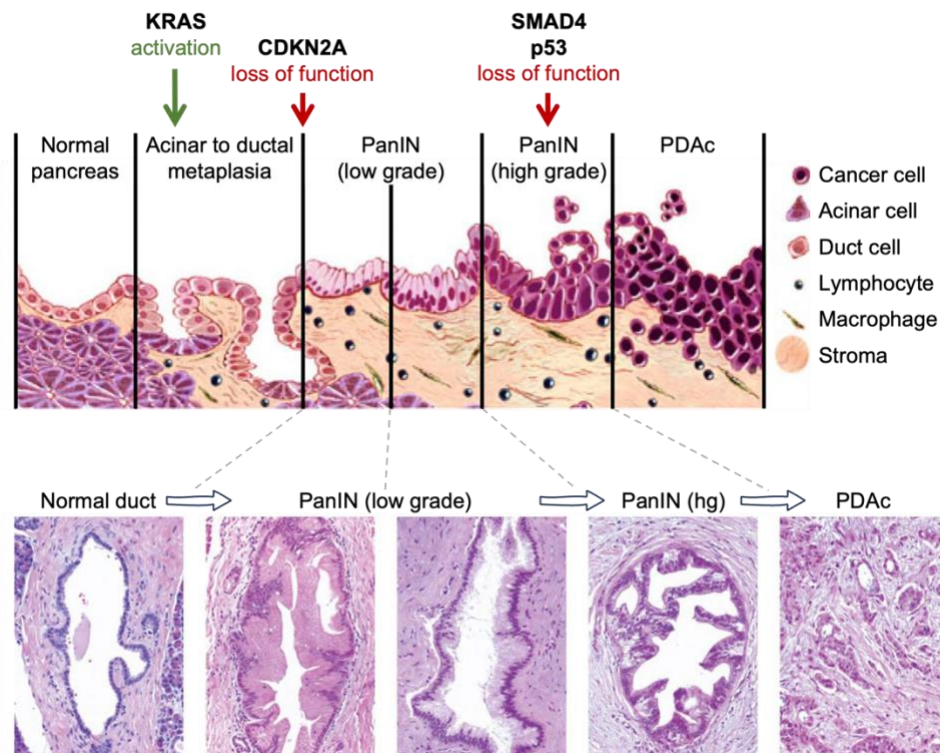


Figure 1.03 Schematic representations and hematoxylin & eosin-stained tissue images of progression of normal pancreas to invasive PDAC.

Particular focus is made on acquisition of key mutations and development of desmoplastic stroma as disease progresses. **Top)** Schematic illustration of normal exocrine pancreas progressing through ADM and into increasingly dysplastic PanIN and PDAC. **Bottom)** Hematoxylin-eosin-stained tissue sections. hg = high grade. Figure was inspired by Škoda, 2016 [71]. Images originally appeared in Logsdon, Ji, and Hwang, 2010 [72], and Hezel et al., 2006 [73] and have been adapted and reprinted with permission from the publisher and authors under the Creative Commons license, respectively. To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>.

KRAS

KRAS, a member of the RAS family of GTPases, plays a pivotal role in regulating cell proliferation, differentiation, and survival through various signaling pathways, including the *RAF/MAPK/ERK* and *PI3K/AKT* signaling cascades (**Fig. 1.04A** [74]). *KRAS* activating mutations are the most pervasive genetic alteration—an estimated 40% prevalence—across all cancers [75–77]; but they are a truly *defining* feature of PDAC pathogenesis [45–47, 78]. In fact, an innovative three-dimensional genomic mapping study recently identified at least one *KRAS* mutation in 97% of PanIN precursor lesions [50], further emphasizing their importance in the early stages of PDAC tumorigenesis. These mutations occur predominantly at codon 12 (e.g. *KRAS*^{G12D}) [50, 78], and promote cell survival via constitutive activation of the *KRAS* protein independent of receptor tyrosine kinase (RTK) signaling [74]. Activated *KRAS* can lead to activation of downstream transcription factors. For example, activated *KRAS* engages *RAF*, leading to the sequential activation of *MEK1/2* and *ERK1/2*. In turn, activated *ERK1/2* translocates into the nucleus, where it phosphorylates and activates major transcription factors like *c-MYC* (*MYC*) [79, 80], driving gene expression associated with cellular proliferation and survival [74, 81, 82].

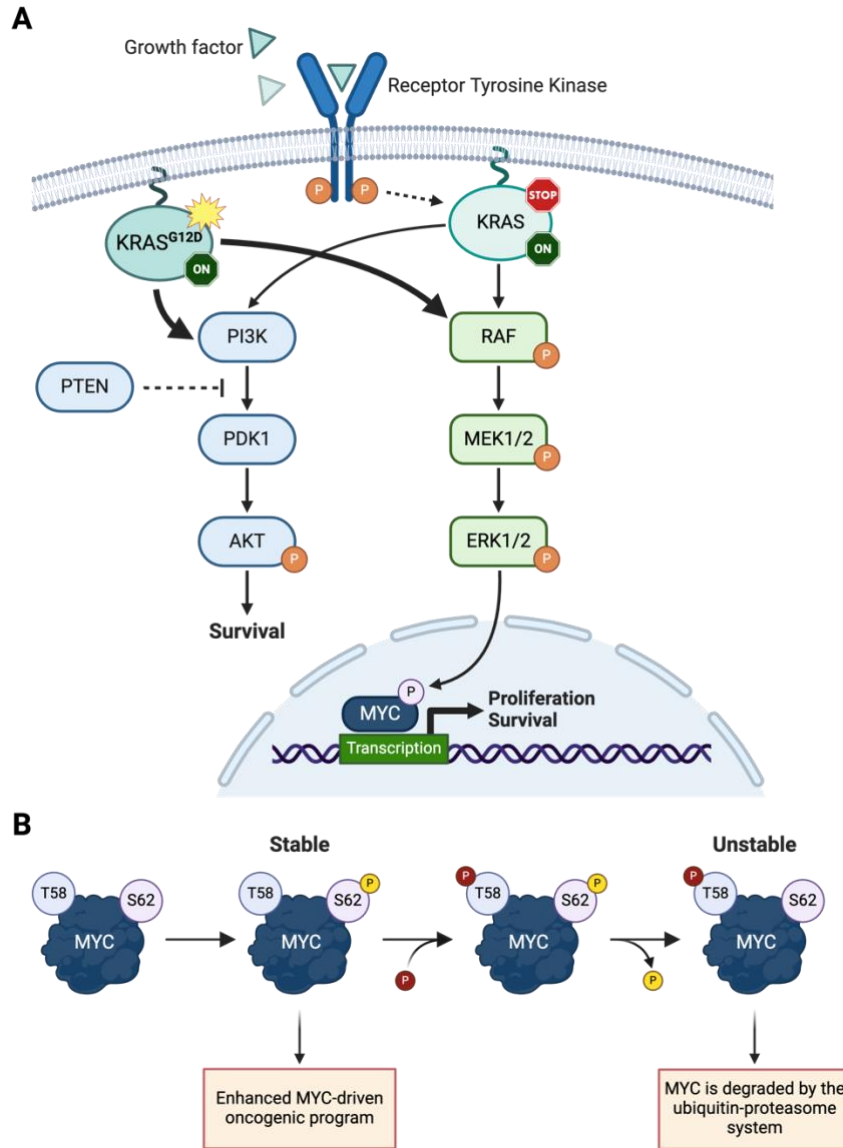


Figure 1.04 KRAS-mediated MYC activation and stabilization in pancreatic ductal adenocarcinoma.

A) *KRAS* signaling pathways leading to MYC activation in PDAC. The *KRAS*^{G12D} mutation causes constitutive activation of the *KRAS* protein (marked as "ON"), independent of receptor tyrosine kinase (RTK) signaling. Shown in the schematic are multiple pathways through which *KRAS*^{G12D} promotes cell survival and growth in PDAC. B) *MYC* stability and oncogenic function. Schematic showing how MYC stability is influenced by phosphorylation at two key residues: Threonine 58 (T58) and Serine 62 (S62). Panel B originally appeared in Boi, Int J Mol Sci, 2023 [104] and has been adapted and reprinted with permission from the authors under the Creative Commons license. Both panels were created and/or adapted with BioRender.com.

c-MYC

MYC is a proto-oncogene that encodes a potent master transcription factor, a protein that binds to DNA and regulates the expression of other genes. In normal cells, *MYC* is involved in numerous cellular processes, including cell growth, differentiation, and

apoptosis [83]. MYC protein expression is controlled at several levels, including gene transcription, mRNA stabilization, translational control, and changes in protein stability [84, 85]. Our lab has elucidated a RAS-regulated signaling pathway that post-translationally controls MYC protein stability and transcriptional activity [86–88], as is depicted by Boi et al., in **Figure 1.04B**. Normal MYC expression is very tightly regulated by a number of protein kinases, phosphatases, and isomerases. MYC stability increases with phosphorylation at serine 62 (S62) by ERK or CDKs [89]. Its subsequent phosphorylation at threonine 58 (T58) by GSK3 β begins the process for downregulation and degradation of the protein [80]. Overall, the phosphorylation state of MYC determines its stability and activity, with stable MYC enhancing proliferation, while unstable MYC is targeted for degradation to maintain cellular homeostasis.

Alongside its essential role in normal cellular processes, *MYC* is also one of the most commonly deregulated genes across cancers [90, 91]. When deregulated, *MYC* can promote tumor aggressiveness and therapeutic resistance in cancers [92–97]. Its overexpression has been observed in many types of cancer, including lymphoma [98, 99], leukemia [100], breast [101–106], lung [104–106], colon [107], and PDAC [94, 108, 109]. The dominant alteration in MYC across cancers involves an increase in copy number, but even when RNA levels remain unchanged, protein stabilization is often observed [101, 110, 111]. Multiple post-transcriptional mechanisms—the dysregulation of RNA binding proteins that stabilize MYC mRNA [112, 113], the repression of MYC-targeting microRNAs such as miR-29 [114], and the inhibition of the tumor suppressor PP2A [115], which normally regulates MYC stability—have been shown to contribute to observed MYC overexpression in pancreatic cancer. As *KRAS* is an upstream activator of *MYC*, increasing its stability and DNA binding [80], these two oncogenes cooperate in driving multiple cancers, including lung cancer [106, 116] and PDAC [93, 105, 117–119]. A study

by Walz et al. showed that genetic loss of endogenous *MYC* in mutant *KRAS* models inhibits PanIN progression to PDAC [120].

The PDAC tumor microenvironment & desmoplastic response

PDAC is characterized by an exceptionally complex and dynamic tumor microenvironment (TME), which is pivotal not only in the progression of the disease but also in its notorious resistance to therapy. As PDAC progresses from PanIN to invasive carcinoma, profound alterations occur within the TME, driven by both cancer cell intrinsic genetic mutations and extrinsic microenvironmental factors. Key mutations in genes such as *KRAS*, *TP53*, *SMAD4*, and *CDKN2A* that initiate and sustain the malignant transformation of pancreatic ductal cells also contribute to changes in the TME [25]. For example, the activation of *KRAS* triggers the recruitment and activation of PSCs, leading to the formation of a dense extracellular matrix (ECM) [121, 122]. Intrinsic signals, including those from mutated *KRAS*, can also modulate the immune response within the TME [22, 123], often leading to an immunosuppressive environment that further complicates effective treatment. Together, the altered TME in PDAC is not just a passive backdrop but rather an active participant in shaping the physical and biological behavior of the tumor.

The desmoplastic response in the PDAC TME is a hallmark feature that involves the extensive proliferation of stromal cells and the excessive deposition of ECM components, particularly collagen [121, 124, 125]. This dense, fibrous stroma both provides structural support to the tumor while simultaneously promoting tumor growth, chemoresistance, and immune evasion [126]. This fibrotic stroma acts as a physical barrier, impeding the penetration of therapeutic agents and contributing to the hypoxic and nutrient-deprived conditions within the tumor, thereby fostering an environment conducive to tumor survival and progression [127–129]. The cancer-associated fibroblasts (CAFs) within the TME are crucial drivers of this desmoplastic response. As depicted in **Figure 1.05**, CAFs, which

can arise from a variety of sources including resident fibroblasts, pancreatic stellate cells (PSCs), and bone marrow-derived mesenchymal stem cells (MSCs), play a central role in maintaining the tumor-supportive microenvironment [128, 130–132]. Single cell approaches to analyzing the TME have revealed multiple subtypes of CAFs that are spatially and temporally regulated during tumor development [122, 128, 130, 133, 134]. Myofibroblast-like CAFs (myCAFs) are primarily involved in ECM deposition, contributing to the creation of the dense fibrotic stroma that hinders immune cell infiltration and supports tumor growth through intricate cell-to-cell interactions [122, 124, 130]. In contrast, inflammatory or immunomodulatory CAFs (iCAFs) secrete pro-inflammatory cytokines and growth factors such as TGF β , VEGF, IL-6, and CXCL12, which promote tumor progression by enhancing immunosuppression, angiogenesis, and tumor cell proliferation [130, 135, 136]. Distinct functions of CAF subtypes in both structural support and immune modulation can make them key players in the progression of PDAC. For example, PSC-derived CAFs comprise approximately 10-15% of the CAF population [128, 137]. In their quiescent state, PSCs store retinoids as lipid droplets, but upon activation by oncogenic signals, they transform into myofibroblast-like cells that generate the dense fibrotic stroma characteristic of PDAC [138–140]. PSC-derived CAFs have a unique function in establishing an aggressive TME, providing unique components of the ECM [137].

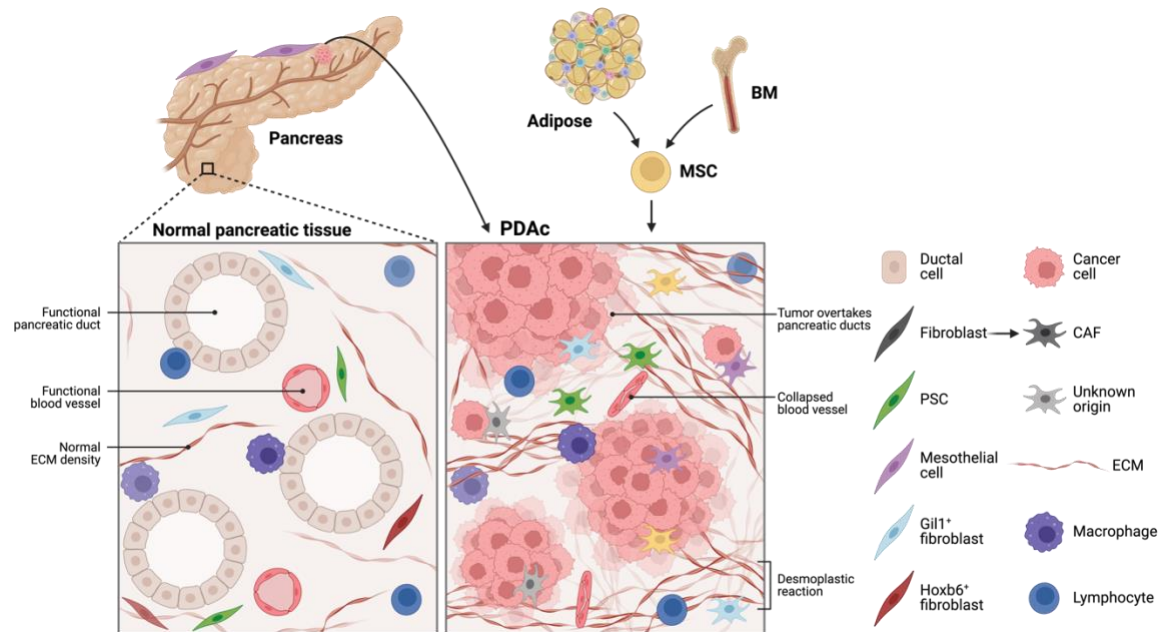


Figure 1.05 Histopathological changes and cellular composition of PDAC.

Model depicting histological features and cellular composition of PDAC, highlighting CAF heterogeneity and cellular origins. Upper panel shows a schematic of the pancreas, with an inset highlighting the transition from normal pancreatic tissue to PDAC. Lower left panel represents a detailed view of normal pancreatic tissue, including pancreatic ducts surrounded by acinar cells, along with normal stromal cells, such as fibroblasts and immune cells. Lower right panel details the characteristic dense desmoplastic stroma of PDAC, infiltrated by various cell types, including CAFs, immune cells, and endothelial cells. CAFs in the TME are shown to arise from tissue-resident mesenchymal populations as well as cell types recruited into the microenvironment during tumor progression. Determinants of the relative contributions of these diverse cell types to the CAF population include but are likely not limited to tumor genotype, as well as cytokine and growth factors gradients. Abbreviations: BM, bone marrow; CAF, cancer-associated fibroblast; ECM, extracellular matrix; MSC, mesenchymal stem cell; PDAC, pancreatic ductal adenocarcinoma; PSC, pancreatic stellate cell; TME, tumor microenvironment. Figure and figure legend originally appeared in Sherman and Pasca di Magliano, 2023, and are adapted and reprinted with permission from the publisher. Figure further adapted with BioRender.com. and figure legend originally appeared in Sherman and Pasca di Magliano, 2023 [132], and has been adapted and reprinted with permission from the authors under the Creative Commons license. To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>.

PDAC also employs sophisticated mechanisms to subvert the immune system both locally within the tumor microenvironment and systemically throughout the body. Locally, PDAC creates an immunosuppressive microenvironment by recruiting and activating various immune cells such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) [141–143]. These cells secrete immunosuppressive cytokines, including TGF- β , IL-10, and CXCL12, which inhibit the activation and infiltration of cytotoxic T lymphocytes (CTLs), the primary immune cells responsible for attacking tumor cells [28, 144, 145]. Additionally, the dense desmoplastic

stroma acts as a physical barrier, further preventing immune cell infiltration and fostering an immune-privileged site where the tumor can evade detection and destruction [132, 146, 147]. Systemically, PDAC induces a state of chronic inflammation and immune dysregulation, characterized by elevated levels of circulating immunosuppressive cytokines and the expansion of immunosuppressive cell populations [142, 148, 149]. This systemic immunosuppression not only blunts the immune system's ability to mount an effective anti-tumor response but also promotes the establishment of pre-metastatic niches in distant organs, facilitating metastasis [145, 150]. Furthermore, PDAC manipulates the immune checkpoint pathways, such as PD-1/PD-L1, to inhibit T cell activation and sustain immune tolerance to the tumor, thereby undermining the effectiveness of immune surveillance and contributing to the overall immune evasion strategy of the cancer [30, 144, 151].

Despite the challenges posed by the desmoplastic stroma and suppressive tumor immune microenvironment (TiME), emerging evidence suggests that a subset of PDAC tumors exhibits moderate to high T cell infiltration, challenging the traditional view of PDAC as an immunologically "cold" tumor [30, 152]. This evidence indicates that immunotherapy could be more broadly applicable in PDAC, provided that the appropriate patient populations can be identified. Identifying these populations and unraveling the complexities of the TME will be critical for enhancing the effectiveness of immunotherapies and developing strategies that can overcome the inherent resistance of PDAC to current treatments [26, 153–155].

MODELS OF PDAC

Since defining the genetic features of PDAC, researchers have sought to mimic these changes in a laboratory setting in order to further elucidate the biology of the disease and to test therapeutic strategies.

Increasing the complexity of a model can increase its biological relevance, but this often comes with a trade-off in throughput, limiting our ability to reproduce and validate results in a timely and cost-efficient manner. Bearing that in mind, research questions are often approached from different angles and answered using multiple models of disease (**Fig. 1.06**).

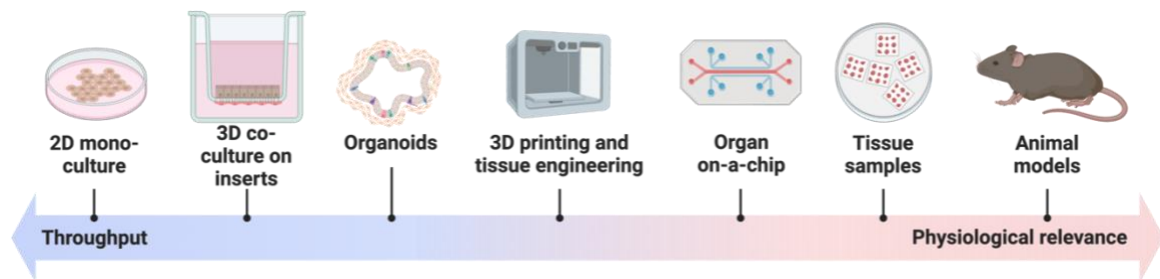


Figure 1.06 Pancreatic cancer tissue culture and engineering platforms.

Model systems for pancreatic cancer research displayed on a continuum from highest throughput to most physiologically relevant. Created with Biorender.com.

In vivo models

Our understanding of pancreatic cancer biology has been greatly advanced with mouse models of the disease. Ever since the early development of genetically modified mouse models (GEMMs) to study PDAC [22, 82, 156, 157], GEMMs have proven to be an indispensable resource for investigating various aspects of PDAC biology, its microenvironment, and translational research. Some of the many emerging areas of study that are being explored using GEMMs include early detection of tumors [158], identification of essential biomarkers [156], evaluation of novel therapeutics [159, 160], and investigation into the processes underlying drug resistance [161–163]. As previously mentioned, the understanding of pancreatic carcinogenesis has been advanced through the examination of *KRAS* activation and the gradual loss of tumor suppressor genes such as *CDKN2A*, *p53*, and *SMAD4*. Mouse models utilizing *KRAS*^{G12D} expression in the pancreas have been developed to effectively replicate the observed initiation and progression in human PDAC [82, 156, 164]. These models have greatly contributed to our

comprehension of the involvement of high-frequency driver genes and biochemical pathways in the development of pancreatic tumors.

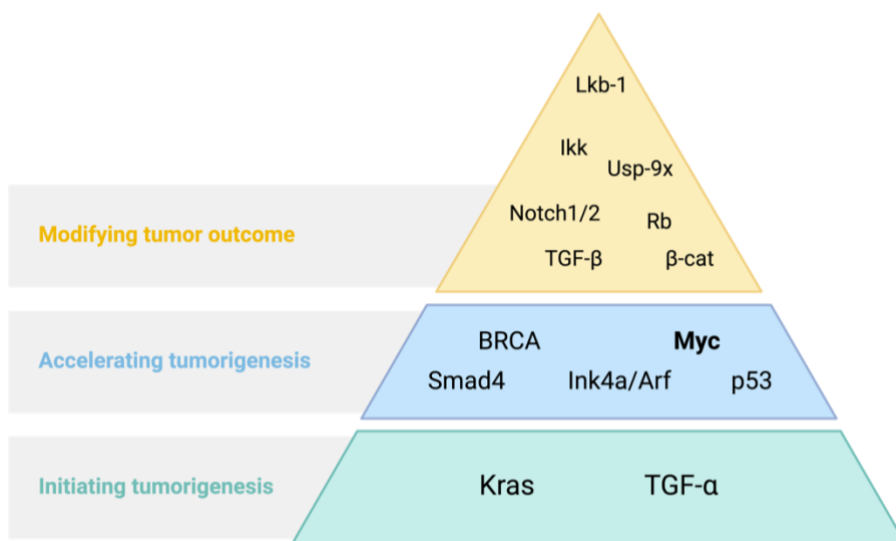


Figure 1.07 Genetic alterations that act as drivers of human pancreas cancer used for the generation of PDAC GEMMs.

Similar figure originally appeared in Mota-Reyes et al., *Pancreapedia*, 2021 [164], and is adapted with the addition of MYC and reprinted with permission from the publisher.

Today, a majority of GEMMs of pancreatic cancer are built on one known as the "KC" mouse. The KC mouse features the conditional activation of an endogenous knocked-in lox-stop-lox- (LSL-) *KRAS*^{G12D} oncogene in the pancreatic lineage that is activated after excision of the stop codon upon Cre expression [82]. These mutant *KRAS*^{G12D} mice can be bred with transgenic strains expressing either *Pdx1*-Cre or *Ptf1a*/p48-Cre to specifically target pancreatic progenitor cells for Cre-recombination. *KRAS* activation increases mitogen activity and activates downstream proteins like ERK and MYC. The *KRAS*^{G12D} mutation was identified as the initial event in PDAC, as the pancreata of KC mutant mice developed the entire spectrum of human PanIN lesions with complete penetration. In advanced age (12–15 months), invasive tumors emerged following a prolonged latent period, in line with the corresponding human condition [82]. The utilization of KC mice in translational therapy strategies is significantly constrained due to the delayed and unpredictable onset of pancreatic cancer. Over the past decade, researchers have

developed various GEMMs to expedite the progression of tumors toward invasive PDAC. This has been achieved by introducing additional genetic modifications in loci that encode tumor suppressor genes commonly found mutated in human pancreatic cancer, including *CDKN2A/p16^{Ink4a}*, *TP53*, *BRCA2*, and *SMAD4* (**Fig. 1.07**) [67, 68, 156, 165].

Arguably the most widely used PDAC GEMM, the KPC mouse, was developed in the same lab as, and just a couple of years after, that of the KC mouse [156]. It utilizes the same mutant *KRAS^{G12D}* allele (K) [82] in conjunction with a mutant *Trp53* allele (P) that not only produces a non-functional p53 protein, but also interferes with the activity of any remaining wild-type p53 present in the cell [166]. To initiate recombination in this model, researchers incorporated either the Pdx1-Cre or Ptf1a-Cre promoter, providing insights into how different cellular origins contribute to PDAC progression. Pdx1-Cre drives tumorigenesis in both acinar and ductal cells, while Ptf1a-Cre is more restricted to the acinar lineage, highlighting the impact of cell origin on tumor behavior [167].

Additionally, the KPC model has been expanded over the years with numerous iterations that allow for the study of specific mutations and their effects on tumor biology [168]. For example, comparing p53 mutation versus complete loss has shed light on the role of p53 in tumor progression and metastasis [169]. Other iterations have combined genetic alterations in *Kras* other tumor suppressors like *Cdkn2a* [68] or *Smad4* [67], or with environmental factors like chronic pancreatitis (induced by caerulein)[22], smoking [170], and obesity [171], to better understand their combined effects on tumor development and therapy resistance. Furthermore, inducible versions of these models, such as those using tamoxifen-inducible Cre^{ERT2} [172–174], offer temporal control over gene activation, enabling studies on the timing of oncogenic events in PDAC development. These models are invaluable for studying the stepwise progression from PanINs to invasive PDAC and

for dissecting the roles of cell of origin, additional mutations, and environmental insults in this process [48, 68, 156, 175].

In conclusion, the KC and KPC mouse models and their variants have provided valuable insights into the role of *Kras*, *p53*, and other drivers in PDAC development. They have been instrumental in identifying potential therapeutic targets and evaluating the efficacy of various treatment modalities, making them invaluable tools for translational research in PDAC. As new models continue to be developed, like the one we will discuss in Chapter 2, our understanding of PDAC's heterogeneity and complexity will only deepen, providing greater insights into this devastating disease.

In vitro models

In vitro models of cancer include the relatively simple cell line tissue cultures, which allow researchers high-throughput, cost-effective tools to study the behavior of cancer cells in a controlled laboratory setting. These models provide valuable information on tumor biology, drug response, and potential therapeutic targets. However, these 2D-*in vitro* monoculture models cannot fully replicate the complexity of the TME and may not accurately reflect the behavior of cancer cells *in vivo*.

To improve upon this simplistic model, researchers have used co-cultures of cancer cells together with cells of the TME to model the complex interactions between the different types. For example, a study published by Jiang and colleagues [176] found that co-culturing pancreatic cancer cells with pancreatic stellate cells resulted in increased cancer cell proliferation and invasion. This work allowed for the identification of NGF/TrkA secreted by the PSCs as a driver of this cancer cell proliferation and invasion through activation of the PI3K/AKT/GSK signal cascade, thus providing a potential therapeutic target for pancreatic cancer patients [176]. Culturing, or co-culturing, cells in spheroids with or without matrix proteins also increases the relevance of the cell line models.

Spheroids provide a platform that is still high throughput and relatively low cost, but enables the formation of 3D cell-cell interactions, which are crucial for tumor growth, invasion, and metastasis. A recent study demonstrated that cancer cells grown as tumor spheroids in a collagen matrix co-cultured with PSCs undergo EMT and become therapy resistant [177]. These types of studies together point toward the importance of tumor-stromal cell interactions in PDAC progression.

Organoids, self-organizing 3D structures that are grown in specialty media embedded in a basement membrane matrix, are also widely used as a model system. Organoids begin to resemble the architecture and function of their tissue of origin, and several studies have demonstrated the utility of organoid cultures in modeling pancreatic cancer [178–183]. Pancreatic tumors are known for their genetic and phenotypic diversity, which can make it challenging to identify effective therapies; but organoids can be derived from individual tumor cells, allowing researchers to study the heterogeneity of the tumor at the single-cell level. This has been demonstrated in a study by Boj and colleagues [184], who used organoids to identify subpopulations of pancreatic cancer cells with different drug sensitivities. Further, a study by Tiriác and colleagues used patient-derived organoids to investigate the effects of chemotherapy on pancreatic cancer [185]. This study and one by Huang and colleagues [186] compared organoid and traditional cell line model systems. Both found that chemoresistance observed in organoids more closely mirrored that seen in patient populations, highlighting the importance of using more clinically relevant models. This information could be used to understand mechanisms of drug resistance or to develop personalized therapies based on the genetic makeup of the tumor.

Engineered models

More deliberately engineered models of cancer include various techniques, such as complex 3D-cell culture systems, organ-on-a-chip, or 3D-bioprinting technologies. These

models enable researchers to mimic specific aspects of the tumor microenvironment and the function of human tissues and organs in ways that traditional models cannot. For instance, the incorporation of complex immune milieu [187] and the addition of shear stress forces to simulate the circulation of circulating tumor cells (CTCs) [188–190] provide a more accurate representation of cancer cell behavior. Organ-on-a-chip models, which consist of tiny channels and chambers lined with living cells, allow for the study of cellular responses to various stimuli in a highly controlled environment. A notable example in pancreatic cancer research is a study by Kramer and colleagues [191], where an organ-on-a-chip model was developed to mimic intratumoral pressure in PDAC using interstitial flow. This model and others demonstrate superior predictive capabilities for drug resistance compared to conventional 2D cell culture models [192–194], yet still leave room for improvement. One particular drawback of these types of models is the inability to collect cells for further analysis after experimentation, necessitating assays with exclusively visual results.

3D-bioprinting models, on the other hand, are designed to create three-dimensional structures that replicate not only the function but also the morphology of human tissues and organs. This technology employs specialized printers to construct intricate tumor models using bioinks made from living cells within a printable substrate. Among the various 3D-bioprinting techniques, extrusion-based bioprinting has gained attention for its ability to replicate the tumor microenvironment with precise control, particularly in pancreatic cancer models. For example, a recent study developed a bioprinted “sea-and-island” multicellular model that dissects pancreatic tumor-stroma reciprocity and adaptive metabolism [195]. Another study created novel 3D micro-tissues (μ tissues) that mimic the fibrotic stroma in pancreatic cancer, offering a platform to study cellular interactions and test stroma-modulating therapeutics [196]. Work in our lab, led by Drs. Langer and Allen-

Petersen, has also contributed to this field by creating PDAC models that capture the heterogeneity and complexity of tumor-stroma interactions [197]. This dissertation will further elaborate on this extrusion-based bioprinting technology and its capacity to replicate additional aspects of the tumor microenvironment and facilitate investigations into tumor-stromal-immune interactions in Chapter 3.

Overall, the use of organ-on-a-chip and 3D-bioprinting models has revolutionized the study of pancreatic cancer. These models provide researchers with innovative tools and techniques to develop more effective treatments and bridge the gap between preclinical models and clinical trials. As the field of cancer biology continues to evolve, these engineered models are likely to play an increasingly critical role in the fight against pancreatic cancer and other malignancies. By integrating these models with *in vivo* studies using animal models or patient-derived xenografts, researchers can achieve a more comprehensive understanding of the complex interactions between cancer cells and their surrounding environment.

Patient-derived models of cancer

Patient-derived models of cancer (PDMCs) vary greatly in their methodology and utility, but each one is first initiated by extracting cancer cells from consenting patients who have generously allowed us to learn from their tissue. PDMCs result from the direct implantation of small pieces of intact tumor into mice as patient-derived xenografts (PDXs) or carefully established as tissue explants. Patient samples can also be dissociated for expansion into primary cell lines to be used in *in vitro* assays, propagated as organoids, or integrated into more complex 3D micro-models (**Table 1.01**).

							
	Genomic Sequencing	Metabolomics/Proteomics	Patient-Derived Cell Lines	Patient-Derived Xenografts	Patient-Derived Tissue Explants	Patient-Derived Organoids	Patient-Derived 3D Micro-Models
Predictive Power	+++	++	+	+++	++	+++	+++
Predictable Therapies	T	T	C/R	T/C/IO*/R	T/C/IO/R	T/C/R	T/C/IO/R
Speed	+++	+++	+	-	+++	++	+++
Establishment Success from Biopsy	+++	++	-	+	-	+	++
Throughput	+++	+++	+	-	-	+	+++
TME	-	-	-	++*	+++	-	++
Reproducibility	+++	+++	++	+++	-	++	+++
Cost	+	+	+	+++	++	++	+

T = Targeted Therapies, C = Chemotherapies, R = Radiation, IO = Immuno-oncology Therapies, TME = Tumor Microenvironment
*PDX models include mouse stromal cells in the TME and require immune reconstitution with humanized mouse models to adequately recapitulate IO therapies.

Table 1.01 Comparison of advantages and disadvantages of current patient-derived tumor models.

Table originally appeared in Bose et al., Cancer Cell, 2022 [198], and is adapted and reprinted with permission from the publisher.

There has been much work devoted to developing PDMCs specifically for pancreatic cancer. Classical PDAC models include well-characterized cell lines, such as Panc-1 [199] and MIAPaCa-2 [200], which continue to serve as foundational research models [201]. More advanced systems like patient-derived organoids have also been utilized to reflect the tumor heterogeneity observed in patients, offering a more robust platform for studying drug responses [184]. Additionally, PDX models have helped recapitulate key aspects of PDAC biology, including the tumor-stromal interactions that play a pivotal role in drug resistance [202, 203].

These PDMCs, in combination, allow researchers to compare the behavior and treatment sensitivities of heterogeneous models that harbor different genetic and phenotypic characteristics [204]. In addition, each model offers a more accurate representation of a single patient's specific cancer and can aid in personalized medicine approaches. For example, patient-derived cell lines (PDCLs) offer great opportunities to test therapeutic response *ex vivo* [205], though they are often limited in their ability to recapitulate the stromal dynamics of desmoplastic expansion [206]. While each PDMC has its own

advantages and drawbacks, as highlighted in **Table 1.01**, PDMCs provide an advantage that no other tumor models can: contextualized clinical data.

As these models continue to evolve, the combination of experimental results with clinical context (treatment history, survival data, genetics, and transcriptomics) will continue to improve our ability to fully assess new therapeutic approaches and tumor responses, offering more personalized and effective treatment options for pancreatic cancer patients.

THERAPEUTIC TREATMENT OF PDAC

Chemotherapy

While specific targeted inhibitors and immune-based therapeutic agents have improved patient outcomes for some cancer types, PDAC has historically been resistant to these therapies, and standard-of-care (SoC) for patients with metastatic disease (>53%) relies on combination chemotherapy regimens that only provide a modest (months) increase in median survival [207–213]. These SoC treatments for PDAC consist of one of two cocktails of harsh chemotherapies—Gemcitabine/nAb-paclitaxel (nanoparticle albumin-bound paclitaxel, also referred to as Abraxane) or modified FOLFIRINOX (**Fig. 1.08**) [207–213]. Patients are frequently sensitive to one of these therapies, but not the other, demonstrating one challenge in treating this clinically and biologically heterogeneous disease [31, 33, 59, 214–217].

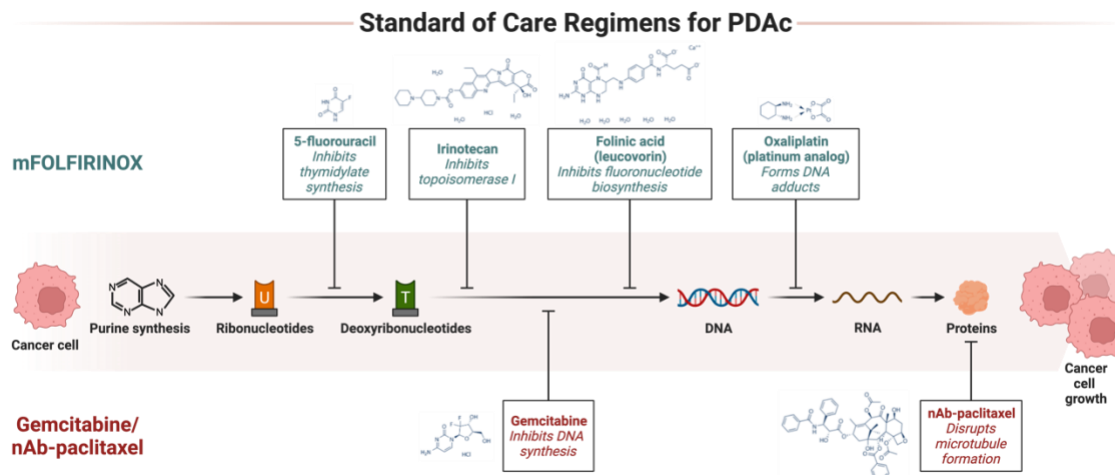


Figure 1.08 Standard-of-Care Regimens for PDAC.

The majority of patients diagnosed with PDAC are given one of two options for neoadjuvant therapy, modified FOLFIRINOX (mFOLFIRINOX) or Gemcitabine with nAb-paclitaxel. This figure highlights the four-drug combination that makes up the mFOLFIRINOX regimen and the two-drug combination of Gemcitabine/nAb-paclitaxel. Their chemical structures are shown along with their point of interference in cancer cell proliferation. Figure created by author with BioRender.com.

The first cocktail is Gemcitabine/nAb-Paclitaxel. Gemcitabine is a commonly used chemotherapeutic agent that works by interfering with DNA synthesis. This disruption of DNA synthesis leads to the accumulation of DNA damage and the inhibition of cancer cell proliferation. nAb-paclitaxel is another commonly used chemotherapy used in several cancers. nAb-paclitaxel interferes with cancer cell proliferation by binding to the beta-tubulin subunits of microtubules and stabilizing them, preventing them from disassembling during mitosis (cell division). This leads to the formation of abnormal microtubule structures and prevents the formation of the mitotic spindle, which is required for proper chromosome segregation and cell division. FOLFIRINOX is a four-drug ‘cocktail’ of Leucovorin (FOL), 5-Fluorouracil (F; 5-FU), irinotecan (IRIN), and oxaliplatin (OX), that is used as both first- and second-line therapeutic regimens in metastatic PDAC [208, 212]. Each of these drugs has a different mechanism of action to shut down cancer cell proliferation. Cancer cells have a high rate of cell division, which means they require a greater amount of folic acid than normal cells. Leucovorin, also known as folinic acid, is used in combination with 5-FU to enhance its effectiveness. By acting as a substitute for

folic acid, which is essential for DNA synthesis and cell division, leucovorin helps support the normal function of healthy cells and reduces the side effects of chemotherapy. 5-FU inhibits thymidylate synthase, reducing the availability of thymidine, and leading to a decrease in DNA synthesis and cell division. Irinotecan inhibits topoisomerase I, an enzyme involved in DNA replication and transcription, leading to the formation of stable cleavage complexes where it would otherwise have resealed the cleaved DNA strands. Finally, oxaliplatin, is a platinum-based drug that forms adducts within DNA strands. These adducts cause the DNA strands to bend and kink, leading to the formation of cross-links, which, in turn, prevent the normal replication and transcription of DNA, ultimately leading to the inhibition of cancer cell proliferation. Though these are all efficient cancer-cell-killing drugs, they are also fairly indiscriminate as they are systemic treatments interfering with processes occurring in any dividing cell of the body. The unfortunate result of this is a high incidence of painful side effects that patients endure when going through treatment [207, 209, 210].

Targeted inhibitors

In an effort to reduce toxic side effects as well as to directly treat mutations that drive a patient's tumor, researchers are continuously developing and testing a wide range of targeted inhibitors. These selectively target specific molecules that are involved in cancer growth and progression. In the case of PDAC, targeted therapies that have been approved or are in clinical trials for PDAC include those that target EGFR, BRAF, MEK, and PARP [218, 219]. These therapies are often employed in a subset of patients who carry specific mutations. Recently, several targeted inhibitors have been developed that aim to inhibit MYC or KRAS signaling in PDAC [220–222], two key targets our lab focuses on and that are altered in a majority of PDAC patients. In this section, we will focus on the current state of these inhibitors and their potential as therapeutic agents.

KRAS inhibition

Targeting mutant-RAS has been a long-sought after goal as it is mutated in upwards of 40% of all cancers (**Table 1.02**). Many approaches have sought to inhibit KRAS indirectly, targeting the downstream effectors of KRAS signaling, inhibiting KRAS membrane association, inhibiting metabolism or synthetic lethal interactors with RAS [223, 224]. After decades of relentless effort, the field has successfully developed therapies that are poised to fundamentally change the approach to treating RAS-driven cancers.

Tumour types	Sample	KRAS mutations (%)							Top 1
		Total rate	Mutation sites						
			G12	G13	Q61	A146	Other		
Pancreatic adenocarcinoma	1207	67.61	62.30	0.83	4.23	0.08	0.17	G12D	26.84
Colorectal adenocarcinoma	3953	35.77	22.82	6.68	1.67	2.76	1.85	G12D	9.87
Nonsmall-cell lung cancer	7135	20.42	17.39	0.85	0.31	0.06	1.81	G12C	8.38
Cholangiocarcinoma	1072	12.69	8.96	1.12	1.12	1.87	0.37	G12D	4.29
Uterine endometrial carcinoma	1907	14.11	10.38	1.78	0.63	0.10	1.21	G12D	4.20
Testicular germ cell cancer	506	11.66	6.92	0.00	1.98	1.58	1.19	G12V	2.77
Cervical squamous cell carcinoma	607	4.28	2.47	0.99	0.00	0.49	0.33	G12D	1.32
Myelodysplastic	6940	3.83	1.86	0.75	0.29	0.23	0.71	G12D	0.84
Data sources from cBioPortal.org. G12: codon 12 encoding glycine; G13: codon 13 encoding glycine; Q61: codon 61 encoding glutamine; A146: codon 146 encoding									

Table 1.02 The frequency of KRAS mutations in cancer

Table originally appeared in Huang, Guo, Wang, and Fu, 2021 [75], and has been adapted and reprinted with permission from the authors under the Creative Commons CC BY license. To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>.

Recent advances in drug discovery have led to the development of several promising direct mutant KRAS inhibitors. AMG 510 is a small molecule inhibitor that can bind directly to the *KRAS*^{G12C} mutant, which is found in approximately 1-3% of PDAC cases [223, 225–227]. This inhibitor can lock *KRAS*^{G12C} in an inactive state, preventing its downstream signaling and inhibiting cancer cell growth. AMG 510, has shown single-agent efficacy in immune-competent mice. Treatment with this specific inhibitor improved CD8⁺ T cell infiltration and upregulation of proinflammatory cytokines. Moreover, synergy with immune checkpoint blockade therapies with lower doses of AMG 510 was demonstrated, providing an exciting path to the clinic for patients with that mutation [228]. An inhibitor of the more common pancreatic cancer mutation, *KRAS*^{G12D} (MRTX1133), has recently been

published with extremely positive results. MRTX1133 leads to tumor regression in multiple mouse models of PDAC as a monotherapy by blocking cancer cell proliferation, inducing cancer cell death, and promoting immune infiltration and activation [229].

Two additional advancements in RAS targeting have recently come from Revolution Medicine. Revolution Medicine currently has two RAS inhibitors in phase 1/1b trials for advanced or resistant solid tumors: a Pan-RAS inhibitor RMC-6236, for which patients must contain one of five possible mutations at KRAS^{G12} (potentially treating 62% of PDAC patients) [NCT05379985, [230]] and another, RMC-6291, that more specifically targets KRAS^{G12C} (potentially treating 1-3% of PDAC patients) [NCT05462717, [231]]. While there is much excitement around the potential to inhibit mutant KRAS, it is expected that resistance to KRAS inhibition will develop over time. This has been observed in early studies with the KRAS^{G12C} targeting agents [232, 233]. Of note, studies from both the Olive and Der labs have shown MYC to be the primary resistance mechanism to direct [234] and indirect [117] KRAS signaling pathway inhibition in PDAC, respectively. As *KRAS* mutations are a defining feature in PDAC, and KRAS activation leads to the stabilization and activation of MYC (**Figure 1.09**), it will be of particular importance to study the development of resistance and determine strategies for overcoming resistance as these drugs head to clinical trials for the treatment of PDAC.

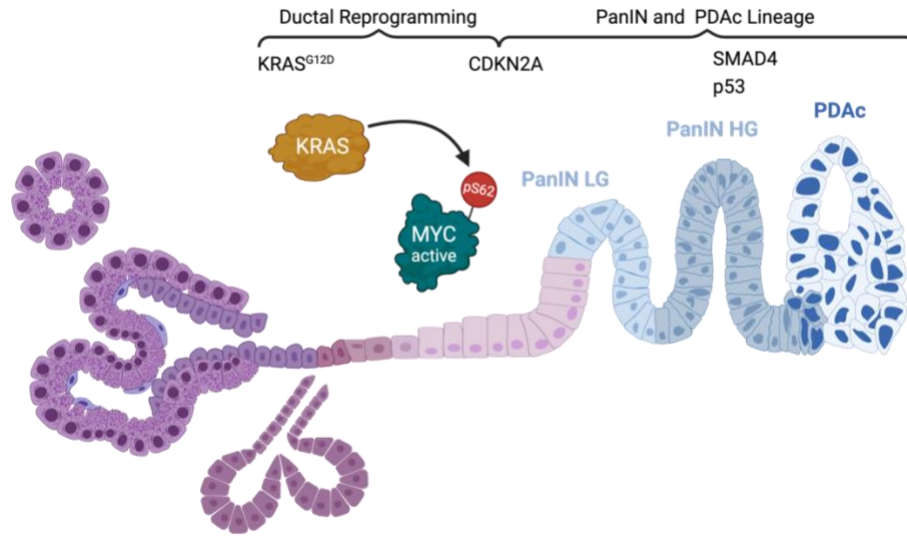


Figure 1.09 KRAS and MYC cooperate to drive PDAC progression.

Molecular progression of pancreatic ductal adenocarcinoma (PDAC) from normal acinar cells through pancreatic intraepithelial neoplasia (PanIN) stages to fully developed PDAC. The role of KRAS mutations in driving this progression is highlighted, showing how KRAS activation leads to the phosphorylation of MYC (at serine 62), stabilizing and activating MYC to promote oncogenic transcriptional programs. This cooperative interaction between KRAS and MYC is depicted as a critical event in the transition from low-grade (LG) to high-grade (HG) PanINs and eventually to invasive PDAC. The figure emphasizes the stepwise accumulation of genetic alterations that drive the transformation and progression of pancreatic cells. Figure originally appeared in English and Sears, *Cancer Discovery*, 2020, and was further adapted by author with BioRender.com, and reprinted with permission from the publisher under the Creative Commons license. To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>.

MYC inhibition

MYC has long been considered an attractive target for cancer therapy due to its central role in cancer progression. However, developing drugs that can directly target MYC has been challenging due to its intrinsically disordered structure and lack of a well-defined drug binding pockets [235]. Nonetheless, several approaches have been explored, including small molecule inhibitors [236], antisense oligonucleotides [237], RNA interference [238], and a dominant-negative of *MYC* transcription function [239, 240]. While some of these strategies have shown promise in preclinical models [241], there have been significant challenges to developing effective MYC inhibitors. Historically, one of the key challenges has been the lack of specificity of MYC inhibitors. Because MYC is involved in many normal cellular processes, inhibiting its activity might result in challenging side effects in patients. Moreover, since *MYC* is a transcription factor, its inhibition can

affect the expression of many other genes, some of which may be critical for normal cellular function [242].

MYC plays a crucial role in the later stages of PDAC progression, acting downstream of KRAS signaling to enhance oncogenic transcriptional programs (**Figure 1.09**). Targeting MYC in PDAC, therefore, represents a significant therapeutic challenge, but also a promising avenue for intervention. The discovery and use of Omomyc, a dominant-negative of *MYC* transcriptional function, has been an promising development in the treatment of PDAC and other solid tumors. When treated with Omomyc over extended periods of time, adult mice tolerated the effects of Myc inhibition with minimal side effects [239, 241]. In the last two years, Drs. Elena Garralda, Laura Soucek, and Marie-Eve Beaulieu have successfully led OMO-103, a first-in-modality anti-MYC miniprotein therapeutic, based on Omomyc, through a Phase 1a trial [NCT04808362, [243]]. Patients reported minimal toxicity or side effects, with some patients achieving stable disease and one PDAC patient even showing tumor shrinkage [244, 245]. OMO-103 is now in Phase 1b [NCT06059001], dose-finding, after Peptomyc (the biotech spinoff manufacturing the drug) received a €5 million grant from Horizon Europe EIC Accelerator Program to scale up its manufacturing process [246].

Other approaches to inhibiting MYC have targeted the proteins that regulate its expression or stability. For example, BET inhibitors such as JQ1 can block MYC expression by inhibiting the function of bromodomain-containing protein (BRD4) as an epigenetic reader [247, 248]. This has recently shown exciting results in a model of MYC-driven medulloblastoma in combination with histone deacetylases [248]. Another example is the use of small molecule activators of PP2A (SMAPs), as highlighted in **Appendix A**, which drive the dephosphorylation and subsequent degradation of MYC [99, 249–252]. Other

drugs such as ATR inhibitors can induce MYC degradation by activating the DNA damage response pathway [253].

Targeting of MYC in preclinical research has shown that it can also have a profound effect on the tumor microenvironment [103, 105]. To that end, the Evan lab (highlighted in **Appendix B**) and the Stanger lab have each recently demonstrated important connections between immune suppression in PDAC and the tumor cell-intrinsic master transcription factor, MYC [108, 254, 255]. Li, Byrne, and colleagues isolated tumor clones from the KPC mouse model of PDAC [156], and classified them by their ability to drive a T-cell-inflamed and non-T-cell-inflamed tumor microenvironment after orthotopic implantation. The authors identified an intriguing correlation between non-T-cell-inflamed clones (by IF) and high MYC and G2M cancer Hallmark signatures (by RNAseq) [255].

The Evan Lab mouse model incorporated a mutant *Kras* with a 'switchable' expression of MYC, allowing them to show a massive, yet reversible, immune response to MYC activation and deactivation in the same pancreata [108]. More specifically, they demonstrated that MYC activation initiated an influx of immunosuppressive macrophages and neutrophils, accumulation of tumor peripheral B-lymphocytes, eradication of CD3⁺ T cells, and activation of PSCs to CAFs, along with subsequent desmoplasia, hypoxia, and loss of vascularity. When they modeled MYC loss or inhibition by removing doxycycline, they saw complete regression of disease, including the collapse of the desmoplastic, inflamed stroma, and immune remodeling, including an influx of T cells and death or transdifferentiation of neoplastic duct cells. In their model, MYC loss even restored a state of normal acinar cells and early PanINs, along with recovery of blood vessel patency and local tissue oxygenation [108]. Together, these data are a clear indication of the viability of MYC inhibition as a targeted, therapeutic approach for PDAC.

However, significant gaps remain in modeling MYC-driven PDAC, particularly in capturing the full heterogeneity of the tumor microenvironment and the interplay between MYC signaling and stromal interactions during progression and metastasis. The Evan lab's model does not fully address molecular and subtype heterogeneity, nor does it assess metastasis in a comprehensive manner. For instance, while their model demonstrates changes in immune infiltration and stromal features, the absence of a detailed exploration of MYC's role in promoting subtype diversity or transcriptional heterogeneity limits the insights gained into MYC-driven tumor progression. Furthermore, their model focuses more on immune remodeling and regression than on capturing how MYC-driven tumors metastasize or respond to various therapeutic challenges.

In contrast, our KMC^{ERT2} model allows for a more granular exploration of MYC's role in promoting tumor heterogeneity, including distinct transcriptional clusters and phenotypic diversity within the PDAC landscape. The model enables a deeper understanding of MYC's impact on metastatic potential, tumor subtypes, and therapeutic responses. This work provides new avenues to study the specific mechanisms of MYC-driven tumor progression, while addressing the molecular heterogeneity that remains unexplored in previous MYC models. Together, these models complement each other, offering a broader perspective on how MYC influences various facets of pancreatic tumor biology.

In summary, *MYC* is a critical gene involved in cancer development, and understanding its role in cancer is essential for developing effective cancer therapies. While several approaches have been explored for targeting MYC there are significant challenges to developing other effective inhibitors. Continued research into MYC and its signaling pathways will remain critical for developing new and more effective cancer therapies.

In conclusion, targeted inhibitors represent a promising approach to the treatment of PDAC. MYC and KRAS inhibitors have shown efficacy in preclinical studies, and some

have even entered clinical trials. However, the development of effective targeted inhibitors for PDAC remains a challenge, and further research is needed to improve their efficacy and reduce toxicity. Nevertheless, the potential of targeted inhibitors to revolutionize cancer therapy continues to make them an exciting area of study for cancer researchers. This dissertation will focus on addressing these challenges by utilizing advanced model systems, such as a novel GEMM and 3D-bioprinting approaches, to better understand the role of MYC in PDAC and explore therapeutic responses.

RATIONALE AND HYPOTHESIS

Great advances in scientific knowledge and understanding have come from existing models of cancer, ranging from 2D culture of immortalized cell lines all the way to GEMMs, which serve as the foundation for most clinical trials to date. Unfortunately, fewer than half of clinical trials today produce actionable results in the form of FDA-approved regimens, with only 11% of phase three trials in PDAC being successfully incorporated into clinical practice [256]. This is due, at least in part, to the limitations of current models in fully replicating complexity and heterogeneity of human disease.

GEMMs, which integrate both tumor-intrinsic factors (e.g. genomic and epigenomic changes) and tumor-extrinsic factors (e.g. stromal interactions, immune infiltration, hypoxia, and low nutrient availability), provide a sophisticated *in vivo* platform for studying cancer progression and therapeutic responses. Yet, even these models often fall short of recapitulating the full diversity of tumor microenvironments observed in human PDAC. This includes the variability in stromal interactions, immune infiltration patterns, and the physical and structural characteristics of the tumor microenvironment, which all influence disease progression and response to therapies. To address these gaps, there has been significant development of engineered platforms, such as patient-derived models of cancer (PDMCs), organ-on-a-chip technologies, and 3D-bioprinting, which provide a more controlled setting to examine tumor behavior and microenvironmental dynamics.

These technologies offer an opportunity to model the cellular and molecular complexity of tumors in a reproducible and scalable manner. They allow researchers to explore specific tumor–TME interactions, including the effects of various immune cells, extracellular matrix composition, and signaling factors. However, the limitations of engineered models in replicating systemic responses make them ideal complements to animal models like GEMMs. The combination of GEMMs with engineered platforms represents a promising

approach to more accurately reflect the complexities of PDAC, especially in the context of therapeutic resistance and immune evasion.

To this end, the following dissertation will outline our expansion of existing models of PDAC in the development and characterization of a novel GEMM and the use of a unique bioprinting technology. **The central hypothesis of this dissertation is that models which better represent tumor and tumor microenvironment heterogeneity—particularly the interactions between tumor cells, stromal elements, and immune cells—will offer superior platforms for investigating PDAC progression and will enable more accurate testing of therapeutic responses.**

We developed a novel inducible Ptf1a-cre^{ERT2} mouse model of *Myc*-deregulated PDAC (KMC^{ERT2}), driven by the *Kras*^{G12D} activating mutation in conjunction with a physiologic low-expressing *Myc* on the *Rosa26* promoter [110]. Recombination was induced in the pancreatic acinar cell lineage, ensuring the model faithfully mimics the progression of pancreatic ductal adenocarcinoma. This novel autochthonous model of PDAC, together with orthotopic models using autochthonous tumor-derived cell lines, effectively mirrors the tumor and tumor microenvironment heterogeneity, including challenges of therapeutic resistance and metastatic potential, observed in clinical PDAC populations. We expect this KMC^{ERT2} model will serve as a valuable resource in the pancreatic cancer research field, providing an *in vivo* system that reflects both the tumor complexity and behavior of human PDAC.

In parallel, our lab has developed expertise in an extrusion-based 3D-bioprinting technology that allows us to reliably produce manipulable, heterotypic, scaffold-free human tumor tissues with defined architecture [197]. These bioprinted tissue structures mimic human tumors in key ways, including immune- and stromal cell interactions. Cells within these structures exhibit tissue-like cellular density, and they proliferate, deposit

ECM, migrate, and respond to extrinsic signals. This dissertation will elaborate on our efforts to advance this technology by integrating immune populations into these bioprinted tissues and leveraging PDMCs to compare phenotypes of tissues printed with distinct PDCLs.

The interplay between tumor cells and the tumor microenvironment is critical to understanding the evolution of tumor phenotypes and therapeutic response. A comprehensive understanding of the molecular mechanisms underlying these dynamic interactions requires complex, manipulable models. Together, the novel KMC^{ERT2} mouse model and the 3D-bioprinting approaches developed and described in this dissertation add to the available model systems for investigating the biology of pancreatic cancer and predicting therapeutic outcomes.

CHAPTER 2: THE KMC^{ERT2} MOUSE MODEL OF PDAC

MYC AND KRAS COOPERATE TO DRIVE PHENOTYPIC HETEROGENEITY, METASTASIS, AND THERAPEUTIC RESISTANCE IN A NOVEL PANCREATIC CANCER MOUSE MODEL

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The following chapter contains the majority of my graduate research and is the basis of a manuscript in preparation.

Personal contribution

IAE's contributions to this chapter are as follows:

Figure 2.01: A) ASF performed IF for pS62-MYC on human pancreas tissue. B) ASF performed initial quantification of pS62-MYC IF; JE performed subsequent statistical analysis. C-D) VS and MT compiled the hPDAC progression TMA, performed pS62-MYC IHC, and quantified staining. E) KAH performed the survival analysis of TEMPUS samples based on MYC V1 hallmark expression. F-G) IAE performed the analysis of PDAC disease-specific and progression-free survival based on altered MYC in publically available datasets through cBioPortal.

Figure 2.02: A) IAE created schema for KMC^{ERT2} genetics. B) ASF, BAP, IAE, PJW, XW, CJD, and MCT all contributed to KMC^{ERT2} breeding and colony maintenance, therefore contributing to the autochthonous model survival curve; BAP, IAE, and PJW performed end of study takedowns (data collection); IAE performed data curation and analysis.

Figure 2.03: A) IAE & the OHSU HSR performed tissue sectioning and H&E staining. IAE scanned all slides and curated data and images. B) ALK performed the CODA classification and initial analysis of provided H&E images. D) IAE performed additional analyses to create heatmap and hierarchical clustering of CODA data output.

Figure 2.04: A) PJW scraped tumor areas from FFPE tissue slides and extracted RNA for RNAseq. RNAseq was performed by the OHSU MPSSR. CP performed initial RNAseq TPM analysis. IAE further analyzed mRNA expression of key genes. B) JLP performed p53 IHC on murine tissues. C) JE performed the quantification of P53; IAE performed statistical analysis. D-E) KAH performed differential expression analysis and created volcano plot. F) IAE performed GO term analysis between KMC^{ERT2} and KPC models. G) VS performed mIHC on murine tissues, with the guidance of CB. H) CB and SS performed analysis of mIHC images to quantify cell types. H-I) IAE performed downstream analyses for %CD45⁺ population and cell density comparisons.

Figure 2.05: A-C) KAH performed PCA, RNAseq DE, and GSVA analyses for KMC^{ERT2} tumors. B-C) CP performed PurIST and COMBAT analyses on RNAseq data to classify KMC^{ERT2} tumors by subtype. D) IAE scanned and curated representative images of each KMC^{ERT2} tumor cluster.

Figure 2.06: A) IAE generated and validated KMC^{ERT2} tumor-derived cell lines and created cell line generation schema. B) IAE performed the western blots for key protein expression. C-D) KAH performed the RNAseq DE and PCA analyses for KMC^{ERT2} cell lines. E-K) IAE and PJW performed orthotopic model studies (including surgeries and ultrasound monitoring) with the help of JLP and JMC; IAE performed data curation and analysis.

Figure 2.07: A-F) IAE and PJW performed *in vivo* implantations, treatments, and collections for orthotopic SoC studies with both 171J and 314J KMC^{ERT2} tumor-derived cell lines. IAE performed analyses.

Table 2.01: IAE performed data curation and analysis of the the autochthonous KMC^{ERT2} model.

Table 2.02: IAE performed data curation and analysis of the the KMC^{ERT2} tumor-derived orthotopic model.

Supplementary material not directly extended from main figure data:

Supplementary Figure 2.01: A-B) ASF carried out the autochthonous KMC model study.

Supplementary Figure 2.08: A-B) PJW scraped tumor areas from FFPE tissue slides and extracted cDNA for WGS. WGS was performed by the OHSU MPSSR. MBH performed CNA analysis and created CNA frequency summary histogram and heatmap from WGS data.

Supplementary Figures 2.10–2.12: A-C) IAE performed top 20 GO term and GSEA hallmark pathway analyses between autochthonous KMC^{ERT2} tumor RNAseq Clusters 1 and 2.

Supplementary Figure 2.13: A) IAE created the BioRender schema for orthotopic model w/ SoC. B) IAE and PJW assessed liver metastasis progression in the orthotopic model.

Supplementary Figure 2.14: A-G) PJW and MT performed the individual cell line tumor growth kinetics studies; IAE performed data analysis.

Supplementary Figure 2.15: A-D) IAE, PJW, and MT performed *in vivo* implantations, treatments, and collections for orthotopic tumor growth kinetics studies with the help of JLP and JMC; IAE performed data analyses.

Supplementary Figure 2.16: A-D) MT performed *in vivo* implantations, treatments, and collections for orthotopic SoC studies with both 171J and 314J KMC^{ERT2} tumor-derived cell lines. IAE performed data analyses.

Supplementary Table 2.02: PJW scraped tumor areas from FFPE tissue slides and extracted cDNA for WES. WES was performed by CD Genomics. MBH performed WES data analysis for SNV point mutation calling. IAE performed downstream analysis and data curation to create summary table.

Supplementary Table 2.04: IAE extracted cDNA from KMC^{ERT2} tumor-derived cell lines for WES. WES was performed by CD Genomics. MBH performed WES data analysis for SNV point mutation calling. IAE performed downstream analysis and data curation to create summary table.

All figures for this chapter were prepared and finalized by IAE.

INTRODUCTION

Despite accounting for only 3% of new cancer diagnoses in the United States annually, pancreatic ductal adenocarcinoma (PDAC) is arguably the most aggressive cancer as it is responsible for 8% of cancer-related deaths, an estimated 66,440 new cases and 51,750 deaths in 2024 [24]. PDAC is currently the third leading cause of cancer-related deaths in the United States, with a five-year survival rate of only 13%, and it is projected only to increase in incidence over the coming years [24]. Standard-of-care chemotherapeutic regimens offer only modest survival benefits (on the order of months), and targeted inhibitors and immune-based therapies that have improved patient outcomes in other adenocarcinomas have had disappointing results in PDAC [207–213]. In order to better study this disease, models that effectively recapitulate not only the molecular and cellular heterogeneity, but also metastasis and therapeutic responses observed in human populations are needed.

Kirsten rat sarcoma viral oncogene homologue (*KRAS*), is mutated in over 84-95% of PDAC tumors [45–47]. *KRAS* mutations occur early in pancreatic tumorigenesis, driving early-stage lesions known as pancreatic intraepithelial neoplasia (PanIN) [257]. While *KRAS* mutation is sufficient for PanIN development, it alone is not sufficient for progression to PDAC, and requires accumulation of additional alterations [258]. Other common mutations in PDAC include *TP53* (56-72%), *CDKN2A* (30-50%), and *SMAD4* (32-50%) [53–64]. The use of GEMMs selectively expressing mutant *Kras*^{G12D/+} and *p53*^{R172H/+} via Cre recombination in the pancreas ("KPC" model [156]), has been a common approach to study the natural history of PDAC. KPC mice develop pancreatic tumors that can metastasize throughout the body [156]. This model has paved the way for many discoveries in PDAC research; however, *P53* mutations typically occur late in human PDAC progression [56, 64, 77], which limits the utility of this model in understanding tumor

evolution and phenotypic diversity that might arise during stepwise progression of the disease. Other systems have combined oncogenic *Kras* with mutations in canonical tumor suppressors, including *Smad4* [67, 259] and *Cdkn2a* (*Ink4a/Arf*) [65, 68], to drive pancreatic tumors, which along with approaches to alter cell of origin [48], have increased the diversity of available models. However, given the lack of translatable progress in human outcomes, it is clear that additional preclinical models are needed to encompass the breadth of disease biology and to gain further insight into effective therapeutics.

MYC is a potent transcription factor that drives cell proliferation and many other hallmark cancer phenotypes, and plays an important role in tumor progression. Its overexpression has been correlated with aggressive tumor behavior and therapeutic resistance in diverse human malignancies [95]. While rarely mutated itself, *MYC* gene amplification, transcriptional upregulation, and increased protein stability are common in PDAC [84, 85, 94, 260, 261]. [84, 85]. *KRAS* is a known upstream activator of *MYC* that drives the phosphorylation of *MYC* at phospho-serine 62 (pS62) [86]. This phosphorylation event stabilizes *MYC* protein and increases its activity in cancer cells, therefore promoting its oncogenic properties [80, 101, 110]. *MYC* and *KRAS* cooperate in driving PDAC; and genetic loss of endogenous *Myc* in mutant *Kras* models inhibits PanIN formation and progression to PDAC [120, 262].

To further investigate the in vivo connection between *KRAS* and *MYC* in pancreatic cancer, we generated a novel genetically engineered mouse model of PDAC driven by Cre-inducible expression of mutant *Kras*^{G12D} and low deregulated *Myc* [110] in pancreatic acinar cell populations (KMC^{ERT2} : *LSL-Kras*^{G12D/+}; *Rosa-LSL-Myc*^{+/+}; *Ptf1a-Cre*^{ERT2}). We performed genomic, transcriptomic, and histologic analyses of pancreatic tumors arising in this model that revealed molecular, cellular, and phenotypic heterogeneity among tumors, with high fidelity to the natural history and clinical features of human PDAC.

Reflecting phenotypic heterogeneity in human disease, multiple subtypes of autochthonous tumors were observed. We generated KMC^{ERT2} tumor-derived cell lines from different subtype tumors, which in the orthotopic setting, demonstrate line-specific metastatic patterns to the liver, lungs, and lymph nodes, and display differential sensitivity to SoC. Together this novel GEMM and the orthotopic cell lines provide a new model system in which to study biologic responses and novel therapeutic approaches in PDAC.

RESULTS

MYC is deregulated during the development of human PDAC and is associated with worse overall survival

Large scale molecular analyses have found increased MYC activity in primary human PDAC [36, 263, 264] and have associated its activity with metastatic disease and poor prognosis [31, 265]. To better understand whether stabilized, active MYC protein expression correlates with PDAC tumor progression, we performed immunofluorescent (IF) staining for phosphorylated Serine 62 c-MYC (pS62-MYC) on PanIN and PDAC samples from eight patients along with matched adjacent normal tissues (**Fig. 2.01A**). The staining intensity in low and high-grade PanIN lesions and PDAC was quantified relative to patient-matched adjacent normal ducts, and this analysis showed significantly increased pS62-MYC expression with disease progression (**Fig. 2.01B**). Because of the observed correlation in these initial eight specimens, we confirmed our findings in a PDAC tissue microarray (TMA) with 27 normal, 8 PanIN, 30 PDAC, and 3 metastatic site PDAC samples. We stained the TMA by immunohistochemistry (IHC) to assess nuclear expression of pS62-MYC across disease stages (**Fig. 2.01C**) and found again that the percent of positive nuclear staining for pS62-MYC was significantly higher in PDAC and metastasis when compared to normal tissue (**Fig. 2.01D**).

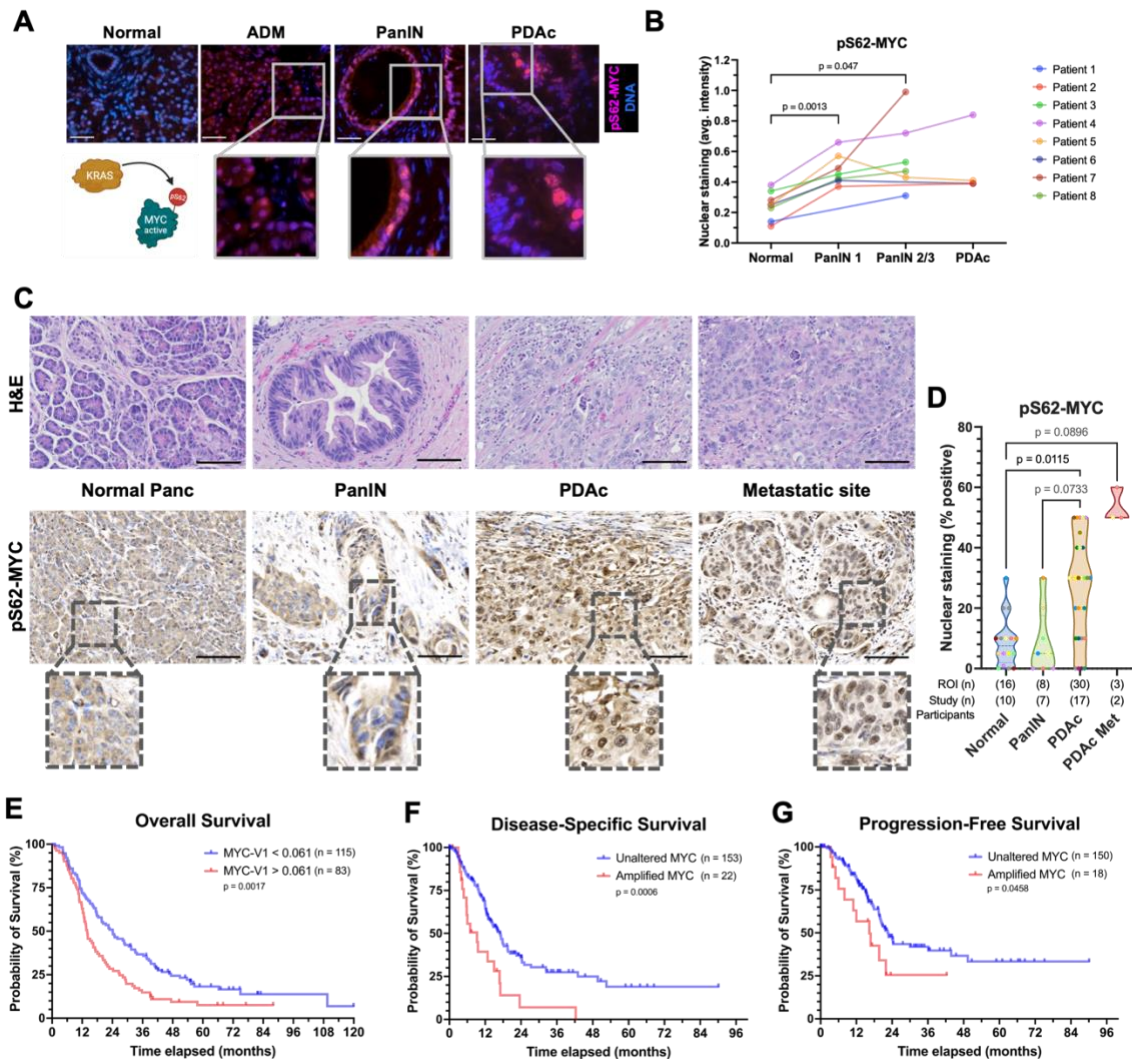


Figure 2.01 MYC is deregulated during the development of human PDAC and is associated with worse overall survival.

A) Representative images of human PDAC patient tissue samples stained for pS62-MYC (red) and DAPI (blue). Scale bars represent 50 μ m (top) and equally magnified among insets. **B)** Nuclear pS62-MYC staining intensity was quantified in 10 randomly identified individual epithelial cells in each of 5-10 selected regions of distinct histology (Normal, PanIN1, PanIN2/3, and PDAC) and averaged for each of eight patient samples. Significance was assessed by paired t-tests and corrected with Benjamini-Hochberg FDR. **C)** Representative H&E images of human PDAC patient tissue samples from a tissue microarray (n = 96 tissues) with corresponding tissue sections stained for pS62-MYC (brown; n = 86 tissues) by IHC. Scale bars represent 200 μ m and 50 μ m (insets). **D)** Nuclear pS62-MYC staining intensity was quantified in 86 tissues. Significance was assessed by 2-way ANOVA and corrected with Dunnett's multiple comparisons test. **E)** Kaplan-Meier curve showing probability of overall survival between patient groups with high vs. low MYC V1 score by RNAseq DESeq2 analysis. Significance was assessed by Log-rank (Mantel-Cox) test. **F)** Kaplan-Meier curve showing probability of disease-specific survival between patient groups with and without copy number (CN) gain in MYC. Significance was assessed by Logrank (Mantel-Cox) test. **G)** Kaplan-Meier curve showing probability of progression-free survival between human patient groups with and without CN gain in MYC. Significance was assessed by Logrank (Mantel-Cox) test.

We examined expression of the Hallmark MYC gene signature in a PDAC patient primary tumor RNAseq dataset established at the Brenden Colson Center for Pancreatic Care at Oregon Health and Science University (BCCPC, OHSU, n=212) [29]. Using gene set enrichment analysis (GSEA), we observed a significantly shorter overall survival in patients with high Hallmark MYC_V1 activity scores when compared to those with low MYC_V1 activity scores (**Fig. 2.01E**) [266]. This finding is consistent with the results of previous studies showing MYC to be a core gene program in squamous [31] (overlapping with quasi-mesenchymal [33] and basal-like [214] subtypes) tumors, which in turn have been correlated with poor prognoses in the clinic [267]. To expand on these findings, we performed additional survival analyses using pancreatic cancer datasets from The Cancer Genome Atlas (TCGA, PanCancer Atlas) [268–277] and the University of Texas Southwestern using cBioPortal (UTSW, Nature Communications 2015) [278, 279]. Patients with MYC amplifications were found to have a significantly shorter duration of disease-specific and progression-free survival (**Fig. 2.01F-G**). Together, these data suggest that MYC stabilization occurs early in pancreatic carcinogenesis and that its increased expression and dysregulation play important roles in the progression and severity of disease.

Deregulated MYC and KRAS^{G12D} in the adult pancreas drives PDAC in a new inducible genetic mouse model

To model the effects of MYC activation in pancreatic carcinogenesis and progression, we sought to create a mouse model of the disease using our *Myc* knock-in mice, which express a deregulated, but physiological level of *Myc* from the *Rosa26* locus (*Rosa26-LSL-Myc*) following tissue-specific expression of Cre recombinase [110]. We have previously demonstrated that combining this low deregulated *Myc* with mutant oncogenes drives accelerated cancer progression [101, 102, 110, 262]. Therefore, we crossed the *Rosa26-LSL-Myc* (M) mice with *LSL-Kras^{G12D};Ptf1a-Cre* mice (KC) [82] to generate *LSL-*

Kras^{G12D};Rosa26-LSL-Myc;Ptf1a-Cre (KMC) mice (Supp. Fig. 2.01A). In contrast to previous reports using high levels of *Myc* expression driven by *Pdx1-Cre;CAGtTA;TetO-Myc* [280], two copies of low-level, deregulated *Myc* alone [108], driven by either *Ptf1a*- or *Pdx1-Cre*, did not result in pancreatic tumors (Supp. Fig. 2.01B) [262]. In combination with mutant *Kras^{G12D}*, expression of one copy of *Myc* significantly decreased the latency of disease when compared to KC mice, and survival was further reduced with two copies of knock-in *Myc* (Supp. Fig. 2.01B). By initiating disease during embryogenesis however, we were unable to restrict intended mutations to a specific pancreatic cell of origin. In turn, these mice developed a wide spectrum of pancreatic lesions, including well-differentiated and poorly-differentiated PDAC, often mixed with neuroendocrine (NE) lesions (data not shown).

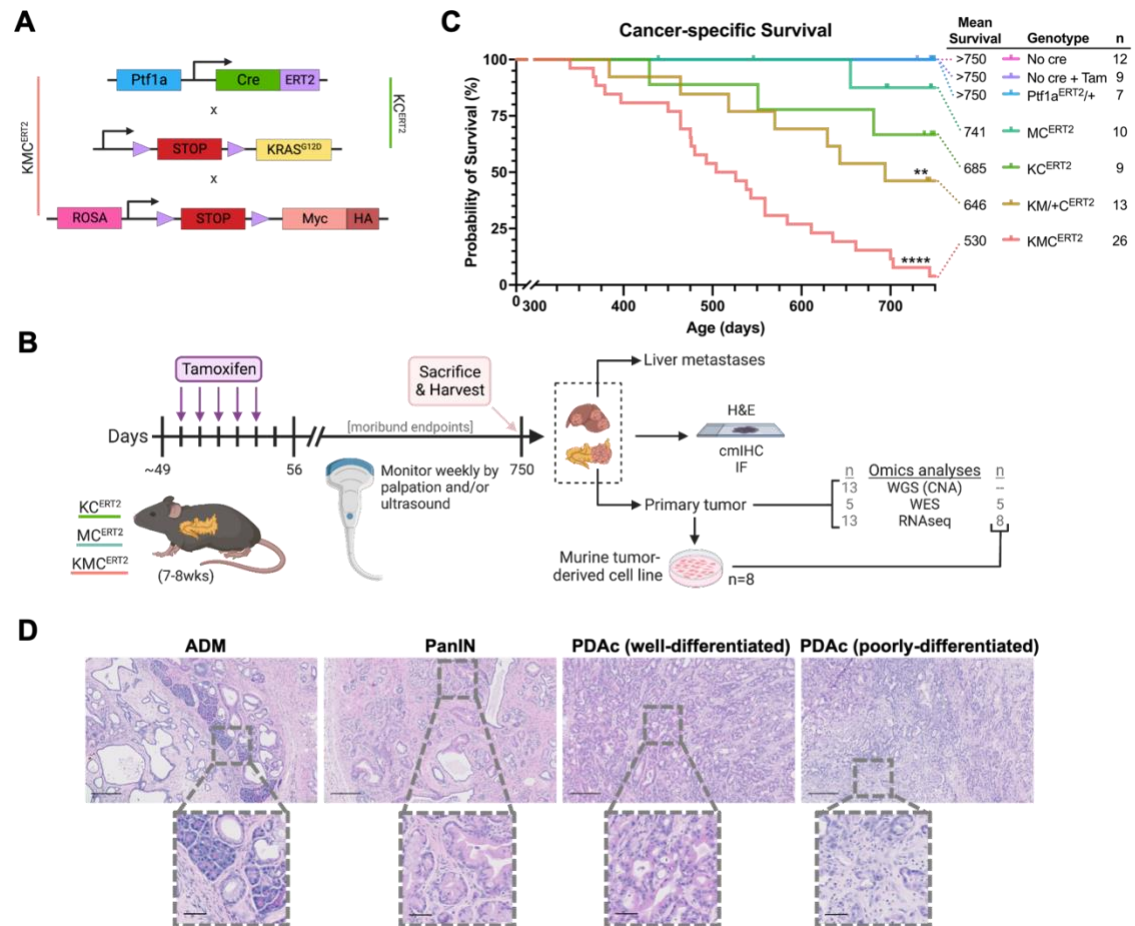


Figure 2.02 Deregulated MYC combined with KRAS^{G12D} in the adult pancreas drives PDAC in a new inducible spontaneous mouse model.

A) Genetics schema depicting alleles of the LSL-Kras^{G12D};ROSA-LSL-Myc-HA;Ptf1a-Cre^{ERT2} (KMC^{ERT2}) and LSL-Kras^{G12D};Ptf1a-Cre^{ERT2} (KC^{ERT2}) GEMMs. **B)** Experimental design schema for KMC^{ERT2} GEMM generation, monitoring, downstream processing, and analysis. **C)** Kaplan-Meier curve showing cancer-specific survival between KMC^{ERT2} GEMM study groups. All data are presented, with censored data points indicated by upwards tick. Significance versus No-cre controls is shown on lines, and versus KC^{ERT2} is shown in legend. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant. **D)** Representative H&E images of PDAC progression in KMC^{ERT2} mice, including ADM, PanIN, and PDAC. Scale bars represent 200 μ m (top) and 50 μ m (insets). (Panels A and B were created with BioRender.com.)

Adult acinar cells are a known cell of origin of PDAC, transdifferentiating into a ductal phenotype through a process known as acinar to ductal metaplasia (ADM), subsequently progressing to PanINs [48, 281, 282]. To determine *Myc*'s contribution to PDAC development from an acinar cell of origin, we crossed the *Rosa26-LSL-Myc* and *LSL-Kras^{G12D}* alleles with the conditionally inducible *Ptf1a-Cre^{ERT2}*. This generated a novel KMC^{ERT2} GEMM of PDAC, with tamoxifen-inducible acinar cell-specific expression of

mutant *KRAS*^{G12D} and physiologically relevant levels [110] of deregulated *MYC* (**Fig. 2.02A**). To determine how deregulated *Myc* affects *Kras*-driven PanIN and PDAC lesion development in this model, we aged mice in seven genetic cohorts, sacrificing them when they reached predefined humane endpoints or at 750 days (**Fig. 2.02B-C**, **Table 2.01**, Supp. Fig. 2.02A-B). Tamoxifen induced expression of these mutations in adult mice (7-8 weeks of age), abrogates islet hyperplasia and neuroendocrine lesions seen in the constitutive model in Farrell et al [262]. Progression in affected cells progress with high histologic fidelity through known human disease stages including ADM and PanIN, before developing into various grades of frank ductal adenocarcinoma (**Fig. 2.02D**).

Table 2.01 Autochthonous KMC^{ERT2} PDAC model summary

Autochthonous Tumor Endpoint Data							Omics Data Availability			Derived Models	
Mouse ID	Sex	Genotype	Tumor subtype	Overall survival (days)	Relative pancreas weight (% bodyweight)	Macro metastases noted on necropsy	RNAseq	WGS	WES	Cell line grown	Orthotopic growth
011T	M	KMC ^{ERT2}	Not assessed	635	7.36%	-	-	-	-	+	Not assessed
078J	F	KMC ^{ERT2}	Squamous	700	Not available	+	+	+	-	-	N/A
106L	F	KMC ^{ERT2}	Not assessed	584	6.90%	-	-	-	-	+	Not assessed
136J	F	KMC ^{ERT2}	Squamous	369	9.35%	+	+	+	-	-	N/A
167J	F	KMC ^{ERT2}	Squamous	493	5.82%	+	+	+	-	-	N/A
171J	M	KMC ^{ERT2}	Squamous	611	5.98%	+	+	+	+	+	Consistent
222J	M	KM/+C ^{ERT2}	Pancreatic Progenitor	629	2.80%	-	+	+	-	-	N/A
255J	F	KMC ^{ERT2}	Squamous	480	8.01%	+	+	+	+	+	Inconsistent
301J	M	KMC ^{ERT2}	Pancreatic Progenitor	559	5.34%	-	+	+	+	+	Consistent
303J	M	KMC ^{ERT2}	Pancreatic Progenitor	366	6.73%	+	+	+	+	+	Consistent
304J	F	KM/+C ^{ERT2}	Pancreatic Progenitor	464	7.11%	-	+	+	-	-	N/A
313J	F	KMC ^{ERT2}	Pancreatic Progenitor	379	6.76%	-	+	+	-	-	N/A
314J	M	KMC ^{ERT2}	Squamous	504	8.10%	+	+	+	+	+	Consistent
352J	M	KM/+C ^{ERT2}	Pancreatic Progenitor	518	5.04%	-	+	+	-	-	N/A
369J	F	KM/+C ^{ERT2}	Pancreatic Progenitor	384	5.70%	-	+	+	-	-	N/A
808J	F	KM/+C ^{ERT2}	Not assessed	543	5.64%	-	-	-	-	+	Not assessed

Tumor subtype: Squamous subtype, defined by Bailey et al., 2016, overlaps with basal-like and quasi-mesenchymal, as defined by Collisson et al., 2011 [33] and Moffitt et al., 2015 [214], respectively. Pancreatic progenitor, defined by Bailey et al., 2016 [31], overlaps with classical, as defined by both Collisson et al., 2011 and Moffitt et al., 2015.

In comparison to combined controls without oncogene expression and to either MC^{ERT2} or KC^{ERT2} alone, KMC^{ERT2} mice have a significantly increased relative pancreas weight at endpoint, indicating a larger tumor burden (Supp. Fig. 2.02C-E). These mice were also seen to exhibit signs of cachexia, a secondary disease characterized by weight loss via skeletal muscle and adipose tissue loss, that is observed in as many as 87% of pancreatic cancer patients [283–285]. Specifically, KMC^{ERT2} mice showed muscular atrophy and

significant fat lipolysis—as measured in the gastrocnemius muscle and in the gonadal fat pad and brown adipose tissue, respectively (Supp. Fig. 2.02F-H). Together, these findings indicate that the KMC^{ERT2} model drives rapid development of PDAC from acinar cells, and tumor development is associated with a cachectic phenotype, as observed in pancreatic cancer patients (Supp. Fig. 2.02) [286–289].

KMC^{ERT2} tumor tissues exhibit cellular heterogeneity

To further characterize tumor progression and endpoint pancreatic tissue composition, we used CODA, a semantic segmentation deep learning model previously developed for quantification of human pancreas histology by our collaborators at Johns Hopkins University (**Fig. 2.03A-B**, Supp. Fig. 2.03, Supp. Table 2.01) [290]. For this project, CODA was retrained for mouse histology, and a subset of resulting output was then validated by blinded pathologist (L.D.W.) examination (Supp. Fig. 2.03A).

We used CODA to classify the histology of representative H&E sections from nine control, three MC^{ERT2}, seven KC^{ERT2}, eleven KM/+C^{ERT2}, and seventeen KMC^{ERT2} tumors. Analyzing the results from CODA by unsupervised clustering, we identified four histologically diverse groups across all genotypes, which we designated ‘acinar’, ‘cellular’, ‘stromal’, and ‘mixed’. (**Fig. 2.03C**). ‘Acinar’ histology was primarily composed of non-tumor bearing pancreata (Supp. Fig. 2.04). The ‘cellular’ histology group was identified to have high tumor cellularity, and included tumors of multiple genotypes (Supp. Fig. 2.05). The largest group of tumors, designated the high ‘stroma/ECM’ group, contained increased stromal desmoplasia, a common feature of human PDAC [95, 142, 291, 292]. This group (Supp. Fig. 2.06), was composed mainly of pancreatic tissue from KMC^{ERT2} and KM/+C^{ERT2} mice (**Fig. 2.03C**, Supp. Table 2.01). The last group included tumors partially composed of cellular PDAC, stroma/ECM, and necrosis, and is therefore referred to as the mixed group (Supp. Fig. 2.07). Occasional areas of necrosis were also observed

in other groups, likely where nutrients were lacking as a result of loss of tumor vascularity, or speed of tumor growth, as has been previously reported [108, 254]. For comparison, we included six KPC tissues in our CODA analysis. We found the KMC^{ERT2} model did show a trend toward more desmoplasia, less necrosis, more immune aggregates, and more adipose, though our analysis was underpowered to detect significant differences. Both KPC and KMC^{ERT2} tumors exhibited heterogeneous stromal desmoplasia, PanINs, and PDAC.(Fig. 2.03D, Supp. Table 2.01).

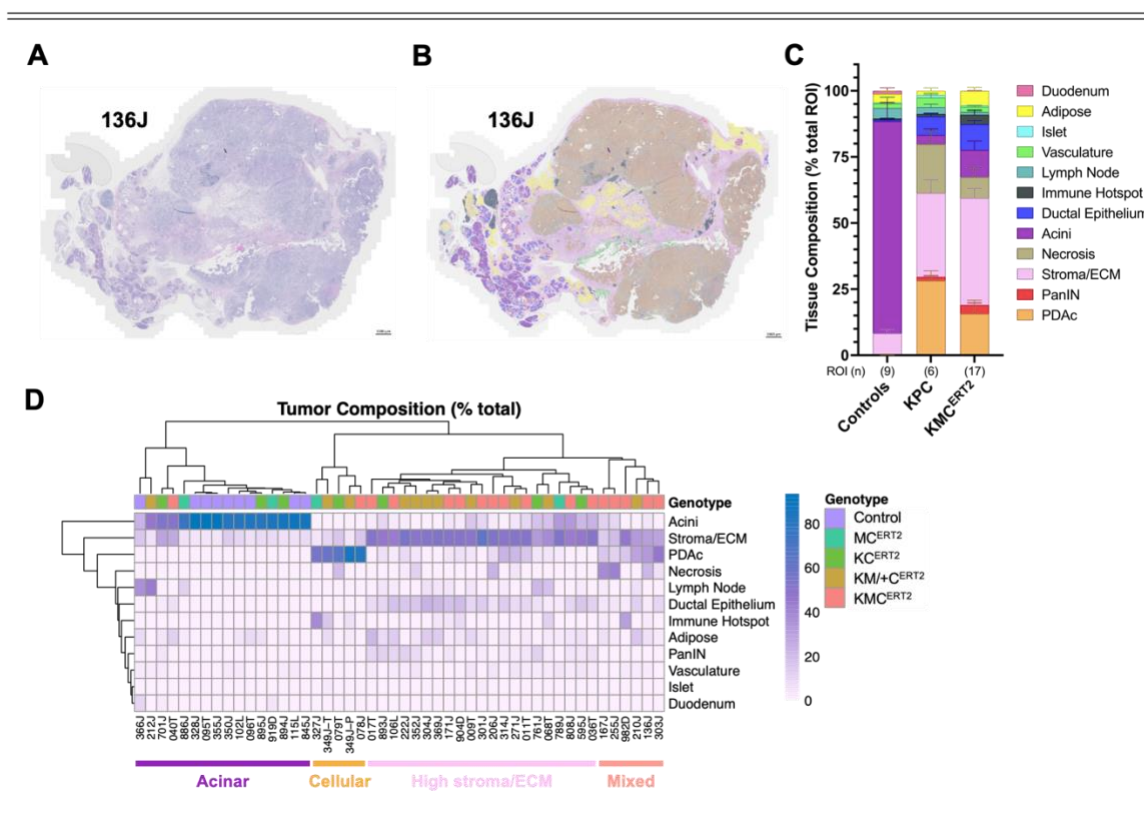


Figure 2.03 KMC^{ERT2} tumor tissues exhibit cellular heterogeneity.

A) H&E of KMC^{ERT2} pancreas tumor tissue from mouse 136J. **B)** H&E of KMC^{ERT2} pancreas tumor tissue from mouse 136J overlaid with CODA classification colors outlined in **C)**, which shows tumor composition as percent total tumor area in stacked bar plot. **D)** Heatmap depicting tumor composition as assessed by CODA analysis in pancreas tumor tissues from all arms of KMC^{ERT2} survival study.

KMC^{ERT2} tumors model unique genetic, transcriptional, and tumor immune microenvironmental signatures of human PDAC disease progression

Mutant *KRAS* expression and dysregulated *MYC* are present in early PanIN lesions, and represent early events in pancreatic cancer development. To determine whether our

model follows the well-established mutational progression of the human disease [56], we analyzed tissues at end-stage by histology to assess expression of tumor suppressors and by whole exome sequencing (WES) to assess acquisition of driver mutations (**Table 2.01**).

KMC^{ERT2} tumors showed a low mutational burden in high-impact genes, much like recent analyses of the KPC model [293]. No mutations were observed in *Trp53*, *Cdkn2a* or *Smad4* (Supp. Table 2.02). Because we did not observe alterations in high-impact genes and PDAC patients are known present with highly variable copy number aberrations in clinic [31, 60, 294], we used low-pass whole genome sequencing (WGS) to analyze copy number changes in our model. Consistent with human disease, we observed genomic alterations including heterogeneity in gene copy number across the entirety of the murine PDAC genome, especially in *Rosa-Myc* homozygous animals (Supp. Fig. 2.08) [31, 60, 294]. To address transcriptional differences, we performed RNA sequencing on spontaneous KMC^{ERT2} and KPC tumors. Given the spatial heterogeneity and multifocality of autochthonous tumors in GEMMs, we identified regions of adenocarcinoma on histopathology and enriched for these with microdissection. RNA was extracted, assessed for quality, sequenced, and assessed for expression of *Cdkn2a*, *Trp53*, and *Smad4*. This approach showed a stark loss of *Cdkn2a* expression in KMC^{ERT2} tumors as compared to other model genotypes and KPC tumors. (**Fig. 2.04A**). While mean expression of *Trp53* and *Smad4* were not significantly different between KMC^{ERT2} and KPC, the KMC^{ERT2} tumors displayed a wide range of expression, as has been observed in patient PDAC samples (**Fig. 2.04A**) [56, 60, 64, 70, 295]. Because the KPC model directly alters *Trp53* stability by genetic modification and the KMC^{ERT2} tumors showed no acquired mutation in the gene, we further interrogated potential post-transcriptional changes to *Trp53* in the KMC^{ERT2} model to understand whether this key tumor suppressor was being controlled to

allow for tumor progression. We stained FFPE KMC^{ERT2} PDAC tissue by immunohistochemistry and observed a clear loss of TRP53 (p53) nuclear staining (**Fig. 2.04B-C**, n = 6 (KMC^{ERT2}), n = 4 (KPC)). Recent studies have associated this staining pattern (complete absence) with poor prognosis in gastric and endometrial cancers [157, 296–299], likely indicating post-translational protein destabilization.

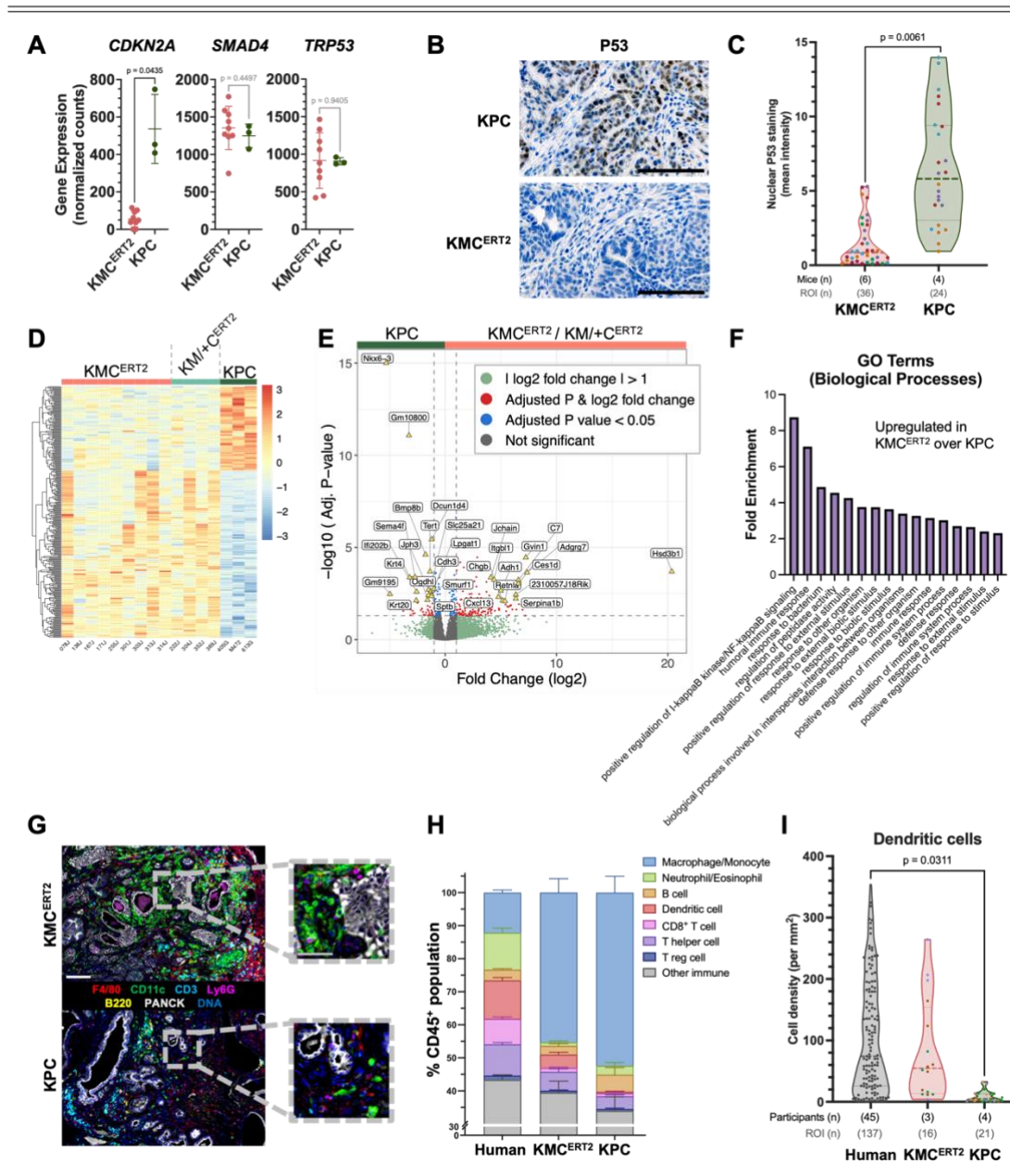


Figure 2.04 KMC^{ERT2} tumors model unique transcriptional signatures and tumor microenvironments of PDAC disease progression.

A) mRNA expression of Cdkn2a, Smad4, and p53 in endpoint KMC^{ERT2} and KPC pancreatic lesions from DE analysis of RNA-seq. **B)** Representative images of p53 protein expression in endpoint KMC^{ERT2} and KPC pancreatic lesions, by immunohistochemical staining. **C)** Dots in the graph are color coded to represent the variability across ROIs of individual mice within each cohort. Significance was assessed by unpaired t-test with Welch's correction. **D)** Heatmap of differentially expressed genes in PDAC GEMM tumors, by RNAseq differential expression (DE) analysis using DESeq2 ($|E| > 1.0$, $p\text{-adj} < 0.05$). **E)** Volcano plot of differentially expressed genes between KMC^{ERT2} & KM/+C^{ERT2} when compared to KPC. Each point represents a gene. One outlier gene (Rps13, adjusted p-value = $2.14e^{-43}$) upregulated in KPC lesions was removed for visualization purposes. **F)** Significantly enriched ($E > 0.5$, $p\text{-adj} < 0.05$) gene ontology (GO) terms of genes differentially expressed in endpoint KMC^{ERT2} over KPC pancreatic tumors in biological processes. **G)** Representative pseudocolor images of multiplexed immunohistochemical (mIHC) staining of intratumoral immune and other cell populations in endpoint KMC^{ERT2} and KPC pancreatic lesions, highlighting antibodies against F4/80, CD11c, CD3, Ly6G, B220, PanCK, and DNA (DAPI) (full list in Supp. Table 2.06). **H)** Frequency of distinct immune cell types as determined from mIHC is shown as % of total CD45+ cells. **I)** Frequency of dendritic cells per mm² as determined from mIHC. Dots in the graph are color coded to represent the variability across ROIs of individual mice. Significance was assessed by unpaired t-test with Welch's correction.

Differential gene expression analysis of KMC^{ERT2} and KPC tumors demonstrated clear differences between GEMM cohorts (**Fig. 2.04D-E**). We performed gene ontology (GO) enrichment analysis on genes differentially expressed in tumors of distinct genotypes. Due to substantially fewer genes being overexpressed in KPC tumors versus KMC^{ERT2} tumors at endpoint, there were no biological process GO terms significantly upregulated in KPC tumors over KMC^{ERT2}. In KMC^{ERT2} tumors, however, endpoint tumors showed enrichment of 19 immune-related biological process GO terms when compared to KPC tumors (**Fig. 2.04F**). This result is consistent with recent work underscoring MYC's key role in immune suppression in multiple cancers, including PDAC [103, 104, 108, 116, 300–303].

To examine how MYC deregulation impacts the immune milieu in the resulting PDAC tumors, we performed multiplexed immunohistochemistry (mIHC) on a subset of spontaneous KMC^{ERT2} tumors ($n = 3$) and compared them to KPC tumors ($n = 4$; **Fig. 2.04G-I**). By sequentially staining sections of endpoint KMC^{ERT2} and KPC pancreatic tumors, we were able to identify major immune cell types, including macrophages, B cells, T cells, and dendritic cells (DCs), within the tumors (**Fig. 2.04H-I**, Supp. Table 2.03) [304]. There were many similarities across the examined model immune microenvironments, including the large population of macrophages/monocytes and the sparsity of CD8⁺ T-cells

in the mouse models as compared to the human disease. One clear difference between the two mouse models was the presence of DCs in KMC^{ERT2} tumors and their near absence in KPC tumors (**Fig. 2.04I**). DCs are a class of resident tissue antigen presenting cells and have been implicated in the complex immune response to pancreatic cancer [305]. Intratumoral T-cell activity relies on antigen presentation by dendritic cells and recent models have been developed specifically to overcome the lack of neoantigen elaboration in the KPC model [306]. The presence of dendritic cells in our model is more similar to human disease and suggests a better reflection of this immune cell type [30]. Together, these data demonstrate two distinct PDAC TMEs in the KPC and KMC^{ERT2} GEMMs, with our new mouse model offering a distinct and complimentary means of understanding the immune response and molecular complexities in the human disease.

KMC^{ERT2} PDAC model displays intertumoral transcriptional and phenotypic heterogeneity

We investigated transcriptional heterogeneity across KMC^{ERT2} genotypes (both homo- and heterozygous for ROSA-Myc), by principal component analysis (PCA) and sample-to-sample distance analysis on normalized mRNA expression profiles (**Fig. 2.05A**, Supp. Fig. 2.09A). Tumors separated into three distinct clusters (Cluster 1, C1; Cluster 2, C2; Cluster 3, C3) regardless of *Rosa26-Myc* zygosity. Multiple molecular subtyping classifications have identified two main subtypes of PDAC: pancreatic progenitor/classical and squamous/basal-like/quasi-mesenchymal [32, 33, 214, 307, 308]. MYC pathway overexpression has been associated with the basal-like/squamous subtype in PDAC, as well as in other cancers [31, 97, 309]. We applied Purity Independent Subtyping of Tumors (PuriST), a single-sample classifier designed for robust classification of low-input tumor samples, with gene sets from Bailey et al., 2016, to categorize each tumor by subtype (**Fig. 2.05B-C**) [31, 34].

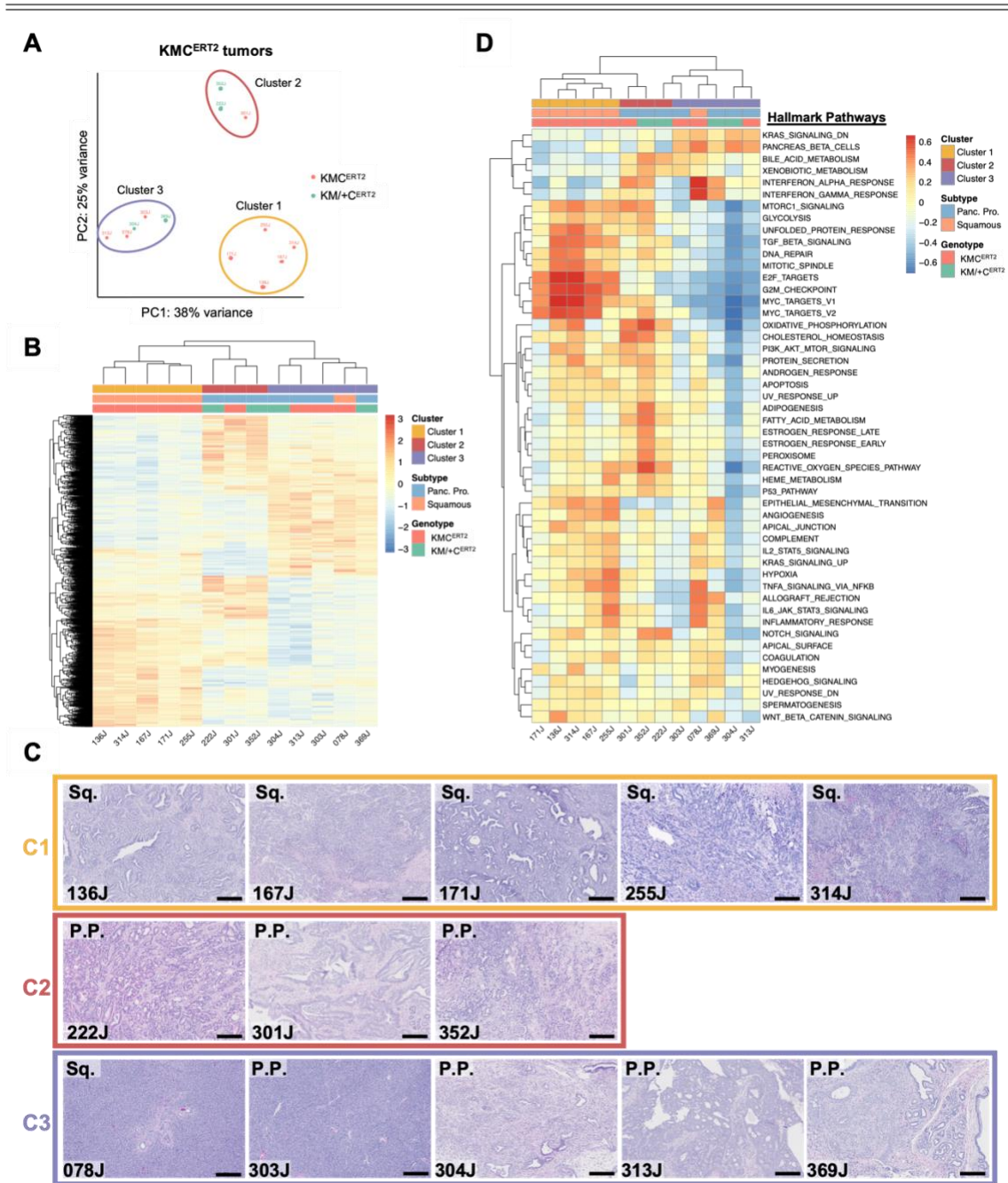


Figure 2.05 KMCERT2 PDAC model displays intertumoral transcriptional and phenotypic heterogeneity.

A) Principal component plot of PDAC GEMM tumors; clusters circled genotypes color-coded. **B)** Unsupervised hierarchical clustering of KMCERT2 tumor RNAseq data with labels indicating genotype, cluster, and molecular subtype. **C)** H&E images of KMCERT2 tumors showing phenotypic heterogeneity between clusters, as defined in (A). Scale bars depict 200 μ m. **D)** Heatmap of differentially enriched cancer hallmarks in KMCERT2 tumors by GSVA.

We found consistency with mRNA principle component clustering, with squamous-like tumors primarily in Cluster 1, while pancreatic progenitor-like tumors occupied Clusters 2

and 3 (**Fig. 2.05**, Supp. Fig. 2.09B). These clusters also have distinct expression of diverse cancer Hallmark pathways (**Fig. 2.05D**, Supp. Fig. 2.10-12). As expected, in the squamous cluster, Cluster 1, tumors showed enriched expression of genes in key cancer Hallmarks MYC_V1 and MYC_V2 (Supp. Fig. 2.10). Cluster 1 tumors additionally showed enriched expression in Hallmarks for E2F targets, G2M checkpoint, mitotic spindle, epithelial to mesenchymal transition (EMT), TGFbeta signaling, and DNA repair. When differentially expressed gene lists were compared and categorized by GO Term analysis, similar themes of cell cycle, DNA replication, and repair biological processes were enriched in Cluster 1 tumors (Supp. Fig. 2.10). Cluster 2 tumors were upregulated in GO terms and cancer Hallmark pathways for oxidative phosphorylation and metabolic biological processes, along with estrogen and androgen response (**Fig. 2.05D**, Supp. Fig. 2.11). Cluster 3 tumors were significantly downregulated in KRAS signaling pathways and upregulated the normal pancreas beta cell and interferon-gamma Hallmarks, joined by GO terms in immune-modulatory and tyrosine kinase biological processes, pointing to an important role for immune signaling within this cluster of tumors (**Fig. 2.05D**, Supp. Fig. 2.12, Supp. Data). Taken together, these data demonstrate the heterogeneous nature of our novel KMC^{ERT2} PDAC model, providing a unique challenge and opportunity to study therapeutic response in a study population that better reflects the diversity of disease seen in the clinic.

KMC^{ERT2} tumor-derived cell lines recapitulate autochthonous tumor heterogeneity and exhibit subtype-driven plasticity *in vitro*

We generated eight distinct KMC^{ERT2} tumor-derived cell lines by harvesting primary tumors via surgical resection followed by enzymatic disaggregation and *in vitro* expansion (**Fig. 2.06A**). To ensure additional driver mutations did not occur in the resulting cell lines, we performed WES on the established KMC^{ERT2} tumor-derived cell lines (171J, 301J, 303J, and 314J). Similar to what we observed in the autochthonous tumors, the KMC^{ERT2} cell

lines contained zero or one SNV in genes with high probable impact (Supp. Table 2.04).

These results confirmed the genomic stability of these cell lines and their consistency with the autochthonous tumors from which they were derived.

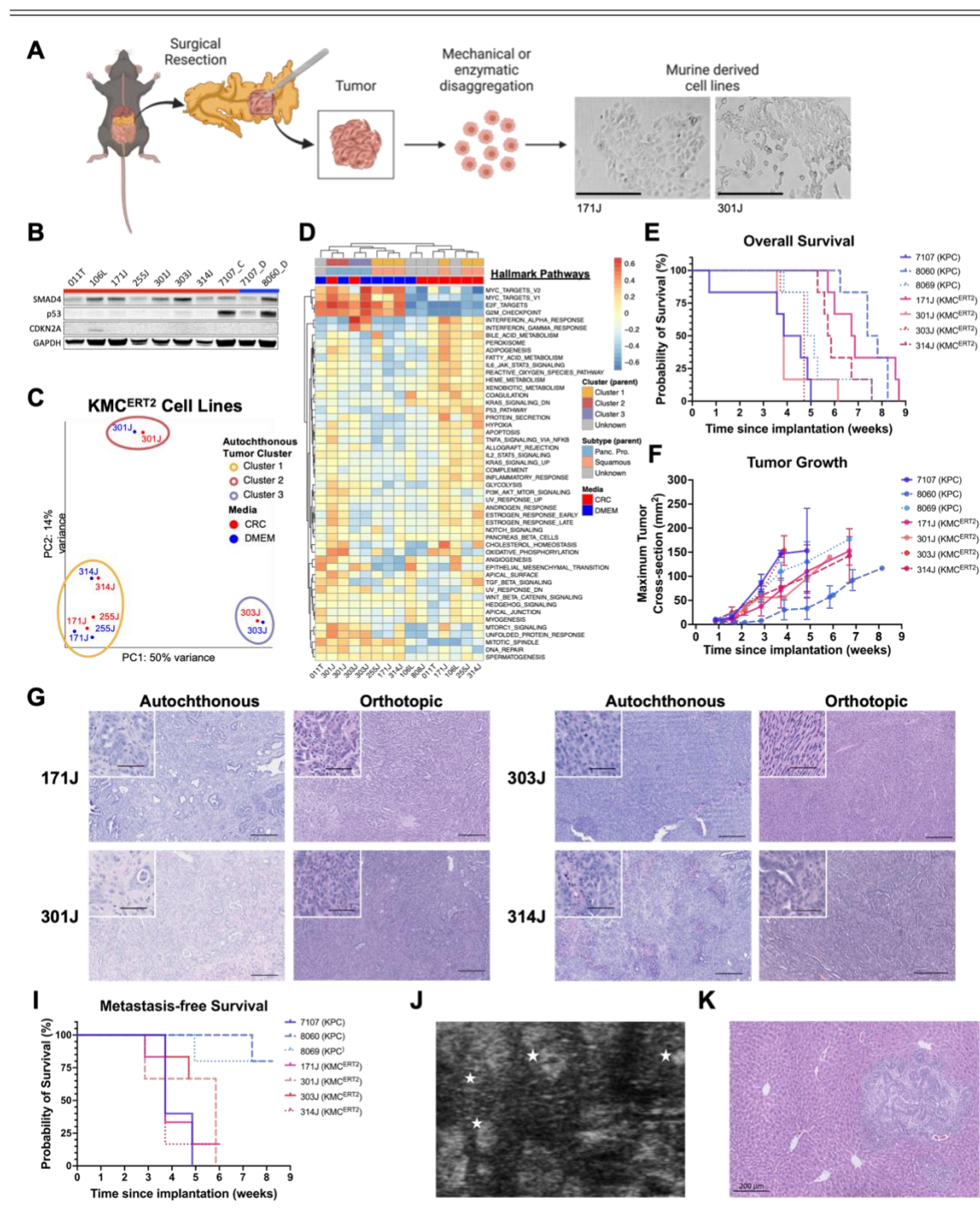


Figure 2.06 KMCERT2 tumor-derived cell lines recapitulate the heterogeneity observed within their respective autochthonous tumors, histologically and molecularly.

A) Schematic depicting generation of autochthonous KMC^{ERT2}-tumor derived cell line. Made with BioRender.com **B)** Western blot of SMAD4, p53, and CDKN2A expression in 10 distinct PDAC GEMM tumor-derived cell lines. Red bar indicates cells were grown in CRC media. Blue bar indicates cells were grown in DMEM + 10% FBS. **C)** Principal component plot of KMC^{ERT2}-tumor derived cell lines grown in either CRC media or DMEM + 10% FBS; corresponding autochthonous tumor clusters and subtypes are circled. **D)** Heatmap of differentially enriched cancer hallmarks in KMC^{ERT2} tumor-derived cell lines grown in either CRC media or DMEM + 10% FBS, by GSVA. **E)** Overall survival of B6129SF1/J mice orthotopically implanted with each of 4 distinct KMC^{ERT2}- and 3 distinct KPC-tumor derived cell lines. **F)** Tumor growth of 4 distinct KMC^{ERT2}- and 3 distinct KPC-tumor derived cell lines orthotopically implanted in B6129SF1/J mice. **G-H)** Representative H&E images showing histology of orthotopic tumors as compared to their respective autochthonous tumors and autochthonous tumor-derived cell lines. Scale bars depict 200 μ m on larger images and 50 μ m on insets. **I)** Metastasis-free survival of B6129SF1/J mice orthotopically implanted with each of 4 distinct KMC^{ERT2}- and 3 distinct KPC-tumor derived cell lines. Metastasis presence identified by weekly or bi-weekly ultrasound, shown in **J)**. **K)** Representative H&E of PDAC liver metastasis from orthotopic implantation of KMC^{ERT2}-tumor derived cell line.

To further validate the conservation of parent tumor phenotype, we examined protein expression and transcriptional signatures in the KMC^{ERT2} cell lines. Having previously shown a loss of p53 expression in autochthonous KMC^{ERT2} tumor epithelial cells, we decided to examine protein expression levels of the three major tumor suppressors by Western blot (**Fig. 2.06B**). As expected from our IHC result (see **Fig. 2.04B**), p53 protein levels were low in KMC^{ERT2} tumor-derived cell lines when compared to KPC-tumor-derived cell lines (**Fig. 2.06B**). Additionally, SMAD4 protein expression levels were variable, but CDKN2A levels remained consistently low. To examine transcriptional differences of our KMC^{ERT2} tumor-derived cell lines, we sequenced five representative KMC^{ERT2} cell lines grown in two different media conditions: CRC media [310] and DMEM + 10% FBS (**Fig. 2.06C-D**). Differential media conditions were evaluated by sequencing to evaluate best practices for in vitro culture and to probe differences in these novel lines. As with the autochthonous tumors (above) principal component analysis (PCA) of the mRNA expression profiles separated the tumor-derived cell lines into three distinct clusters regardless of growth media, and these reflected their respective autochthonous parent tumor clusters (Cluster 1; Cluster 2; Cluster 3) (**Fig. 2.06C**). Unsupervised clustering of gene set variation analysis (GSVA) of the cancer Hallmark pathways from these samples, however, showed two major groups broadly divided by media conditions [311]. The disparity in clustering between PCA and unsupervised clustering of GSVA results may be

partially explained by the fact that the two principal components reflected in the PCA plot only account for a total of 64% of sample variance. It is possible that the two outer clusters in the PCA plot, C2 and C3, have a third component in common that would have brought them together had it been accounted for—supported by the fact that the 301J and 303J cell lines clustered closely together in our GSVA analysis, regardless of growth media (**Fig. 2.06D**). When compared to those grown in DMEM, the majority of cells grown in CRC media showed significantly diminished expression of top Hallmarks: MYC targets V2, MYC targets V1, E2F targets, and G2M checkpoint (**Fig. 2.06D**).

Interestingly, in cell lines with matched autochthonous parent tumors with sequencing data, response to growth media seemed to be dependent on parent tumor subtype. Squamous KMC^{ERT2} tumors—171J, 255J, and 314J—gave rise to media-responsive cell lines, with the top hallmark pathways enriched in the autochthonous tumors being downregulated in the cell lines in CRC media and upregulated in the cell lines in DMEM + 10% FBS (**Fig. 2.06D**). Conversely, pancreatic progenitor KMC^{ERT2} tumors—301J and 303J—gave rise to media-resistant cell lines with high expression in MYC, E2F, and G2M checkpoint Hallmark pathways (**Fig. 2.06D**). These data together highlight the potential impact of growth media on experimental results as well as increased *in vitro* plasticity in the squamous subtype derived cell lines. These KMC^{ERT2} tumor-derived cell lines provide a new opportunity to study the effects of important cancer Hallmarks MYC, E2F, and G2M checkpoints on drug response in an *in vitro* system.

KMC^{ERT2} tumor-derived cell lines reliably metastasize to the liver

To examine the *in vivo* growth capacity of our MYC-driven KMC^{ERT2} cell lines, we performed a number of orthotopic experiments evaluating growth kinetics, metastatic potential, and autochthonous fidelity. We implanted five of our KMC^{ERT2} cell lines (171J, 255J, 301J, 303J, and 314J) and three KPC cell lines (7107, 8060, 8069) into immune-

competent 129J x BL/6-F1 mice. Cell line transplantation demonstrated reproducible tumor growth kinetics (Supp. Fig. 2.13) in seven of the eight cell lines assessed, with KMC^{ERT2} cell line 255J only growing in one of three orthotopic studies. As a result, 255J was not included in further experimentation. Additional studies showed that orthotopic growth of the remaining four KMC^{ERT2} cell lines was independent of both cell number and host sex (Supplementary Fig. 2.14-2.15). These KMC^{ERT2} cell lines show consistent *in vivo* growth with varying degrees of desmoplastic and heterogeneous histology, mirroring human pathology (**Fig. 2.06E-H**, **Table 2.02**, Supp. Table 2.05), providing new reliable options for modeling heterogeneous tumor drug response. When comparing orthotopic tumor histology to the autochthonous tumors from which they were derived, we observed similar grossly similar histologic characteristics, but specific composition as percent of the region of interest (ROI) on CODA typing varied. While our autochthonous KMC^{ERT2} tumors ranged from 29-62% Stroma/ECM and 4-49% cellular PDAC in composition, their orthotopic counterparts ranged from 10-20% and 48-86%, respectively (Supp. Table 2.05).

Table 2.02 KMC^{ERT2}-derived cell line and orthotopic model summary

Autochthonous Tumor Endpoint Data			Omics Data Availability			<i>In vivo</i> (orthotopic model)			
Mouse / Cell Line ID	Sex	Tumor subtype	RNAseq	WGS	WES	Growth consistency	Liver metastatic frequency	Gemcitabine/Abraxane sensitivity	FOLFIRINOX sensitivity
011T	M	N/A	-	-	-	Not assessed	Not assessed	Not assessed	Not assessed
106L	F	N/A	-	-	-	Not assessed	Not assessed	Not assessed	Not assessed
171J	M	Squamous	+	+	+	Consistent	73%	Resistant	Resistant
255J	F	Squamous	+	+	+	Inconsistent	50%	Not assessed	Not assessed
301J	M	Pancreatic Progenitor	+	+	+	Consistent	40%	Sensitive	Not assessed
303J	M	Pancreatic Progenitor	+	+	+	Consistent	40%	Not assessed	Not assessed
314J	M	Squamous	+	+	+	Consistent	76%	Resistant	Resistant
808J	F	N/A	-	-	-	Not assessed	Not assessed	Not assessed	Not assessed

Tumor subtype: Squamous subtype, defined by Bailey et al., 2016, overlaps with basal-like and quasi-mesenchymal, as defined by Collisson et al., 2011 [33] and Moffitt et al., 2015 [214], respectively. Pancreatic progenitor, defined by Bailey et al., 2016 [31], overlaps with classical, as defined by both Collisson et al., 2011 and Moffitt et al., 2015.

Using serial transabdominal ultrasound, we followed both orthotopic primary tumor growth and development of liver metastases over the course of 6-8 week studies (Supp. Fig. 2.13). All four lines consistently metastasized to the liver (**Fig. 2.06I-K**). Metastases from orthotopic KMC^{ERT2} tumors typically appear within four weeks of implantation. Orthotopic

growth and overall survival of three KPC cell lines were comparable to those of KMC^{ERT2} tumor-derived cell lines but two of the three KPC lines had greatly reduced metastasis to the liver (**Fig. 2.06E, F, I**). Together, these data demonstrate the promise of using KMC^{ERT2} tumor-derived cell lines in an orthotopic setting to study diverse PDAC phenotypes, disease progression and metastasis, and therapeutic efficacy.

Response to standard-of-care therapy is correlated to molecular subtype in KMC^{ERT2} orthotopic PDAC model

To determine *in vivo* therapeutic response, we performed additional orthotopic studies with selected KMC^{ERT2} tumor-derived cell lines with differing PurIST subtypes: one squamous, 171J, and one pancreatic progenitor, 301J (Supp. Fig. 2.16). Once orthotopic tumors were established, we treated mice with SoC gemcitabine 100 mg/kg and abraxane 15 mg/kg (Gem/Abraxane) via intraperitoneal injection, twice weekly. We observed clear differences between squamous- (171J) and pancreatic progenitor-derived (301J) orthotopic tumor growth and overall survival (Supp. Fig. 2.16). Further validating previously reported subtype-dependent drug responses [312], the squamous 171J-implanted tumors were resistant to Gem/Abraxane, and the pancreatic progenitor 301J-implanted tumors showed a clear response (Supp. Fig. 2.16).

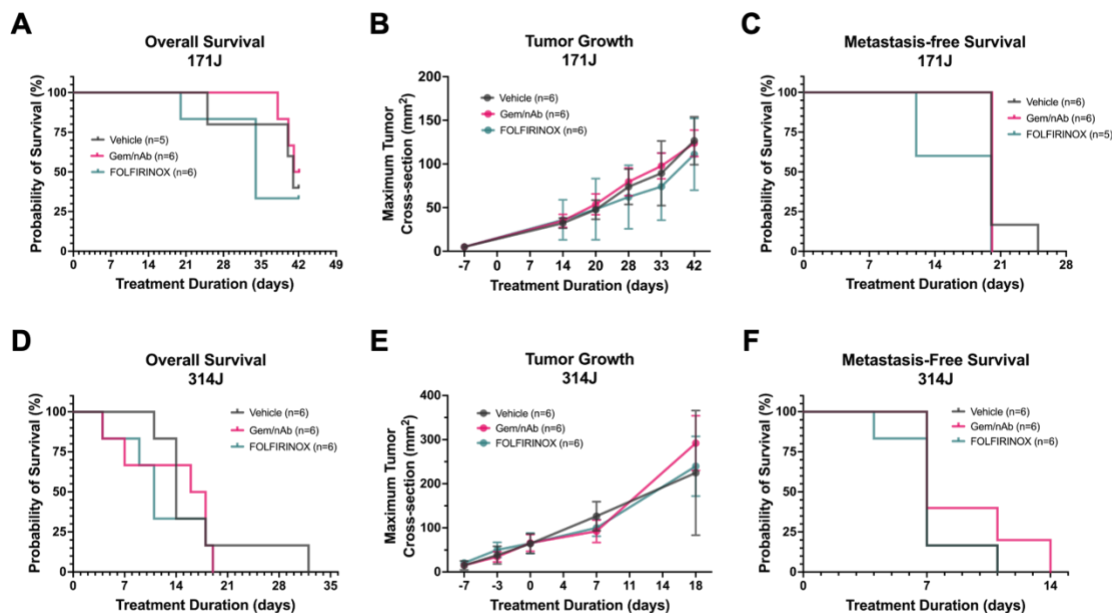


Figure 2.07 KMC^{ERT2} orthotopic model of PDAC is variably resistant to standard-of-care therapy.

A-C) Kaplan-Meier curve showing probability of overall survival, graph showing tumor growth, and Kaplan-Meier curve showing probability of metastasis-free survival in B6129SF1/J mice orthotopically implanted with murine PDAC cell line 171J when treated with standard of care (SoC) therapy Gemcitabine/nAb (50mg/kg & 0.5mg/kg ; I.P. weekly) or FOLFIRINOX (100 mg/kg folinic acid (FOL) + 5 mg/kg 5-fluorouracil (F) + 50 mg/kg irinotecan (IRIN) + oxaliplatin 5 mg/kg (OX) ; I.V. d14 & d16), or vehicle. **D-F)** Kaplan-Meier curve showing probability of overall survival, graph showing tumor growth, and Kaplan-Meier curve showing probability of metastasis-free survival in B6129SF1/J mice orthotopically implanted with murine PDAC cell line 314J when treated with SoC therapy Gemcitabine/nAb (50mg/kg & 0.5mg/kg ; I.P. weekly) or FOLFIRINOX (100 mg/kg folinic acid (FOL) + 5 mg/kg 5-fluorouracil (F) + 50 mg/kg irinotecan (IRIN) + oxaliplatin 5 mg/kg (OX) ; I.V. d14 & d16), or vehicle.

To assess whether or not Gem/Abraxane-resistant cell lines would be sensitive to the alternative SoC chemotherapy FOLFIRINOX, we implanted larger cohorts of mice with one of two squamous-derived cell lines, 171J or 314J (**Fig. 2.07**). Again, we saw no significant change to survival with either Gem/Abraxane or FOLFIRINOX treatment (**Fig. 2.07A, D**). There was also no difference in tumor growth or metastasis rate between treatment conditions (**Fig. 2.07B, D, E, F**). While additional studies with larger cohort numbers will be required to validate these therapy response results and determine significance, these initial studies demonstrate the potential of our KMC^{ERT2} tumor-derived cell lines as a new set of tools with which to study and overcome therapy resistance in PDAC.

DISCUSSION

Pancreatic cancer is a disease with a high rate of mortality, as it is generally diagnosed in advanced stages when no curative treatments are available. Developing new models that can investigate the mechanisms of initiation, dissemination, and resistance to therapy is of the utmost importance. More effective treatment regimens are critically needed, as nearly two decades of standard-of-care therapies have not substantially improved survival [208, 313]. To efficiently assess the efficacy of new drug candidates, *in vivo* models that accurately reflect the complex features of human pancreatic cancer, including its subtypes, treatment responses, and metastatic potential, are paramount.

We and others have shown that elevated levels of pS62-MYC occur in early pancreatic precursor lesions in human pancreatic cancer [169, 314, 315]. In this study, we have demonstrated that even low levels of deregulated *Myc*, in combination with mutant *Kras*, significantly accelerate PDAC progression in the novel KMC^{ERT2} autochthonous model. This model also faithfully represents the molecular heterogeneity seen in human PDAC, generating distinct subtypes of tumors, including classical and squamous/quasi-mesenchymal variants. These subtypes not only differ in their molecular characteristics but also show differential responses to chemotherapy. For instance, while classical PDAC tumors tend to be more responsive to gemcitabine and nAb-paclitaxel [313], squamous-like tumors exhibit higher resistance to treatment, reflecting similar findings in patient populations [31–33, 214].

The Evan lab's MYC model [108] has provided critical insights into the dynamic role MYC plays in both tumorigenesis and the TME. By employing a switchable MYC system in a *Kras*-driven PDAC model, they demonstrated that MYC activation alone is sufficient to drive rapid tumor growth, promote desmoplasia, and induce a highly immunosuppressive TME [108]. Importantly, they observed a reversible effect when MYC expression was

halted, with tumors regressing and TME remodeling, leading to the restoration of normal tissue architecture. This reversible model underscored MYC's importance in maintaining the malignant state. However, the Evan lab's model largely focused on MYC's influence on tumor cell proliferation and immune modulation without thoroughly addressing the full molecular heterogeneity or metastatic potential that characterizes PDAC. Moreover, their system's emphasis on rapid onset of MYC overexpression limits its ability to model steady low-MYC-driven tumorigenesis, which is critical in certain PDAC subtypes.

In contrast, the KMC^{ERT2} model captures these aspects by modeling low-level deregulated MYC expression alongside mutant KRAS, more accurately reflecting the heterogeneous nature of PDAC. Our model also allows for a detailed study of MYC-driven metastasis, which was less emphasized in the Evan lab's system. Notably, we observed significant rates of spontaneous liver metastasis in KMC^{ERT2} tumors, an important hallmark of PDAC progression that the Evan model is unable to fully explore due to the limitations of long-term doxycycline treatment. In turn, the KMC^{ERT2} model is a more comprehensive tool for studying both early tumorigenesis and later-stage disease, including the molecular drivers of metastasis. Additionally, by developing a series of cell lines from these tumors, we can further investigate subtype-specific plasticity, an area that the Evan lab's model did not address.

Our characterization of the KMC^{ERT2} model also revealed interesting insights into the plasticity of these tumors. Cell lines derived from the tumors demonstrate differential plasticity when grown in vitro under varying conditions, which mirrors the heterogeneity of PDAC in vivo. Squamous lines exhibited changes in global transcriptional profiles when grown in CRC media vs. DMEM 10%, indicating a propensity to adapt to environmental signals. This subtype-specific plasticity reflects the dynamic nature of PDAC and could be key to understanding how these tumors resist standard treatments and develop metastatic

potential. Interestingly, pancreatic progenitor-derived cell lines maintained their high MYC activity regardless of culture conditions, correlating with their rapid in vivo growth and sensitivity to proliferative-targeting therapies like gemcitabine and Abraxane [109, 208, 316].

Our data show that common downstream molecular aberrations observed during human PDAC, including p53 inactivation and loss of CDKN2A expression [169], also occur during the progression of KMC^{ERT2} tumors. These genetic alterations are critical for the transition to advanced disease and may contribute to the high rate of liver metastasis observed in this model [59, 317]. The KMC^{ERT2} tumors histologically and molecularly resemble human PDAC, particularly in their metastatic behavior. This aspect of the model offers a unique advantage, as it allows researchers to study the mechanisms underlying PDAC metastasis and evaluate how early events drive the disease's aggressive nature.

Durable cure in pancreatic cancer remains rare and requires both surgical resection of the tumor and systemic chemotherapy. Recent studies assessing correlations between tumor subtype and patient outcome have shown that squamous PDAC tumors are more aggressive and often correlate with worse prognosis [31–33, 214]. Squamous/quasi-mesenchymal tumors were often sensitive to adjuvant gemcitabine, but sometimes reported as highly resistant to mFOLFIRINOX regimens [32, 33, 214]. Our data is consistent with these findings. The divergent PurIST subtypes of KMC^{ERT2} tumors and cell lines derived from this model reflect the ability of early-event alterations such as *KRAS* mutation and *MYC* dysregulation to provide high fidelity modeling of human disease. As with human PDAC, the therapeutic responsiveness of these lines, when grown orthotopically, seems related to transcriptional subtype—with classical subtypes more sensitive to gemcitabine, while squamous subtypes exhibited significant resistance to mFOLFIRINOX.

Our characterization of cell lines from this model also revealed interesting results about the plasticity of tumor derived models. We observed subtype-specific plasticity of the cell lines when grown in culture in different medias. The squamous lines exhibited a change in global transcriptional profiles when grown in CRC media vs. DMEM 10%, indicating their propensity to respond or adapt to extrinsic signals or stress. Interestingly, in contrast to that result and to recent studies showing that squamous tumors typically exhibit higher MYC activity [109, 316], pancreatic progenitor cell lines maintain their high expression of MYC_V1 and MYC_V2 target hallmarks regardless of growth condition, potentially explaining their rapid growth *in vivo*. This observation may also relate to their sensitivity to Gem/Abraxane, which acts primarily on proliferating cells. Further work will be needed to link the cell line subtype plasticity in differential media conditions to the plasticity of PDAC that drives resistance, but this would be a significant development in pre-clinical modelling and would provide tools for mechanistic understanding and therapeutic interception.

This model also provides critical insights into the tumor immune microenvironment. Despite PDAC's general resistance to immunotherapies, recent studies the presence of cytotoxic T cells within PDAC tumors [30, 255] or demonstrating the presence of neoantigens [31, 153, 318] indicate the potential promise of therapeutic interventions that could restore T cell responses in PDAC. In our studies, we observed a similar density of dendritic cells DCs in KMC^{ERT2} tumors as observed in human PDAC samples. This is significant, as recent research implicates the paucity of DCs in dysfunctional immune surveillance in PDAC [306, 319–321]. Interestingly, KMC^{ERT2} tumors displayed a higher density of DCs compared to KPC controls, suggesting that this model may be ideal for studying DC involvement in PDAC and exploring immune-based therapies. Further interrogation into the link between MYC deregulation and increased DC presence in the

tumor immune microenvironment is necessary. Determining whether a subset of PDAC patients with *MYC* amplifications could respond to T cell-activating immunotherapies could lead to novel therapeutic avenues. The KMC^{ERT2} mouse model provides an excellent preclinical system for these investigations.

The generation and characterization of our KMC^{ERT2} model offers a valuable platform for understanding pancreatic cancer biology and performing preclinical studies. The ability to generate heterogeneous, metastatic PDAC from a series of tumor-derived cell lines will be critical for high-throughput in vivo experimentation. The reliable metastatic capacity of KMC^{ERT2} tumors will be valuable to those wishing to explore the nuances of metastatic disease without forceful initiation by hemi-spleen or intravenous injection. Reliable understanding of early events in pancreatic cancer metastasis are difficult in humans given the rarity of early diagnosis and temporal and spatial challenges associated with biopsy. This model provides an exciting opportunity to evaluate metastatic disease in pancreatic cancer at all stages of dissemination, colonization, and progression. Additionally, differential metastatic potential in this model will provide tools for understanding mechanisms of tropism and pancreatic cancer metastasis. Moreover, the cell lines generated recapitulated subtype-specific responses to chemotherapy in these initial studies. In addition to further studies on chemotherapy resistance, this model is well-positioned for investigations into novel therapeutic targets, including *KRAS* and *MYC* inhibitors [229, 245, 322, 323]. The KMC^{ERT2} model will also be useful in studying responses to immunotherapy, given its physiological relevance to human PDAC. Moving forward, continued development and characterization of the model's cell lines, alongside a more detailed exploration of the tumor microenvironment, will enhance its utility in preclinical studies.

We are excited to present this first report of the novel KMC^{ERT2} model, which offers significant promise for advancing pancreatic cancer research and identifying new therapeutic strategies for a disease that remains highly resistant to treatment.

MATERIAL AND METHODS

Relevant methods are provided below; for more detailed information and catalog numbers, see **Appendix C** (Key Resources Table).

Primary patient tissue acquisition and patient consent

Primary patient data and tissue were collected through the IRB-approved Oregon Pancreas Tissue Registry (OPTR) (IRB00003609) in accordance with the Declaration of Helsinki. De-identified tissue was assigned a Participant ID and utilized for relevant experiments (IRB00003330).

OHSU Human PDAC progression TMA

The human progression TMA was designed and generated by the Brenden-Colson Center for Pancreatic Care at Oregon Health & Science University (OHSU). Tissue regions of interest were manually marked by a trained pathologist (T.M.) on FFPE sample blocks from surgically resected primary pancreatic tissue samples from pancreas patients at OHSU. Using a TMA Master II (3DHistech, Hungary) for drilling recipient block and MTA-1 (Estigen Tissue Science, Estonia) for tissue coring, 1.5 mm tissue cores, two cores per surgical specimen (with 4 exceptions where tissue was too small, so only one core was punched), were punched from representative areas containing normal pancreas, pancreatic intraepithelial neoplasia (PanIN, grades 1-3), adenocarcinoma (PDAC, grades 1-3), intraductal papillary mucinous neoplasia (IPMN, grades 1-3), islet cell tumor, and cholangiocarcinoma. Tissue cores were randomized and divided between two paraffin blocks (TMA-1 and TMA-2). The resulting TMAs consisted of 109 unique tissue samples (213 tissue core punches, including duplicates) from 53 patients. Tissue cores included:

normal pancreas (n = 17), PanIN grades 1, 2, 3 (n = 10, 7, 1); adenocarcinoma grades 1, 2, 3 (n = 3, 16, 22); IPMN grades 1, 2, 3 (n = 4, 8, 15); islet cell tumor (n = 1); and cholangiocarcinoma (n = 5) samples. There were an additional 6 cores of normal placenta tissue as controls.

Immunohistochemical staining of OHSU Tissue Micro Array (TMA-1)

TMA-1 was used for the assessment of nuclear pS62-MYC by immunohistochemistry (IHC). The stained section was comprised of tissue samples from 25 patients, including normal pancreas (n = 20), PanIN (n = 7), PDAC (n = 17), and PDAC metastasis (n = 2). Heat-induced antigen retrieval was performed where slides were immersed in target-retrieval solution of citric acid buffer (pH 6.0) for 10 min at 120°C. After antigen retrieval, endogenous peroxide activity was blocked using 3% H₂O₂ solution for 15 min at room temperature (RT), followed by protein blocking using 3% FBS/goat serum for 30 min at RT. Next, TMAs were incubated overnight at 4°C primary pS62-MYC antibody (Sears Lab (1:200)). After primary antibodies, TMAs were incubated with anti-rat horseradish peroxidase (HRP) conjugated secondary antibody (Vector Laboratories, cat# BA-9401, 1:150) for 1 hour and ABC Kit (Vector Laboratories, catalog# PK-7100) for 30 min at RT, followed by detection using 3,3'-Diaminobenzidine (Vector Laboratories, catalog# SK-4100) according to manufacturer's instructions and counterstained using hematoxylin. IHC expression of pS62-MYC was scored as the product of expression level multiplied by percentage of nuclear staining in positive cell.

Genetically Engineered Mouse Model Generation & Care

All animal studies were conducted in compliance with ethical regulations and were approved by OHSU institutional animal care and use committee (IACUC) (protocol number TR01_IP00001014). The generation and characterization of the Rosa-LSL-Myc mouse (*Mus musculus*) has been previously described by our lab [110]. The LSL-Kras^{G12D}; Pdx1-Cre mice [82] were obtained from Dr. Kerry Campbell at Fox Chase Cancer Center. The

Ptf1a-Cre^{ERT2} mice have been previously described [315]. Our Tamoxifen-inducible KMC^{ERT2} PDAC GEMM was generated by crossing the Rosa-LSL-Myc-HA and LSL-Kras^{G12D} alleles with the conditional Ptf1a-Cre^{ERT2}. All mice were on a mixed Bl/6-129 background, and both males and females were used. Allele recombination was induced in mice between 7-9 weeks old with 50 mg/kg Tamoxifen (MilliporeSigma, Burlington, MA) once daily for 5 days by oral gavage.

Murine Tissue Harvest & Processing

Mice were euthanized when they first appeared moribund or when their pancreatic tumors reached 2 cm² at a maximum tumor cross-section, as measured by ultrasound. Mice in the long-term GEMM survival study not having reached the two aforementioned endpoints at 750 days of age were euthanized at that point. Mice were perfused with 15 mL PBS (Fisher Scientific, Cat. # SH3002802) containing 0.005% heparin (Sigma Aldrich, Cat. # H3393) under general anesthesia. Pancreatic, liver, lung, spleen, gastrocnemius muscle (GM), gonadal fat pad (GFP), and brown adipose (BAT) tissues were harvested and weighed. GM, GFP, BAT and pieces of pancreas, liver, lung, and spleen were preserved in RNAlater and/or flash-frozen in liquid nitrogen. Remaining tissues were then fixed in 10% formalin (4% paraformaldehyde) for 48 hours, then transferred to 70% EtOH. Tissue blocks and slides were made and H&E stained by the Histopathology Shared Resource Core at OHSU or by lab members. Stained slides were scanned using a Zeiss AxioScan microscope at the Advanced Light Microscopy Core at OHSU. Relative pancreas weight was calculated using the following formula:

$$\left(\frac{\text{tumor weight (g)}}{\text{body weight (g)}} \times 100\% = \text{relative pancreas weight} \right)$$

CODA Tissue Composition Methodology

Mouse pancreas histological composition was quantified using a semantic segmentation deep learning model previously developed for quantification of human pancreas histology

[290]. Manual annotations were performed in 30% of the images to label 10 microanatomical tissue components in pancreatic histology: islets of Langerhans, normal ductal epithelium, pancreatic cancer precursor lesions, pancreatic cancer, vasculature, acinar tissue, fat, collagen, immune hotspots, and non-tissue. A deep learning algorithm was trained to fully label the 10 tissue components in all H&E images to a resolution of 1 $\mu\text{m}/\text{pixel}$. Lymph nodes and duodenum were manually labeled on images where they appeared. Testing annotations were performed in seven histological images and validated by a board-certified pathologist specializing in pancreatic cancer, revealing an independent accuracy of 96% (Supp. Fig. 2.03A). Following testing, all images were fully segmented by the trained model, and pancreatic compositions were quantified and compared between conditions.

Immunofluorescence (IF) Tissue Staining

For IF of human samples, formalin-fixed paraffin-embedded tissue sections from patients with matched normal and pancreatic cancer tissues were obtained from archives of the OHSU Department of Pathology, (IRB00002086). These sections were then de-paraffinized, rehydrated, and blocked in 3% BSA. They were incubated in primary antibody (rabbit polyclonal pS62-MYC) [324] overnight at 4°C. Sections were then incubated with AlexaFluor 488 secondary antibody (Life Technologies, Grand Island, NY) (1:1000) and mounted using SlowFade Gold Antifade mountant with DAPI (Life Technologies, Grand Island, NY). Images were taken with a Hamamatsu digital camera mounted on a fluorescence microscope. Immunofluorescence density was analyzed using the Measure Density tool in OpenLab 5.5 software. pS62-MYC staining intensity was then measured in 10 randomly selected individual cells in each of 5-10 random fields of view and averaged for each sample.

For IF of murine samples, formalin-fixed paraffin-embedded tissue sections were de-paraffinized, rehydrated, and antigen retrieval performed in pH 6 Citrate buffer (Sigma, location) in a pressure cooker for 4 min at high pressure and allowed to de-pressurize for 16 minutes. A second antigen retrieval step was then performed with pH 9 IHC Antigen Retrieval solution (Life Technologies) for 15 min. After rinsing slides in dH₂O to cool to room temperature and equilibrating in PBS, tissues were blocked with 10% normal goat serum in PBS for 30 min. Primary antibodies (see Key Resources) were diluted in 5% normal goat serum with 1% BSA in PBS and applied to tissues for overnight incubation at 4°C. Secondary antibodies conjugated to Alexa Fluorophores 488, 555, 568, 594, 647, or 750 were diluted 1:200 in 5% normal goat serum with 1% BSA in PBS and applied to tissues for 1 hr at room temperature. Slides were mounted with SlowFade Gold Antifade mountant with DAPI (Life Technologies).

Immunohistochemistry (IHC)

Unstained sections were de-paraffinized, rehydrated, and placed in Target Retrieval Solution (Dako, Carpinteria, CA) for 10 min at 100°C. After cooling for 20 min, slides were quenched with 3% hydrogen peroxide for 5 min, followed by blocking with 3% goat serum and primary antibody incubations overnight at 4°C. Primary antibodies used were: CDKN2A (p16-INK4A) (Invitrogen, Waltham, MA), Smad4 (Abcam, Cambridge, UK), p53 (Cell Signaling Technology (CST), Danvers, MA). Sections were then incubated with anti-biotin secondary antibodies (1:1000) and labeling was then detected with the Vectastain ABC kit (Vector Laboratories, Newark, CA). Slides were mounted using Vectamount mounting media (Vector Laboratories).

IHC Image Processing and Analysis

SMAD4, CDKN2A and P53 IHC single cell staining quantification were performed using color deconvolution in ImageJ followed by the Mesmer algorithm for nuclear segmentation

[325] and a custom algorithm for duct segmentation and signal intensity quantification (https://github.com/engjen/KMC_Mouse). Briefly, CDKN2A and p53 duct segmentation was automated by selecting pixels with DAB and hematoxylin intensity greater than the 0.3 quantile, removing objects smaller than 500 pixels and performing a morphological opening with a 3-pixel disk. SMAD4 duct segmentation was automated by selecting pixels with a hematoxylin intensity greater than the 0.3 quantile, removing holes smaller than 2000 pixels and performing a morphological closing with an 8-pixel disk. ROIs dominated by tumor were manually selected for staining quantification.

Multiplexed Immunohistochemistry (mIHC), Image Acquisition, and Analysis

Multiplexed IHC was performed on 5 μ m formalin-fixed paraffin-embedded (FFPE) sections, as previously described [30, 304, 326–328]. Briefly, slides were deparaffinized and stained with CSF-1R (Santa Cruz Biotechnology, Dallas, TX), F4/80 (Bio-Rad, Hercules, CA), and hematoxylin (Dako, Santa Clara, CA), followed by digital whole-slide scanning at 20X magnification on an Aperio AT2 scanner (Leica Biosystems, Wetzlar, Germany). Tissues then underwent 20 minutes heat-mediated antigen retrieval in pH 6.0 Citra Plus solution (BioGenex, Fremont, CA), followed by endogenous peroxidase blocking in either 0.6% H₂O₂ for 20 minutes or Dako Dual Endogenous Enzyme Block (Dako, Santa Clara, CA) for 10 minutes. Protein blocking was performed for 10 minutes with 5% normal goat serum and 2.5% BSA in PBS. Slides were incubated with primary antibody for a duration of 30 to 60 minutes at room temperature or overnight at 4°C. Antibodies and staining conditions are listed in Supplementary Table 2.06. Slides were then washed in TBST and either anti-rat, anti-rabbit, or anti-mouse secondary antibody was applied for 30 minutes at room temperature, followed by signal detection with AEC chromogen (Vector Laboratories, Burlingame, CA). Slides were then digitally scanned before chromogen removal in 100% ethanol. For staining cycles with two rounds of

antibody development, protein blocking was repeated after chromogen removal and tissue sections were taken through all steps listed above from primary antibody application through chromogen development, slide scanning, and chromogen removal. The next cycle was then started at the heat-mediated antigen retrieval step, in order to ensure removal of all antibodies from the previous cycle.

Tissue Annotation and Region of Interest (ROI) Selection

Whole-slide digitally scanned images for each case were reviewed by a pathologist (TKM), and tumor-enriched tissue regions were digitally annotated. Areas of necrosis were excluded. One FFPE tissue section per GEMM PDAC tumor (n = 3 for KMC^{ERT2} cohort; n = 4 for KPC cohort) was used for staining by a combined mouse cell lineage and functional state mIHC biomarker panel. ROI number and size were maintained across samples. To calculate cell densities of immune populations in an individual sample, cell counts for a given cell type (e.g. Dendritic cells) from each ROI were added to get total cell count, and areas of each ROI were also added. Cumulative density was then calculated with the following equation:

$$\frac{\text{Total cell number } (n)}{\text{Total area } (mm^2)} = \text{Cumulative cell density } (mm^2)$$

mIHC Image Processing and Analysis

Image co-registration and processing was performed using methods adapted from the Coussens lab's previously described image processing workflow [304, 326, 329, 330]. Briefly, selected ROIs from each RGB marker image were registered to the hematoxylin stained RGB image using the detectSURFfeatures algorithm in the Computer Vision Toolbox in Matlab (version R2018b, The MathWorks, Inc., Natick, MA) to identify matching keypoints in the blue or red channel of the hematoxylin image with keypoints in the blue channel of the AEC stained image. Using estimateGeometricTransform in the Computer

Vision Toolbox, a geometric transformation was estimated using similarity that was applied to all channels of the AEC-stained image. This process was used to register each marker-stained image region to the same region of the hematoxylin-stained image.

Image processing, cell quantification, and image cytometry were performed using FIJI (FIJI Is Just ImageJ) [330], CellProfiler (version 3.5.1) [331], and FCS Express 6 Image Cytometry RUO (De Novo Software, Glendale, CA), respectively. AEC signal was extracted for quantification and visualization in FIJI using a previously described custom macro [30] for color deconvolution. The resulting data is a csv containing the mean intensity of every cell for each marker. These data are subsequently used in image cytometry single-cell gating, hierarchical identification and quantification in FCS Express (De Novo Software). Hierarchical cell classifications are shown in Supplementary Table 2.03. For mIHC visualization, signal-extracted images were overlaid in pseudo-color in FIJI [330].

MYC genomic alteration survival analysis in published human patient cohort

cBioPortal for Cancer Genomics (<http://cbioportal.org>) provides a framework for multidimensional cancer genomics data analysis and visualization [279, 332]. In this study, we performed survival analyses based on mutations and copy number alterations (amplifications) in c-MYC appearing in dataset downloaded from cBioPortal: The Pancreatic Adenocarcinoma (TCGA, PanCancer Atlas).

DNA and RNA isolation

DNA and RNA were isolated from FFPE end-stage murine pancreatic tumor tissue by first identifying tumor-dominant ROIs on full tissue scans of H&E slides for each tumor. These ROIs were used as reference microdissection maps while carefully scraping tumor-rich regions from freshly cut 10 μ m sections from slides under a dissection microscope. RNA was then isolated from the microdissected FFPE tissue using an AllPrep® DNA/RNA

FFPE kit (Cat. # 80234) with on-column DNase treatment (Qiagen, Hilden, Germany), per manufacturer's protocol.

KMC^{ERT2} cell lines were cultured to 80% confluence on 10 cm cell culture dishes in CRC media or DMEM + 10% FBS. DNA and RNA were isolated directly from 80% confluent 10 cm cell culture dishes for each cell line, using an AllPrep® DNA/RNA mini kit (Cat. # 80204) with on-column DNase treatment (Qiagen, Hilden, Germany), per manufacturer's protocol.

Whole Genome Sequencing (WGS) for Copy Number Variant Calling

Library preparation and sequencing were done by the OHSU Massively Parallel Sequencing Shared Resource (RRID SCR_009984). Sequencing libraries for FFPE GEMM tumors were constructed using the Illumina TruSeq Nano Library Prep kit (Illumina, San Diego, CA). Sample libraries were diluted and applied to an Illumina NovaSeq 6000 (RTA version 3.4.4) flow cell at a concentration appropriate to generate about 400 million reads. All libraries were prepared with indexing barcodes to permit multiplexing in a single lane. The 150-cycle paired-end read sequencing was used to assemble the reads into standard FastQ formatted data.

Low-pass whole genome sequencing was performed on tumor/normal pairs first by aligning short-read sequencing data with BWA mem with default settings [333, 334]. Second, ichorCNA (<https://github.com/broadinstitute/ichorCNA>, Broad Institute, Cambridge, MA) [335] was used to predict CNVs and estimate tumor fraction with default settings.

Whole Exome Sequencing (WES) for Somatic Point Mutation Calling

Library preparation and sequencing were done by CD Genomics (Shirley, NY). Briefly, sequencing libraries were generated using SureSelectXT Mouse All Exon Library Prep Kit following manufacturer's recommendations (Agilent Technologies, Folsom, CA) to enrich

genomic DNA for coding exons. Index codes were added to attribute sequences to each sample. Sample libraries were then subjected to paired-end whole exome sequencing using Illumina SBS instruments with a raw sequencing coverage of 200 x. Across samples, the average sequencing depth was 1.28×10^{10} base pairs sequenced per sample.

The sequenced reads were aligned to the mouse genome with BWA mem with default settings [333, 334]. Tumor/normal mutation calling was performed with the NF-Core/Sarek (<https://nf-co.re/sarek>) [336, 337] pipeline for detection of somatic variants with exome data. The resulting mutation calls were filtered using the Filters for Next Generation Sequencing (FiNGS) package (<https://github.com/cpwardell/FiNGS>) [338] using standard settings.

RNA Sequencing (RNAseq)

Library preparation and sequencing were done by the OHSU Massively Parallel Sequencing Shared Resource (RRID SCR_009984).

Sequencing libraries for FFPE GEMM tumors and KMC^{ERT2} cell lines were constructed using the Stranded RNA Prep, Ligation, with the Illumina Ribo-Zero Plus kit or the Illumina TruSeq Stranded mRNA Library Prep kit (Illumina, San Diego, CA), respectively. Briefly, poly(A)+ RNA was isolated from 400 ng of total RNA (per sample) using oligo-dT-coated magnetic beads. The recovered RNA was then chemically fragmented. First-strand cDNA was generated using random hexamers as primers for reverse transcriptase. Strand-specific second-strand cDNA synthesis was performed and a single 'A' nucleotide was then added to each end. Illumina adaptors were ligated to the cDNAs. Limited-round (15-cycle) PCR was used to amplify the material to yield the final libraries. Library concentration was determined using real-time PCR with primers complementary to the Illumina adaptors. Sample libraries were diluted and applied to an Illumina NovaSeq 6000 (RTA version 3.4.4) flow cell at a concentration appropriate to generate about 400 million

reads. All libraries were prepared with indexing barcodes to permit multiplexing in a single lane. The 100-cycle paired-end read sequencing was used to assemble the reads into standard FastQ formatted data.

RNA-seq analysis was performed similar to previous description [339]. Briefly, paired-end FastQ sequences were trimmed using trim-galore (version 0.6.3) and default parameters. Pseudoalignment was performed with kallisto (version 0.44.0) [340] using genome assembly GRCm39 (release M26) and gencode (version 24) annotation; default parameters were used other than the number of threads. The Bioconda package bioconductor-tximport (version 1.12.1) was used to create gene level counts and abundances (TPMs). Quality checks were assessed with FastQC (version 0.11.8) and MultiQC (version 0.1.7). Quality checks, read trimming, pseudoalignment, and quantitation were performed via a reproducible snakemake pipeline, and all dependencies for these steps were deployed within the anaconda package management system [340, 341].

Differential Expression Analysis

All differential expression analysis was performed in R (version 4.1.0) [342] using the package DESeq2 (version 1.34.0) [343]. To compare KMC^{ERT2} mice to KPC mice, we first summarized Kallisto transcript abundance outputs for all mice into a gene-level expression dataset via tximport (version 1.22.0) [344], then ran DESeqDataSetFromTximport() to construct the DESeq object used for downstream analysis. Genes with less than 10 cumulative read counts summed across samples in the DESeq object were excluded. Differential expression was performed using the DESeq() function set to compare KMC^{ERT2} mice with KPC mice (KMC^{ERT2} and KM/+C^{ERT2} mice were regarded as a single group). Volcano plots of differential protein-coding genes were created with ggplot2 (version 3.4.1) [345]. For heatmap visualization, we applied variance-stabilizing transformation (VST) to expression counts using DESeq2 and plotted the transformed counts with pheatmap

(version 1.0.12) [346]. Heatmap values were scaled by row/gene, and only protein coding genes called differential by an adjusted p-value of 0.05 or less were included. For differential expression between KMC^{ERT2} and KM/+C^{ERT2} samples, as well as differential expression between KMC^{ERT2}/KM/+C^{ERT2} clusters, we performed the same computational workflow but excluded KPC mice from the DESeq object and model.

Analysis of KMC expression clusters

To identify distinct clusters within our KMC^{ERT2} PDAC model, we analyzed RNA-seq profiles of homozygous and heterozygous ROSA-MYC-expressing mice (KMC^{ERT2} and KM/+C^{ERT2}). We applied the R function `dist()` to a matrix of VST-transformed expression counts generated from the KMC^{ERT2}/KM/+C^{ERT2} DESeq object. Sample-to-sample distances generated through `dist()` were visualized and clustered with `pheatmap`. Hierarchical clustering results from `pheatmap` indicated three major clusters that we used for further analysis. To perform PCA, we applied the DESeq2 function `plotPCA()` on the same VST-transformed count data used for clustering. PCA settings in DESeq2 was set to use the top 500 variable genes. Differential expression between clusters was tested in pairwise fashion via DESeq2. Protein-coding genes called significant (adjusted p-value of 0.05 or less) within each differential expression test were collected and visualized as a heatmap via `pheatmap`. VST-transformed counts were used as input for `pheatmap()` and heatmap values were scaled by row/gene.

Gene ontology (GO) enrichment

All GO [347–349] enrichment analysis was done using the PANTHER [350, 351] statistical overrepresentation test provided by their website (<http://www.pantherdb.org>). Protein coding genes identified as differentially expressed (min. adjusted p-value 0.05 and abs. log2-fold change 1.0) were used as the test set while the reference set included all protein coding genes with at least 10 read counts summed across samples. PANTHER overrepresentation test settings used were: Fisher's Exact as the test type, FDR for p-

value correction, and “GO biological process complete” as the annotation data set (GO annotation release version 2023-03-06).

Gene set variation analysis (GSVA)

All GSVA was performed with the GSVA R package (v.1.42.0) [311] on MSigDB [352] hallmark gene sets imported with msigdbR (v.7.5.1) [353]. To assign GSVA scores to each sample, VST-transformed counts obtained from DESeq2 were inputted into the `gsva()` function using default settings. VST-transformation and GSVA were performed on mouse and human RNA-seq datasets separately. Heatmap visualization of GSVA results for KMC^{ERT2}/KM/+C^{ERT2} samples was done with pheatmap. To improve visual interpretability, dendrogram nodes for samples/columns were rotated to keep clusters 1-3 together without affecting meaning.

Gene set enrichment analysis (GSEA)

GSEA tool [354, 355] was used with default settings (1000 permutations) on RNAseq clusters data to compare enrichment to gene ontology and hallmark gene sets in the MSigDB database (version 7.5.1) (see Supplementary dataset 3) [266].

Survival analysis of human pancreatic cancer RNA-seq profiles

To analyze the impact of MYC on overall survival in PDAC, we used existing human pancreatic tumor RNA-seq profiles obtained from the Oregon Pancreas Tissue Registry under IRB study # IRB00003609. Informed consent was obtained from all subjects and experimental protocols were approved by the OHSU Institutional Review Board. The collection, processing, and sequencing of these samples are described by Link JM, et al. [29]. Clinical information pertaining to specimen site, time from diagnosis to death or follow-up, and vital status at last follow-up was collected from the OHSU Cancer Registrar on 01-26-2023. Samples were included for the present analysis if the sequenced specimen was obtained from a primary tumor and the clinical annotation specifying tumor site included “Pancreas”. Two samples with conflicting survival information recorded were

excluded, resulting in a final list of 196 human primary pancreas tumors for downstream analysis. RNA-seq profiles (formatted as Kallisto transcript abundance files) for each sample were imported into a DESeq object through the same general tximport workflow we used for our mouse differential expression analysis. To perform Kaplan-Meier survival estimation, we used the R package survival (version 3.3-1) [356] and plotted the resulting survival curves with survminer (version 0.4.9) [357]

Primary Murine Cell Line Generation

Cell lines 011T, 106L, 171J, 255J, 301J, 303J, 314J, and 808J were propagated from primary murine PDAC tumor tissue and cultured in conditionally reprogrammed cell (CRC) media (Ham's F-12:DMEM (1:3) media supplemented with 5% FBS, 0.4 $\mu\text{g/mL}$ hydrocortisone, 5 $\mu\text{g/mL}$ human insulin, 8.4 ng/mL cholera toxin, 10 ng/mL EGF, 24 $\mu\text{g/mL}$ Adenine, 10 μM ROCK inhibitor Y-27632, 0.25 $\mu\text{g/mL}$ Amphotericin B, and 50 mg/mL Primocin) [184]. Tumors were minced with razorblades and further mechanically dissociated in CRC media using a Medimachine instrument (BD Biosciences, Franklin Lakes, NJ). Resulting suspension was then transferred to a 6-well dish and incubated for 24-48 hrs before further manipulation. Serial trypsinization with 0.05% Trypsin was performed every 3 days or when plate reached 90% confluency in order to remove fibroblasts and select for epithelial cancer cell propagation. Serial trypsinization was stopped when exclusively epithelial cancer cells were observed. Cell lines were considered established when doubling efficiently after 3 passages. Once established, cell lines were confirmed mycoplasma-free and used between passages 5-10 for orthotopic experiments and before passage 15 for *in vitro* experiments. 2D cultures were maintained in CRC media unless otherwise noted.

Orthotopic Allograft Model Generation and Methodology

Pancreatic orthotopic allografts were performed as previously described [358]. Briefly, 8-12 week-old B6129SF1/J mice (Cat. # 101043, The Jackson Laboratory, Bar Harbor, ME) were given meloxicam as an analgesic and then anesthetized with isoflurane. A small abdominal flank incision was then made, and the spleen exteriorized. Cells were resuspended in 50 μ L CRC media and Matrigel (1:1) and 25 μ L were injected into the tail of the pancreas using a 28-gauge needle (injection numbers and n for each experiment in text). After injection, the needle was held in place for 30 seconds to allow for partial solidification of cell suspension, and the injection site was swabbed with a sterile cotton swab to prevent leakage. All exteriorized tissue was then placed back into the body cavity, the peritoneum sutured shut, and the epidermis closed with Vetbond tissue adhesive (3M, Saint Paul, MN). Animals were monitored daily for 1 week post-injection for overall health and wound healing.

In Vivo Drug Studies

After the tumors reach a size of ~ 60 mm² (~ 2 wks), mice were divided into cohorts based on average tumor size and rate of tumor growth. All therapeutic agents were obtained from SelleckChem (Houston, TX) except for Gemcitabine (LC Laboratories, Woburn, MA). For Gem/nAb-pac (nAb-paclitaxel) treatments [163, 359], mice were treated IP with 50 mg/kg Gemcitabine (LC Laboratories) and 0.5 mg/kg Abraxane (nAb-paclitaxel) once weekly. For mFOLFIRNOX treatments [360], mice were treated IP with 100 mg/kg folinic acid (FOL, also known as leucovorin) + 5 mg/kg 5-fluorouracil (F) + 50 mg/kg irinotecan (IRIN) + oxaliplatin 5 mg/kg (OX) in 10% DMSO on days 14 and 16. Note: typical administration of these drugs is performed in 30-day cycles; a second cycle of treatment would be administered on days 44 and 46.

Tumor Size and Metastatic Disease Assessment

Tumor size and presence of metastases were monitored over time by ultrasound using a Vevo-LAZR 2100 high-frequency/high-resolution linear array Ultrasound (FUJIFILM

VisualSonics, Toronto, CA). Upon detection, tumors were imaged and measured using the Vevo 770 image system software. Tumors were continuously monitored by ultrasound over time (weekly for long-term survival study; biweekly for orthotopic allograft studies).

Western Blotting

Cells were lysed in AB Lysis buffer and Western blot analysis was performed. Briefly, equal amounts of protein were fractionated on 4% to 12% Tris-glycine polyacrylamide gels (Criterion gels, Bio-Rad, Hercules, CA), transferred to polyvinylidene difluoride membranes (PVDF), and probed with primary antibodies against CDKN2A (p16INK4A (E5F3Y), 1:1000), SMAD4 (D3R4N, 1:1000), P53 (D2H9O, 1:1000), and GAPDH (6C5; 1:5000). Antibodies were diluted in blocking buffer (1:1 Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) : PBS with 0.001% Tween20). Primary antibodies were detected with secondary antibodies (1:10,000) labeled with the near-infrared fluorescent dyes IRDye800 (Rockland, Philadelphia, PA) and Alexa Fluor 680 (Molecular Probes, Eugene, OR) diluted in blocking buffer. Blots were visualized and bands quantified on an Odyssey imaging system (LI-COR Biosciences).

Software

R (version 4.2.3) was used with R packages DESeq2, GSVA, msigdb, and ggplot. GSEA was run using GSEA tool (version 4.3.2) [354, 355]. Western blots were quantified and analyzed using Image Lab software (version 6.1.0, Bio-Rad, Hercules, CA).

Statistical Methods

All statistical tests were performed with GraphPad Prism 9 or R (Version 4.2.3). A Mantel-Cox test was used to compare all Kaplan-Meier survival and recurrence curves. ANOVA was used for plots with multiple comparisons and t-tests were used for plots with single comparisons.

Supplementary Information:

Supplementary Dataset 1: RNA-sequencing TPM values for all genes

Supplementary Dataset 2: DESeq2 analysis of RNA-seq

Supplementary Dataset 3: GSEA analysis of RNA-seq

Supplementary Dataset 4: WGS Raw FastQ files

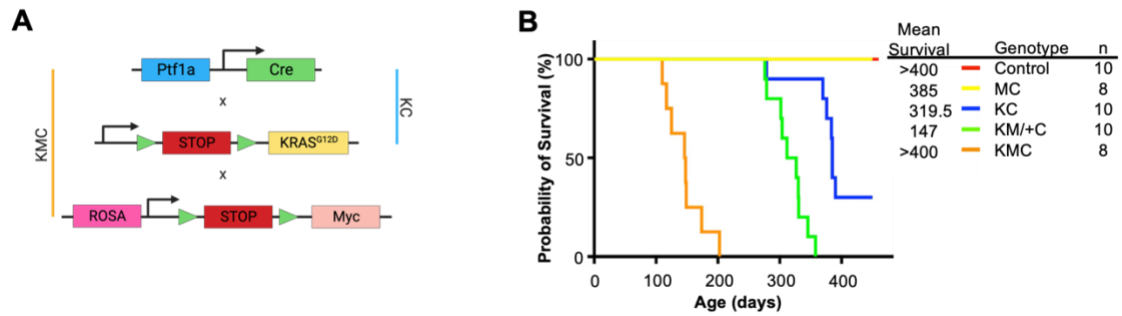
Supplementary Dataset 5: CNA analysis of WGS

Supplementary Dataset 6: WES Raw FastQ files

Supplementary Dataset 7: SNV analysis of WES

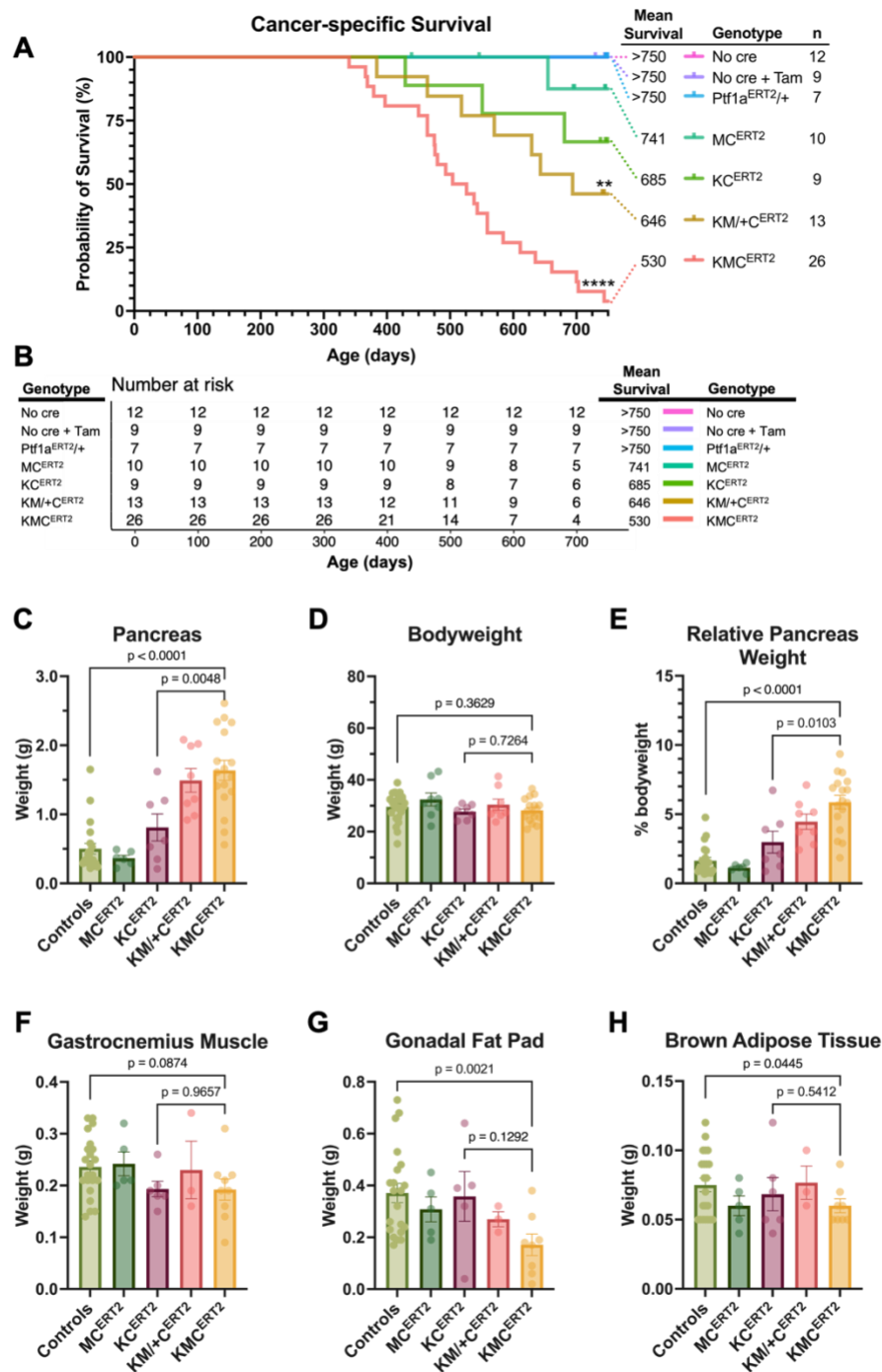
The datasets generated during this study will be available at the NCBI Gene Expression Omnibus; submission and accession codes in progress.

SUPPLEMENTARY FIGURES



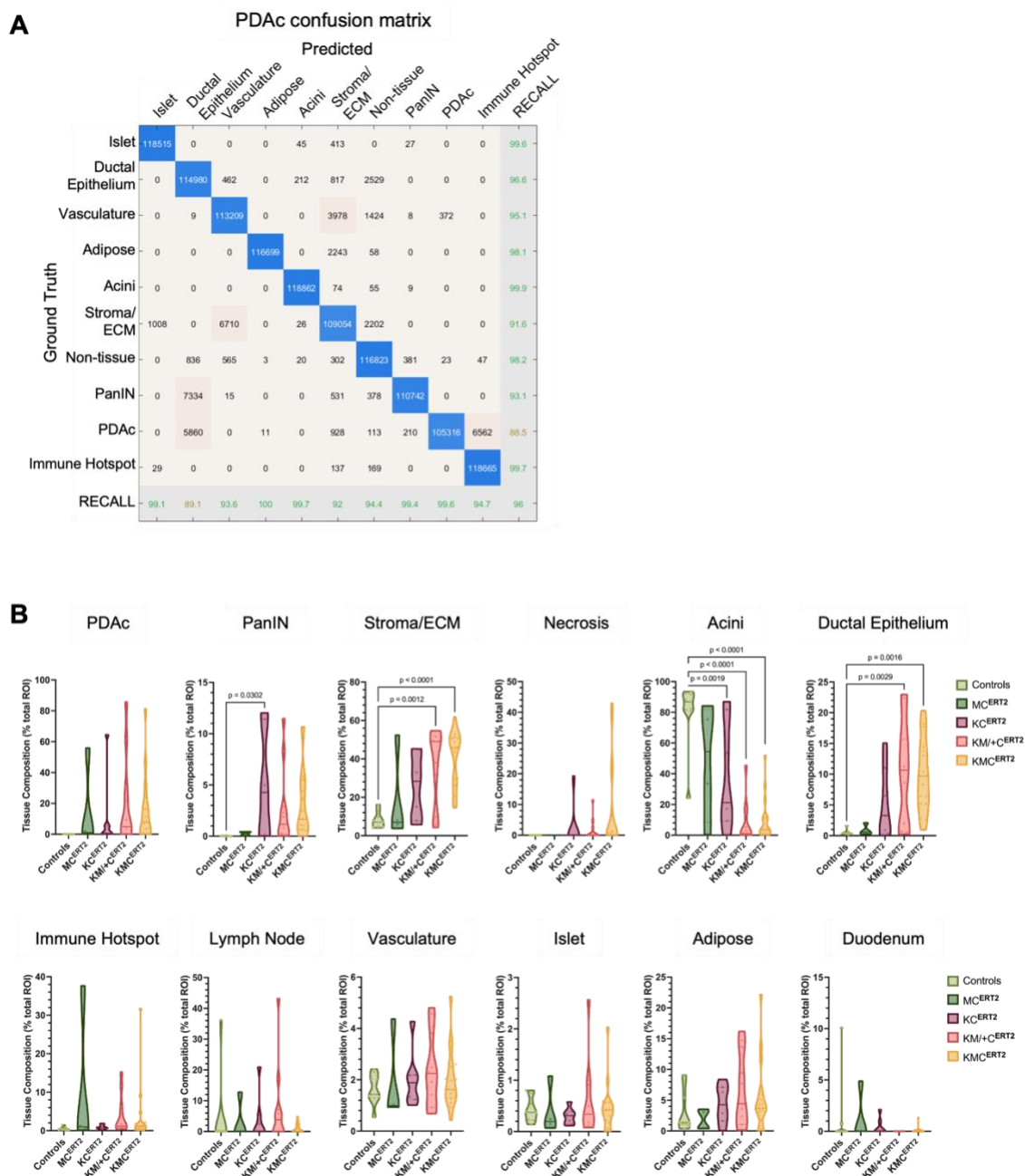
Supplementary Figure 2.01 Deregulated Myc expression in combination with oncogenic Kras accelerates pancreatic cancer initiation and progression.

A) Schematic depicting generation of the LSL-Kras^{G12D}; LSL-Rosa-Myc; Ptf1a-Cre mice. **B)** Kaplan-Meier curve showing expression of deregulated Myc in combination with oncogenic Kras reduces overall survival in the Ptf1a-Cre KMC GEMM. The number of mice and median survival of each arm is shown in the legend.



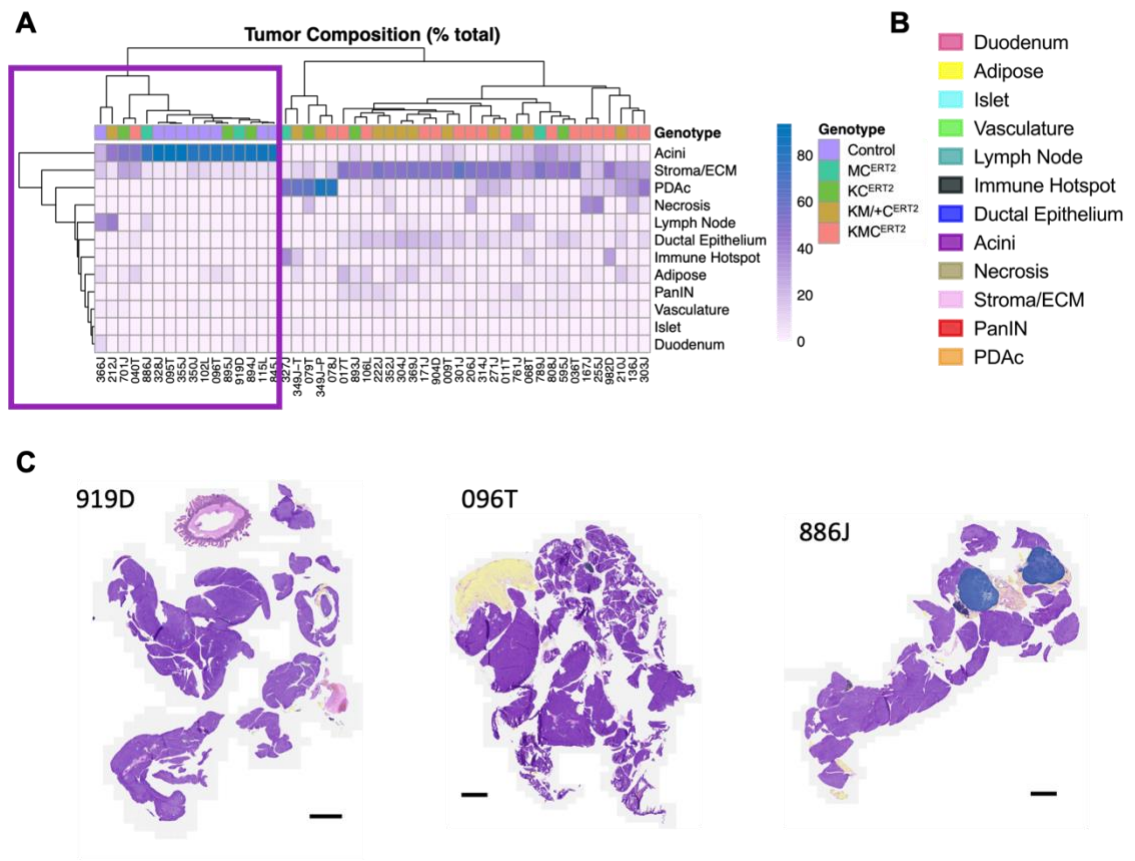
Supplementary Figure 2.02 Cachectic phenotype and at-risk survival in KMC^{ERT2} model.

A) Kaplan-Meier curve showing cancer-specific survival between KMC^{ERT2} GEMM study groups. All data are presented, with censored data points indicated by upwards tick. Significance versus No-cre controls is shown on lines, and versus KC^{ERT2} is shown in legend. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant. **B)** Risk table for KMC^{ERT2} GEMM study cancer-specific survival **(E)**. **C-H)** Endpoint tissue weights and relative pancreas weights from KMC^{ERT2} GEMM study. Significance (p-value) between KMC^{ERT2} and KC^{ERT2} or combined controls is shown for each tissue (p < 0.05).



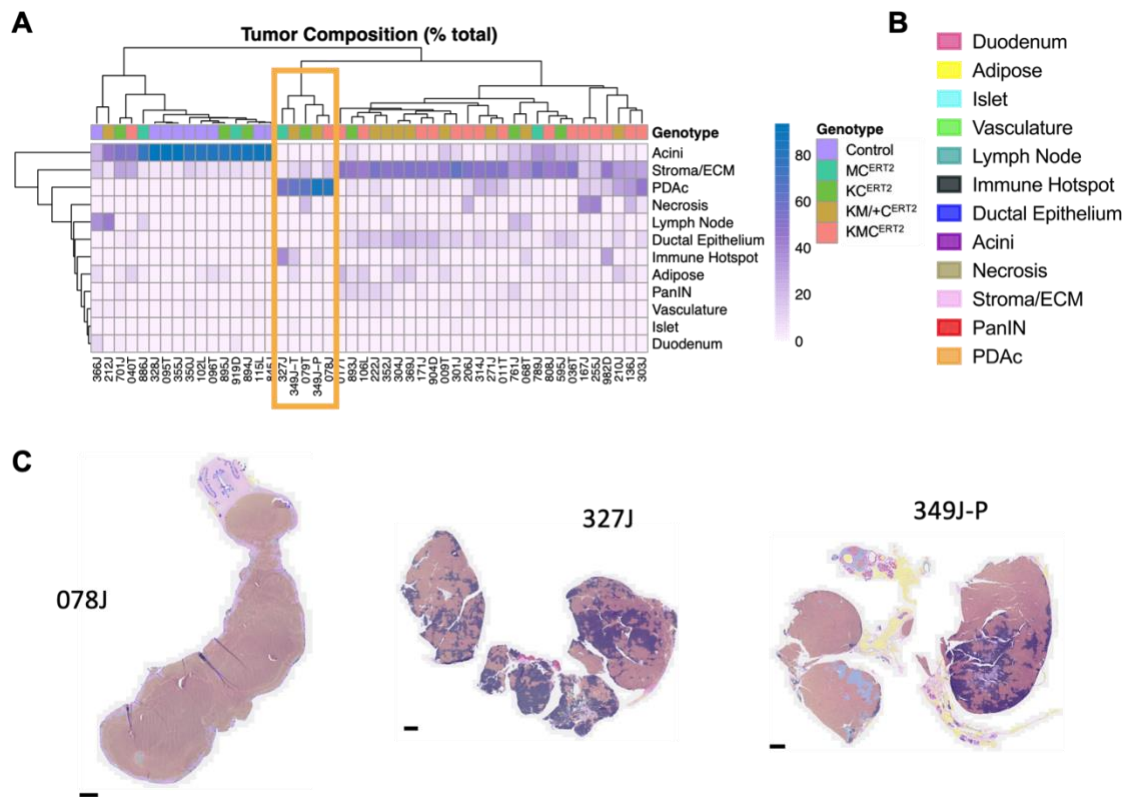
Supplementary Figure 2.03 CODA pancreatic tissue composition analysis.

A) Confusion matrix for murine PDAC tissue used in CODA analysis. **B)** Pancreatic tumor composition in each study group broken down by individual composition components.



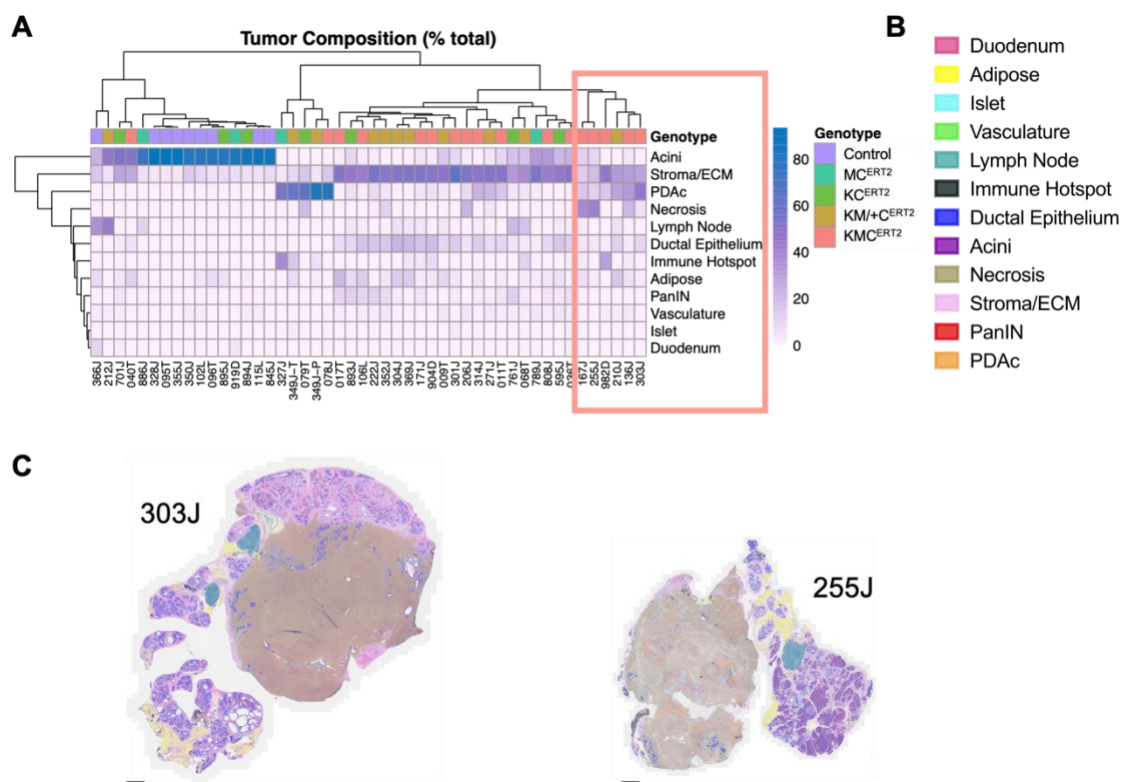
Supplementary Figure 2.04 CODA pancreatic tissue composition analysis ‘acinar’ pancreata

A) Heatmap depicting tumor composition as assessed by CODA analysis in pancreas tumor tissues from all arms of KMCERT2 survival study. Violet box highlights tissues composed primarily of healthy pancreatic acini. **C)** Representative H&E images of KMCERT2 study pancreas tissues in violet box overlaid with CODA classification colors outlined in **B)**.



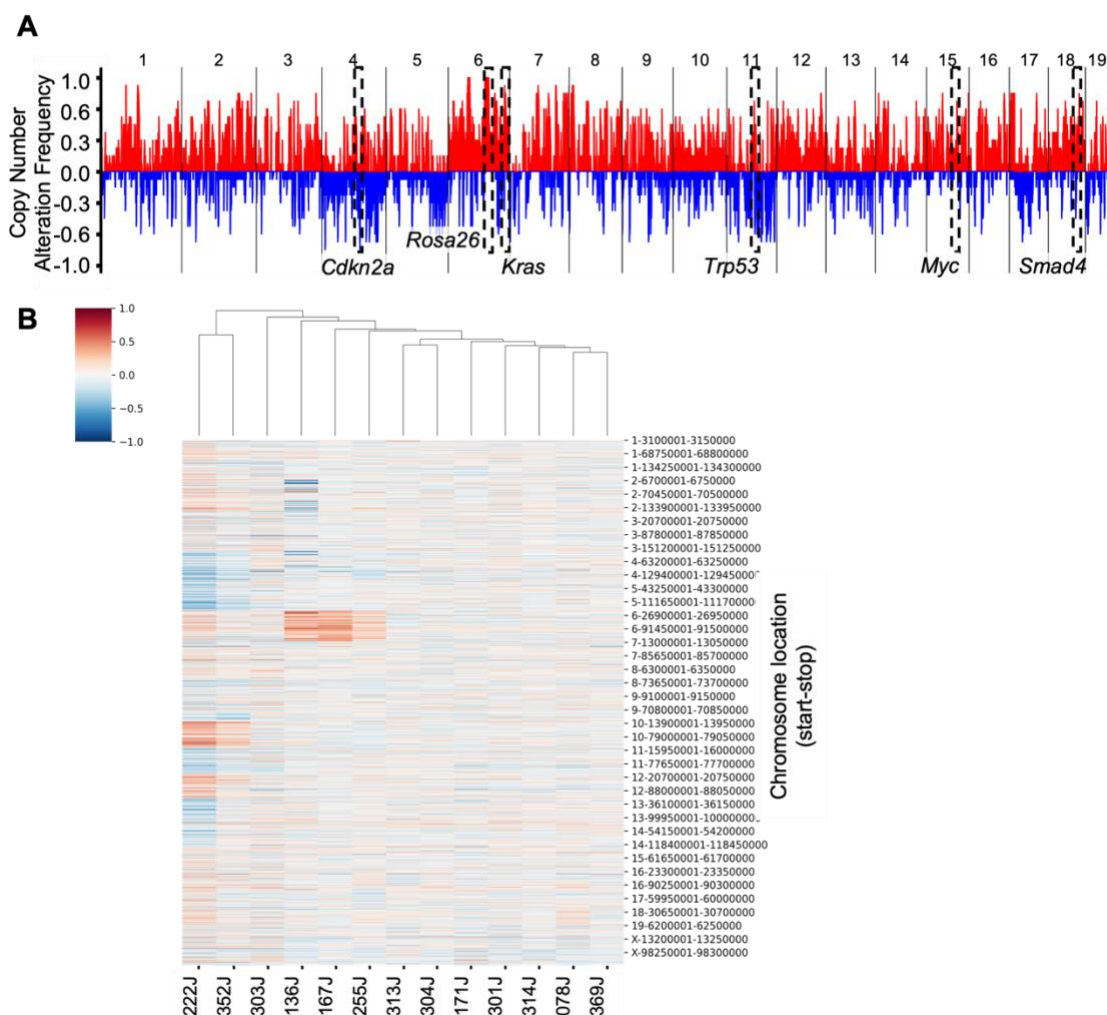
Supplementary Figure 2.05 CODA pancreatic tissue composition analysis 'low stroma/ECM' pancreata

A) Heatmap depicting tumor composition as assessed by CODA analysis in pancreas tumor tissues from all arms of KMC^{ERT2} survival study. Gold box highlights tissues composed primarily of low stroma/ECM PDAC. **C)** Representative H&E images of KMC^{ERT2} study pancreas tissues in gold box overlaid with CODA classification colors outlined in **B)**.



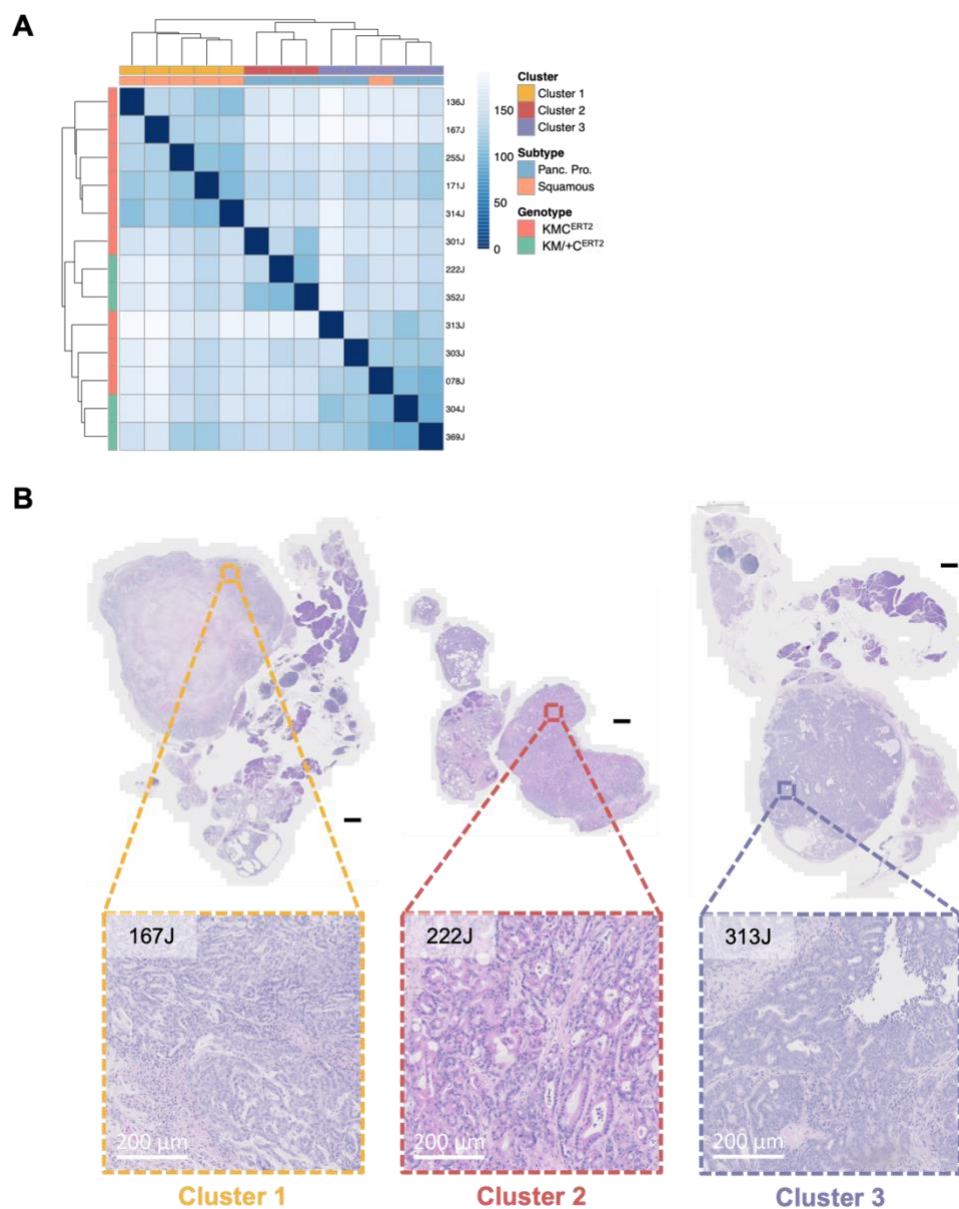
Supplementary Figure 2.07 CODA pancreatic tissue composition analysis ‘mixed’ pancreata

A) Heatmap depicting tumor composition as assessed by CODA analysis in pancreas tumor tissues from all arms of KMC^{ERT2} survival study. Pink box highlights tissues composed primarily of highly desmoplastic PDAC. **C)** Representative H&E images of KMC^{ERT2} study pancreas tissues in orange box overlaid with CODA classification colors outlined in **B)**.



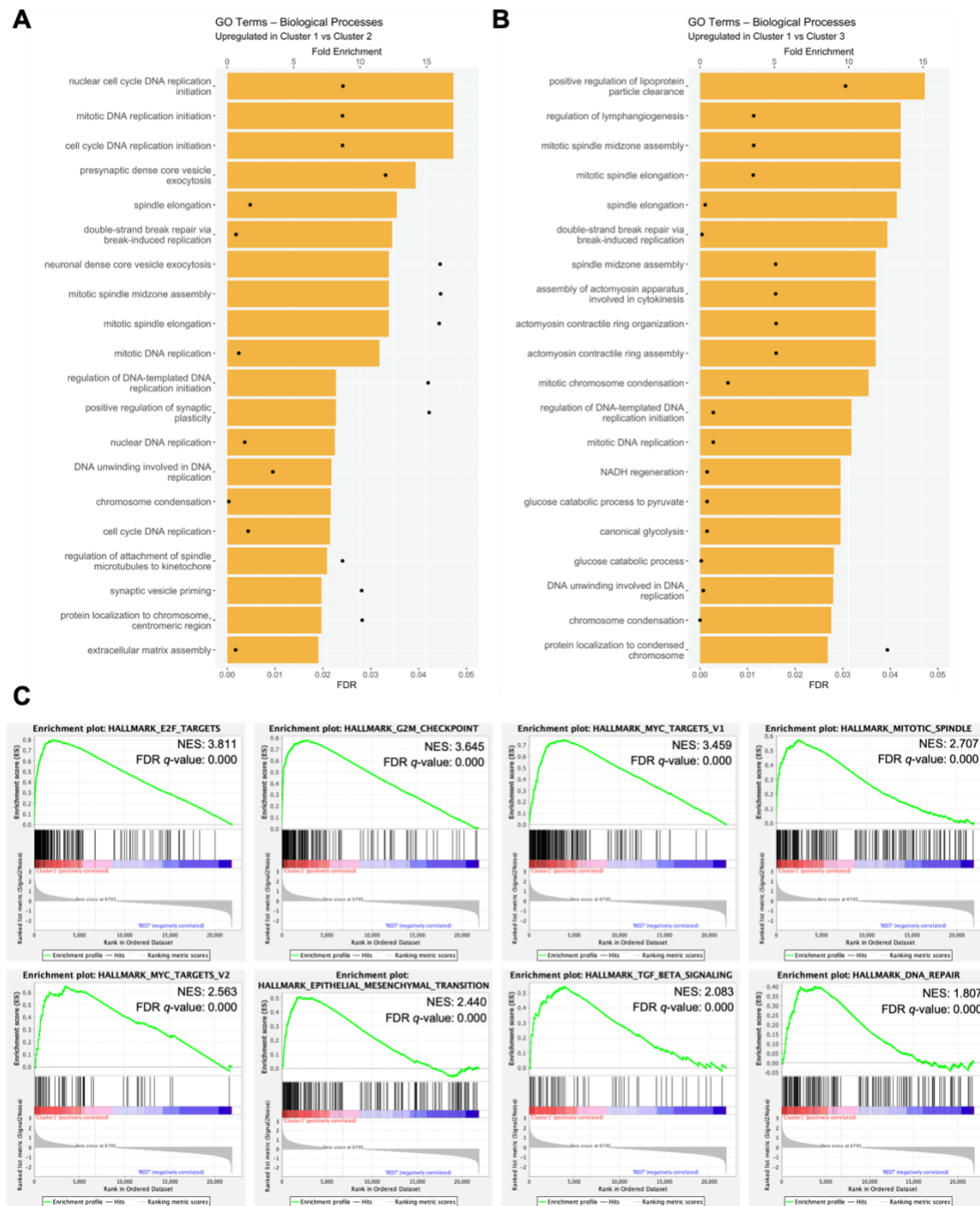
Supplementary Figure 2.08 Copy number analysis of KMC^{ERT2} tumors.

A) Summary plot of copy number variance in endpoint KMC^{ERT2} pancreatic lesions. Boxes highlight location of key genes: *Cdkn2a*, *Rosa26*, *Kras*, *Trp53*, *Myc*, *Smad4*. **B)** Unsupervised hierarchical clustering of whole genome sequencing (WGS) copy number analysis (CNA) of KMC^{ERT2}.



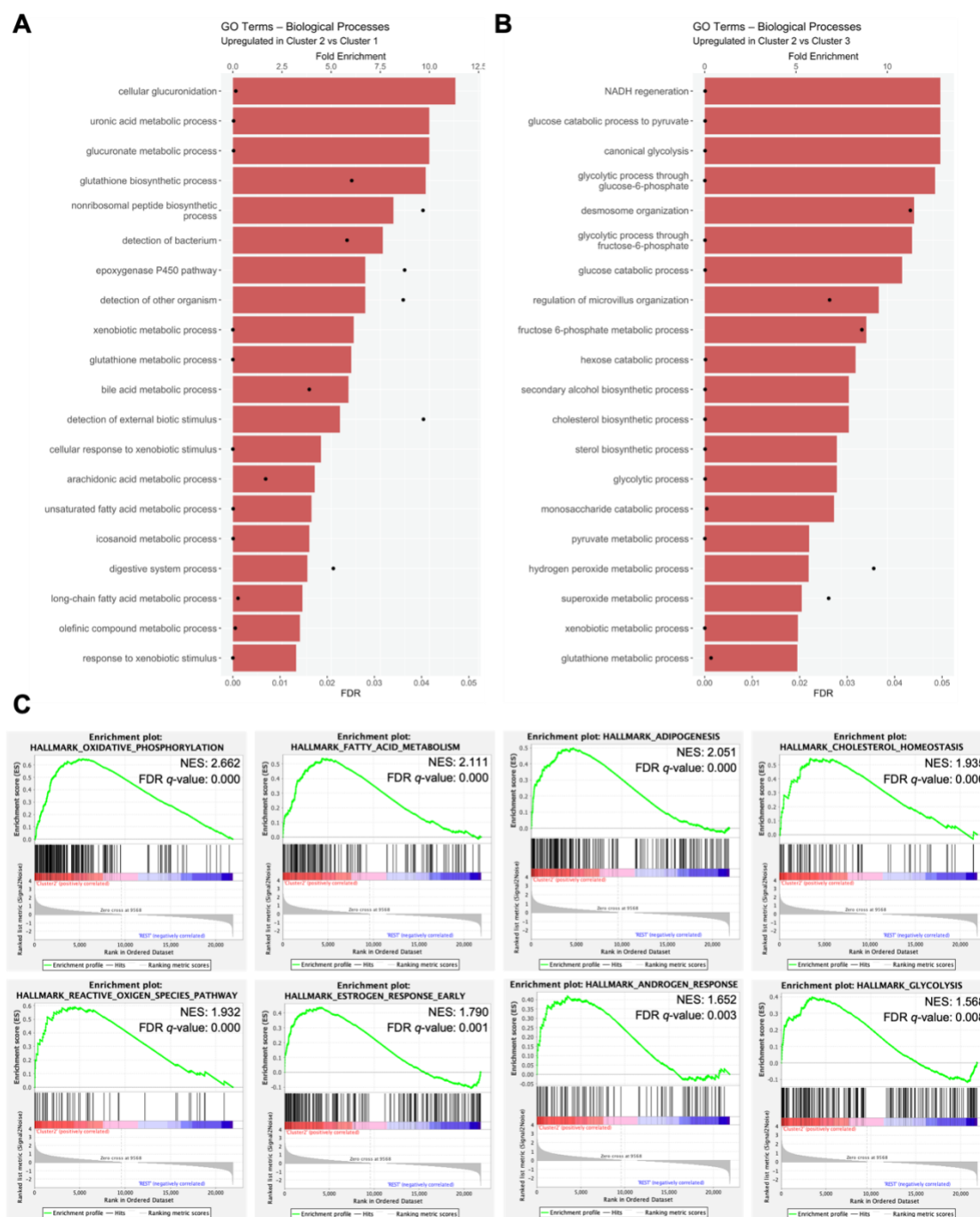
Supplementary Figure 2.09 KMCERT² PDAC model displays intertumoral transcriptional and phenotypic heterogeneity.

A) Heatmap of sample-to-sample distances between PDAC GEMM tumors, as seen in **Fig. 2.05**. **B)** Representative H&E images of phenotypic heterogeneity seen among KMCERT² tumors. Scale bars depict 200 μ m.



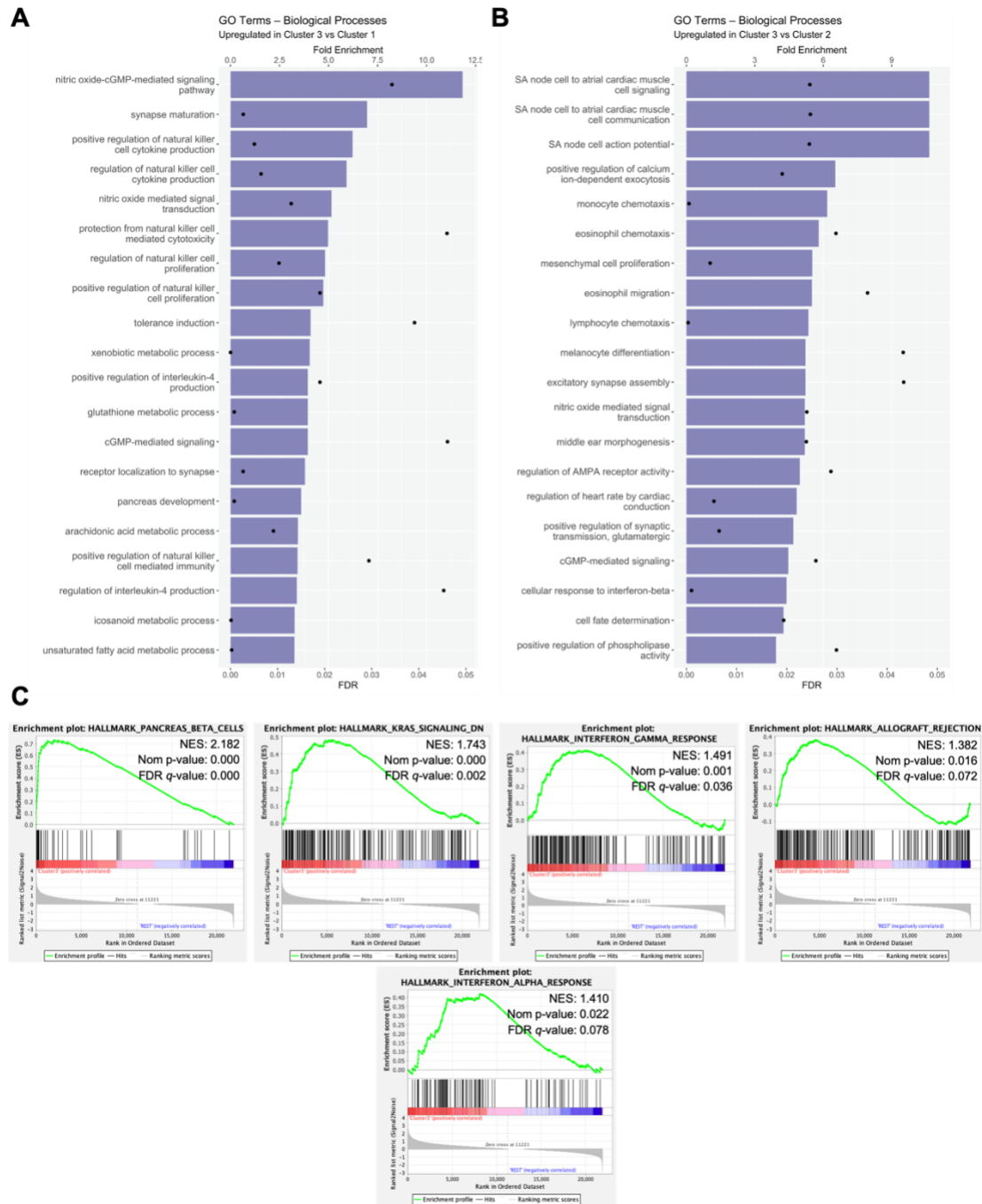
Supplementary Figure 2.10 Cluster 1 tumors are upregulated in cell cycle, DNA replication and repair biological process GO Terms and GSEA hallmark pathways.

A-B) Top 20 significantly enriched ($E > 2.0$, $FDR < 0.05$) biological process gene ontology (GO) terms of genes differentially expressed in endpoint KMC^{ERT2} tumor cluster 1 over clusters 2 and 3, respectively. Fold enrichment depicted by gold bars; FDR shown as overlaid black circles. **C)** GSEA Enrichment plots showing significantly enriched Cancer Hallmarks in tumors from cluster 1 over clusters 2 and 3. Normalized enrichment score (NES) and FDR q-value shown on each plot. See Supplementary Data Set 3 for full GSEA results.



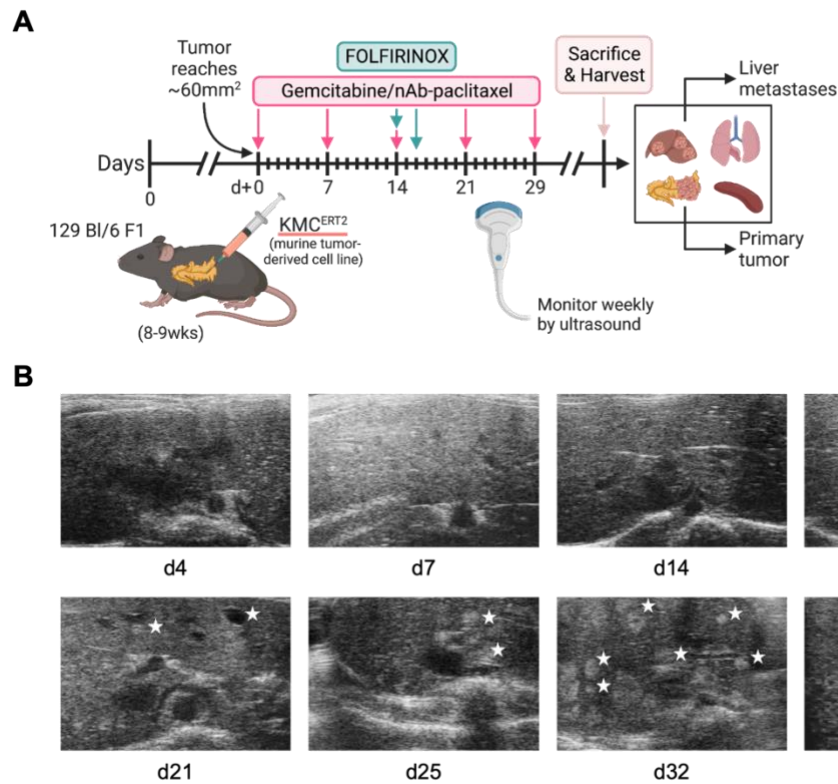
Supplementary Figure 2.11 Cluster 2 tumors are upregulated in oxidative phosphorylation and metabolic biological process GO Terms and GSEA hallmark pathways.

A-B) Top 20 significantly enriched ($E > 2.0$, $FDR < 0.05$) biological process gene ontology (GO) terms of genes differentially expressed in endpoint KMC^{ERT2} tumor cluster 2 over clusters 1 and 3, respectively. Fold enrichment depicted by red bars; FDR shown as overlaid black circles. **C)** GSEA Enrichment plots showing significantly enriched Cancer Hallmarks in tumors from cluster 2 over clusters 1 and 3. Normalized enrichment score (NES) and FDR q-value shown on each plot. See Supplementary Data Set 3 for full GSEA results.



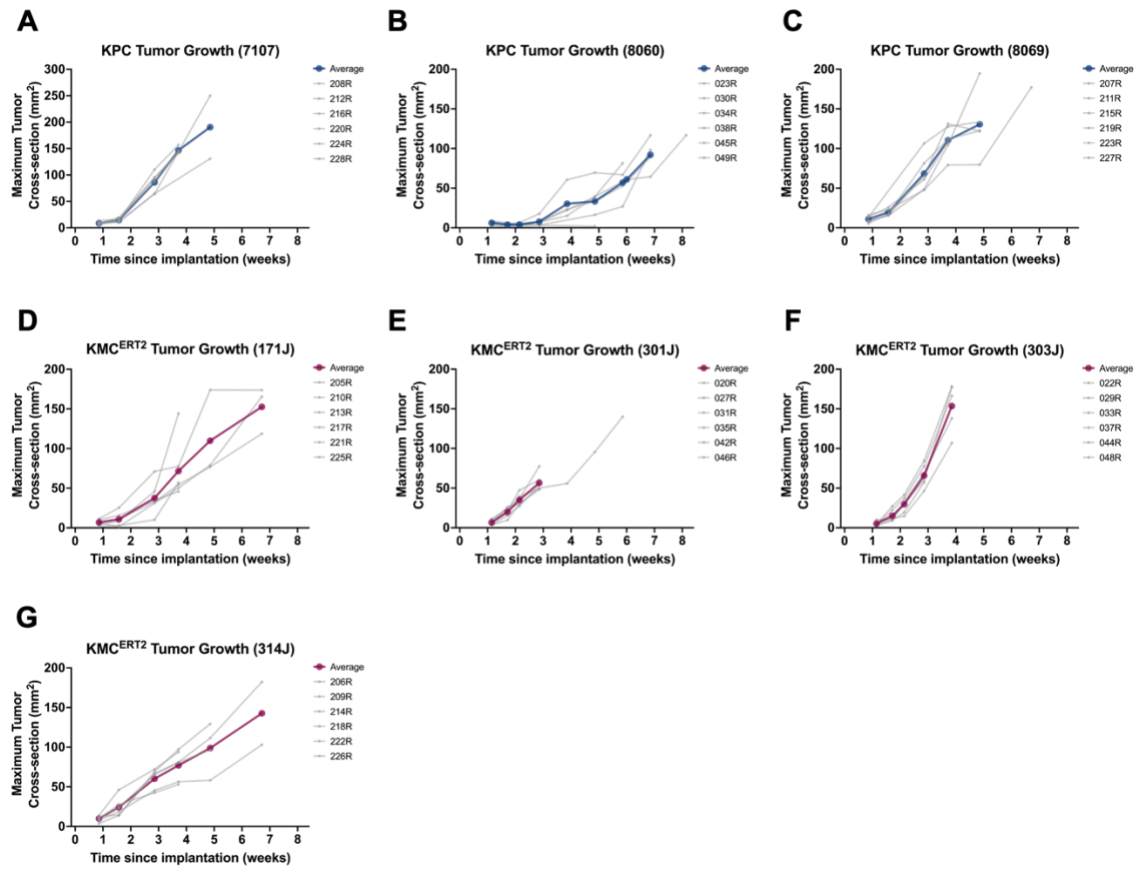
Supplementary Figure 2.12 Cluster 3 tumors are upregulated in immune-modulatory and tyrosine kinase biological process GO Terms and GSEA hallmark pathways.

A-B) Top 20 significantly enriched ($E > 2.0$, $FDR < 0.05$) biological process gene ontology (GO) terms of genes differentially expressed in endpoint KMC^{ERT2} tumor cluster 3 over clusters 1 and 2, respectively. Fold enrichment depicted by purple bars; FDR shown as overlaid black circles. **C)** GSEA Enrichment plots showing significantly enriched Cancer Hallmarks in tumors from cluster 3 over clusters 1 and 2. Normalized enrichment score (NES) and FDR q-value shown on each plot. See Supplementary Data Set 3 for full GSEA results.



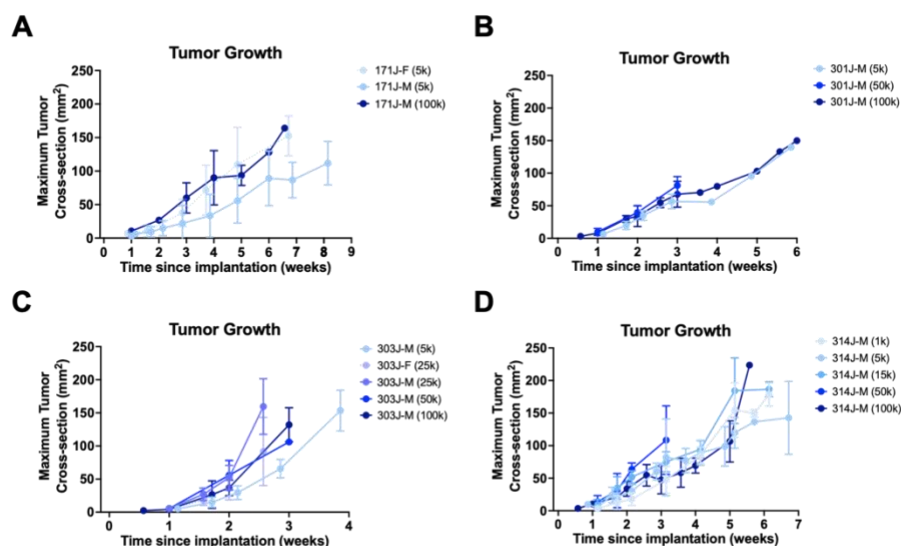
Supplementary Figure 2.13 Orthotopic KMCERT2 cell line tumors give rise to liver metastases.

A) Schematic depicting standard-of-care (SoC) treatment of orthotopic allograft of KMCERT2 tumors in B6129SF1/J mice, tumor progression monitoring, and tissue harvest. Schematic made with BioRender.com **B)** Typical development and progression of liver metastasis in orthotopic KMCERT2 tumors (implanted cell line shown: 171J), visualized by ultrasound.



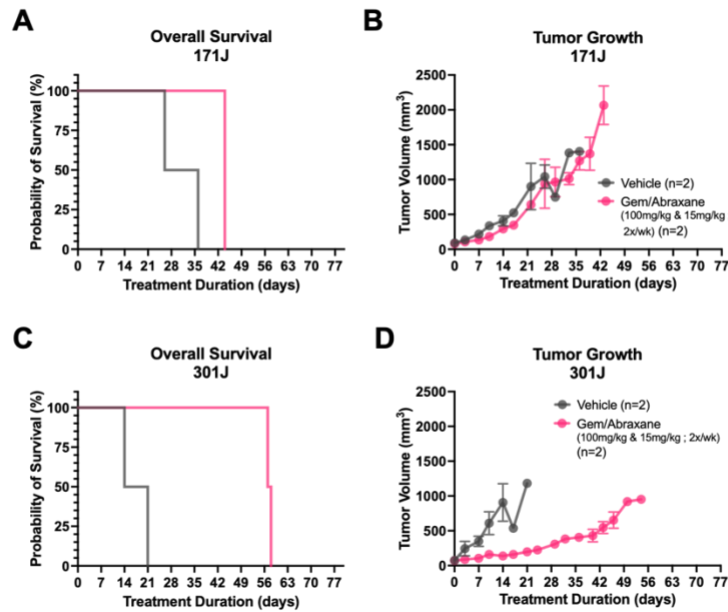
Supplementary Figure 2.14 KMCERT² orthotopic model of PDAC reliably grows in B6129SF1/J mice.

A-G) Pancreas tumor growth over time in B6129SF1/J mice orthotopically injected with murine PDAC-tumor derived cell lines 7107 (KPC), 8060 (KPC), 8069 (KPC), 171J (KMCERT²), 301J (KMCERT²), 303J (KMCERT²), and 314J (KMCERT²), respectively. Color line shows average growth, individual mouse PDAC tumor growth is shown in grey.



Supplementary Figure 2.15 KMC^{ERT2} orthotopic models of PDAC reliably grow in B6129SF1/J mice, regardless of host sex or cell number.

A-D) Pancreas tumor growth over time in B6129SF1/J mice orthotopically injected with murine PDAC-tumor derived KMCERT2 cell lines 171J, 301J, 303J, and 314J, respectively. “[Mouse ID]-F” indicates cell line implanted into female mice. “[Mouse ID]-M” indicates cell line implanted into male mice. Number in parentheses indicates number of cells implanted orthotopically.



Supplementary Figure 2.16 KMC^{ERT2} orthotopic models of PDAC are variably resistant to standard-of-care therapy.

A-B) Kaplan-Meier curve showing probability of overall survival and graph showing tumor growth, as measured by ultrasound, in B6129SF1/J mice orthotopically implanted with primary murine PDAC cell line 171J or **C-D)** 301J when treated with standard of care (SoC) Gemcitabine/Abraxane (100mg/kg & 15mg/kg ; I.P. weekly) or vehicle.

Supplementary Table 2.01 Autochthonous PDAC GEMM pancreatic tissue composition (as % ROI)

Genotype	(n)	PDAC	PanIN	Stroma/ ECM	Necrosis	Acini	Ductal Epithelium	Immune Hotspot	Vasculature	Islet	% Total ROI
Control											
p48 ^{ERT2} /+ only	(3)	0.0%	0.0%	11.3%	0.0%	65.9%	0.9%	0.3%	1.9%	0.6%	80.9%
No cre + Tam	(3)	0.1%	0.0%	7.1%	0.0%	85.2%	0.3%	0.3%	1.7%	0.2%	94.9%
No cre	(3)	0.0%	0.0%	6.3%	0.0%	89.5%	0.3%	1.0%	1.3%	0.5%	98.9%
Average	(9)	0.1%	0.0%	8.2%	0.0%	80.2%	0.5%	0.5%	1.6%	0.4%	91.5%
Experimental											
KPC	(6)	28.2%	1.4%	31.7%	18.5%	3.4%	7.0%	0.9%	3.6%	0.9%	95.6%
MC ^{ERT2}	(4)	14.6%	0.3%	17.5%	0.0%	48.4%	0.9%	9.9%	1.8%	0.4%	93.8%
KC ^{ERT2}	(7)	10.8%	4.7%	25.7%	2.8%	38.6%	5.3%	0.9%	2.0%	0.3%	91.1%
KM/+C ^{ERT2} (het)	(11)	20.1%	2.7%	36.3%	1.7%	8.2%	10.0%	4.0%	2.3%	0.6%	85.9%
KMC ^{ERT2}	(17)	15.7%	3.3%	40.5%	7.9%	10.2%	9.7%	3.5%	2.0%	0.5%	93.3%

Supplementary Table 2.02 KMC^{ERT2} lesion WES single nucleotide variant summary

Sample ID	High Probable Impact (# [name])	Moderate Probable Impact (#)	Total non-syn SNVs (#)
171J	1 (FGFR2)	19	27
301J	1 (ITGA2)	32	38
303J	0	24	31
314J	3 (FRAS1, MYO15, TLE6)	23	37

Supplementary Table 2.03 Hierarchical mouse cell lineage Identification and functional state classification by mIHC

Immune cell type identification by marker expression	
<u>Lineage</u>	<u>Identification (All populations are CD45⁺)</u>
Th0 (naïve) helper T cells	CD3 ⁺ CD4 ⁺ CD8 ⁻ Foxp3 ⁻ RORgt ⁻ Tbet ⁻ GATA3 ⁻
Regulatory T cells (Treg)	CD3 ⁺ CD4 ⁺ CD8 ⁻ Foxp3 ⁺
Th17 helper T cells	CD3 ⁺ CD4 ⁺ CD8 ⁻ RORgt ⁺
Th2 helper T cells	CD3 ⁺ CD4 ⁺ CD8 ⁻ GATA3 ⁺
CD8 ⁺ T lymphocytes (all)	CD3 ⁺ CD8 ⁺
CD8 ⁺ T lymphocytes (memory)	CD3 ⁺ CD8 ⁺ EOMES ⁺
B cells	CD3 ⁻ B220 ⁺
Granulocytes	CD3/B220 ⁻ Ly6G ⁺
Pro-tumor TAM	CD3/B220/Ly6G ⁻ F4/80 ⁺ CSF1R ⁺ CD206 ⁺
Re-programmed TAM	CD3/B220/Ly6G ⁻ F4/80 ⁺ CSF1R ⁺ CD206 ⁻ MHCII ⁺
Other pro-tumor myeloid	CD3/B220/Ly6G ⁻ F4/80 ⁺ CSF1R ⁺ CD206 ⁺
Other myeloid	CD3/B220/Ly6G ⁻ F4/80 ⁺ CSF1R ⁺ CD206 ⁻
Conventional DC (DC1)	CD3/B220/Ly6G ⁻ F4/80 ⁻ CD11c ⁺ MHCII ⁺ CD11b ⁺
Cross presenting DC (DC2)	CD3/B220/Ly6G ⁻ F4/80 ⁻ CD11c ⁺ MHCII ⁺ CD11b ⁻ CD207 ⁺
Interrogation of functional state of immune cells	
<u>Marker</u>	<u>Classification</u>
Proliferation	Ki67
Cytotoxicity	Granzyme-B ⁺
T cell activation	TCF1/TCF7
Peripheral tolerance	PD-L1
Identification of non-immune cells	
<u>Cell type</u>	<u>Identification (All populations are CD45⁻)</u>
Activated fibroblast + SMCs	αSMA ⁺
Endothelial cells	CD31 ⁺

Supplementary Table 2.04 KMC^{ERT2} tumor-derived cell line WES single nucleotide variant summary

Sample ID	High Probable Impact (# [name])	Moderate Probable Impact (#)	Total non-syn SNVs (#)
171J	0	32	40
301J	0	20	20
303J	1 (Ryr1)	35	52
314J	1 (Ccdc15)	27	34

Supplementary Table 2.05 Autochthonous and orthotopic PDAC model pancreatic tissue composition

Mouse ID	Model Type	(n)	PDAC	PanIN	Stroma/ ECM	Necrosis	Acini	Ductal Epithelium	Immune Hotspot	Vasculature	Islet	% Total ROI
171J	Autochthonous		3.8%	5.5%	51.3%	1.1%	5.2%	18.2%	4.7%	1.6%	0.22%	91.8%
	Orthotopic	(3)	48.9%	0.04%	9.1%	13.8%	17.1%	2.0%	0.55%	2.9%	1.2%	95.5%
301J	Autochthonous		0.65%	3.7%	61.8%	1.3%	10.6%	13.7%	0.82%	1.6%	2.0%	96.3%
	Orthotopic	(5)	69.5%	0.03%	18.2%	0.00%	8.4%	0.97%	0.90%	1.3%	0.39%	99.7%
303J	Autochthonous		46.4%	0.6%	29.3%	6.4%	0.35%	10.4%	0.53%	1.3%	0.17%	95.4%
	Orthotopic	(1)	86.0%	0.02%	11.8%	0.00%	0.10%	0.02%	0.46%	1.5%	0.05%	100.0%
314J	Autochthonous		23.8%	2.5%	48.6%	0.00%	0.86%	14.4%	1.2%	1.1%	0.43%	93.0%
	Orthotopic	(5)	49.6%	0.1%	19.5%	10.9%	11.4%	2.6%	0.6%	3.7%	0.65%	99.1%

Supplementary Table 2.06 mIHC protocol for mouse cell lineage and functional state identification

	Cycle 1		Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	Cycle 7
	(Pre-Antigen Retrieval)							
	Round 1	Round 1	Round 1	Round 1	Round 1	Round 1	Round 1	Round 1
Primary Antibody or Stain	CSF-1R	Hematoxylin	CD11c	CD4	BTK	PDL1	CD3	CD206
Clone	E2412	N/A	D1V9Y	D7D2Z	Polyclonal	E1L3N	SP7	Polyclonal
Vendor	Santa Cruz	Dako	Cell Signaling	Cell Signaling	LS Bio	Cell Signaling	Thermo	Abcam
Catalog #	sc-692	S3301	97585S	25229	LS-C180161	13684S	RM-9107-S	64693
Host Species	Rabbit	N/A	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit
Dilution	1:500	N/A	1:100	1:100	1:200	1:50	1:300	1:1000
Incubation Time	1 hr @ RT	1 min @ RT	1 hr @ RT	4 hr @ RT	1 hr @ RT	O/N @ 4°C	1 hr @ RT	O/N @ 4°C
AEC Development Time	40 min	N/A	25 min	40 min	13 min	25 min	27 min	15 min
	Round 2			Round 2	Round 2	Round 2	Round 2	Round 2
Primary Antibody or Stain	F4/80			MHC II	CD45	CD8	CD207	B220
Clone	Cl:A3-1			M5/114.15.2	30-F11	4SM15	eBioRMUL.2	R13-6B2
Vendor	BioRad			eBioscience	eBioscience	eBioscience	eBioscience	BD
Catalog #	MCA497G			eBi14-5321	14-0451-82	14-0808-82	14-2073-82	550286
Host Species	Rat			Rat	Rat	Rat	Rat	Rat
Dilution	1:200			1:100	1:50	1:100	1:100	1:100
Incubation Time	1 hr @ RT			4 hr @ RT	1 hr @ RT	O/N @ 4°C	1 hr @ RT	O/N @ 4°C
AEC Development Time	40 min			14.5 min	15 min	25 min	19 min	40 min
	Cycle 8	Cycle 9	Cycle 10	Cycle 11	Cycle 12	Cycle 13	Cycle 14	Cycle 15
	Round 1	Round 1	Round 1	Round 1	Round 1	Round 1	Round 1	Round 1
Primary Antibody or Stain	RORgt	GATA3	CD11b	TCF1/TCF7	TIM3	EOMES (TBR2)	Granzyme B	Ki67
Clone	EPR20006	EPR16651	EPR1334	C63D9	D3M9R	EPR19012	Polyclonal	Polyclonal
Vendor	Abcam	Abcam	Abcam	Cell Signaling	Cell Signalling	Abcam	Abcam	Abcam
Catalog #	ab207082	199428	133357	2203S	83882S	183991	4059	15580
Host Species	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit
Dilution	1:100	1:100	1:3000	1:100	1:200	1:1000	1:200	1:5000
Incubation Time	1 hr @ RT	30 min @ RT	1 hr @ RT	30 min @ RT	30 min @ RT	1 hr @ RT	O/N @ 4°C	30 min @ RT
AEC Development Time	20 min	25 min	10 min	15 min	25 min	25 min	10 min	17 min
	Round 2						Round 2	Round 2
Primary Antibody or Stain	Foxp3						Ly6G	Pan Cytokeratin
Clone	FJK16S						1A8	AE1/1E3
Vendor	eBioscience						eBioscience	Abcam
Catalog #	14-5773-82						551459	ab27988
Host Species	Rat						Rat	MOUSE
Dilution	1:100						1:200	1:100
Incubation Time	30 min @ RT						O/N @ 4°C	30 min @ RT
AEC Development Time	30 min						20 min	10 min

CHAPTER 3: PRECLINICAL MODELING OF PDAC

IN VITRO PRECLINICAL MODELING OF PDAC TUMOR CELLS AND THEIR MICROENVIRONMENT USING 3D-BIOPRINTING

Personal contribution

IAE's contributions to this chapter are as follows:

Figure 3.03 A) MAT and EML grew cells and bioprinted tissues; IAE performed IF for α SMA and CD31 on bioprinted tissues; VS performed quantification of CD31⁺ staining. **B)** XW performed IHC on KPC;*Pin1*^{+/+} and KPC;*Pin1*^{-/-} tissues; VS performed quantification of CD31⁺ staining.

Figure 3.04 MAT and EML grew cells and bioprinted tissues. **A)** IAE performed IHC for CD45 and **B)** IF for Vimentin, PanCK, CD31, and CD45 on bioprinted tissues.

Figure 3.05 A) IAE created BioRender schema for bioprinting methodology using patient-derived cells and PBMCs. **B)** CP performed initial RNAseq analysis of pancreatic PDCLs; JML performed Z-score calculations for *MYC* mRNA expression; IAE curated and visualized data. **C-E)** IAE selected PDCLs, grew cells, and bioprinted tissues. IAE dissociated bioprinted tissues and performed flow cytometry. IAE performed all downstream analyses.

Initials above represent the following people:

CP: Carl Pelz

EML: Ellen M. Langer

IAE: Isabel A. English

JML: Jason M. Link

MAT: Megan A. Turnidge

VS: Vidhi Shah

XW: Xiaoyan Wang

All figures for this chapter were prepared and finalized by IAE.

INTRODUCTION

Despite significant advances in cancer research, pancreatic ductal adenocarcinoma (PDAC), remains one of the deadliest, and most challenging cancers to treat [24, 361, 362]. To effectively develop and test novel therapeutic agents and/or new combinations of existing therapies, we must (1) better understand the complex mechanisms behind the disease, and (2) have access to applicable preclinical models of our target disease for testing therapeutics. One of the biggest challenges that cancer biologists face is creating accurate models for both of these purposes. The better a model mimics its human counterpart, the more likely our interpretation of results can be translated to clinical responses in patients.

***In vivo* models of cancer**

As highlighted in Chapter 2, *in vivo* models play a crucial role in pancreatic cancer research as they incorporate the complex interactions between cancer cells and their microenvironment within a living organism [137, 156, 168, 363–370]. These models allow researchers to study the progression of tumors, evaluate the efficacy of potential therapies, and investigate the underlying mechanisms driving cancer development and metastasis [169, 371]. Additionally, *in vivo* models provide valuable insights into the pharmacokinetics and toxicology of anti-cancer drugs, aiding in the development of more effective treatment strategies for patients [161, 368, 372]. There are several types of *in vivo* models used in cancer research, including carcinogen-induced models, xenograft models, allograft models, and genetically engineered mouse models, as described in Chapter 2. Each of these models has distinct advantages and disadvantages depending on the research question one is asking.

Carcinogen-induced models are often used in the study of skin and lung cancers, using physical irritants of UV radiation and cigarette smoke, respectively [373–376]. These

models reflect realistic outcomes as they induce inflammation and/or DNA damage from which mutated cells develop, eventually leading to carcinogenesis in a similar way to what might be expected in a patient population. Smoking is a risk factor for pancreatic cancer, and protocols for cigarette smoke exposure have been used in conjunction with genetic modifications or caerulein treatment to induce pancreatitis to interrogate mechanisms underlying tumor development [43, 377, 378]. Xenograft models are defined by the implantation of human tumor cells into immunodeficient mice. These models have allowed researchers to study the behavior of a large array of human cancer cells, including PDAC [379], in living organisms. Unfortunately, xenograft models do not allow interrogation into the immune response. Allograft models, such as we described in Chapter 2, use mouse tumor cells implanted into immunocompetent mice, allowing the study of tumor biology along with the immune response. For grafted models of PDAC, tumor cells are implanted either orthotopically into the pancreas, as we did with our KMC cell lines, or into another specified site, such as subcutaneously in the flank, overlying the upper thigh and lower part of the back and abdomen. Finally, GEMMs facilitate the study of specific tumor-initiating genetic alterations and/or secondary genetic hits, and allow for studies of tumor development since they undergo spontaneous tumorigenesis [380]. GEMMs of cancer are typically driven by engineered genetic alterations in oncogenes or tumor suppressors expressed in all cells in a target population. In PDAC modeling, for example, driving mutant Kras, as we did in Chapter 2, can lead to the formation of PanINs [82]; but it's the additional loss of tumor suppressors that typically leads to further disease progression [56, 156, 259]. Depending on how and when the genetic alteration is introduced, GEMMs can model cancer progression from initial mutation through late-stage disease.

In vivo models—especially mouse models—are invaluable in biomedical research and have led to countless scientific discoveries. Several advantages of these models include

the ability to elucidate complex biological processes in a whole organism and the availability of a wide range of genetic tools for manipulation [381, 382]. They play a vital role in our ever-expanding comprehension of disease mechanisms and in the evaluation of novel treatments, as they increasingly replicate human disease progression and response to therapies. Mouse models, however, have some significant limitations as well, such as their high cost, time-consuming nature, and difficulty to manipulate [381, 382].

The inability of mouse models to consistently translate therapeutic success to human clinical trials is a serious problem. New therapeutic regimens that exhibit promising outcomes in mice often lead to disappointment in the clinic when they fail to significantly improve human disease [383]. Cancer researchers frequently lament that although we have “cured” mouse cancer a hundred times over, these treatments have failed when brought to the clinic.

“If you have cancer and you are a mouse, we can take good care of you.”
– Judah Folkman [384]

Among the many reasons for this discrepancy are genetic, physiological, and immunological differences between mice and humans, as well as the increased heterogeneity seen in the patient population compared to our controlled mouse studies. As a result, the treatment efficacy can be overestimated since mouse models do not replicate the full human disease state. Improved engineering and the use of more representative disease models may greatly enhance the predictive power of preclinical models.

Considering these limitations, however, it is imperative to complement *in vivo* studies with other preclinical modeling platforms. Researchers, including myself, have been using patient-derived models of cancer (PDMCs) to evaluate the impact of patient heterogeneity and incorporating them into more complex engineered models to maintain specific

components of the human pancreatic tumor microenvironment (TME) [197, 379, 385–392]. The goal of this integrative approach is to produce heterogeneous and manipulable models, which will ultimately improve the predictive value of preclinical research and facilitate the translation of therapeutic discoveries to clinical settings.

Patient-derived models of cancer

Patient-derived models of cancer (PDMCs) include cell lines, organoids, and patient-derived xenograft (PDXs) [392]. The generation of commercial or established human cancer cell lines provided researchers the very first PDMCs, and these remain a critical part of contemporary cancer research [393, 394]. These cell lines were originally isolated and propagated from a patient's tumor (e.g. HeLa cells derived from the cervical cancer of Henrietta Lacks in 1951) [395, 396], or other bodily fluids (e.g. ascites or peripheral blood). In fact, the comprehensive characterization of hundreds of cell lines has demonstrated their ability to preserve the genetic intertumoral heterogeneity and some tumor-specific properties of their tumors of origin [397, 398]. As a result, these cell lines have been collected and preserved in biobanks (such as the American Type Culture Collection, or ATCC), and are effectively disseminated and employed as preclinical *in vitro* models for studying cancer in an inexpensive and easily replicable system [399]. One foundational PDAC study by Collisson et al. used established cell lines to compare basal and classical subtype treatment responses [33]. It has been shown, however, that cell lines generally have insufficient capacity to accurately replicate the more specific drug sensitivity and behavioral characteristics observed in an oncology patient [400]. Additionally, established cell lines have undergone countless rounds of cell division and have accumulated additional mutations as a result of their prolonged cultivation *in vitro* resulting in clonal drift which are often distinct from the mutations gained in a native site [393, 401–404]. Consequently, additional primary cell lines are required to have more representative models for human tumors.

Patient-derived cell lines (PDCLs) from primary patient samples are being generated and used in an effort to accurately represent tumor features and therapeutic responses of individual patients. In order to establish primary PDCLs, tumor specimens such as resection material or biopsies are dissected into small pieces (1-3 mm³) and dissociated by either physical or enzymatic means to obtain a single-cell suspension [310]. A recent study conducted by Kim et al. derived PDCLs from a cohort of 77 patients diagnosed with advanced lung cancer, with a success rate of 24%, and demonstrated that the established PDCLs retained patient tumor driver mutations [405]. Similar approaches have been initiated in pancreatic cancer research at OHSU by the Brenden Colson Center for Pancreatic Care (BCCPC) as well as by others with promising results. In both the Kim et al. study [405] as well as another by Peschke et al. [406], the efficacy of anticancer therapies as single agents and in combination were assessed in PDCLs through *in vitro* studies. Based on their findings, both studies proposed innovative combinatorial therapeutic regimens for patients with advanced lung and pancreatic cancer [405, 406]. Unfortunately, in contrast to using previously established and commercial cell lines, the establishment of primary PDCLs is relatively challenging, and initial proliferation is often delayed [407, 408]. As more research groups develop these resources, they hold potential to uncover new biological insights and enhance our understanding of therapeutic responses.

When dissociated patient samples or murine tumors are cultured with key extracellular matrix (ECM) components in specialized media, they can develop into self-organizing organotypic structures known as organoids [184, 186, 409, 410]. Organoids are three-dimensional cellular structures that exhibit some features and functionality of *in vivo* tissues [181, 185, 411, 412]. Though they are more challenging to culture than PDCLs or traditional cell lines, patient-derived pancreatic cancer organoids (PDOs) better replicate

the structural characteristics and architecture of the primary tumor [413–416]. In recent years, PDOs have emerged as a promising approach for identifying individual patient therapies in specialized trials [181, 182, 406, 411, 412, 417]. For example, Seppälä et al. demonstrated that organoids established from PDAC tissues could be used to improve results of next-generation sequencing (NGS) and to screen patient samples for chemotherapeutic sensitivity [417]. The PDOs' responses to chemotherapy predicted clinical responses, suggesting this as a valid method to accurately identify optimal therapeutic strategies for individual PDAC patients.

While organoids are proving to be a valuable new option in personalized cancer treatment, they are still limited in their ability to model more global (full-body) responses and toxicities to these therapeutics. PDXs, however, allow for the *in vivo* expansion and study of patient tumor cells following transplantation of patient-derived tumor tissues into immunodeficient mice [388, 418]. When these patient-derived tissues are successfully engrafted—a non-trivial challenge—they can be further propagated and transplanted into additional mice, allowing researchers to study patient-specific responses to different therapeutic regimens along with potential toxicities to healthy tissues [180, 182, 416, 419, 420]. However, PDXs are expensive and time-consuming and still do not give full insight into the native human tumor stroma or immune microenvironment, as they are generated in immune-incompetent mice [203, 421].

Engineered models of cancer

Engineered models, such as organ-on-a-chip and 3D-bioprinting, have emerged as promising complements to other *in vitro* and *in vivo* models as they provide the opportunity to model and manipulate the TME and often incorporate primary PDMCs [422, 423]. Organ-on-a-chip microfluidic devices can be engineered to mimic the physiological conditions of specific organs. These models have been used in cancer research to study

phenomena such as fluid shear stress on cells [424, 425], the effects of drugs on specific organ systems [426, 427], and sensitivity of model tumors to chemotherapies [428, 429]. For example, Nguyen et al. recently reported a novel organotypic PDAC-on-a-chip model that mimics vascular invasion and tumor-blood vessel interactions [430]. In this study, the authors used confocal microscopy to observe the ablation of endothelial cells by cancer cells. In another study, Hye-ran Moon et al. developed a microfluidic pancreatic tumor model using heterogeneous mouse PDAC cells to study how interaction between heterogeneous cancer cell subpopulations exposed to gemcitabine influenced drug resistance [431]. Organ-on-a-chip models provide opportunities for impressive visualization of a simplified tumor microenvironment, but can often be limited by their complexity and the difficulty of model production.

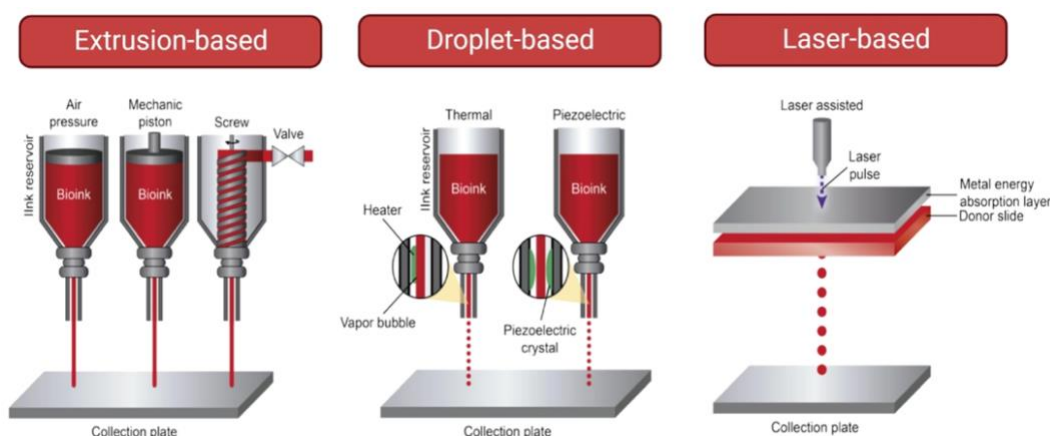


Figure 3.01 Three-dimensional bioprinting techniques.

Extrusion-based: filaments of the bioink are deposited in response to pressure. Droplet-based: external energy causes fine droplets of the bioink to be ejected from the nozzle. Laser-based: a pulsed laser beam scans the surface of the reservoir and generates a specific cure of the bioink. Figure and figure legend originally appeared in Sharma et al., *Biomater. Sci.* 2023 [432], and were adapted and reprinted under the Creative Commons Attribution License (CC BY) (<https://creativecommons.org/licenses/by/4.0/>).

3D-bioprinting uses 3D printing technology—depositing structural layers in a predefined pattern with a bottom-up methodology—to create living tissue structures [385, 433]. Three main categories of bioprinting technologies are characterized by their method of cell deposition: laser-based, droplet-based, or extrusion-based (**Fig. 3.01**) [432, 434]. Laser-based bioprinters utilize a pulsed laser beam to generate a specific pattern in the bioink.

Droplet-based bioprinters use external energy (usually from heat or electricity) to eject fine bioink droplets onto a collection plate or vessel. The most widely adapted and least costly method, however, is extrusion-based bioprinting [434]. In this setting, bioink filaments are deposited in response to pressure—from a screw and valve, a mechanical piston, or air pressure. Importantly, the bioinks that biomedical researchers use must be biocompatible and allow for the resulting structure to hold its intended shape and morphology. Bioinks can contain hydrogels that can be synthesized explicitly for this purpose [435] or can originate from naturally occurring products, including alginate [436–438], hyaluronic acid (HA) [439], gelatin [439], or fibrinogen [440, 441]. Scientists can bioprint tissue structures with bioinks that contain permanent scaffolds, scaffolds that are reversible, or without established scaffolds at all to selectively address particular research questions. A recent study proposed a method using decellularized adipose tissue based hydrogels in 3D cancer modeling [442]. Many scaffolds, both artificially synthesized and naturally occurring, remain in place for the duration of a study, preserving printed structure, but often preventing the model from achieving a more native tissue-like cellular density or morphology when their concentration is too great [437, 443, 444]. Scaffold-free bioprinting methods can be generated by using hydrogel-based bioinks that allow for cellular ECM deposition and tissue densification [445–448].

Many bioprinting methodologies have greatly contributed to our understanding of cellular function in a three-dimensional context, but these approaches have traditionally been limited by the availability of appropriate biomaterials, the small size of the resulting tissues, and the difficulty of replicating the cellular and extracellular matrix heterogeneity of the original tumor [449–452]. Control over these aspects of an experiment greatly affects the biological behavior of tissue models and can augment our ability to replicate *in vivo* conditions. Recently, research groups have made strides toward solving some of these

obstacles, advancing tissue fabrication for transplantation, but also building tumor tissue models in multiple ways. Advances in 3D-bioprinting, such as laser direct-write, now enable precise control over the spatial patterning and size of cell-encapsulating microbeads and other biomaterials, significantly impacting the biological behavior of tissue models and improving our ability to mimic *in vivo* conditions [453, 454].

Tumor modeling with bioprinted technology is also advancing. For example, in PDAC, Liu et al. used HA to mimic key aspects of the tumor matrix [439]; Puls et al. developed a tumor-ECM model to examine the effects of ECM composition and stiffness on PDAC EMT [455]; and Osuna de la Peña et al. recently established a tunable bioengineered 3D PDAC model using patient-derived cells for patient-specific *in vivo* drug studies [387]. A study from our lab, led by Drs. Ellen Langer and Brittany Allen-Petersen, successfully established an extrusion-based 3D-bioprinting methodology to reliably fabricate manipulable, heterotypic, scaffold-free human tumor tissues with well-defined tissue architecture in both breast and pancreatic cancers [197]. These bioprinted structures are printed using an additive manufacturing, or bottom-up, method: first the base of a 'box' is printed, followed by its side panels or 'walls;' next, the cancer cell mixture, or bioink, is extruded into the partially built structure, and then the 'box' is sealed with the stromal cell bioink on the top (**Fig. 3.02**). Using this methodology, our 3D-bioprinted tissues are rapidly (less than one minute per structure) and reproducibly generated by surrounding PDAC cells with a dense stromal bioink [197]. Our stromal bioink for pancreatic tumor models is typically composed of primary human endothelial cells (human umbilical vein endothelial cells, HUVECs) and primary human pancreatic stellate cells (hPSCs), which have been shown to contribute to the well-documented desmoplasia during tumorigenesis [140]. Cells within relatively large printed structures (> 500 μm in diameter) exhibit tissue-like cellular density; they actively proliferate, deposit ECM, migrate, and respond to extrinsic

signals from their microenvironment [197]. Further increasing the relevance of the model to human tumors, printed HUVECs formed contiguous vascular networks through bioprinted tissues and the addition of other microenvironmental cell types (e.g. bone marrow-derived mesenchymal stem cells or adipocytes) affected tissue phenotypes [197]. Our 3D-bioprinted tissues can be cultured *in vitro* on transwells for over two weeks, allowing time for experimentation [197].

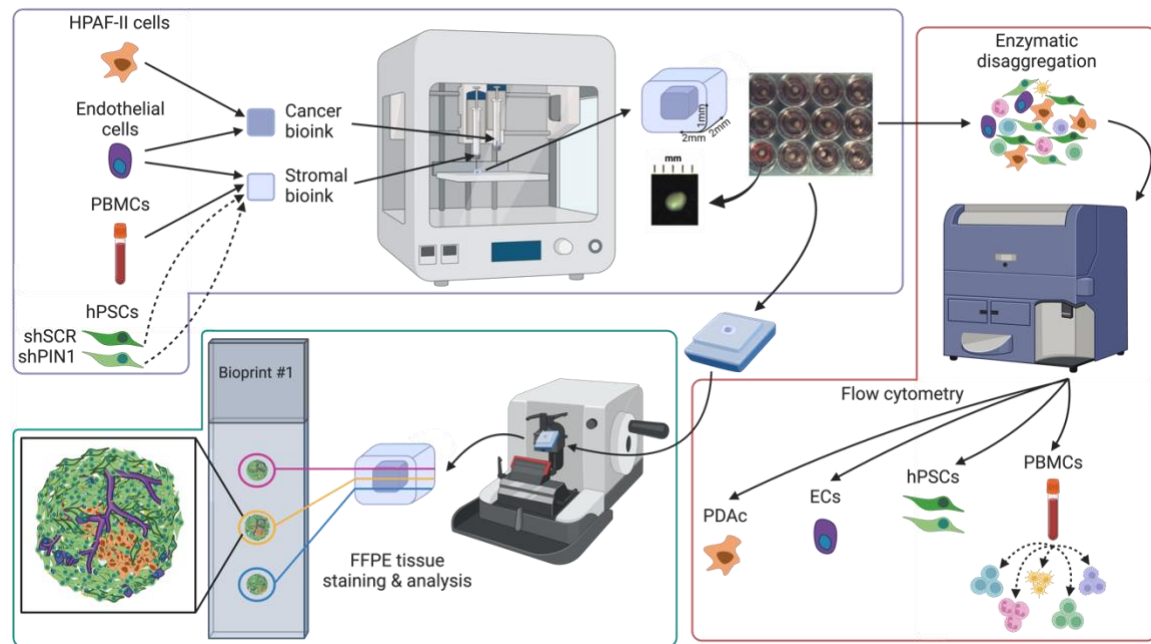


Figure 3.02 Overview of 3D-bioprinting methodology and downstream analyses.

Purple box shows a schematic depiction of 3D-bioprinting tumor models composed of HPAF-II pancreatic cancer cells, endothelial cells, peripheral blood mononuclear cells (PBMCs), and human pancreatic stellate cells (hPSCs) with or without *PIN1* knockdown (*shPIN1* or *shSCR*). Red and green boxes show downstream analyses of 3D-bioprinted tissues, either by enzymatic disaggregation and flow cytometry or by formalin-fixation and paraffin-embedding for histologic examination, respectively. Figure created with Biorender.com.

While each preclinical model of cancer has its own advantages and drawbacks [386, 388, 412, 456–463], the use of these models together can provide a more comprehensive understanding of pancreatic cancer biology and lead to the development of more effective treatments for PDAC. **We hypothesized that by incorporating and manipulating specific stromal cell types, in our 3D-bioprinted tissues, we could recapitulate *in vivo* microenvironmental, specifically endothelial and immune, responses to PDAC**

tumor growth and treatment. To that end, in this chapter, we will present two vignettes in which we explore how 3D-bioprinting allows investigation of heterogeneous fibroblast and immune phenotypes in pancreatic cancer. The first will focus on an approach to alter fibroblasts included in the print to determine how changing fibroblast state influences tissue phenotypes. In this study, we eliminate PIN1 expression in the PDAC fibroblasts in our *in vitro* model system to examine resulting immune and vascular phenotypes. The second vignette will show the beginning of our work to understand how heterogeneity of PDCLs impacts immune complexity. We will discuss the exciting opportunities provided by combining PDCLs and 3D-bioprinting to achieve complex, but manipulable tumor models to study the tumor immune microenvironment.

MATERIALS & METHODS

Primary patient tissue acquisition and patient consent

Primary patient data and tissues were obtained with informed consent, in accordance with the Declaration of Helsinki, through the IRB-approved Oregon Pancreas Tissue Registry (OPTR) (protocol #: IRB00003609).

Cell culture

HPAF-II cells were obtained from ATCC and grown in DMEM + 10% FBS. Primary hPSCs were obtained from ScienCell (Carlsbad, CA) and grown in complete Stellate Cell Medium on PLL-coated plates. Human umbilical vein endothelial cells (HUVECs) were purchased from MilliporeSigma (Burlington, MA) and cultured in PeproGrow MacroV media (Peprotech, Rocky Hill, NJ). Peripheral blood mononuclear cells (PBMCs) were purchased from ZenBio (Cat. # SER-PBMC-200-F; Durham, NC).

All patient-derived cell lines (PDCLs) discussed in this chapter were propagated from dissociated primary PDAC tumor tissue and cultured in PDCL media (F-12:DMEM (1:3) media supplemented with 5% FBS, 0.4 µg/mL hydrocortisone, 5 µg/mL human insulin, 8.4 ng/mL cholera toxin, 10 ng/mL EGF, 24 µg/mL Adenine, 0.25 µg/mL Amphotericin B, and 50 mg/mL 1X Primocin) with 10 µM ROCK inhibitor Y-27632 (Cat. # NC1629868; Fisher Scientific, Waltham, MA). Each one has an official publication ID (e.g. ST-00015919-T-CRC or ST-00018136-T-CRC) but is referred to in the text as 15919-T or 18136-T, respectively, for ease of reading. The prefix “ST-000” is simply the beginning of its official publication ID, while the “-CRC” refers to its state as a PDCL (previously referred to as a Conditionally Reprogrammed Cell line). The “-T” that remains in IDs used in the text refers to the origin of the PDCL, in this case it indicates the primary PDAC tumor as the source. STR profiling (Genetica, Burlington, NC) matched that of the respective patient’s

peripheral blood leukocytes, and mutant KRAS was confirmed in each PDCL prior to being bioprinted.

PIN1 knockdown

hPSCs with stable knockdown of PIN1 were generated by lentiviral infection of pLKO-Pin1 shRNA (shPIN1) or an shSCR control (MilliporeSigma, Burlington, MA), followed by selection with 2-5 µg/mL puromycin. Stable knockdown pools were assessed to confirm knockdown by Western blot and then used in further experiments.

All cells, with the exception of PBMCs which were used directly from thaw, were passaged a minimum of two times after thawing before experimental use.

Bioprinted tissue models

Bioprinted tissue models were fabricated as previously described [197] using a Novogen MMX Bioprinter Instrument (Organovo, Solana Beach, CA). Briefly, a stromal cell bioink composed of hPSCs and HUVECs (3:1 ratio) with or without PBMCs (Zen-Bio Inc., Durham, NC) or a cancer cell bioink of cancer cells (HPAF-II, 15919-T, or 18136-T) and HUVECs (3:1 ratio of cancer:HUVEC) was resuspended at 37°C in a reversibly cross-linkable hydrogel comprising 1.5% Pronova UP LVG Sodium Alginate (NovaMatrix, Sandvika, Norway) and 5% porcine gelatin (MilliporeSigma, Burlington, MA) in PBS. The final concentration of the cells within the bioink was 1.5×10^8 cell /mL. To generate tissues, bioinks were loaded into Hamilton syringes fitted with 250 µm needles and bioprinted onto 3µm Transwell clear polyester membrane inserts (Cat. # 353181; Corning Life Sciences, Corning, NY) in a 12-well plate (**Fig. 3.02**). Tissues were treated for 2 minutes with 104 mM CaCl₂ (MilliporeSigma, Burlington, MA) to crosslink the alginate-containing hydrogel and then were cultured at 37°C in a combination of media reflecting relative number of each component cell type. Crosslinked tissues were treated with Alginate Lyase (340 mg/mL, MilliporeSigma, Burlington, MA) 48 hours after printing, and subsequent culture

at 37°C allowed for complete removal of the hydrogel. Tissues were maintained in culture with media changes every 24 hours. Tissues were either fixed in 2% PFA for two hours and subsequently embedded in paraffin for sectioning for other projects, or for this work were dissociated in 3 mL media with 0.014 mg/mL Liberase (Cat. # 05401135001; Roche, Basel, Switzerland). Dissociated cells were assessed by flow cytometry on day 0 (pre-printed bioinks) or day 4, 7, 10, or 14 after printing.

Study design for examination of tumor-stromal interactions in bioprinted PDMCs

This study was conducted with two unique PDCLs, 15919-T (n = 18) and 18136-T (n = 16), both printed onto transwells in each of three 12-well plates. Three tissues from each group were fixed on days 4, 7, 10, and 14 for histological examination. One tissue on each day for each PDCL bioink was dissociated for flow cytometric analysis.

Flow cytometry

Enzymatic dissociation of bioprinted tissues

Bioprinted tissues were carefully lifted from transwells and placed directly into 50 mL Erlenmeyer flasks in 3 mL media with 0.014 mg/mL Liberase and a sterile stir bar. The flask was covered with foil and placed on a stir plate in a 37°C incubator for 60-90 minutes. Once digested, the cell suspension was transferred to a 15 mL conical tube and pelleted at 1000 RPM for 5 minutes. Pelleted cells were resuspended in PDCL media with ROCK inhibitor, counted, and appropriately diluted for experimentation.

Flow cytometry staining and analysis

Dissociated bioprinted tissue samples and pre-printed bioinks were washed with 1X FACS buffer (1.5% FBS, 2 mM EDTA, 25 µg/mL DNase, 0.1% sodium azide, in 1X PBS (Ca/Mg²⁺ free)) and resuspended in 200 µL PBS with 5 µL Human Fc block (Cat. # 422302; BioLegend, San Diego, CA) and 1 µL live/dead SyTox Blue and incubated for 15 minutes on ice in the dark. Samples were spun at 300 x g for 5 minutes, then resuspended

in 200 μ L Antibody cocktail (detailed below in **Table 3.01**) diluted in 1X FACS buffer, and incubated again on ice in the dark for 30 minutes. Samples were spun down, washed in 1X FACS buffer, and spun down again at 300 x g for 5 minutes before fixation. Samples were resuspended in 200 μ L of fixation buffer (2% PFA in 1X PBS (Ca/Mg₂⁺ free)) and incubated on ice in the dark for 10 minutes. Samples were spun down, washed in 1X FACS buffer, and spun down again at 300 x g for 5 minutes before being resuspended a final time in 1X FACS buffer for analysis using a BD Symphony flow cytometer. Data was analyzed using FlowJo software. Veri-Cells PBMCs were purchased from BioLegend (Cat. # 425003) and used as a control.

<i>Antibody target</i>	<i>Fluorophore</i>	<i>Clone</i>	<i>Dilution</i>	<i>Manufacturer</i>	<i>Catalog #</i>
CD3	BUV661	UCHT1	1:50	BD Horizon	565066
CD4	BUV496	SK3	1:50	BD Horizon	564652
CD8	PerCP	SK1	1:50	BioLegend	344708
CD16	BV786	3G8	1:150	BD Horizon	563690
CD19	BB700	SJ25C1	1:150	BD Horizon	566396
CD45	BV711	HI30	1:250	BD Horizon	564358
CD14	BUV805	M5E2	1:50	BD Horizon	612902
CD33	APC	Polyclonal	1:15	BD Pharmingen	551378
CD11b	PE	Polyclonal	1:75	BD Pharmingen	555388
CD11c	BUV395	B-ly6	1:50	BD Horizon	563787
CD68	PE-CF594	Y1/82A	1:75	BD Horizon	564944
HLA-DR	BV650	G46-6	1:75	BD Horizon	564231
CD90	PeCy7	5E10	1:150	BD Horizon	328124
CD31	APC-Cy7	WM59	1:50	BD Horizon	303120
SyTox Blue (dead cell stain)	(405nm) Pacific Blue		1:75	Invitrogen	S34857

Table 3.01 Antibody panel used for flow cytometric analysis of 3D-bioprinted tissues

Immunostaining of bioprinted tissues

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) sections were de-paraffinized, rehydrated, and placed in Target Retrieval Solution (Dako, Carpinteria, CA) for 10 min at 100°C. After cooling for 20 min, slides were quenched with 3% hydrogen peroxide for 5 min, followed

by blocking with 3% goat serum and then incubated with primary antibody against CD45 (see **Table 3.02**) overnight at 4°C. Sections were then incubated with anti-biotin secondary antibodies (1:1000) and labeling was detected with the Vectastain ABC kit (Vector Laboratories, Newark, CA). Slides were mounted using Vectamount mounting media (Vector Laboratories).

Immunofluorescence (IF)

FFPE tissue sections were de-paraffinized, rehydrated, and then placed in antigen retrieval pH 6 Citrate buffer (Sigma, Burlington, MA) in a pressure cooker for 4 min at high pressure and allowed to de-pressurize for 16 minutes. A second antigen retrieval step was then performed with pH 9 IHC Antigen Retrieval solution (Life Technologies) for 15 min. After rinsing slides in dH₂O to cool to room temperature and equilibrating in PBS, tissues were blocked with 10% normal goat serum in PBS for 30 min. Primary antibodies (see **Table 3.02**) were diluted in 5% normal goat serum with 1% BSA in PBS and applied to tissues for overnight incubation at 4°C. Secondary antibodies conjugated to Alexa Fluorophores 488 or 750 were diluted 1:200 in 5% normal goat serum with 1% BSA in PBS and applied to tissues for 1 hr at room temperature. Slides were mounted with SlowFade Gold Antifade mountant with DAPI (Life Technologies).

Cyclic immunofluorescence (cIF)

cIF staining was performed as previously described [464] to assess the presence of and spatial relationship between constituents of the tumor microenvironment. All antibodies used to stain 3D-bioprinted tissues are detailed below, in **Table 3.02**, with bold text highlighting staining displayed in **Figure 3.04B**.

<i>Antibody target</i>	<i>Fluorophore wavelength</i>	<i>Clone</i>	<i>Dilution</i>	<i>Manufacturer</i>	<i>Catalog #</i>	<i>Staining protocol</i>
CD45	unconjugated	30-F11	1:50	eBioscience	14-0451-82	IHC
αSMA	unconjugated [#]	EPR5368	1:100	Abcam	ab202295	IF
CD31	unconjugated ^{\$}	PECAM-1	1:3200	Cell Signaling	3528S	IF

Rabbit (IgG)	#488	[secondary]	1:200	Thermo Fisher	A21206	IF
Mouse (IgG)	\$750	[secondary]	1:200	Invitrogen	A21037	IF
αSMA	488	1A4	1:500	Invitrogen	53-9760-82	cIF (R1)
panCK	555	AE1/AE3 + 5D3	1:100	Abcam	ab86734	cIF (R1)
CD45	647	2D1	1:50	R&D Systems	FAB1430R	cIF (R1)
FN1	750*	F14	1:200	Abcam	ab206928	cIF (R1)
KI67	488	Polyclonal	1:100	Novus Biologicals	NB500-170	cIF (R2)
VIM	555	D21H3	1:50	Cell Signaling	9855S	cIF (R2)
CD31	647	EPR3094	1:100	Abcam	ab218582	cIF (R2)
COL-I	750	EPR7785	1:200	Abcam	ab215969	cIF (R2)
PCNA	488	PC10	1:100	Cell Signaling	8580S	cIF (R3)
cCASP3	555	D3E9	1:50	Cell Signaling	9604S	cIF (R3)
COL-IV	647	1042	1:100	Invitrogen	50-112-4582	cIF (R3)
FAP	750	EPR20021	1:100	Abcam	ab222924	cIF (R3)

Table 3.02 Antibodies used in immunostaining of 3D-bioprinted tissues

Bold text indicates antibody used for data discussed in this chapter. * indicates in-house conjugation. # and \$ indicate specific secondary antibody used. cIF (R#) indicates staining round of cIF.

Statistical analysis

For comparison of CD31⁺ immunofluorescent staining between shPIN1⁻ and shSCR-hPSC-containing bioprints (**Fig. 3.03A**), significance was determined by unpaired, two-tailed t-test. For comparison of CD31⁺ immunohistochemical staining between KPC Pin1^{-/-} and KPC Pin1^{+/+} mouse pancreata (**Fig. 3.03B**), significance was determined by unpaired, two-tailed t-tests. All calculations were performed using GraphPad Prism software. Qualitative assessments were reported for remaining experiments, as they did not have a sufficient number of replicates for statistical analyses.

RESULTS

The PDAC TME is composed of many cell types including CAFs, PSCs, endothelial cells, and immune cells, as well as a wide range of non-cellular constituents that make up the ECM [30, 124, 465]. Our lab and others have shown that PIN1, a phosphorylation-directed prolyl isomerase, plays an important role in peritumoral stroma [339, 466–468]. Specifically, we showed that PIN1 plays a role in regulating TGF β -driven activation of hPSCs to a myofibroblast state *in vitro*. This was consistent with results *in vivo*, where *Pin1*^{-/-} KPC tumors had significantly less α SMA expression in the TME [469].

Manipulating fibroblast phenotypes using a regulator of cellular differentiation states, *PIN1*, to study the effects on the PDAC tumor microenvironment

Having identified a role for PIN1 in hPSC differentiation to a myofibroblast state *in vitro* [339], we wanted to understand how loss of PIN1 specifically in fibroblasts affected the larger tumor ecosystem. Unfortunately, our *Pin1*^{-/-} mouse model was a full-body knockout, which limited our ability to differentiate TME changes resulting from altering PIN1 expression in fibroblasts alone *in vivo*. Therefore, we chose to utilize our recently established 3D-bioprinting model system [197]. Our 3D-bioprinting methodology allows us to generate heterotypic *in vitro* tissues from multiple cell types that model tissue-like cellular organization including vascular networks, cell-cell interactions, and stromal-epithelial crosstalk [197]. For this study, we bioprinted tissues with a cancer cell bioink that included a human PDAC cell line (HPAF-II) and a stromal cell bioink that included hPSCs with or without *PIN1* knockdown (shPIN1 or shSCR, respectively). HUVECs were added to each bioink at a ratio of 25% HUVEC and 75% HPAF-II or hPSC.

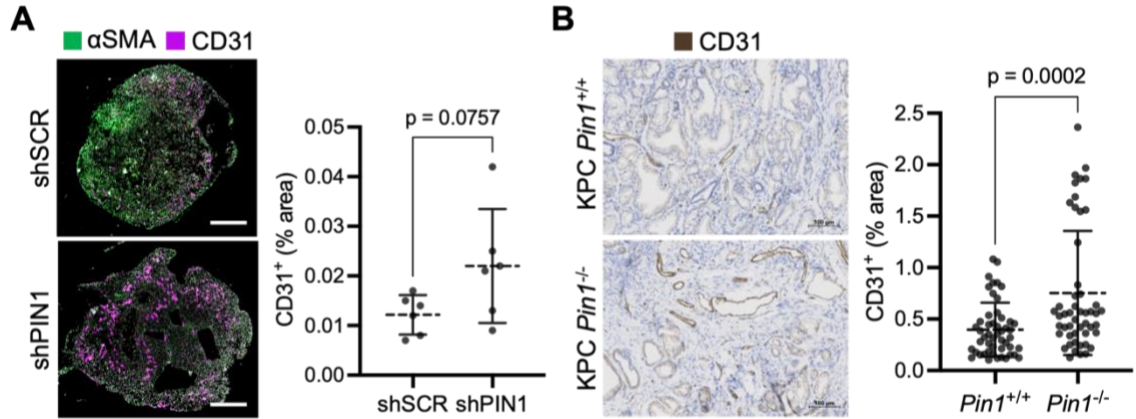


Figure 3.03 *PIN1* loss in fibroblasts affects the PDAC tumor microenvironment.

A) Representative images of CD31⁺ (purple) and αSMA (green) staining in day 4 bioprints containing shSCR (top) or shPIN1 (bottom) hPSCs. Scale bars represent 500 μm. Quantification of CD31⁺ staining showing mean ± SD of multiple sections of two biological replicates is to the right. **B)** Representative IHC images and quantification of CD31⁺ staining in KPC;*Pin1*^{+/+} (top) or KPC;*Pin1*^{-/-} (bottom) tumors. Scale bars represent 100 μm. Mean ± SD of 4 representative images each of n=6 (*Pin1*^{+/+}) or n=4 (*Pin1*^{-/-}) tumors. Significance was determined with unpaired, two-tailed t-tests.

Four days after printing, tissues were fixed and stained for CD31, an endothelial marker, and αSMA, a protein marker of PSC activation. Tissues with sh*PIN1* hPSCs showed a decrease in αSMA expression compared to those with shSCR hPSCs, indicating diminished PSC activation in these tissues. Furthermore, we observed an increase in endothelial cell network formation in the bioprinted tissues with sh*PIN1* hPSCs (**Fig. 3.03A**). This suggested that downregulation of PIN1 in hPSCs may promote a more favorable microenvironment for endothelial cell growth and organization within a tumor. We then stained KPC;*Pin1*^{+/+} and KPC;*Pin1*^{-/-} tumor tissues to determine whether vasculature was also impacted by loss of PIN1 expression in an *in vivo* setting, and we found a significant increase in CD31 staining in the KPC;*Pin1*^{-/-} tumors (**Fig. 3.03B**). Though the KPC;*PIN1*^{-/-} mice exhibited a loss of PIN1 in all tissues, the comparable outcome in both the KPC;*PIN1*^{-/-} tumors and the bioprinted tissues implies that the loss of PIN1 specifically in fibroblast populations is sufficient to affect PDAC tumor vasculature.

Understanding the complex relationship between PIN1 expression in fibroblasts and tumor vasculature also raises questions about the immune response within the TME. It is possible that alterations in the vasculature due to PIN1 loss in the fibroblasts impact

immune cell infiltration and/or function [470, 471], ultimately influencing tumor progression [472, 473]. To further examine these complex stromal relationships in our *in vitro* system, we first needed to develop a method to include immune populations in our bioprinted tissues. We began by adding commercially available peripheral blood mononuclear cells (PBMCs) to the stromal cell bioink in our bioprints (**Fig. 3.02**). Resulting bioprinted tumor tissues were composed of a cancer cell bioink with HPAF-II cells and HUVECs (75%:25%), and a stromal cell bioink of hPSCs with or without *PIN1* knockdown (shPIN1 or shSCR, respectively), HUVECs, and PBMCs (50%:16.7%:33%).

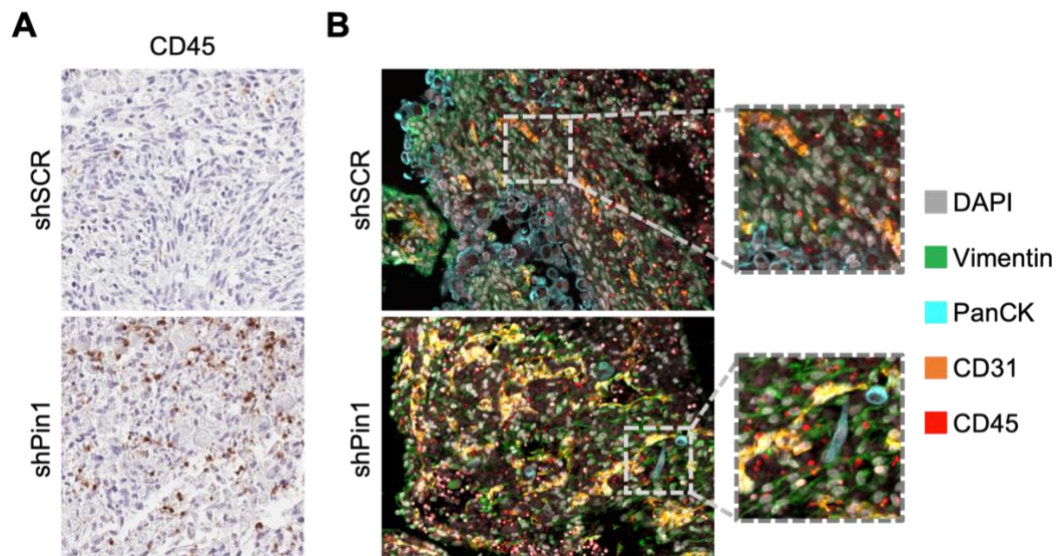


Figure 3.04 *PIN1* expression in pancreatic stellate cells is inversely correlated with presence of immune cells in 3D-bioprinted PDAC tumors.

Representative staining of 3D-bioprinted tumors composed of PDAC cells (HPAF-II), HUVECs, PBMCs, and hPSCs with or without *PIN1* knockdown (shPIN1 (n = 2 tissues) or shSCR (n = 2 tissues), respectively) 4 days post-print **A**) by IHC for CD45 (brown) or **B**) by cIF for DAPI (grey), Vimentin (green), PanCK (cyan), CD31 (orange), and CD45 (red).

Tissues were fixed and embedded, and then stained for CD45 by immunohistochemistry. Tissues containing PIN1 knockdown PSCs showed increased immune cell presence over those with wildtype PIN1 (**Fig. 3.04A**), although additional biological replicates will be required to check for statistical significance in this observation. Given that these tissues were stained only for the presence of CD45⁺ cells, we cannot determine whether the loss of PIN1 expression in fibroblasts affected immune cell survival, proliferation, or both.

Alone, these data suggest that PIN1 expression in pancreatic stellate cells negatively impacts immune cell populations. Interestingly, however, this is distinct from our observation of similar numbers of CD45⁺ cells in our autochthonous KPC;*Pin1*^{-/-} mice when compared to KPC;*Pin1*^{+/+} controls (data not shown), and further work will be needed to determine reasons for this difference.

Modeling tumor immune microenvironmental changes in 3D-bioprinted tissues containing patient-derived cancer cells

Previous work in our lab [102, 254, 474] and by others [105, 255, 475–479] suggests that cancer cell-intrinsic MYC expression affects the tumor immune microenvironment, with high levels of MYC typically being correlated with immune evasion. A study published by Li & Byrne, et al., in 2018 [255], showed that pancreatic cancer cell-intrinsic factors, including MYC levels, significantly impact the tumor immune microenvironment and in murine PDAC tumors. Having studied some effects of MYC on the TME in *in vivo* murine settings myself (Chapter 2), I wanted to determine whether MYC drives similar gene programs and resulting phenotypes in human tissues.

To do so, we needed to first identify and establish an appropriate model system in which we could incorporate patient-derived tissues and/or cells. PDCLs offer a promising opportunity to test therapeutic responses *ex vivo* [205], but they are often limited in their capacity to recapitulate stromal reactions like desmoplastic expansion as many PDCLs are grown in monocultures [206]. For this work, we leveraged the pancreatic cancer PDCLs that have been generated by the BCCPC. We sought to assess the effects of cancer cell-intrinsic MYC expression on immune cell population survival in bioprinted tissues.

Figure 3.05A illustrates the process of deriving pancreatic PDCLs from surgically resected tumors and incorporating these cells into a 3D-bioprinted tumor model with PBMCs. Our

goal is to not only use PBMCs, but to use patient-matched PBMCs from blood draws; eventually using *all* patient-matched donor cells in our bioprints, including hPSCs and endothelial cells. It is important to carefully consider, however, each use of patient cells as they remain primary and finite resources, generously donated by patients. Therefore, we began assessing bioprints that contained commercially available primary human stellate, endothelial, and immune cells. In the following studies, cells were unmatched, and neither MHC nor HLA-type was considered at the time of purchase.

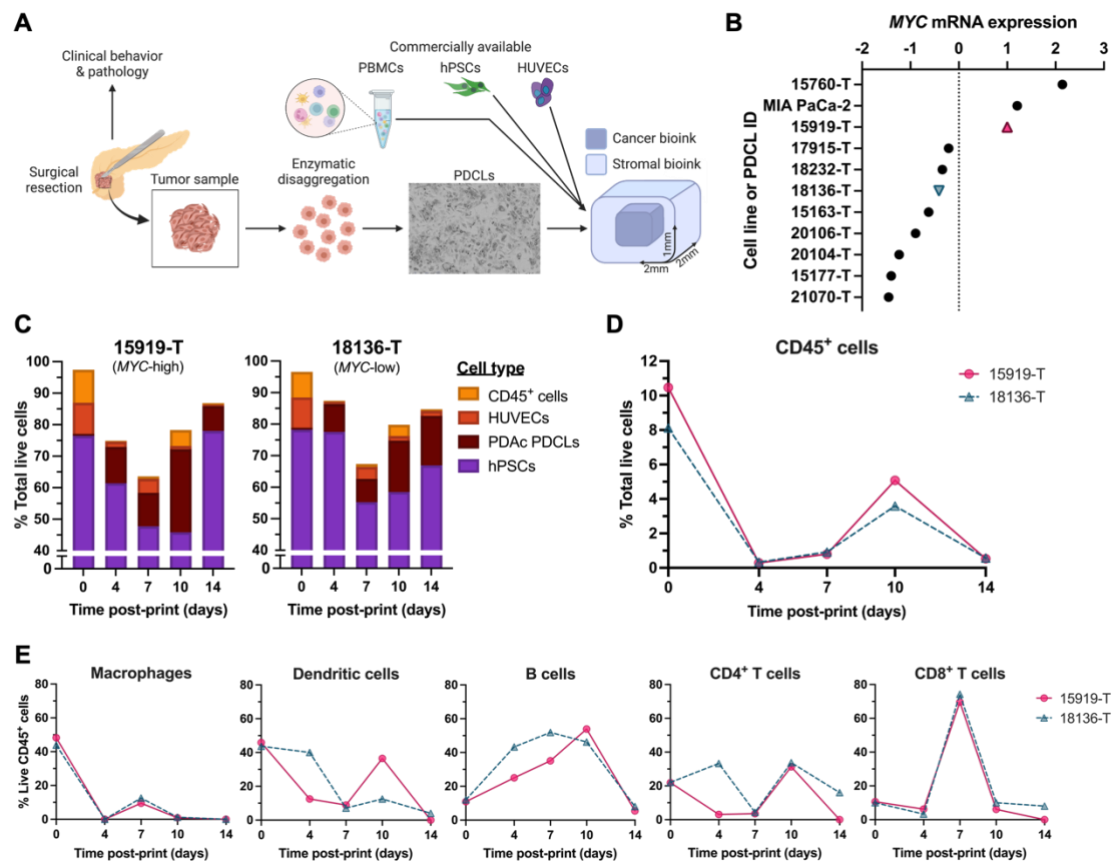


Figure 3.05 MYC expression in patient-derived cancer cell lines has minimal effect on immune cell survival in 3D-bioprinted tissues

A) Schematic depiction of derivation of pancreatic PDCLs from surgically resected tumors and a 3D-bioprinted tumor model utilizing these PDCLs in conjunction with commercially available primary PBMCs, hPSCs, and HUVECs. Created with BioRender.com. **B)** MYC mRNA expression by RNAseq in pancreatic PDCLs. MIA PaCa-2 included as commonly used cell line reference. Pink and blue triangles highlight 15919-T and 18136-T as our selected MYC-high and MYC-low PDCLs, respectively. **C)** Major cell type populations, identified by flow cytometry, shown as percent of total live cells in bioinks prior to print on day 0 and in the 3D-bioprinted tissues on days 4, 7, 10, and 14. Tissues with 15919-T PDCLs on the left and those with 18136-T PDCLs are on the right. **D)** Surviving CD45⁺ cells over time shown as percent total live cells, identified by flow cytometry. **E)** Surviving major immune cell type populations, identified by

flow cytometry, shown as percent live CD45⁺ cells in bioinks prior to print on day 0 and in the 3D-bioprinted tissues on days 4, 7, 10, and 14.

We stratified PDCLs from the BCCPC by mRNA *MYC* expression from RNAseq analysis and, aiming for physiological relevance [110], selected two PDCLs, 15919-T and 18136-T, due to their relatively high and low baseline *MYC* expression levels (**Fig. 3.05B**; Z-scores: 1.00 and -0.41, respectively). We bioprinted these into tissues with hPSCs, HUVECs, and PBMCs. Given the substantial presence of desmoplastic stroma in PDAC tumors [126, 140, 291, 480], our bioink composition for the cancer compartment was adapted to also include hPSCs (cancer bioink: 50% cancer, 35% hPSCs, 15% HUVECs). Additionally, to mimic the often low immune infiltration into PDAC tumor cell clusters [481, 482], only the surrounding stromal cell bioink included PBMCs (stromal bioink: 80% hPSCs, 15% HUVECs, 5% PBMCs). We employed a 17-color flow cytometry panel to distinguish major functional cell types and assess lymphoid and myeloid lineages (as described in methods) to study the response of specific immune cell subtypes to *MYC*-high and *MYC*-low tumor settings in bioinks on day 0 and bioprinted tissues on days 4, 7, 10, and 14 (**Fig. 3.05C-E**).

We observed variability in major cell type populations within bioinks using PDCLs 15919-T (*MYC*-high) or 18136-T (*MYC*-low) prior to print on day 0 and in the resulting 3D-bioprinted tissues over the course of this experiment (**Fig. 3.05C**). On day 0, before bioprinting, the quantitated cell populations in the bioinks, expressed as percent of total live cells identified by flow cytometry, were consistent with the intended compositions. However, by day 4, there was a noticeable decrease in the proportion of cancer cells, particularly in the *MYC*-low (18136-T) bioprints. The proportions of hPSCs and HUVECs appeared relatively stable across all time points in both *MYC*-high and *MYC*-low settings, although some variability was observed. The percentage of CD45⁺ cells generally showed a decreasing trend from day 0 to day 14 in both *MYC*-high and *MYC*-low settings (**Fig.**

3.05D). Specifically, day 4 prints showed a decrease in CD45⁺ cell percentages. Day 7 and day 10 prints had higher percentages of CD45⁺ cells than day 4 prints, though still lower than the Day 0 input; and day 14 prints showed few CD45⁺ cells, reaching near zero for all immune populations by day 14. Comparing *MYC*-high and *MYC*-low PDCLs revealed no significant differences in the survival rates of CD45⁺ cells, suggesting that signaling from these two cell lines did not markedly affect overall immune cell viability.

Before printing, both bioink mixes displayed a higher proportion of myeloid (macrophages and dendritic cells) compared to lymphoid (B cells, CD4⁺ T cells, and CD8⁺ T cells) lineages (**Fig. 3.05E**). By day 4, a significant reduction in both immune cell lineages was observed, with myeloid cells showing a more pronounced decline. This trend persisted through the day 7, 10, and 14 prints, although the magnitude of these changes varied. Macrophages, in particular, showed a particularly substantial decrease from day 0 to day 4. A slight increase was observed in day 7 prints, but macrophages in both *MYC*-high and *MYC*-low settings showed minimal survival by day 14. Dendritic cells also exhibited a decrease in proportion from day 0 to day 4, with a slightly more stable population in the *MYC*-low setting. By day 14, the proportion of dendritic cells had significantly decreased in both *MYC*-high and *MYC*-low settings, with no significant differences observed between the two groups. While these observations suggest potential dynamic changes, the variability in the data indicates that additional experiments are needed to optimize the system for reliably studying dynamic changes over time.

Among the lymphoid lineage, CD4⁺ T cell population showed a decline from day 0 to day 4 in the *MYC*-high, but this was not seen in the *MYC*-low setting (**Fig. 3.05E**). Despite small differences in CD4⁺ T cells day 7, the two groups did not exhibit significant differences in overall CD4⁺ T cell survival, suggesting that the difference in paracrine signaling from these two PDCLs does not substantially impact these cells' viability. CD8⁺

T cells exhibited a similar pattern to CD4⁺ T cells, but with larger numbers in the day 7 prints. The survival of CD8⁺ T cells was relatively consistent between the *MYC*-high and *MYC*-low settings, and by day 14 the proportion of CD8⁺ T cells was near zero in both groups. Interestingly, B cells as a percentage of CD45⁺ cells did not show the sharp decrease seen with T cells and monocytes at day 4. The survival of B cells was relatively consistent between the *MYC*-high and *MYC*-low settings, with slightly lower numbers at the earlier time points in the *MYC*-high setting. Again, though, by day 14, the proportion of B cells was near zero in both groups.

Together, these data suggest that the difference in baseline *MYC* mRNA expression in our selected PDCLs does not significantly impact the survival of immune cells within 3D-bioprinted pancreatic tumor models, but the small sample size necessitates additional studies to confirm our findings. Important to note, however, both *MYC*-high and *MYC*-low PDCLs 15919-T and 18136-T, were able to endure the bioprinting process, and some immune cells of both myeloid and lymphoid lineages survived until day 10 post-print in both settings. These findings provide valuable preliminary insights for future studies aimed at incorporating patient-derived models and immune cells into manipulable experimental systems, potentially advancing our understanding of tumor-immune interactions in pancreatic cancer.

DISCUSSION AND FUTURE DIRECTIONS

In the seventy-two years since the very first patient-derived cancer cell line (HeLa) was established and immortalized [395, 483], cancer models have significantly advanced to better mimic the complex nature of heterogeneous tumors and interactions between the cancer cells themselves and the tumor microenvironment [392, 418, 484–486]. These models now give researchers the ability to incorporate various components such as stromal cells, immune cells, and extracellular matrix components to recreate a more realistic tumor microenvironment [181, 182, 197, 487]. By integrating these elements, researchers can now gain a deeper understanding of how different cell types interact within the tumor, allowing for more accurate predictions of drug response and the development of personalized treatment strategies.

In this chapter, we took advantage of a manipulatable 3D-bioprinting methodology that allowed us to alter individual components of printed tumor models. First, by selectively altering the expression of PIN1 in fibroblasts, we characterized the specific effect of fibroblast *PIN1* knockdown on the overall model system and began to observe how altering fibroblasts impacted other proximal cell types. We showed that loss of *PIN1* expression in PSCs resulted in decreased PSC activation and increased vascular network formation (**Fig. 3.03**). Further studies, both in our bioprinted tissues and in conditional knockout mice, could investigate the specific mechanisms by which PIN1 loss in fibroblasts affects endothelial cell growth and vascular organization. It would be interesting to also determine if the observed immune changes were driven directly by changes in the fibroblasts or were a result of changes in vascular signaling or organization. Notably, this increase in tumor vascularity provides a chance for therapeutic treatments to more thoroughly penetrate PDAC tumors—an opportunity that warrants further investigation with PIN1 inhibitors.

We also demonstrated an inverse correlation between *PIN1* expression in bioprinted PSCs and the presence of CD45⁺ cells in the TME (**Fig. 3.04**). Given that these tissues were stained only for the presence of CD45⁺ cells, we were unable to determine whether the loss of *PIN1* expression in fibroblasts improved immune cell survival, proliferation, or both. Further studies also are needed to examine specific subsets of immune cells and their functional states (e.g., activation markers, exhaustion markers), and it would also be valuable to compare cytokine and chemokine profiles between bioprint conditions to reveal differences in immune cell signaling pathways. Interestingly, while the loss of *PIN1* increased the presence of CD45⁺ cells in our bioprinted tissues, the number of CD45⁺ cells appeared similar between our autochthonous *KPC;Pin1*^{-/-} mice and their *KPC;Pin1*^{+/+} counterparts (data not shown). There are a few potential explanations for this discrepancy, including the fact that our bioprinted model is generated and tracked for a relatively short period of time, and it does not model the natural tumor progression like the GEMM. It also does not require immune cells to actively infiltrate the tumor, navigating through the dense stroma with minimal vascular access. In turn, the controlled environment of our bioprinted model may allow for increased interaction between PSCs and immune cells, which may lead to a more pronounced response than would be observed in a more complex *in vivo* setting. Alternatively, the creation of a new autochthonous mouse model with a conditional *PIN1* knockout specifically in PSCs would help isolate the effects of *PIN1* loss in these cells without the confounding factors or compensatory mechanisms present in a full-body knockout.

The compatibility with patient-derived cells highlights the potential of our bioprinting model to study the interactions between heterogeneous cancer cells with different cell types within the tumor microenvironment in a purely human cell structure. By incorporating primary PDMCs and manipulating various components, we aim to understand how

individual patient's cancer cells influence the tumor microenvironment and immune response. We specifically examined how cancer cells with varying levels of *MYC* expression influence the immune cells within the bioprinted tumor tissues. Our results demonstrated that both *MYC*-high and *MYC*-low PDCLs could endure the bioprinting process, and that immune cells of both myeloid and lymphoid lineages survive until day 10 post-print. Our findings showed small differences in specific immune cell subsets, but largely suggest that the differential paracrine signaling from our chosen PDCLs with different *MYC* levels does not significantly impact the overall survival of immune cells in the bioprinted models. Additional experimental replicates are needed to confirm these findings, and extensions of these experiments may help reveal the underlying biology.

For example, our study in Chapter 2, along with a number of other studies, indicates important shifts across immune cell populations—including T cells, B cells, NK cells, mast cells, M2 macrophages, and dendritic cells—correlated to *MYC* expression across cancers, including lung, breast, and pancreas [103, 104, 116, 255, 300, 301, 476, 477, 488, 489]. While our initial bioprinting experiments didn't find changes in populations of T cells, B cells, or myeloid cells with different levels of *MYC* expression, we did not examine subsets of these populations. It will be important to study the activation states, for example, of CD8⁺ cytotoxic T cells versus regulatory T cells (Tregs) to show how *MYC* influences the balance between immune activation and suppression. Similarly, investigating the roles of different macrophage phenotypes (M1 vs. M2) could elucidate how *MYC* affects tumor-promoting versus tumor-fighting activities.

We also performed this experiment with only two PDCLs, but could extend to a greater number. In addition to bioprinting with multiple PDCLs with varied *MYC* expression, we could also directly engineer *MYC* expression in cell lines with dox-inducible *MYC* overexpression or knockdown in PDCLs. This approach would allow us to control *MYC*

expression dynamically and study both gain-of-function and loss-of-function scenarios. Additionally, it would give us a much more controlled approach to minimize the impact of other differences between PDCLs.

The bioprint studies discussed in this chapter were the first steps toward our tumor immune microenvironment (TiME) modeling goals. Importantly, however, the effective display of mutant peptide fragments by the human leukocyte antigen class I (HLA-I) genes is crucial for most immune activation, and, therefore, the immune-mediated eradication of cancer cells [161, 162]. In **Figure 3.05**, the HLA-I class of neither the commercially available PBMCs nor the PDCLs was known, as we remained in the troubleshooting phase of this research, and almost certainly resulted in an HLA mismatch between the two in the bioprinted tissues. There was also likely a mismatch with the hPSCs and/or HUVEC. That said, the continued use of PDCLs may give us the ability to include autologous stromal cell populations in our studies, therefore eliminating this confounding factor. In addition to selectively isolating epithelial cancer cells from PDAC biopsies, as shown in this chapter, we have begun isolating cancer associated fibroblasts alongside their cancer cell counterparts to create a more extensive biobank of matched patient-derived cells. Long term, we intend to bioprint tissues with autologous PDCLs and PBMCs from tumor biopsies and blood draws, respectively. Utilizing autologous cells should significantly improve the applicability of our results to patients in a clinical setting. Contrary to the lymphocyte lineage, myeloid cells do not necessitate the recognition of HLA molecules to become activated [490, 491]. Hence, until we have fully matched bioprints, future experiments could focus solely on immune cells from a myeloid lineage. Toward this end, current approaches in the lab are 3D-bioprinting tissues with PDCLs and CD3⁺ cell-depleted PBMCs.

Finally, in using only PBMCs in our bioprints, we have neglected to account for the many tissue-resident immune cells typically observed in PDAC tumors [119, 160]. Unfortunately, this bioprinting system may never be able to fully recapitulate a complete immune response, as disturbing the cells to generate each bioprint inevitably alters any previously printed or ‘tissue-resident’ immune cells. As we continue using and improving our bioprinting methodology to study the TiME, we will need to ensure that our models match immune phenotypes and effects seen in PDAC patients. This might be done by comparing our results to immune phenotypes seen in cultured patient tissue slices [492–494] or in bioprints with directly dissociated patient samples, as was shown in Langer & Allen-Petersen et al. [197].

In summary, *in vitro* preclinical modeling of the tumor microenvironment using 3D-bioprinting is a promising and highly tailorable approach, adding models to our arsenal that can be customized to address specific research questions. These models utilize patient-derived tumor cells, primary stromal components, and immune cells such as patient (or healthy donor) peripheral blood mononuclear cells (PBMCs), providing an accurate reflection of the human tumor microenvironment. By replicating key aspects of the tumor microenvironment, we can better study the mechanisms that drive tumor growth, immune evasion, and therapeutic resistance. With continued refinement, these models hold the potential to identify novel treatments and significantly improve patient outcomes, contributing to our understanding of PDAC and other cancers.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

OVERVIEW AND CONTEXT

Pancreatic ductal adenocarcinoma remains one of the most aggressive and lethal forms of cancer, characterized by late-stage diagnosis and poor responses to therapy. The overarching goal of this dissertation was to address some of the critical gaps in preclinical modeling that hinder the translation of research findings into effective clinical treatments. By developing a novel *Kras*- and *Myc*-driven genetically engineered mouse model and leveraging advanced 3D-bioprinting technologies, this work seeks to provide more accurate models of PDAC that can be used to better understand the disease's complex biology and improve therapeutic strategies.

The novel KMC^{ERT2} mouse model described in this dissertation was designed to mimic the molecular and histological complexity of human PDAC. By integrating the deregulated expression of MYC with an activating *Kras*^{G12D} mutation, this model provides a powerful tool for studying the early stages of pancreatic tumorigenesis and the subsequent development of a desmoplastic and immunosuppressive tumor microenvironment. Additionally, the use of 3D-bioprinting to create patient-specific tumor models offers a promising approach to personalized medicine, allowing for the testing of therapeutic strategies in a setting that closely mimics human disease.

KEY FINDINGS AND FUTURE DIRECTIONS

MYC as a Driver of Heterogeneous PDAC

MYC's role as an oncogenic driver is well established across multiple cancer types, including lymphoma, breast cancer, and lung cancer [91, 98, 101, 102, 105, 106]. Its overexpression is frequently observed in PDAC, and is closely linked with poor prognosis, increased metastatic potential, and resistance to standard therapies [107, 120, 262]. The

central role of MYC in PDAC is underscored by its ability to act as a master regulator of gene expression, driving the transcriptional programs that underpin both tumor initiation and progression.

In Chapter 2, we demonstrated that even low levels of deregulated MYC, when combined with mutant *KRAS*^{G12D}, are sufficient to initiate PDAC development in the KMC^{ERT2} mouse model. This finding is consistent with the growing body of evidence that MYC is not only critical for the maintenance of established tumors but that it also plays a pivotal role in the early stages of pancreatic tumorigenesis [108, 109, 309, 495].

Compared to other MYC-driven mouse models of PDAC, our KMC^{ERT2} model presents key distinctions. For example, the Evan lab's MYC model [108] used a reversible, switchable MYC system in combination with mutant KRAS to explore MYC's role in pancreatic tumorigenesis. Their model demonstrated that MYC overexpression is sufficient to drive aggressive tumor formation, with MYC deactivation leading to complete tumor regression, including remodeling of the TME, stromal collapse, and reactivation of immune surveillance. However, this model focused on rapid onset of MYC overexpression, which does not fully reflect the typical, steady low MYC expression found in human PDAC.

In contrast, our KMC^{ERT2} model integrates low-level MYC deregulation alongside mutant KRAS, more accurately mimicking human PDAC in which KRAS mutations are a key initiating event and MYC expression is often elevated but not to the extreme levels seen in the Evan lab model. This distinction is crucial for modeling how MYC and KRAS interact at levels that more closely resemble those observed in patient tumors, especially when studying therapeutic resistance and tumor plasticity.

In a recent study led by Maddipati and colleagues [109], the authors identified high MYC expression in metastatic tumors and cell lines, showing that MYC overexpression in non-metastatic lines could drive metastasis through macrophage recruitment and increased

intravasation. However, their model relied on heterozygous p53 loss and higher MYC overexpression levels, which produced more uniform and widespread metastatic disease. In contrast, our KMC^{ERT2} model allows for the study of metastatic PDAC with low MYC expression and without genetic changes to p53, offering a more physiologically relevant platform to investigate spontaneous metastasis and tumor heterogeneity.

Further validation of MYC's necessity in PDAC comes, again, from Sodikov and colleagues [105]. Their study highlighted the importance of MYC in maintaining tumor growth and an immunosuppressive microenvironment [105]. Importantly, MYC loss in their model not only halted tumor growth but also induced a state of tumor dormancy, suggesting that continuous MYC expression is required to maintain the aggressive phenotype of PDAC, a finding that aligns with our observations in the KMC^{ERT2} model but under conditions of low MYC expression. These comparisons underscore how MYC-driven models, including ours, contribute to understanding how MYC regulates both intrinsic tumor growth and extrinsic factors such as stromal and immune components.

Tumor heterogeneity is a critical factor in pancreatic cancer progression, as it reflects the diversity of lesions seen in patient populations and impacts both prognosis and therapeutic response [33, 496–498]. Recapitulating this heterogeneity in preclinical models is essential for developing therapeutic strategies that can effectively target the broad spectrum of tumor subtypes seen in clinical settings. Our KMC^{ERT2} model further adds to the field by providing a heterogeneous, desmoplastic PDAC model that allows for spontaneous progression in the absence of tumor suppressor mutational loss. By establishing this autochthonous model, as well as a series of cell lines with distinct phenotypes, we provide additional resources to understand the heterogeneous progression of this deadly disease.

Using CODA, a semantic segmentation deep learning model, we were able to identify four histologically diverse groups within KMC^{ERT2} study mouse pancreata: acinar, cellular, high stroma/ECM, and mixed. Pancreata from KMC^{ERT2} and KM/+C^{ERT2} mice showed a trend toward a desmoplastic phenotype, falling primarily within the high stroma/ECM group, consistent with progression to advanced PDAC in clinical cohorts. Future work on this model will characterize the desmoplastic stroma and compare phenotypes of, for example, the CAFs in this and other PDAC models. This work could leverage flow cytometry approaches or single-cell sequencing to quantify composition of myCAFs, iCAFs, and apCAFs at different stages of tumor progression or upon transplantation of the distinct cell lines.

To further understand the heterogeneity of this model on the molecular level, we performed RNAseq on microdissected tumor areas. We identified three distinct transcriptional tumor clusters, each with unique gene expression profiles that included distinct MYC-dependent pathways in the different clusters (Fig. 2.05A, Supp. Fig. 2.10-2.12). Cluster 1 tumors were significantly enriched for cell cycle regulation, DNA replication, and repair pathways, as well as glycolysis. The enhancement of glycolysis is a major MYC-driven pathway where MYC upregulates the expression of glycolytic enzymes, increasing ATP and biosynthetic precursor availability to support rapid cancer cell proliferation [499]. In addition, MYC-driven transcriptional reprogramming also includes the upregulation of nucleotide biosynthesis pathways, essential for DNA replication and repair, ensuring a continuous supply of nucleotides for sustained proliferation [83].

The significant enrichment of DNA replication and repair pathways in Cluster 1 tumors provides further evidence of MYC's role in regulating these processes. These metabolic alterations not only provide building blocks for cell growth while creating a tumor

microenvironment that fosters immune evasion and metastasis. For example, the increased glycolytic flux often leads to lactate production, which acidifies the tumor microenvironment, promoting immune evasion [499, 500]. Cluster 2 tumors were upregulated in oxidative phosphorylation and metabolic pathways, consistent with MYC's promotion of ribosome biogenesis by regulating the transcription of ribosomal RNA and protein genes [500]. MYC exerts its oncogenic effects through multiple mechanisms, and the transcriptional signatures we identified further support MYC's role in reprogramming key cellular processes to fuel tumor growth. Interestingly, the upregulation of metabolic pathways in Cluster 2, particularly oxidative phosphorylation, is coupled with increased estrogen and androgen response signaling, suggesting distinct metabolic reprogramming that supports sustained tumor growth through enhanced mitochondrial activity and hormonal signaling. This highlights MYC's role in driving anabolic processes that ensure energy production and biosynthesis, crucial for tumor cell survival.

Cluster 3 tumors exhibited a unique transcriptional profile characterized by upregulation of inflammatory response and hypoxia-associated pathways. This cluster showed enrichment for genes involved in epithelial-mesenchymal transition (EMT), which is often associated with increased invasiveness and metastatic potential in PDAC. The hypoxia signaling pathway, in particular, drives tumor adaptation to low oxygen conditions, promoting angiogenesis and further contributing to immune evasion. The distinct signature of EMT and hypoxia in Cluster 3 suggests that these tumors may be more aggressive and capable of early dissemination, emphasizing diverse array of pro-tumorigenic processes orchestrated by MYC.

Given MYC's multifaceted role, the KMC^{ERT2} model presents an opportunity to continue to explore how MYC drives its downstream pathways. Moreover, targeting MYC's downstream metabolic pathways may present a promising therapeutic strategy.

Additionally, while MYC itself has traditionally been considered "undruggable," recent advances in small-molecule inhibitors and MYC-targeting strategies offer new therapeutic avenues [108, 116]. The pathways we identified in Chapter 2, such as the upregulation of oxidative phosphorylation and cell cycle pathways, suggest that combination therapies targeting both MYC and glycolysis may be especially effective.

Heterogeneity of the Tumor Microenvironment

The heterogeneous and desmoplastic TME is a defining feature of PDAC and plays a critical role in the disease's notorious resistance to therapy and its variable clinical outcomes [36, 214]. By developing models that accurately recapitulate this complexity, we can better understand how interactions between tumor cells and the TME drive disease progression and therapeutic resistance. In the KMC^{ERT2} model, MYC-driven alterations to epithelial cells not only promoted aggressive tumor growth but also led to profound changes within the TME, including increased stromal desmoplasia and immunosuppression—hallmarks of aggressive PDAC [36, 116, 130, 214].

In comparison, the Evan lab's MYC model [108] highlighted how MYC inactivation in their system led to tumor regression, stromal collapse, and reactivation of immune surveillance. However, this model focused on the effects of a rapid onset of MYC overexpression and reversible MYC activity, which, while informative, does not fully mimic the persistent, low-level MYC deregulation typically observed in human PDAC. The KMC^{ERT2} model extends this work by demonstrating that chronic low-level MYC expression, in conjunction with mutant *Kras*, can drive sustained tumor growth and immune suppression, more closely aligning with the MYC/KRAS interaction seen in patients.

To analyze the TME in our model, we performed analyses of the KMC^{ERT2} model both with CODA and multiplex IHC, analyzing full sections of multiple mouse tumors. This analysis revealed spatial and functional heterogeneity of the TME. Some regions exhibited an

immune-rich microenvironment with a high density of infiltrating immune cells, while others were dominated by dense stromal components with minimal immune cell infiltration. While more analysis is needed, as described above to define the composition of the TME in this mouse model, it will also be interesting to continue spatial analyses to connect this work to the distinct spatial neighborhoods that have been described in human PDAC [481, 501, 502]. Together, these results demonstrate the power of the KMC^{ERT2} model for studying how MYC shapes the tumor microenvironment to promote survival and therapy resistance.

A novel finding in this study is the identification of a dendritic cell (DC)-heavy immune response within the KMC^{ERT2} model, which is distinct from the commonly used KPC model that we used for comparison. Therefore, this model provides a platform by which we may be able to further understand the signaling involved in recruiting and/or maintaining presence of these critical immune cells. DCs play a pivotal role in initiating and modulating adaptive immune responses [153], acting as the bridge between innate and adaptive immunity. Their presence within the TME is particularly important in shaping the immune landscape of PDAC [503, 504]. Our results indicate that MYC, in conjunction with mutant KRAS, may drive a TME that is highly conducive to DC recruitment and activation. This MYC-driven environment could have profound implications for tumor progression and response to therapy. Of note, the Evan lab's MYC model did not focus on DCs in the immune remodeling process following MYC inactivation, further underscoring the value of the KMC^{ERT2} model in exploring how MYC impacts DC recruitment and how this immune cell population interacts with the TME.

While DCs are generally associated with promoting anti-tumor immunity by presenting tumor antigens to T cells, their role in PDAC appears to be more complex and context-dependent. Recent studies suggest that in the PDAC TME, DCs can adopt a tolerogenic phenotype, contributing to immune evasion by inducing regulatory T cells (Tregs) and

promoting an immunosuppressive environment [306, 505]. The crosstalk between DCs and other immune components within the TME, such as Tregs and MDSCs, further complicates the immune landscape in PDAC. This interaction creates a feedback loop that reinforces immune suppression, making the tumor more resistant to conventional therapies, including chemotherapy and emerging immunotherapies [506]. The abundance of DCs observed in the KMC^{ERT2} model may indicate a similar shift towards a tolerogenic state. Understanding the molecular cues that drive this shift in DC function, particularly the role of MYC, could reveal new therapeutic targets aimed at reprogramming these cells to restore their anti-tumor activity. The KMC^{ERT2} model, with its robust DC infiltration, provides an ideal platform to explore these dynamics in detail and to test strategies that could disrupt this immunosuppressive network.

There remains much to analyze in the TME of the KMC^{ERT2} model, including characterizing the state of DCs present, the infiltration and programming of other immune populations such as Tregs and myeloid-derived suppressor cells (MDSCs), as well as a characterization of stromal components like CAFs, which also play a significant role in shaping the immune landscape. While a detailed characterization of CAF subtypes (myCAFs and iCAFs) was beyond the scope of this initial study, future analysis of CAFs in this model should also address whether changes to CAFs could affect the changes observed in the immune microenvironment. The interplay between CAFs and immune components, such as DCs, Tregs, and MDSCs, is particularly complex. For example, the ECM produced by myCAFs not only serves as a physical barrier to immune cell infiltration but also actively modulates immune cell function, often promoting a suppressive microenvironment that limits the effectiveness of immunotherapies [507]. The increase in stromal desmoplasia observed in the KMC^{ERT2} model raises the intriguing possibility that

CAFs are influencing immune cell behavior, either through direct interactions or by reshaping the extracellular matrix (ECM) to create a more immunosuppressive niche.

Our findings suggest that the KMC^{ERT2} model may be useful to dissect these intricate stromal-immune interactions, offering insights into how the TME contributes to therapeutic resistance. For instance, as mentioned above, performing single-cell sequencing at early and late timepoints during tumor initiation and progression could illuminate differences in stromal cell populations and TME composition. Further, targeting specific components of the ECM or modulating CAF activity could potentially enhance the efficacy of existing therapies. Emerging therapeutic strategies aimed at reprogramming CAFs from a tumor-promoting to a tumor-suppressing phenotype are particularly promising [507]. Understanding these stromal dynamics in greater detail will be crucial for developing more effective and personalized therapeutic approaches for PDAC.

An additional promising avenue for future research involves combining MYC inhibitors with agents that can modulate DC function, potentially enhancing the effectiveness of immunotherapies. For example, recent studies have suggested that targeting pathways involved in DC maturation or antigen presentation could reinvigorate the immune response against MYC-driven tumors [303, 479]. Exploring how the KRAS–MYC axis affects other immune modulatory pathways could provide a broader understanding of how these oncogenes contribute to the immunosuppressive environment in PDAC. This knowledge could lead to the development of combination therapies that simultaneously target tumor cell-intrinsic factors and the surrounding immune context, thereby overcoming the multifactorial resistance observed in PDAC.

Mechanistic Studies on MYC-Driven Immune Responses

While in our KMC^{ERT2} model, a higher density of DCs was observed compared to the widely used KPC model, suggesting that MYC may influence DC recruitment, our study

did not delve into the activation status of these DCs or the specific mechanisms driving their recruitment, leaving important questions unanswered.

To build on these findings, future research should focus on characterizing the phenotype and functional status of DCs within the KMC^{ERT2} model. It will be crucial to determine whether the increased DC presence correlates with enhanced immune surveillance or contributes to an immunosuppressive environment that supports tumor growth. Furthermore, exploring the interactions between MYC-driven signaling pathways and immune cell recruitment could reveal potential therapeutic targets. For example, understanding how MYC signaling intersects with immune checkpoint pathways could inform strategies to reprogram the TME to enhance anti-tumor immunity [506].

Additionally, employing single-cell mass cytometry, as utilized in the Brouwer study [504], could provide detailed insights into the interactions between MYC-driven pathways and immune cells, revealing how these dynamics influence therapeutic resistance. Integrating these approaches with our findings could lead to novel combination therapies that target both MYC signaling and immune modulation, potentially improving outcomes for patients with PDAC.

Subtype-Specific Therapeutic Responses

PDAC is characterized by significant molecular and phenotypic heterogeneity, which translates into varying therapeutic responses among its subtypes. This heterogeneity is a major factor contributing to the difficulty in treating PDAC effectively, as different subtypes respond differently to standard-of-care (SoC) therapies [33, 508]. For instance, squamous-like subtypes of PDAC, which are generally more aggressive and characterized by higher levels of epithelial-to-mesenchymal transition (EMT) markers, have shown poor responses to SoC therapies such as gemcitabine and FOLFIRINOX. These subtypes often present with extensive stromal desmoplasia and immune evasion mechanisms,

further complicating treatment [36]. On the other hand, classical subtypes, which retain more epithelial characteristics and have lower levels of EMT, tend to be more responsive to these therapies, though they still pose significant treatment challenges [496].

Excitingly, the KMC^{ERT2}-tumor derived orthotopic model developed in this dissertation also demonstrated heterogeneity in treatment response, confirming that tumors can exhibit a broad spectrum of sensitivities to SoC therapies based on their molecular subtype. We observed that squamous-like subtype-derived KMC^{ERT2} tumors, which are often more aggressive, showed poor responses to SoC, while classical (pancreatic progenitor) subtypes were more responsive, albeit still challenging to treat (**Fig. 2.07**).

Our work demonstrates that the KMC^{ERT2} model may be useful for testing novel therapies targeted at specific subtypes [33, 36, 508], potentially leading to more personalized treatment approaches for PDAC patients. For example, recent studies have suggested that targeting the molecular pathways unique to squamous-like subtypes, such as the NOTCH or TP63 pathways, could enhance therapeutic efficacy [33, 509]. Combining SoC therapies with agents that modulate the TME, such as ECM-targeting drugs or immune checkpoint inhibitors, could also improve outcomes for patients with the more resistant subtypes [507]. Moreover, this model may be useful for identifying and validating biomarkers that can predict a tumor's subtype and, therefore, its likely response to therapy. Such biomarkers could be used to stratify patients and guide the selection of the most appropriate treatment regimen from the outset, thereby improving overall survival rates and reducing unnecessary exposure to ineffective treatments [510].

Modeling Metastasis

Perhaps the most devastating hallmark of PDAC is that of its metastatic tendency as this significantly contributes to the poor prognosis associated with the disease. Despite advances in understanding primary tumor biology, the mechanisms driving metastasis

remain incompletely understood, particularly how cancer cells navigate and colonize distant organs. Our KMC^{ERT2} mouse model provides a valuable system for investigating the metastatic process due to its ability to spontaneously metastasize to the liver, the most common site for PDAC dissemination.

In Chapter 2, we demonstrated that KMC^{ERT2} tumors are not only phenotypically diverse but also reliably metastasize to the liver, recapitulating the behavior observed in human disease. The orthotopic implantation of tumor-derived KMC^{ERT2} cell lines into the pancreas resulted in consistent development of liver metastases, typically appearing within two to four weeks of implantation. This spontaneous metastasis mirrors the metastatic spread seen in PDAC patients, allowing us to study the sequential steps of the metastatic cascade, from local invasion to distant organ colonization. underlying biology of metastatic colonization and the molecular cues involved in organ tropism.

Future studies using this model will aim to elucidate the mechanisms governing the underlying biology of the steps of the metastatic cascade as well as of signals driving metastatic organ tropism. Focus will be given to why cells metastasize to the liver, a predominant site of metastasis in PDAC, and one that correlates with poor survival. Moreover, integrating transcriptomic analyses of metastatic lesions from the KMC^{ERT2} model could reveal critical genes involved in metastatic seeding, potentially identifying novel therapeutic targets.

In preclinical therapeutic studies not highlighted in Chapter 2, we tested the impact of targeted inhibitors on metastatic progression in the KMC^{ERT2} model. **Figure 4.01** shows that treatment with the MEK inhibitor Trametinib successfully diminished primary tumor size and eliminated liver metastases, whereas treatment with the PARP inhibitor Olaparib had no effect on primary tumor size or liver metastases as compared to the control. Interestingly, treating with a combination of Trametinib and Olaparib, while very effective

at reducing primary tumor size, still allowed for metastatic behavior similar to vehicle treated mice. This suggests a potential bypass mechanism at play. While MEK inhibition may be effective in reducing metastatic burden, further work is needed to understand resistance mechanisms and the role of additional signaling pathways in driving metastasis. In particular, this result suggests caution when combining therapies that reduce primary tumor size without considering metastatic burden.

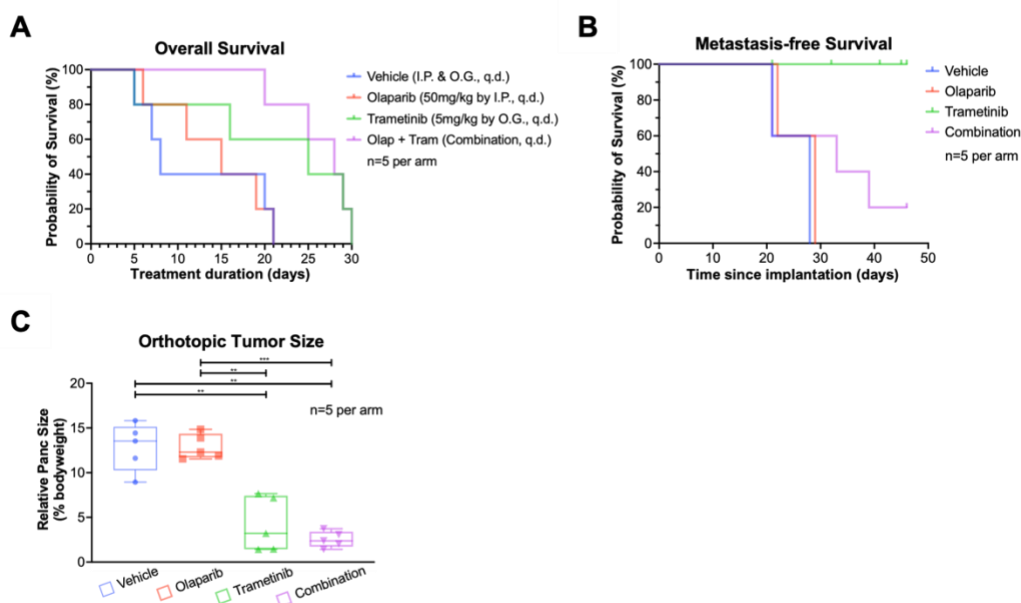


Figure 4.01 Effects of MEK and PARP Inhibition on Metastatic Progression in the KMC^{ERT2} Model

A) Kaplan-Meier curves showing probability of overall or B) metastasis-free survival. C) Box and whiskers plot showing tumor size, as relative pancreas (relative panc) size (% bodyweight at takedown), in B6129SF1/J mice orthotopically implanted with murine PDAC cell line 171J when treated with vehicle, Olaparib (Olap, 50 mg/kg; I.P., q.d.), Trametinib (Tram, 5 mg/kg; I.P., q.d.) or both Olap and Tram (50 mg/kg; I.P. & 5 mg/kg; O.P., q.d.).

The KMC^{ERT2} model offers a unique platform to explore how interactions between tumor cells and the TME contribute to metastasis. As shown in Chapter 2, KMC^{ERT2} tumors exhibit distinct immune landscapes compared to other models, including significant DC infiltration, which may play a role in shaping metastatic potential. It is well established that the immune microenvironment can influence the metastatic process, either by promoting immune evasion or facilitating colonization through immune suppression. Future studies using this model can investigate how immune modulation, specifically the role of DCs,

Tregs, and MDSCs, influences metastatic behavior in PDAC. Building on the current data, the next steps in studying metastasis using the KMC^{ERT2} model could involve tracking metastatic clones through single-cell sequencing to better understand the cellular and molecular changes that occur during dissemination and colonization.

Advancements in 3D-Bioprinting

One of the significant technological advancements highlighted in this dissertation is the application of 3D-bioprinting to create more physiologically relevant PDAC tumor models. 3D-bioprinting technology offers the unprecedented ability to precisely control the architecture and microenvironmental factors of tumor models, enabling the creation of *in vitro* systems that closely mimic the *in vivo* conditions found in human tumors [197, 487]. This capability is particularly crucial for PDAC, where the complexity of the TME—including its dense stroma, hypoxia, and immune cell infiltration—plays a significant role in therapeutic resistance and disease progression.

The bioprinted models developed in this study incorporated patient-derived cells, allowing for the recapitulation of key features of the TME, including ECM composition, stromal interactions, and immune cell infiltration. These models have been used to explore how variations in both cancer cell-intrinsic and stromal components affect therapeutic responses, providing insights into the complex interactions between tumor cells and their microenvironment that are not easily captured in traditional 2D cultures or even *in vivo* models [485].

In Chapter 3, we conducted two pilot studies that demonstrate the versatility of these bioprinted systems. The first pilot study focused on manipulating fibroblast phenotypes by knocking down PIN1, a regulator of cellular differentiation, to understand its impact on the PDAC microenvironment. Our findings showed that PIN1 knockdown in pancreatic stellate cells (PSCs) led to changes in both the vascular and immune landscapes, suggesting that

fibroblast differentiation significantly influences the tumor's ability to recruit immune cells and form new vasculature. The second pilot study modeled tumor immune microenvironmental changes by incorporating patient-derived cancer cells into the bioprinted system. This allowed us to investigate how cancer cell-intrinsic factors, such as MYC expression, affect immune cell survival and function in 3D tissue models. While these studies provided preliminary insights, much more work remains to fully optimize the system and explore the dynamics between stromal and immune components over time.

These pilot studies demonstrate the potential of 3D-bioprinting not only for modeling cell-intrinsic factors but also for examining their effects on cell-extrinsic components, such as immune cell recruitment. A long-term goal of this research is to use systems like these to better understand how MYC-driven alterations in cancer cells affect stromal components and immune cell infiltration, such as dendritic cells (DCs), as described in Chapter 2. This approach will help bridge the gap between cell-intrinsic and extrinsic factors within the tumor microenvironment.

Moreover, 3D-bioprinting holds immense potential for personalized medicine. By using patient-derived cells to create individualized tumor models, this technology enables the testing and optimization of therapeutic strategies tailored to the specific characteristics of a patient's tumor [198]. This approach could be particularly beneficial in PDAC, where tumor heterogeneity and microenvironmental complexity often lead to variable responses to treatment. The incorporation of autologous peripheral blood mononuclear cells (PBMCs) into bioprinted tissues would allow for a customized approach to study immune-tumor interactions. This importance of including patient-specific immune components when considering therapeutic responses, as well as challenges to this approach were discussed in Chapter 3. Ultimately, such an approach could provide customizable bioprinted models to allow for high-throughput drug screening and the identification of the

most effective treatment regimens for each patient, thereby improving therapeutic outcomes [459].

In addition to its application in drug screening, 3D-bioprinting can be used to model tumor evolution and the development of therapeutic resistance. By continuously exposing bioprinted tumor models to various therapeutic agents, researchers can observe the emergence of resistant clones and the molecular adaptations that confer resistance. This process mirrors the clinical scenario and provides a platform for testing combination therapies that could preemptively target resistance mechanisms [511]. In **Chapter 3**, we focused on modeling the tumor microenvironment, particularly by manipulating fibroblast phenotypes and incorporating immune components such as patient-derived cancer cells into the bioprinted tissues. While our studies did not directly explore therapeutic resistance, these pilot models lay the groundwork for future investigations into how different stromal and immune interactions might influence responses to treatment. Further work will be needed to adapt these models for testing resistance mechanisms to standard-of-care therapies.

FUTURE DIRECTIONS

Scaling and Validation of 3D-Bioprinted Models

The initial success of 3D-bioprinted models in replicating the complex architecture and microenvironment of PDAC represents a significant advancement in cancer research. Previous studies have demonstrated the potential of 3D-bioprinting to create physiologically relevant tumor models that closely mimic *in vivo* conditions, offering a more accurate platform for studying tumor biology and therapeutic responses [197, 487]. Our work builds on these findings by incorporating patient-derived cells into the bioprinted models, enabling the recapitulation of key features of the TME, including ECM composition, stromal interactions, and immune cell infiltration.

Despite its promising potential, the application of 3D-bioprinting in cancer research is not without challenges. Scalability, reproducibility, and the integration of more complex elements of the TME, such as vascularization and dynamic immune interactions, remain areas that require further development. Future work will focus on overcoming these challenges by refining bioprinting techniques and integrating more sophisticated microenvironmental factors into the models [512]. The ultimate goal is to create robust, reproducible bioprinted models that can be used not only for research but also as predictive tools in clinical settings, bridging the gap between preclinical findings and patient response.

There is a clear need to scale these experiments and validate their findings across a larger cohort of patient-derived cells. Scaling these models would involve expanding the bioprinting platform to include a broader range of tumor subtypes, ensuring that the models accurately represent the heterogeneity observed in clinical PDAC cases [198]. Incorporating dynamic elements, such as microfluidic systems to simulate blood flow and nutrient gradients [178, 512–515], would further enhance the physiological relevance of the models. This could be achieved by leveraging co-culturing techniques in combination with advanced bioprinting technologies that allow for the precise spatial organization of different cell types [445, 487].

Future research should also focus on integrating high-throughput screening methods with 3D-bioprinted models, which could significantly accelerate the discovery of effective therapies. By enabling the rapid testing of multiple therapeutic agents on patient-specific models, this approach could facilitate the development of personalized treatment plans tailored to the unique characteristics of each patient's tumor. Previous studies have shown that combining 3D-bioprinting with organoid technology can improve the accuracy of drug response predictions [182, 198], making it a promising direction for future work.

To ensure the robustness and reproducibility of these models, it will be crucial to standardize the bioprinting protocols and validate the results through multi-center collaborations. Furthermore, refining bioprinting techniques to integrate more sophisticated microenvironmental factors, such as dynamic blood flow and nutrient gradients, will be essential for creating models that not only replicate the structural complexity of tumors but also their functional behaviors in vivo [487]. By addressing these challenges, 3D-bioprinting has the potential to bridge the gap between preclinical research and clinical application, leading to more effective and personalized therapeutic strategies for PDAC.

By leveraging the KMC^{ERT2} model to explore these biomarkers, researchers can advance the precision medicine approach in PDAC, ultimately leading to more effective and personalized therapeutic strategies.

Clinical Translation

The ultimate goal of this research is to bridge the gap between preclinical findings and clinical practice, ensuring that the insights gained from advanced models like the KMC^{ERT2} and 3D-bioprinted systems translate into improved outcomes for patients with PDAC. Previous studies have shown that integrating patient-derived models with advanced technologies like 3D-bioprinting can enhance the physiological relevance of preclinical studies [184, 185, 198], providing a more accurate platform for testing therapeutic strategies.

To achieve clinical translation, it will be crucial to validate these models through multi-center collaborations and standardize protocols to ensure their reproducibility and applicability in diverse patient populations [487]. One promising approach is the integration of 3D-bioprinting with PDMCs, which can offer a more accurate representation of a patient's tumor and its response to therapy [516]. Incorporating these models into clinical

trials could facilitate the development of personalized treatment plans tailored to the unique characteristics of each patient's tumor.

Furthermore, fostering strong partnerships between academia, industry, and regulatory bodies will be essential for overcoming the logistical and regulatory challenges associated with bringing new therapies from the lab to the clinic. By addressing these challenges, researchers can move closer to realizing the potential of precision oncology in treating PDAC, ultimately improving patient outcomes and advancing the field of cancer treatment.

In conclusion, the findings of this dissertation open several avenues for future research, which are crucial for advancing our understanding of PDAC and improving therapeutic outcomes. The development and utilization of both the KMC^{ERT2} GEMM and the incorporation of PDCLs and immune populations into an advanced 3D-bioprinting technology were central to addressing the critical gaps in preclinical modeling of PDAC. The KMC^{ERT2} model enabled the study of tumor heterogeneity, progression, and immune dynamics in an *in vivo* setting, recapitulating key aspects of human PDAC, such as desmoplasia and metastasis. Additionally, by avoiding the genetic manipulation of tumor suppressors like p53, this model allowed for the interrogation of MYC's role in driving tumorigenesis and its effects on the tumor microenvironment. In parallel, the 3D-bioprinting system provided a manipulable, patient-specific *in vitro* platform enabled the exploration both cancer cell-immune and stromal-immune interactions. By leveraging both models, this dissertation successfully bridged *in vivo* and *in vitro* approaches, adding to the existing toolkit for the investigation of PDAC biology with the goal of improving eventual clinical translation of preclinical studies.

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APPENDIX A: TARGETING MYC THROUGH THE USE OF PP2A ACTIVATION

SELECT STABILIZATION OF A TUMOR-SUPPRESSIVE PP2A HETEROTRIMER

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In cancer, suppression of specific protein phosphatases, like protein phosphatase 2A (PP2A), that normally counteract kinase leads to disruption of signaling pathways. Leonard et al. [251] demonstrate that a novel small molecule activator of PP2A, DT-061, selectively stabilizes a specific PP2A holoenzyme responsible for dephosphorylating critical oncogenic targets including c-MYC. The 3.6Å cryo-electron microscopy map of the heterotrimer assembly provides insight into the druggable structure of PP2A, driving phosphatase therapeutics.

Following the landmark discovery of imatinib (tyrosine kinase inhibitor) for chronic myeloid leukemia, recent decades of cancer research have been focused on kinase inhibition for cancer treatment [517]. Though the most common pathologic mechanisms in cancer do involve activated kinase signaling,[518] they also frequently involve the loss of specific protein phosphatases, such as protein phosphatase 2A (PP2A), that normally counteract kinase signaling [518]. PP2A represents a major family of serine/threonine protein phosphatases responsible for the regulation of about 60% of all serine/threonine phosphorylation events, thus making it a key player in phosphorylation signaling cascades and the regulation of numerous processes, including cell growth, cell cycle, mitosis, differentiation, and apoptosis [519, 520]. Overall, PP2A has been established to function as a tumor suppressor – diminishing the activation of master oncogenic regulators including c-MYC (MYC), extracellular signal-regulated kinases (ERK), protein kinase B (AKT) and B-cell lymphoma 2 (BCL2). Given the ubiquitous role of PP2A, it is not surprising that its dysregulation contributes to disease, making it an attractive target in many clinical settings.[521] Despite PP2A's significant role in multiple disorders, mechanistic insight into targeting PP2A with small molecules remains obscure.

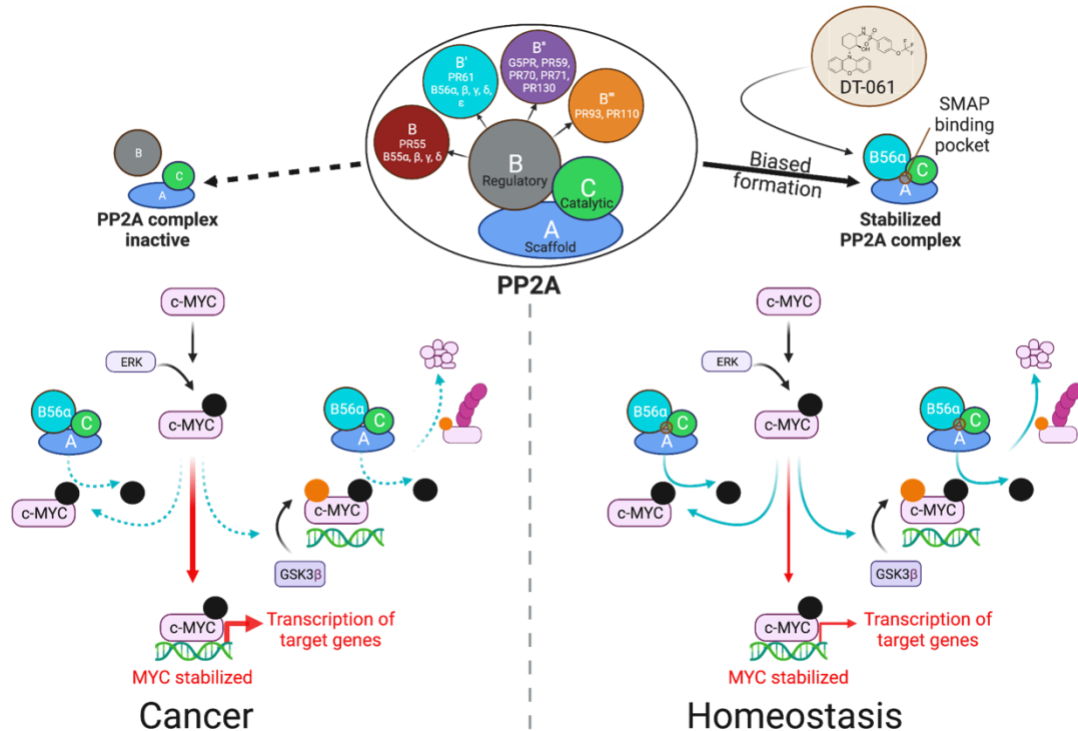


Figure App-A.1 Schematic Depicting Effects of DT-061 on Protein Phosphatase 2A (PP2A) and Its Regulation of c-MYC.

PP2A is a diverse family of heterotrimeric serine/threonine phosphatases containing distinct B subunits from one of four families, where a specific holoenzyme, PP2A-B56α, has an important role in regulating the MYC oncoprotein family. Depicted here, c-MYC is phosphorylated by enzymes, such as extracellular signal-regulated kinase (ERK), at the serine 62 position (pS62; black sphere), which in turn increases MYC protein stability and target gene activation. In cancer, downregulation of PP2A supports increased MYC activity (A). Leonard et al. [11] now show that DT-061, a small-molecule activator of PP2A (SMAP), selectively binds and stabilizes the PP2A-B56α holoenzyme (B). With DT-061, PP2A-B56α dephosphorylates S62-phosphorylated c-MYC to facilitate its inactivation via both immediate S62 dephosphorylation and S62 dephosphorylation subsequent to glycogen synthase kinase 3β (GSK3β)-mediated Threonine 58 phosphorylation (pT58; orange sphere). These regulated phosphorylation events trigger ubiquitin (pink spheres)-mediated degradation of MYC, promoting cellular homeostasis. Dotted lines denote that pathways are suppressed or inactivated, whereas thicker lines indicate increased activity. Figure created using BioRender (<https://biorender.com/>). Abbreviation: c-MYC, cellular MYC.

PP2A's immense role in cellular regulation comes from its unique ability to form over 90 distinct heterotrimeric holoenzymes – each containing a common heterodimeric core comprised of the scaffold A-subunit and catalytic C-subunit bound to one of many regulatory B-subunits (Figure 1). The diversity of holoenzyme formation is made possible by the inherent flexibility of the A-subunit and the large range of B-subunits from four families (B, B', B'', and B'''), which confer broad substrate specificity, cellular location, and tissue distribution (Figure 1). This gives rise to a multitude of activities, which could be influenced by the stoichiometry of the different B-subunit family members [520].

Unlike other tumor suppressors such as tumor protein p53 (p53), BCL2, or phosphatase and tensin homolog (PTEN), PP2A is most frequently inactivated by non-genetic mechanisms, such as phosphorylation and/or demethylation of the carboxyl-terminal tail of the catalytic C-subunit or increased expression of endogenous PP2A inhibitors [519, 520]. These endogenous regulators of PP2A demonstrate oncogenic properties, likely through their targeting of the tumor-suppressive PP2A holoenzymes, and have been the main focus for PP2A reactivating targeting strategies for the past decade [517]. One such example is inhibitor 2 of PP2A (SET), which binds to and inactivates PP2A's catalytic C-subunit, and is frequently overexpressed in cancers. Efforts to target this negative regulator have resulted in effective antagonists of SET, such as the peptide OP449, which causes the intracellular release of SET from PP2A and subsequently increases PP2A activity [522]. Groups studying other endogenous inhibitors, such as cancerous inhibitor of protein phosphatase 2A (CIP2A), and bioactive sphingolipids, such as ceramides or their derivatives, have also shown success in activating PP2A tumor suppressor function [523].

Though these approaches prove promising, they lack the ability to address complexity in the field, where different PP2A holoenzymes can have varied activities in regulating cellular processes due to the diversity of the PP2A family. Selectively targeting specific holoenzymes to direct PP2A activity against specific pathogenic drivers has not previously been possible. Recent studies have shown efficacy of reengineering US Food and Drug Administration (FDA)-approved tri-cyclic neuroleptics, including small molecule activators of PP2A (SMAPs) [249]. These compounds have been shown to induce apoptosis, promote dephosphorylation of PP2A targets, such as MYC and AKT, and suppress tumor growth in numerous mouse models [101, 524]. However, until now, which isoform and subunit is stabilized by SMAPs has been unclear.

In a recent work, Leonard et al. [251] present the structural and biological basis of direct activation of PP2A by DT-061, one of the lead engineered compounds from phenothiazine derivatives [251]. In describing the three-dimensional structure of the DT-061-bound PP2A trimeric complex, Leonard et al. reveal a unique inter-subunit pocket that DT-061 occupies, providing insight into its mechanism of PP2A activation – direct, specific binding and stabilization of the PP2A-B56 α holoenzyme, which drives an essential anti-tumor function of PP2A (Figure 1) [251]. This critical information not only opens the field of cancer therapeutics to a new class of drug targets – tumor-suppressive phosphatases, but also provides a path for the structural design and development of novel and improved drugs for biased targeting of selected PP2A holoenzymes.

Leonard et al. designed a cell-based split luciferase assay to study how DT-061 affects PP2A. They observed a structural constriction in the AC complex, indicative of an increase in B-AC complex binding between 1 and 2 hours of DT-061 exposure. Complex binding then reverted back to baseline within 6 hours. This was further confirmed in xenografted lung adenocarcinoma tumors, where tumors treated with DT-061 showed a significant increase in B56 α -containing holoenzymes, while other B-subunit-containing holoenzymes remained unchanged when compared to vehicle.

To confirm the specific binding of DT-061 to the PP2A-B56 α holoenzyme, a second split luciferase assay separately tagging the amino-terminus of the scaffold A-subunit and the carboxyl-terminus of B56 α was utilized. This, combined with co-immunoprecipitation (co-IP) experimental results, demonstrated that DT-061 selectively enhances the B56 α -containing holoenzyme, increasing specific AB56 α C complex assembly. The authors showed a concentration-dependent association of the AB56 α C complex by size exclusion chromatography coupled with UV absorbance. Importantly, fluorescence polarization and

surface plasmon resonance confirmed that DT-061 stabilized the complex and increased the binding affinity of the B56 α -subunit to the AC dimer.

The authors identified the binding site of DT-061 at the trimeric interface of the AB56 α C complex, elucidating the molecular basis of the biased complex stabilization. The amino-terminus of the A-subunit anchors the B-subunit, whereas its carboxyl-terminus anchors the C-subunit. Here, sandwiched between the A- and B-subunits, a terminally methylated L-309 residue at the carboxyl-tail of the C-subunit is adjacent to the binding site of DT-061. Of note, L-309 methylation is enriched in the DT-061-stabilized PP2A-B56 α C holoenzyme, demonstrating DT-061's nucleation of the active enzyme. Leonard et al. show that the molecular arrangement at the trimeric junction is unique to the B56 α heterotrimer, which explains both the specificity of DT-061 and the increased stability of the assembled complex. Electron microscopy grids prepared with recombinant AB56 α C trimer were unable to maintain the heterotrimeric composition in the absence of DT-061, resulting in monomeric and dimeric two-dimensional class averages, while inclusion of DT-061 yielded a homogeneous distribution of AB56 α C trimers. Thus, the unique stabilizing property of DT-061 allowed the elusive structure of a PP2A heterotrimer to be solved [251].

A key substrate of PP2A-B56 α , MYC, is involved in transcriptional regulation of a multitude of genes that are essential to cellular programs required for both normal and neoplastic cells (Figure 1) [525]. MYC has been intensively studied for its role in driving nearly all types of cancer development, metastasis, and therapeutic resistance [101, 108, 522, 525]. Yet, after decades of research, a way to therapeutically target MYC remains intangible. Importantly, MYC oncogenic activity is post-translationally controlled, providing a potential new route for therapeutic intervention [525]. The best known MYC activating phosphorylation event on Serine 62 is dephosphorylated by PP2A-B56 α , making DT-061

an exciting approach for targeting active MYC through stabilization of PP2A-B56 α (Figure 1). The authors characterized the kinetic loss and recovery of MYC following DT-061 treatment in xenograft tumors, and show an inverse relationship with L-309 methylation and a positive correlation with the induction of apoptosis [251].

While animal studies have demonstrated the potent elimination of tumors upon genetic inactivation of MYC in tumor cells, tumor clearance is dependent upon an intact immune system.[108] PP2A has been shown to control functions of many immune cells, including T cells, natural killer (NK) cells, and myeloid derived suppressor cells (MDSCs) through modulation of upstream signaling and chemokine production [521, 526]. Further, inhibition of specific PP2A holoenzymes such as PP2A-B55 δ can increase anti-tumor immunity [527]. This complexity of PP2A function emphasizes the importance of the current study, where DT-061, a systemically administered drug, could selectively increase the activity of a tumor-suppressive PP2A holoenzyme (PP2A-B56 α) *in vitro*. Given that DT-061 shows clear *in vivo* efficacy in murine tumor models [101, 524], future studies will be needed to determine its effects on the complexity of immune function in disease.

Ultimately, Leonard et al. [251] utilized multiple technologies to structurally identify and validate the biased stabilizing effects of DT-061, a SMAP on PP2A-B56 α , overcoming any concern related to indiscriminate enzyme activation and providing a platform for new SMAP development for other clinical presentations with dysregulated levels of PP2A.

APPENDIX B: TARGETING MYC IN PDAC

DECONSTRUCTING PANCREATIC ADENOCARCINOMA BY TARGETING THE CONDUCTOR, MYC

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The contents of this chapter section are a published In The Spotlight article on a paper by Sodir et al., *Cancer Discovery*, 2020, in *Cancer Discovery*, 2020.

In this issue of *Cancer Discovery*, Sodir et al. present a mouse model with pancreatic expression of mutant KRAS and switchable expression of MYC to demonstrate that MYC acts as a reversible driver of malignant pancreatic ductal adenocarcinoma (PDAC) [108]. MYC activation in KRAS^{G12D}-expressing epithelium instructs malignant progression from the precursor disease in a cell-intrinsic manner, concurrent with induction of cell-extrinsic signals that drive the accompanying desmoplastic stromal expansion and tumor-suppressive immune microenvironment characteristic of human PDAC. Importantly, MYC deactivation *in vivo* triggers rapid regression and disassembly of the ensemble tumor

through both cancer cell-intrinsic and extrinsic mechanisms to restore tissue architecture, as depicted in Figure 1. This, together with recent literature in other cancers, provides strong rationale for therapeutic targeting of MYC.

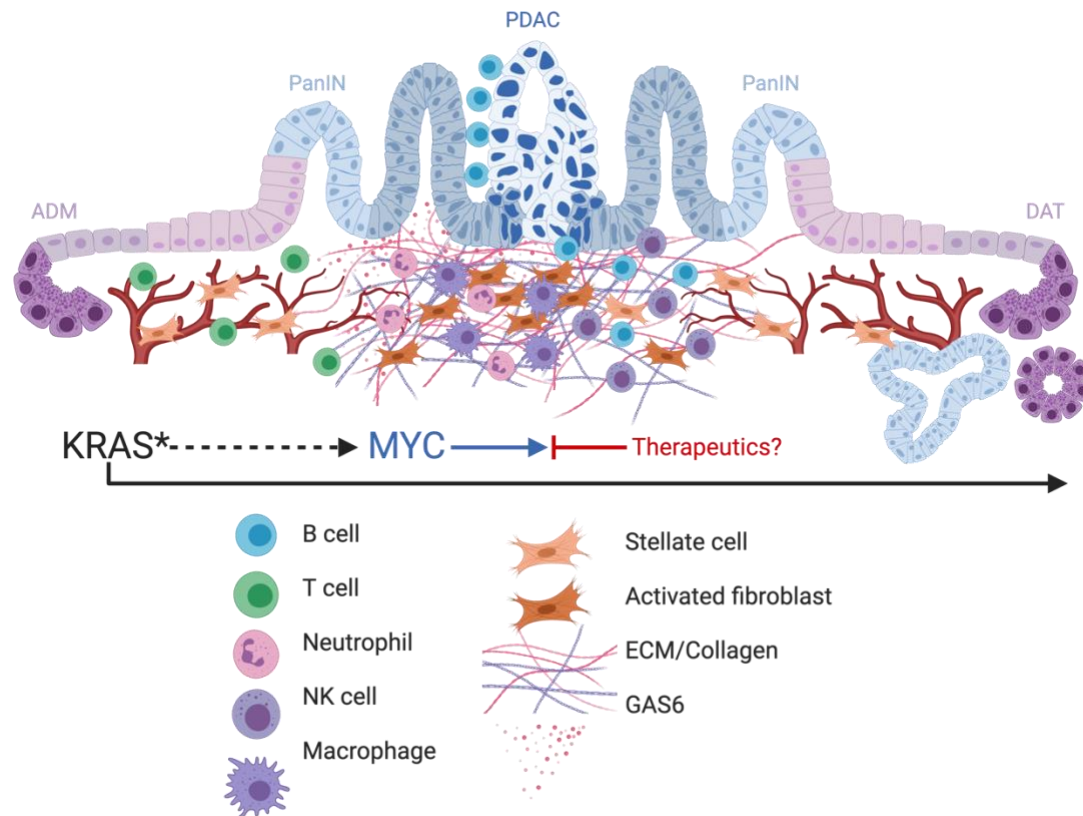


Figure App-B. 1. Schematic representation of the role of MYC as a reversible driver of cell-intrinsic and extrinsic PDAC progression.

Epithelial acinar-to-ductal metaplasia (ADM), pancreatic intraepithelial neoplasia (PanIN), progression to pancreatic ductal adenocarcinoma (PDAC), and reversion to PanIN and ductal-to-acinar transdifferentiation (DAT) are indicated. Desmoplastic stromal and vascular changes and immune cell infiltrates are depicted. Mutationally activated KRAS (KRAS*). Figure created using BioRender.com

Pancreatic ductal adenocarcinoma ranks among the top three most aggressive cancers in the United States and is projected to become the second leading cause of cancer related deaths by 2021, with a 5-year survival rate that the American Cancer Society only just reported has reached 10%. While specific targeted inhibitors and immune-based therapeutic agents have improved patient outcomes for some cancer types, PDAC is generally found to be refractory to these therapies, and standard of care relies on

combination chemotherapy regimens that only provide a modest (months) increase in median survival. The aggressiveness and therapy-resistant nature of this deadly cancer is thought to arise from its signature dense desmoplastic and immune-suppressive tumor microenvironment that comprises the majority of the tumor. In recent years, efforts have focused on strategies to disrupt the dense desmoplasia and diminish immune suppression. Unfortunately, a clinical trial with the hyaluronidase PEGPH20, aimed at reducing stromal density, showed no improvement in overall survival, and mouse models with similar goals have resulted in the unleashing of a more aggressive tumor phenotype, suggesting a tumor suppressive role for the desmoplasia [528]. Immune checkpoint inhibitors have likewise been unsuccessful in treating this recalcitrant cancer. Currently, the only FDA approved immunotherapy in PDAC, pembrolizumab, a PD-1 checkpoint inhibitor, is exclusively indicated for patients with unresectable or metastatic disease that is also microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) – both rare in PDAC. Thus, there is an urgent need to understand the complexity of stromal expansion and dysfunctional tumor immunity to realize meaningful therapies for this aggressive disease. The current study by Sodir et al. demonstrates that the tumor cell-intrinsic master transcription factor, MYC, instructs the PDAC microenvironment and targeting MYC represents an alternative strategy to induce PDAC regression.

MYC is a master transcription factor needed for cell proliferation, and many other hallmark cancer phenotypes. When deregulated, MYC can promote tumor aggressiveness and therapeutic resistance in cancers. RAS is an upstream activator of MYC, increasing its stability and DNA binding [80], and KRAS mutations occur in approximately 95% of PDAC. These oncogenes cooperate in driving PDAC – genetic loss of endogenous MYC in mutant KRAS models inhibits pancreatic intraepithelial neoplasia (PanIN) progression to PDAC [120], and the Der lab has shown MYC to be the primary resistance mechanism to

KRAS signaling inhibition [117]. Recent work to explore the impact of MYC on the tumor microenvironment of multiple cancers, including PDAC, supports a role in suppression of immune surveillance and limiting T cell infiltration [300]. To this end, the Stanger lab showed a positive correlation between MYC overexpression and chemokine (C-X-C motif) ligand 1 (Cxcl1) expression in T cell-low PDAC tumor clones, suggesting that MYC may allow some PDAC tumors to suppress tumor immunity [255].

Sodir et al. crossed their previously developed inducible Rosa-LSL-MycER^{T2} mouse model with mice carrying LSL-KRAS^{G12D} and a pancreas specific promoter (p48) driving CRE recombinase (KM^{p48}). In this model, mutant KRAS expression induces PanIN formation, and tamoxifen treatment in adult mice activates sustained physiological levels of MYC in pancreatic epithelial cells. Signature features of aggressive and spontaneous human PDAC were observed in these mice after 3 weeks of tamoxifen-induced MYC expression. The authors not only observed more proliferating tumor cells, but they also identified stromal alterations, including: an influx of immunosuppressive macrophages and neutrophils, accumulation of tumor peripheral B-lymphocytes, eradication of CD3+ T cells, and activation of stellate cells to cancer associated fibroblasts along with subsequent desmoplasia, hypoxia, and loss of vascularity. The remodeling of the tumor microenvironment began after only 24 hours of tamoxifen exposure suggesting a rapid instruction of these changes by MYC. Molecular analyses revealed that the remodeling involved known pathways of desmoplastic stroma including sonic hedgehog signaling, with neutrophil and macrophage recruitment by CXCL5 and CCL9, respectively. However, the authors also uncovered a novel component of what may be a parallel MYC-driven neutrophil recruitment pathway in PDAC – the ligand/receptor GAS6/AXL pathway, which has previously been implicated in diverse neutrophil dynamics.

Follow-up studies showed that inhibition of GAS6 had little to no effect on MYC-induced T cell depletion and only modest effects on macrophage influx. However, GAS6 inhibition blocked the recruitment of neutrophils and accumulation of peri-tumoral B cells induced by MYC, slowed stellate cell activation and proliferation, and impeded tumor growth. Having previously established that MYC induces PD-L1-expressing macrophage-mediated immune evasion in lung adenocarcinoma [116], the authors also tested PD-L1 blockade in the KM^{p48} tumors, which exhibit PD-L1 expression in the epithelial tumor cells. With PD-L1 blockade, T cells were no longer expelled from the tumors, but no change was observed in tumor growth or PanIN to PDAC progression, pointing away from a prominent role for T cell exclusion and further emphasizing the importance of GAS6 on neutrophil recruitment in their KRAS/MYC-driven PDAC model.

Strikingly, deactivation of ectopic MYC in the KM^{p48} mice through tamoxifen withdrawal caused complete regression of PDAC including collapse of the desmoplastic, inflamed stroma, immune remodeling, including T cell influx and death or transdifferentiation of neoplastic duct cells. MYC loss restored a state of normal acinar cells and early PanINs, along with recovery of blood vessel patency and local tissue oxygenation. The authors showed this phenomenon to occur even after long-term MYC activation – though extensive scarring and cystic phenotypes endured in some cases. To test the importance of endogenous MYC, the authors used expression of OmoMYC [241], a peptide inhibitor of MYC, within a KRAS^{G12D};p53null-driven PDAC mouse model, and observed a similar reversal in PDAC progression and stromal desmoplasia.

In an effort to further define key immune effectors of MYC deactivation-induced regression of PDAC, Sodir et al. depleted the repopulated CD4⁺ and CD8⁺ T cells separately, but this had no effect on the observed tumor collapse. MYC inactivation also shifted CD20⁺ B cells from the periphery toward the center of the tumor, and triggered recruitment of

NKp46+ natural killer (NK-like) cells; and depletion studies of either of these cell types showed both to be essential for cancer cell death caused by MYC withdrawal.

Altogether, Sodik et al. demonstrated the importance of MYC activation in combination with mutant KRAS for PDAC progression and elucidated a novel neutrophil recruitment and fibroblast activation pathway (GAS6/AXL) that could be an important target in the treatment of PDAC. Additionally, the authors compared tissue-specific effects of MYC activation in pancreatic adenocarcinoma to their lung adenocarcinoma model [116]. Both models progressed to adenocarcinoma characterized by influx of immunosuppressive macrophages, expulsion of T cells, and tumor cell proliferation and invasion. However, influx of neutrophils and peri-tumoral B cells was only observed in the PDAC model, and induction of PD-L1 was observed on tumor epithelium in PDAC, while it occurred on incoming macrophages in the lung. Furthermore, desmoplasia caused by fibroblast activation was only observed in the PDAC model. The authors posit that this profound tissue-specificity is indicative of MYC “hacking” an endogenous tissue regenerative program during tumor expansion and that regression of the tumor after deactivation of MYC is enabled by co-opting a tissue-specific injury resolution program.

Systemic therapies for cancer including immune-targeted treatments are plagued by cancer site-agnostic and often debilitating side effects. By highlighting the importance of tumor cell-intrinsic factors in initiating and sustaining immune suppression and stromal remodeling, the work by Sodik et al. may foster alternative approaches to deconstructing tumors and re-engaging the immune system by targeting cancer cell intrinsic drivers that can take advantage of endogenous tissue repair programs. While various strategies to inhibit MYC are in development, many are not cancer cell specific, although studies by the Evan lab suggest that there is little toxicity with inhibiting MYC in adult mice. Alternatively, targeting tumor-specific upstream effectors of MYC activity, such as mutant-KRAS, would

be cancer cell intrinsic. Similar to MYC, targeting mutant-RAS has been a long-sought goal. Exciting progress has recently been made with mutant-KRAS^{G12C} inhibitors. One such inhibitor, AMG 510, has shown single-agent efficacy in immune competent mice [228]. Treatment with this specific inhibitor improved CD8+ T cell infiltration and upregulation of proinflammatory cytokines. Synergy with immune checkpoint blockade therapies with lower doses of AMG 510 was also demonstrated, providing an exciting path in the clinic. The insights from the study by Sodikin et al. encourage the field to closely examine cross-talk between the tumor cell and its microenvironment, which will be essential for effective therapeutic strategies in the treatment of cancers.

APPENDIX C: KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	PRIMARY IDENTIFIER	SECONDARY IDENTIFIER
Antibodies			
Rat monoclonal anti-B220 [R13-6B2]	BD Biosciences	Cat #: 550286	Lot: 1
Rabbit polyclonal anti-BTK	LSBio	Cat #: LS-C180161	
Rabbit monoclonal anti-CD11b [EPR1334]	Abcam	Cat #: 133357	
Rabbit monoclonal anti-CD11c [D1V9Y]	Cell Signaling Technology	Cat #: 97585S	
Rabbit polyclonal anti-CD206	Abcam	Cat #: 64693	
Rat monoclonal anti-CD207 [eBioRMUL.2]	eBioscience	Cat #: 14-2073-82	
Rabbit monoclonal anti-CD3 [SP7]	Thermo Fisher	Cat #: RM-9107-S	
Rabbit monoclonal anti-CD4 [D7D2Z]	Cell Signaling Technology	Cat #: 25229	
Rat monoclonal anti-CD45 [30-F11]	eBioscience	Cat #: 14-0451-82	
Rat monoclonal anti-CD8 [4SM15]	eBioscience	Cat #: 14-0808-82	
Rabbit monoclonal anti-CSF-1R [E2412]	Santa Cruz Biotechnology	Cat #: sc-692	
Rabbit monoclonal anti-EOMES (TBR2) [EPR19012]	Abcam	Cat #: 183991	
Rat monoclonal anti-F4/80 [Cl:A3-1]	BioRad	Cat #: MCA497G	
Rat monoclonal anti-Foxp3 [FJK16S]	eBioscience	Cat #: 14-5773-82	
Rabbit monoclonal anti-GATA3 [EPR16651]	Abcam	Cat #: 199428	
Rabbit polyclonal anti-Granzyme B	Abcam	Cat #: 4059	
Rabbit polyclonal anti-Ki67	Abcam	Cat #: 15580	
Rat monoclonal anti-Ly6G [1A8]	eBioscience	Cat #: 551459	
Rat monoclonal anti-MHCII [M5/114.15.2]	eBioscience	Cat #: eBi14-5321	
Rat monoclonal anti-MYC [4B12]	Rosalie Sears Lab (OHSU)		
Mouse monoclonal anti-p16-1NK4a [1E12E10]	Invitrogen	Cat #: MA5-17142	
Rabbit monoclonal anti-p53 [D2H9O]	Cell Signaling Technology	Cat #: 32532	
Mouse monoclonal anti-Pan-Cytokeratin [AE1/1E3]	Abcam	Cat #: ab27988	
Rabbit monoclonal anti-PDL1 [E1L3N]	Cell Signaling Technology	Cat #: 13684S	
Rabbit monoclonal anti-RORgt [EPR20006]	Abcam	Cat #: ab207082	
Rabbit monoclonal anti-Smad4 [EP618Y]	Abcam	Cat #: ab40759	
Rabbit monoclonal anti-TCF1/TCF7 [C63D9]	Cell Signaling Technology	Cat #: 2203S	
Rabbit monoclonal anti-TIM3 [D3M9R]	Cell Signaling Technology	Cat #: 83882S	

Biological SamplesTumor and adjacent normal
tissue from PDAC patientsOHSU Department of
Pathology Archive

N/A

**Chemicals, Peptides, and
Recombinant Proteins**Accumax Cell Dissociation
Solution

Fisher Scientific

Cat #: NC9870954

Adenine

Sigma

Cat #: A2786

AEC Peroxidase Substrate

Vector Laboratories

Cat #: SK-4200

Alginate Lyase

MilliporeSigma

Cat #: A1603

CAS #: 9024-15-1

AllPrep DNA/RNA FFPE Kit

Qiagen

Cat #: 80234

Amphotericin B

Gibco

Cat #: 15-240-062

Antigen Retrieval Citra Solution
(pH 6.0)BioGenex
BD Biosciences - BD
Horizon

Cat #: HK086-5K

BD Horizon Brilliant Stain Buffer

Horizon

Cat #: 659611

Lot: 9035797

Calcium Chloride (CaCl₂)

MilliporeSigma

Cat #: C1016

Cholera Toxin

Sigma

Cat #: 227036-1MG

Citric acid monohydrate

MilliporeSigma

Cat #: C1909

DMEM, high glucose

Gibco

Cat #: 11965-092

Dual Endogenous Enzyme
Block

Dako

Cat #: S2003

Fetal Bovine Serum - Premium
Select

R&D Systems

Cat #: S11550

Fluorouracil (5-Fluorouracil, 5-
FU)

SelleckChem

Cat #: S1209

Gelatin

MilliporeSigma

Cat #: G2500

CAS #: 9000-70-8
CAS #: 122111-
03-9

Gemcitabine, Hydrochloride salt

LC Laboratories

Cat #: G4177

Ham's F-12 Nutrient Mix

Gibco

Cat #: 11765-054

Hematoxylin (used for mIHC)

Dako

Cat #: S3301

Hematoxylin Counterstain (used
for IHC)

Vector Laboratories

Cat #: H-3401-500

Heparin Sodium (Laboratory),
Fisher Chemical

Fisher Scientific

Cat #: H19

Human TruStain FcX™ (Fc
Receptor Blocking Solution)

BioLegend

Cat #: 422301

Hydrocortisone

Sigma

Cat #: H0888

IHC Antigen Retrieval Solution -
High pH (10X) (eBioscience™)ThermoFisher
Scientific

Cat #: 00-4956-58

ImmoMix™ Red

BioLine

Cat #: BIO-25022

Insulin solution human

Sigma

Cat #: I9278

Irinotecan (CPT-11) HCl

SelleckChem

Cat #: S2217

Batch #: 05

Trihydrate

Leucovorin (NSC -3590)

SelleckChem

Cat #: S1236

Batch #: 04

Calcium Pentahydrate

Liberase TH Research Grade

MilliporeSigma

Cat #: 05401135001

Lipofectamine 3000 Transfection
ReagentLife Technologies
OHSU Department of
Comparative
Medicine

Cat #: L3000008

Meloxicam (Meloxidyl)

N/A

APPENDIX C: KEY RESOURCES TABLE

Olaparib (AZD2281)	SelleckChem	Cat #: S1060	Batch #: 23
OneComp eBeads™	ThermoFisher Scientific	Cat #: 01-1111-42	
Compensation Beads			
Oxaliplatin (NSC 266046)	SelleckChem	Cat #: S1224	Batch #: 12
Paclitaxel (NSC 125973)	SelleckChem	Cat #: S1150	CAS #: 33069-62-4
Pierce™ BCA Protein Assay Kit	Thermo Scientific	Cat #: 23227	
Primocin	InvivoGen	Cat #: ant-pm-2	
Pronova UP LVG Sodium Alginate	NovaMatrix	Cat #: 4200006	
Recombinant Human EGF Protein, CF	R&D Systems	Cat #: 236-EG-200	
Recombinant human TGFβ	PeptideTech	Cat #: 100-21	
Sesame Oil	MP Biomedicals	Cat #: 156621	CAS #: 8008-74-0
SlowFade™ Gold Antifade Mountant with DAPI	Life Technologies	Cat #: S36938	
Tamoxifen	MilliporeSigma	Cat #: T5648	
Trametinib (GSK1120212)	SelleckChem	Cat #: S2673	Batch #: 09
Veri-Cells™ PBMC	BioLegend	Cat #: 425003	Lot: B274266
Veri-Cells Buffer A	BioLegend	Cat #: 76681	Lot: B274237
	3M (supplied by SantaCruz Animal Health)	Cat #: sc-361931	
Vetbond Tissue Adhesive		Cat #: HY-10583	CAS #: 129830-38-2
Y-27632 dihydrochloride (ROCK inhibitor)	MedChemExpress		

Deposited Data

Raw and analyzed data and metadata for WES	This paper	GEO submission in progress
Raw and analyzed data and metadata for low-pass WGS	This paper	GEO submission in progress
Raw and analyzed data and metadata for RNA-seq	This paper	GEO submission in progress

Experimental Models: Cell Lines

171J	This paper	Sex: male	
255J	This paper	Sex: female	
301J	This paper	Sex: male	
303J	This paper	Sex: male	
314J	This paper	Sex: male	
4442-T	This paper	N/A	
4671-T	This paper	N/A	
Capan1	ATCC	Sex: male	RRID: CVCL_0237
Capan2	ATCC	Sex: male	RRID: CVCL_0026
HPAFII	ATCC	Sex: male	RRID: CVCL_0313
Human Pancreatic Stellate Cells (hPSCs)	ScienCell	Cat #: 3830	Lots:10473 and 10295 (both male)
Human Umbilical Vein Endothelial Cells (HUVECs)	MilliporeSigma	Cat #: SCE001	

KPC7107	M. A. Hollingsworth	N/A	RRID: CVCL_0428 RRID: CVCL_0480
KPC8060	M. A. Hollingsworth	N/A	
KPC8069	M. A. Hollingsworth	N/A	
MiaPaCa2	ATCC	Sex: male	
Panc1	ATCC	Sex: male	
Experimental Models: Organisms/Strains			
Mouse: <i>LSL-Kras^{G12D}</i>	Jackson et al, Genes & Development, 2001		RRID: IMSR_JAX:101043
Mouse: <i>ROSA-LSL-Myc</i>	Wang et al, Cancer Research, 2011		
Mouse: <i>P53^{LSL-R172H}</i>	Olive et al, Cell, 2004		
Mouse: <i>Pdx1-Cre</i>	Hingorani et al, Cancer Cell, 2003		
	Fujimori et al, Biochemical & Biophysical Research Communications, 1999		
Mouse: <i>Pin1^{+/-}</i>	Kawaguchi et al, Nature Genetics, 2002		
Mouse: <i>Ptf1a-Cre</i>			
	The Jackson Laboratory		
Mouse: B6129SF1/J		Strain #: 101043	
Oligonucleotides			
siNT ON-TARGETplus Non-targeting pool of: UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUCCUA	Dharmacon	Cat #: D-001810-10-20	
siPIN1 ON-TARGETplus Non-targeting pool of: GCUCAGGCCGAGUGUACUA GAAGACGCCUCGUUUGCGC GAAGAUCACCCGGACCAAG CCACAUCACUAAACGCCAGC	Dharmacon	Cat #: L-003291-00-005, pool of: J-003291-10, J-003291-11, J-003291-12, J-003291-13	
GPO-3_myc For: GGGAGCAAACAGGATTAGAT ACCCT	Integrated DNA Technologies (IDT)	N/A	
MGSO_myc Rev: TGCACCATCTGTCACTCTGTT AACCTC	Integrated DNA Technologies (IDT)	N/A	
ROSA_WT For: CTGGCTTCTGAGGACCG	Integrated DNA Technologies (IDT)	N/A	
ROSA_WT Rev: CAGGACAACGCCACACA	Integrated DNA Technologies (IDT)	N/A	
ROSA_Mut For: AGGGCGAGGAGCTGTTCA	Integrated DNA Technologies (IDT)	N/A	
ROSA_Mut Rev: TGAAGTCGATGCCCTTCAG	Integrated DNA Technologies (IDT)	N/A	
Common_Cre1: ACCAGCCAGCTATCAACTCG	Integrated DNA Technologies (IDT)	N/A	

APPENDIX C: KEY RESOURCES TABLE

Common_Cre2: TTACATTGGTCCAGCCACC Common_Cre3: CTAGGCCACAGAATTGAAAG ATCT Common_Cre4: GTAGGTGGAAATTCTAGCATC ATCC MW636_KRAS_G12D_F:CTAG CCACCATGGCTTGAGT MW637_KRAS_G12D_F:ATGT CTTTCCCCAGCACAGT MW638_KRAS_G12D_R:TCCG AATTCAGTGACTACAGATG MW628_P53_F: AGCTAGCCACCATGGCTTGA GTAAGT MW629_P53_WT_F: TTACACATCCAGCCTCTGTGG MW630_P53_R: CTTGGAGACATAGCCACACT G	Integrated DNA Technologies (IDT) Integrated DNA Technologies (IDT) Integrated DNA Technologies (IDT) Integrated DNA Technologies (IDT) Integrated DNA Technologies (IDT) Integrated DNA Technologies (IDT) Integrated DNA Technologies (IDT) Integrated DNA Technologies (IDT) Integrated DNA Technologies (IDT)	N/A N/A N/A N/A N/A N/A N/A N/A N/A	
Recombinant DNA			
shNon-targeting control (shNT) shMYC target sequence: GATGATGACCGAGTTACTT Source clone ID: V31MMMCG_ 12968867 Vector: piSMART mCMVfTurboGFP pLKO shSCR pLKO shPIN1 target sequences: CCGGCCACCGTCACACATGA TTTATCTCGAGA TAAATACTGTGTGACGGTGGT TTTT	Horizon Discovery Horizon Discovery Sigma Sigma	VSC6570 Cat #: V3SM7671- 233031217 SHC-002 TRCN0000001033	Titer lot #: V21041602 Titer concentration: 6.07 ⁷ TU/mL
Software, Algorithms, & Hardware			
ImageJ	Opensource, Schneider et al., 2012	https://imagej.nih.gov/ij/	Version 1.53
FIJI	Opensource, Schindelin et al., 2012	https://fiji.sc/	Version 2.1.0
CellProfiler	Opensource	https://cellprofiler.org/	Version 2.1.1
Zen	Zeiss Microscopy		Version 2.3
Prism	GraphPad		Version 9
Scitools code repository	Sinnamon et. al., 2019	https://github.com/adeylab/scitools	
R	The R Foundation	https://www.r-project.org/	
chromVAR	Schep et al. 2017	https://github.com/GreenleafLab/chromVAR	
cisTopic	Bravo Gonzalez-Blas et al. 2019	https://github.com/aertslab/cisTopic	

ComplexHeatmap	Gu, Eils, and Schlesner, 2016	https://github.com/joker-goo/ComplexHeatmap	
UMAP	Becht et al., 2018	https://github.com/tkonopka/umap	
Rphenograph	Levine et al., 2017	https://github.com/JinmiaoChenLab/Rphenograph	
BWA-MEM	Li and Durbin, 2010	https://github.com/lh3/bwa	
Macs2	Zhang et al., 2008	https://github.com/mac3-project/MACS3	
ggpubr	Kassambara, A.	https://rpkgs.datanovia.com/ggpubr/	
rstatix	Kassambara, A.	https://rpkgs.datanovia.com/rstatix/	
bcl2fastq	Illumina	https://www.illumina.com/	
Bowtie	Langmead et al., 2009	http://bowtie.cbcb.umd.edu	Version 1.0.0
DESeq2 R package	Love, Huber, and Anders 2014		Version 1.24.0
ClustVis	Metsalu and Vilo, 2015	http://biit.cs.ut.ee/clustvis	
GSEA	Mootha et al., 2003; Subramanian et al., 2005		Version 4.0.3
ImageJ	Opensource	https://imagej.nih.gov/ij/	Version 1.51
FIJI	Opensource	https://fiji.sc/	
BD Medimachine instrument	BD Biosciences	Cat #: 340588	
ichorCNA	Adalsteinsson et al., 2017; Broad Institute	https://github.com/broadinstitute/ichorCNA	
NF-Core/Sarek	Garcia et al., 2020; Ewels et al., 2020	https://nf-co.re/sarek	
Filters for Next Generation Sequencing (FINGS) package	Wardell, Ashby, and Bauer, 2021	https://github.com/cpwardell/FINGS	

APPENDIX D: CURRICULUM VITAE

Isabel A. English

isabel.english93@gmail.com

+1 (503) 805-6484

1928 SW Laurel St. Apt. 2
Portland, OR 97201

EDUCATION

Oregon Health & Science University (OHSU)

□ Ph.D. in Cancer Biology September 2024

Sorbonne Université – Sciences (Formerly : Université de Pierre et Marie Curie (Paris VI))

□ M.Sc. in Cellular Biology June 2018

Scripps College

□ B.A. in Human Biology May 2015

Honors and Awards

○ Sigma Xi Honors Society – Associate Member May 2015 – present

EMPLOYMENT HISTORY

PhD Graduate Student Researcher

September 2018 – September 2024

Oregon Health & Science University

Portland, OR

Graduate Program in Cancer Biology, Dr. Rosalie Sears & Dr. Ellen Langer

□ PhD Dissertation title: Bridging the Gap Between Pre-Clinical Models and Clinical Trials Using a Novel Kras- and Myc-Driven Mouse Model of Pancreatic Cancer and 3D-Bioprinting

Graduate Student Researcher (Master 2)

February – June 2018

INSERM U970 – Équipe 9

Paris, France

PARCC, Dr. Anne Eichmann Lab

□ Master's Thesis title: Arterial flow and the reversibility of arteriovenous malformations in hereditary hemorrhagic telangiectasia

Graduate Student Intern (Master 2)

November 2017 – January 2018

Institut Curie

Paris, France

Institut Pierre-Gilles de Genes, Dr. Matthieu Piel Lab

□ Research project focusing on myosin II rearrangement in nuclear deformation and constriction

Research Assistant II

July 2015 – July 2017

Oregon Health & Science University

Portland, OR

Knight Cancer Institute, Dr. Brian Druker Lab

□ Research focusing on CML, AML, and HNSCC under Dr. Elie Traer

□ Experience with primary patient sample processing, LLS-SCOR database, and mouse handling

CDB Summer Internship (pilot program participant)

June – August 2014

Oregon Health & Science University

Portland, OR

Department of Cell, Developmental, & Cancer Biology

□ Research focusing on FGF2 and FGFR1 expression in HNSCC – under Dr. Elie Traer

□ Research project in conjunction with weekly education sessions and journal clubs

HHMI Summer Research Intern

Summers 2011, 2012, 2013, 2014

Oregon Health & Science University

Portland, OR

Knight Cancer Institute, Dr. Brian Druker Lab

□ Research focusing on FGF2 in various cancers – under Dr. Elie Traer

□ Extensive experience with cell culture, western blotting, and immunohistochemistry

Research Assistant/Intern

September – December 2013

INSERM

Paris, France

CIRB at the Collège de France, Dr. Anne Eichmann Lab

□ Research focusing on angiogenesis – under Laurence Pibouin-Fragner, PhD

□ Extensive experience with gel electrophoresis, PCR, and qPCR

EPIC/McKesson Tutor

June – August 2012

South West Washington Medical Center

Vancouver, WA

- Tutored physicians during implementation of new computer-operated medical documentation systems

PUBLICATIONS

- English, I. A.,** Worth, P. J., MacPherson, K. A., Heskett, M. B., Shah, V., Phipps, J. L., Tsuda, M., Eng, J., Betts, C., Sivagnanam, S., Pelz, C., Waugh, T. A., Farrell, A. S., Allen-Petersen, B. L., Joly, M. M., Daniel, C. J., Wang, X., Thoma, M. C., Chu, J. M., Kiemen, A. L., Wood, L. D., Morgan, T. K., Coussens, L. M., Langer, E. M., Sears, R. C. **A Novel Genetically Engineered Mouse Model of Kras- and Myc-Driven Pancreatic Cancer Recapitulates Phenotypic Heterogeneity, Metastatic Potential, and Therapeutic Resistance Seen in Clinical Settings.** (*In preparation*).
- English, I. A.,** Martinez, J., Javidi-Sharifi, N., El Rassi, E., Schmidt, M., Langer, E., Bornstein, S., Gleysteen, J., Druker, B. J., Wong, M., Traer, E. **FGF2 activation of FGFR1 in head and neck squamous cell carcinoma is associated with more invasive disease and can be attenuated by FGFR inhibition.** (*In preparation*).
- Dobersch, S., Yamamoto, N., Schutter, A., Cavender, S. M., Robertson, T. M., Samraj, A. N., ... **English, I. A.,** ...Kugel, S. **HMG2 and protein leucine methylation drive pancreatic cancer lineage plasticity.** (*In preparation*).
- Link, J. M., Eng, J., Pelz, C., Worth, P. J., MacPherson, K., Sivagnanam, S., ... **English, I. A.,** ... Sears, R. C. (2024). **Ongoing Replication Stress Tolerance and Clonal T Cell Responses Distinguish Liver and Lung Recurrence and Patient Outcomes in Pancreatic Ductal Adenocarcinoma.** *Nature Cancer*, (*In press*).
- Bottomly, D., Long, N., Schultz, A. R., Kurtz, S. E., Tognon, C. E., Johnson, K., ... **English, I.,** ... Tyner, J. W. (2022). **Integrative Analysis of Drug Response and Clinical Outcome in Acute Myeloid Leukemia.** *Cancer Cell*, 40(8), 850-864.e9. <https://doi.org/10.1016/j.ccell.2022.07.002>
- Link, J. M., Pelz, C., Worth, P. J., Owen, S., Keith, D., Langer, E. M., ... **English, I.,** ... Sears, R. C. (2022). **Ongoing Replication Stress Response and New Clonal T Cell Development Discriminate Between Liver and Lung Recurrence Sites and Patient Outcomes in Pancreatic Ductal Adenocarcinoma.** *bioRxiv*, 2022.05.04.490552. <https://doi.org/10.1101/2022.05.04.490552>
- Langer, E., Farrell, A. S., Allen-Petersen, B. L., Daniel, C. J., Kresse, K. M., **English, I. A.,** ... Sears, R. (2021). **The Prolyl Isomerase PIN1 Plays a Critical Role in Fibroblast Differentiation States to Support Pancreatic Cancer.** *SSRN Electronic Journal*, 1–54. <https://doi.org/10.2139/ssrn.3800381>
- Shah, V. M., **English, I. A.,** & Sears, R. C. (2020). **Select Stabilization of a Tumor-Suppressive PP2A Heterotrimer.** *Trends in Pharmacological Sciences*, 41(9), 595–597. <https://doi.org/10.1016/j.tips.2020.06.008>
- English, I. A.,** & Sears, R. C. (2020). **Deconstructing Pancreatic Adenocarcinoma by Targeting the Conductor, MYC.** *Cancer Discovery*, 10(4), 495–497. <https://doi.org/10.1158/2159-8290.cd-20-0046>
- Javidi-Sharifi, N., Martinez, J., **English, I.,** Joshi, S. K., Scopim-Ribeiro, R., Viola, S. K., ... Traer, E. (2019). **FGF2-FGFR1 Signaling Regulates Release of Leukemia-Protective Exosomes from Bone Marrow Stromal Cells.** *eLife*, 8. <https://doi.org/10.7554/elife.40033.001>
- Tyner, J. W., Tognon, C. E., Bottomly, D., Wilms, B., Kurtz, S. E., Savage, S. L., ... **English, I. A.,** ... Druker, B. J. (2018). **Functional Genomic Landscape of Acute Myeloid Leukemia.** *Nature*, 562, 526–531. <https://doi.org/10.1038/s41586-018-0623-z>
- Kurtz, S. E., Eide, C. A., Kaempf, A., Khanna, V., Savage, S. L., Rofelty, A., **English I. A.,** ... Tyner, J. W. (2017). **Molecularly Targeted Drug Combinations Demonstrate Selective Effectiveness for Myeloid- and Lymphoid-Derived Hematologic Malignancies.** *Proceedings of the National Academy of Sciences of the United States of America*, 114(36), E7554–E7563. <https://doi.org/10.1073/pnas.1703094114>

- Traer, E., Martinez, J., Javidi-Sharifi, N., Agarwal, A., Dunlap, J., **English, I.**, ... Druker, B. J. (2016). **FGF2 from Marrow Microenvironment Promotes Resistance to FLT3 Inhibitors in Acute Myeloid Leukemia.** *Cancer Research*, 76(22), 6471–6482. <https://doi.org/10.1158/0008-5472.can-15-3569>
- Pardanaud, L., Pibouin-Fragner, L., Dubrac, A., Mathivet, T., **English, I.**, Brunet, I., ... Eichmann, A. (2016). **Sympathetic Innervation Promotes Arterial Fate by Enhancing Endothelial ERK Activity.** *Circulation Research*, 119(5), 607–20. <https://doi.org/10.1161/circresaha.116.308473>
- Traer, E., Javidi-Sharifi, N., Agarwal, A., Dunlap, J., **English, I.**, Martinez, J., ... Druker, B. J. (2014). **Ponatinib Overcomes FGF2-Mediated Resistance in CML Patients Without Kinase Domain Mutations.** *Blood*, 123(10), 1516–24. <https://doi.org/10.1182/blood-2013-07-518381>

ABSTRACTS, POSTERS, & ORAL PRESENTATIONS

Presenter:

AACR Special Conference Pancreatic Cancer, Boston, MA

September 27-30, 2023

- English, I. A.**, Worth, P. J., Farrell, A. S., Allen-Petersen, B. L., Shah, V., Betts, C., ... Sears, R. C. (2022). **Abstract A067: Myc drives phenotypic heterogeneity, metastasis, and therapy resistance in pancreatic ductal adenocarcinoma** (Vol. 82, pp. A067–A067). Retrieved from <https://doi.org/10.1158/1538-7445.panca22-a067>

Cell, Developmental, and Cancer Biology Annual Retreat, Mini-Symposium, (Internal)

October 10, 2022

- English, I. A.**, Langer E. M., Sears R. C. **Myc drives phenotypic heterogeneity, metastasis, and therapy resistance in pancreatic ductal adenocarcinoma.**

AACR Special Conference: Pancreatic Cancer, Boston, MA

September 13-16, 2022

- English, I. A.**, Worth, P. J., Farrell, A. S., Allen-Petersen, B. L., Shah, V., Betts, C., ... Sears, R. C. (2022). **Abstract A067: Myc drives phenotypic heterogeneity, metastasis, and therapy resistance in pancreatic ductal adenocarcinoma** (Vol. 82, pp. A067–A067). Retrieved from <https://doi.org/10.1158/1538-7445.panca22-a067>

AACR Annual Meeting 2022, New Orleans, LA

April 8-13, 2022

- English, I. A.**, Worth, P. J., Farrell, A. S., Allen-Petersen, B. L., Shah, V., Betts, C., ... Sears, R. C. (2022). **Abstract 880: MYC drives phenotypic heterogeneity, metastasis, and therapy resistance in pancreatic ductal adenocarcinoma** (Vol. 82, pp. 880–880). Retrieved from <https://doi.org/10.1158/1538-7445.am2022-880>

AACR Special Conference: Cancer Metastasis, Portland, OR

January 9-12, 2022

Cancelled due to CoVID-19 omicron wave

- English, I. A.**, Worth P. J., Farrell A. S., Allen-Petersen B. L., Shah V., Betts C., Pelz C., Wang X., Daniel C. J., Thoma M. C., Coussens L. M., Langer E. M., Sears R. C. **Abstract #7741: Myc drives metastasis and therapy resistance in pancreatic ductal adenocarcinoma.**

Patient Derived Models of Cancer Consortium Annual Meeting, Virtual Event

November 8-9, 2021

- English, I. A.**, Langer E. M., Allen-Petersen B. L., Waugh T. A., Agritelley E., Doha Z., Turnidge M. A., Link J. M., Brody J. R., Sears R. C. **Improving patient-derived models of cancer – the use of 3D-bioprinting to study cancer-stromal crosstalk.**

AACR Virtual Special Conference: Pancreatic Cancer, Virtual Event

September 29-30, 2021

- English, I. A.**, Worth, P. J., Farrell, A. T., Allen-Petersen, B. L., Shah, V., Betts, C., ... Sears, R. C. (2021). **Abstract PO-061: Myc Drives Phenotypic Heterogeneity, Metastasis, and Therapy Resistance in Pancreatic Ductal Adenocarcinoma** (Vol. 81, p. PO-061–PO-061). Retrieved from <https://doi.org/10.1158/1538-7445.panca21-po-061>

Cell, Developmental, and Cancer Biology Annual Retreat, (Internal) Virtual Event

September 10, 2021

English, I. A., Langer E. M., Sears R. C. MYC on the move: Illuminating MYC driven metastasis and therapy resistance in pancreatic ductal adenocarcinoma.

ISREC-SCCL Symposium, Lausanne, Switzerland

August 28-31, 2021

****Could not attend due to increased CoVID-19 travel restrictions****

English, I. A., Worth PJ, Farrell AS, Allen-Petersen BL, Joly M, Shah V, Betts C, Wang X, Daniel CJ, Thoma MC, Coussens LM, Langer EM, Sears RC. Myc drives metastasis and therapy resistance in pancreatic ductal carcinoma.

□ Selected for “speed dating” oral presentation and poster session

Patient Derived Models of Cancer Consortium Annual Meeting. Virtual Event

November 4-5, 2020

English, I. A., Waugh TA, Allen-Petersen BL, Link JM, Langer EM, Sears RC. Abstract 16: Improving models of cancer – the use of PDMCs in 3D-bioprinting to study cancer-stromal crosstalk in PDAC.

□ Outstanding Poster Presentation Award

Cell, Developmental, and Cancer Biology Annual Retreat. (Internal) Virtual Event

September 8, 2020

English, I. A., Langer, E. M., Sears, R. C. Studying cancer cell-intrinsic MYC and the tumor stromal and immune microenvironments within PDAC.

Cell, Developmental, and Cancer Biology Annual Retreat. (Internal) Portland, OR

September 4, 2019

English, I. A., Sears, R. C. The Role of PIN1 in the Immune Microenvironment of Pancreatic Ductal Adenocarcinoma.

PARCC Annual Retreat – Poster Session. (Internal) Paris, France

May 31-June 2, 2018

English, I. A., Mathivet, T., Xu, Y, Geraldo, L., Pibouin-Fragner, L., Pardanaud, L., Dubrac, A., Ola, R., Eichmann, A. Arterial flow and the reversibility of arteriovenous malformations in hereditary hemorrhagic telangiectasia.

AACR Annual Meeting 2016, New Orleans, LA

April 16-20, 2016

English, I. A., Martinez, J., Rassi, E. E., Schmidt, M., Langer, E., Bornstein, S., ... Traer, E. (2016). Abstract 1631: FGF2 Activation of FGFR1 in Head and Neck Squamous Cell Carcinoma Is Associated with More Invasive Disease and Can Be Attenuated by FGFR Inhibition (Vol. 76, pp. 1631–1631). Cancer Res. Retrieved from <https://doi.org/10.1158/1538-7445.am2016-1631>

1631

Scripps College Capstone Day, Claremont, CA

May 7, 2015

English, I. A., Martinez, J., Traer, E. Predicting Clinical Response to FGFR Inhibition from Ligand Expression in Head & Neck Squamous Cell Carcinoma.

□ Top-3 Thesis in Biology Award for the Keck Science Department
Undergraduate Thesis Advisor: Dr. Jennifer Armstrong

CDB Summer Internship Program (OHSU) – Poster Session, Portland, OR

August 4, 2014

English, I. A., Martinez J, Traer E. Predicting Clinical Response to FGFR Inhibition from Ligand Expression in Head & Neck Squamous Cell Carcinoma.

Contributor:

AACR Special Conference DNA Damage Repair, Washington, DC

January 9-11, 2024

Park, S. Y., Jeong, K. J., Poire, A., **English, I.**, Brody, J. R., Sears, R. C., & Mills, G. B. (2024).

Abstract B018: RNR inhibitors enhance chemotherapeutic activity in pancreatic cancer

- cells through altering replication stress** (Vol. 84, pp. B018–B018). Retrieved from <https://doi.org/10.1158/1538-7445.dnarepair24-b018>
- AACR Special Conference Cancer Metastasis, Portland, OR*
November 14-17, 2023
- Worth, P. J., **English, I. A.**, Phipps, J. M., Link, J. M., Langer, E. M., Tsuda, M., ... Sears, R. C. (2023). **Abstract PR012: A novel, immune-competent, Myc-dependent murine model of rapid metastatic recurrence of pancreatic cancer after resection** (Vol. 83, pp. PR012–PR012). Retrieved from <https://doi.org/10.1158/1538-7445.metastasis22-pr012>
- AACR Special Conference Pancreatic Cancer, Boston, MA*
September 27-30, 2023
- Phipps, J. L., **English, I.**, Chu, J., Garza, G. S., Link, J., Langer, E., ... Worth, P. (2024). **Abstract C102: Investigating rapid metastatic recurrence of pancreatic cancer after resection in a novel, immune-competent, Myc-dependent murine model** (Vol. 84, pp. C102–C102). Retrieved from <https://doi.org/10.1158/1538-7445.panca2023-c102>
- Society for Surgery of the Alimentary Tract (SSAT) Annual Meeting 2023, Chicago, IL*
May 6-9, 2023
- Phipps, J. L., **English, I. A.**, Chu, J. M., Tsuda, M., MacPherson, K. A., Daniel, C. J., ... Worth, P. J. (2023). **Abstract 803: Surgery accelerates pancreatic liver metastasis in an immune competent mouse model** (Vol. 164, p. S-1490-S-1491). Retrieved from [https://doi.org/10.1016/s0016-5085\(23\)04514-6](https://doi.org/10.1016/s0016-5085(23)04514-6)
- AACR Annual Meeting 2023, Orlando, FL*
April 14-19, 2023
- Phipps, J. L., **English, I. A.**, Chu, J. M., Tsuda, M., MacPherson, K. A., Daniel, C., ... Worth, P. J. (2023). **Abstract 1245: A novel, immune-competent, Myc-dependent model of rapid metastatic recurrence of pancreatic cancer after resection** (Vol. 83, pp. 1245–1245). Retrieved from <https://doi.org/10.1158/1538-7445.am2023-1245>
- AACR Special Conference Pancreatic Cancer, Boston, MA*
September 13-16, 2022
- Phipps, J. L., **English, I. A.**, Chu, J. M., Tsuda, M., Pelz, C., Daniel, C., ... Worth, P. J. (2022). **Abstract B026: Surgery accelerates metastatic recurrence in a novel, immune-competent mouse model of resectable pancreatic cancer** (Vol. 82, pp. B026–B026). Retrieved from <https://doi.org/10.1158/1538-7445.panca22-b026>
- AACR Special Conference Pancreatic Cancer, Virtual Event*
September 29-30, 2021
- Langer, E. M., **English, I. A.**, Kresse, K. M., MacPherson, K., Allen-Petersen, B. L., Daniel, C. J., ... Sears, R. C. (2021). **Abstract LT012: The Prolyl Isomerase PIN1 Plays a Critical Role in Fibroblast Plasticity to Impact Pancreatic Cancer** (Vol. 81, pp. LT012–LT012). Retrieved from <https://doi.org/10.1158/1538-7445.tme21-lt012>
- Langer, E. M., **English, I. A.**, Shah, V., MacPherson, K., Kresse, K. M., Allen-Petersen, B. L., ... Sears, R. C. (2021). **Abstract PO-113: The Prolyl Isomerase PIN1 Plays a Critical Role in Fibroblast Differentiation States to Support Pancreatic Cancer** (Vol. 81, p. PO-113-PO-113). Retrieved from <https://doi.org/10.1158/1538-7445.panca21-po-113>
- AACR Special Conference Pancreatic Cancer, Virtual Event*
September 29-30, 2020
- Langer, E. M., Allen-Petersen, B. L., **English, I. A.**, Link, J. M., & Sears, R. C. (2020). **Abstract IA-06: Heterotypic 3D Bioprinted Tissues to Study Pancreatic Cancer** (Vol. 80, p. IA-06). Cancer Res. Retrieved from <https://doi.org/10.1158/1538-7445.panca20-ia-06>
- AACR Special Conference on the Evolving Landscape of Cancer Modeling, San Diego, CA*
March 2-5, 2020
- Langer, E. M., **English, I. A.**, Turnidge, M. A., Doha, Z. O., & Sears, R. C. (2020). **Abstract A27: Heterotypic 3D Bioprinted Tissues to Interrogate Tumor-Microenvironment Crosstalk in Cancer** (Vol. 80, pp. A27–A27). Cancer Res. Retrieved from <https://doi.org/10.1158/1538-7445.camodels2020-a27>

American Society of Hematology (ASH) 59th Annual Meeting, Atlanta, GA
December 9-12, 2017

Traer, E., Druker, B. J., Martinez, J., Javidi-Sharifi, N., **English, I.**, Agarwal, A., & Dunlap, J. (2017). **Abstract 617: Marrow Stromal Cells Secrete FGF2 in Exosomes, Providing Paracrine Protection for Leukemia Cells from Kinase Inhibitors** (Vol. 130, pp. 306–306). *Blood*. Retrieved from https://doi.org/10.1182/blood.V130.Suppl_1.306.306

American Society of Hematology (ASH) 58th Annual Meeting, San Diego, CA
December 3-6, 2016

Traer, E., Javidi-Sharifi, N., Martinez, J., **English, I.**, Dunlap, J., Agarwal, A., ... Druker, B. J. (2016). **Abstract 617: FGF2 from Bone Marrow Stroma Protects Acute Myeloid Leukemia Cells from the FLT3 Inhibitor Quizartinib and Facilitates Acquisition of Resistance Mutations** (Vol. 128, pp. 1080–1080). *Blood*. Retrieved from <https://doi.org/10.1182/blood.V128.22.1080.1080>

Kurtz, S. E., Eide, C. A., Kaempf, A., Khanna, V., Savage, S. L., Rofelty, A., **English, I. A.**, ... Tyner, J. W. (2016). **Abstract 802: Identification of Effective Combinations of Targeted Drugs for Hematologic Malignancies** (Vol. 128, pp. 836–836). *Blood*. Retrieved from <https://doi.org/10.1182/blood.V128.22.836.836>

American Society of Hematology (ASH) 57th Annual Meeting, Orlando, FL
December 5-8, 2015

Martinez, J., Javidi-Sharifi, N., **English, I.**, Viola, S. A., Jorgens, D., Druker, B. J., et al. **2465 FGF2-Containing Exosomes Secreted from Bone Marrow Stromal Cells Protect Leukemia Cells from Tyrosine Kinase Inhibitors**. American Society of Hematology [Internet]. Orlando, FL; 2015 Dec 6. Available from: <https://ash.confex.com/ash/2015/webprogramscheduler/Paper86673.html>

AACR Annual Meeting 2015, Philadelphia, PA
April 18-22, 2015

Martinez, J., Traer, E., Javidi-Sharifi, N., Agarwal, A., Dunlap, J., **English, I.**, ... Druker, B. (2015). **Abstract 984: FGF2 from the Bone Marrow Promotes Resistance to FLT3 Inhibitors in AML** (Vol. 75, pp. 984–984). *Cancer Res*. Retrieved from <https://doi.org/10.1158/1538-7445.am2015-984>

American Association of Cancer Research (AACR) 106th Annual Meeting, Philadelphia, PA
April 18-22, 2015

Martinez, J., Javidi-Sharifi, N., **English, I.**, Viola, S. A., Jorgens, D., Druker, B. J., & Traer, E. (2015). **Abstract 2465: FGF2-Containing Exosomes Secreted from Bone Marrow Stromal Cells Protect Leukemia Cells from Tyrosine Kinase Inhibitors** (Vol. 126, p. 2465). *Blood*. Retrieved from <https://doi.org/10.1182/blood.v126.23.2465.2465>

American Society of Hematology (ASH) 55th Annual Meeting, New Orleans, LA
December 7-10, 2013

Traer, E., Javidi-Sharifi, N., Agarwal, A., Dunlap, J. B., **English, I.**, Martinez, J., ... Druker, B. J. (2013). **Abstract 631: FGF2 Mediates Resistance in CML Patients in the Absence of Kinase Domain Mutations, and Resistance Is Overcome by Ponatinib** (Vol. 122, pp. 3983–3983). *Blood*. Retrieved from <https://doi.org/10.1182/blood.V122.21.3983.3983>

Traer, E., Javidi-Sharifi, N., Agarwal, A., Dunlap, J. B., **English, I.**, Martinez, J., ... Druker, B. J. (2013). **Abstract 611: FGF2 Promotes Resistance to Quizartinib In Vitro, and FGF2 Increases in the Marrow of Patients Prior to Resistance** (Vol. 122, p. 2541). *Blood*. Retrieved from <https://doi.org/10.1182/blood.v122.21.2541.2541>

COMMUNITY SERVICE AND OUTREACH

OHSU Food Resource Center – Portland, OR October 2020 – present

- Grocery Bag Delivery – responsible for delivering grocery bags with pantry staples and recipes to support student food security, as needed
- FRC Shopping Hours – responsible for supervising resource center shopping hours, replenishing stocks, maintaining clean and organized storage spaces, biweekly

Ronald McDonald House – Portland, OR

August 2016 – present

- Fun Junction – responsible for leading craft projects and entertaining children of families staying at RMHC while parents take a short reprieve, biweekly *paused due to CoVID-19 restrictions*
- Baking – baking ‘home-baked’ treats for families staying at RMHC, biweekly
- Special Events – responsible for set-up, ticket sales, clean-up, etc., as needed

Science Fairs & Expositions – Oregon

- Middle School – Northwest Science Expo (MS-NWSE) – Portland, OR April 2022
 - Middle School Science Competition, Category Judge
- Beaverton-Hillsboro Science Expo (BHSE) – Beaverton, OR Mar. 2021, Feb. ‘22, ‘23
 - High School Science Competition, Category & Finalist Judge
- Aardvark Science Expo (Oregon Episcopal School) – Portland, OR Feb. 2020, ‘21, ‘22, ‘23
 - High School Science Competition, Category & Finalist Judge
- Oregon Junior Science & Humanities Symposium (JSHS) – McMinnville, OR Feb. 2020, ‘21
 - High School Science Competition, Category & Finalist Judge

OHSU CoVID-19 Vaccine Clinic – Portland, OR 2021

- Vaccine Administration – responsible for educating patients about the CoVID-19 vaccine, consenting patients, and administering vaccinations

Visitor (Rotaract Club) – Paris, France September 2017 – May 2018

- Salvation Army – preparing and serving dinner to the houseless
- Hôpital Necker, Enfants Malades – spent time with and entertained children staying in the hospital (3hrs, biweekly)
- Recherche sur Alzheimer Fondation – XIII Gala – helped organize volunteers and sell lottery tickets to attendees

Food Rescue – Inland Empire, CA September 2012 – May 2013

- Responsible for transport of leftover food from dining halls in The Claremont Colleges Consortium campuses to a local homeless shelter

Young & Healthy – Pasadena, CA September 2012 – May 2013

- Taught 1st and 2nd graders in the United Pasadena School District about dental hygiene

National Charity League – Portland, OR May 2005 – May 2011

- Regular volunteering through various organizations focusing on women’s health and houselessness
 - President 2009-2010
 - Treasurer 2006-2007

SKILLS AND ACTIVITIES

Research Techniques:

- | | |
|------------------------------|---|
| ○ Cell culture | ○ Mouse handling |
| ○ Western blotting | □ Colony management |
| ○ PCR | □ Generation of orthotopic HNSCC cell line xenografts |
| ○ qPCR | □ Generation of orthotopic PDAC cell line xenografts |
| ○ cDNA | □ Drug dosing |
| ○ DNA extraction | □ Oral gavage |
| ○ RNA isolation | □ Tail vein injection |
| ○ Gel electrophoresis | □ Retro-orbital injection |
| ○ Immunofluorescent staining | □ Dissection |
| ○ Immunohistochemistry | □ Perfusion |
| ○ siRNA | |
| ○ Microfluidic channels | |
| ○ Fluid shear stress assay | |
| ○ 3D Bioprinting | |

Technology:

- | | | |
|-------------------|-----------------------|----------------|
| ○ Microsoft Word, | ○ Graphpad Prism | ○ CellProfiler |
| Powerpoint, & | ○ Adobe Illustrator & | ○ ImageJ/FIJI |
| Excel | Photoshop | ○ Image Lab |

- LLS-SCOR database
- EPIC
- R

Graduate School Courses – PhD

Practice and Ethics of Science, Advanced Topics in Cancer Biology, Introduction to Immunology, Tissue Biology, Grant Writing & Qualifier Preparation, Principles and Practices of Data Visualization, R Programming, Tumor Microenvironment Journal Club, Cancer Biology Seminar Series

Graduate School Courses – Master's Degree

Cellular Physiology 1 (Experimental and theoretical approaches for resolving scientific enigmas in the laboratory context), Cellular Physiology 2 (Regulation of gene expression: translation and the cell cycle – marine model systems), Advanced Course in Cell Dynamics, Cell Biology and Cancer, Scientific Analysis, Research Proposal, 5-month Cardiovascular Research Laboratory Internship

Undergraduate Upper Division Courses

Cell Biology, Genetics, Organic Chemistry, Applied Biostatistics, Vertebrate Anatomy, Genomics & Bioinformatics, Biology of Cancer

Languages

English (native)
French (conversational/previously fluent)
Spanish (basic/beginner)

Participant in the Following Undergraduate Clubs and Organizations:

- Pre-Health Club
- Women in Science Club
- Mood Swing–Audition-only a cappella group