

Mesenchymal Heterogeneity and Signaling Gradients
underlies Non-Redundant Function of Pancreatic Cancer-
Associated Fibroblasts

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List of Abbreviations

a-SMA	Alpha-smooth muscle actin
ADAM	A disintegrin and metallo-proteinases
apCAF	Antigen-presenting CAFs
CAF	Cancer associated fibroblast
CRE	Recombinase
dFdU	2'2'-difluorodeoxyuridine
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
ECM	Extracellular matrix
Fabp4	Fatty acid binding protein 4
FAK	Focal adhesion kinase
FAP	Fibroblast activation protein
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HSC	Hepatic stellate cell
iCAF	Inflammatory CAFs
IHC	Immunohistochemistry
KIC	$Kras^{LSL-G12D/+}; Ink4a^{fl/fl}; Ptf1a^{Cre/+}$
KPC	$Kras^{LSL-G12D/+}; Trp53^{LSL-R172H/+}; Pdx1-Cre$
KPeCY	$Kras^{+/LSLG12DVgeo}; Trp53^{lox/lox}; ElastTa/tetO-Cre; Rosa^{LSLEYFP}$
LRAT	Lecithin retinol acyltransferase
MHCII	Major histocompatibility complex class II
MMP	Matrix metalloproteinases
mTmG	Rosa26ACTB-tdTomato,-EGFP
OPTR	Oregon Pancreas Tissue Registry program
OSM	Oncostatin M
P-MLC2	Phospho-myosin light chain 2
PanIN	Pancreatic intraepithelial neoplasia
PD/Pk/tox	Pharmacokinetics pharmacodynamics toxicity
PDAC	Pancreatic ductal adenocarcinoma
PDPN	Podoplanin
PEGPH20	Pegvorhyaluronidase alfa
PSC	Pancreatic stellate cell
Shh	Sonic hedgehog
Tie1	Tyrosine kinase with immunoglobulin like And EGF like domains 1
TME	Tumor microenvironment
Treg	T regulatory
TS	Thymidylate synthase

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Chapter 1 : Background and Introduction

Origins of Fibroblasts Heterogeneity in the Pancreatic Tumor Microenvironment.

Abstract

The grim prognosis for pancreatic ductal adenocarcinoma (PDAC) patients impels an improved understanding of disease biology to facilitate the development of enhanced therapies. PDAC often features a remarkably dense stroma established and populated by a pronounced population of cancer associated fibroblasts (CAFs). Stellate cells have been identified as a source of these CAFs. Genetically engineered mouse models and increasingly sophisticated cell culture techniques have demonstrated complex heterogenous roles for fibroblasts in PDAC progression and therapy response. The complex role of fibroblasts are increasingly understood to be dependent on signaling gradients and cell of origin with strong evidence for both tumor supportive and tumor suppressive or homeostatic functions dependent on multiple factors. Here we take a historical perspective, driving into the literature to follow the discovery of stellate cells, reviewing recent literature to investigate the source of fibroblast heterogeneity. This literature along with our studies on stellate cells have improved our understanding of heterogeneity in fibroblast fate and function in this disease including the existence of distinct fibroblast populations and highlight important avenues for future study.

Significance

While the dense stromal reaction of PDAC has long been grasped, the functions of the CAF cells that establish this stromal feature remain unclear. Improving this understanding of transcriptional and functional heterogeneity of PDAC CAFs, and the

tumor suppressive versus tumor supportive capacity, could facilitate the development of anti-stromal therapies. These additional stromal directed therapies will hopefully work in conjunction with current combination therapy to advance efficacy.

Discovery of the Stellate Cell

Since the discovery of the hepatic stellate cell by Kupffer in 1876 and reidentification in 1971 by Wake, much has been observed about this mesenchymal cell while its cousin in the pancreas remained elusive until recently. In 1876 Sternzellen or star cells were found in the liver perisinusoidal space and named for their characteristic star shape. Kupffer revealed these Star-shaped cells in the liver by using a method for identifying nerves by staining with gold chloride, which causes the cells to darken under a light microscope. Kupffer indicated that he thought these cells to be connective tissue cells due to location and shape. However, due to the gold chloride reaction and the ability of these cells to take up special dyes, Kupffer changed his mind calling them phagocytes. For a short time, these cells were called pericytes by Zimmerman. For roughly 75 years, these cells remained mysterious until Ito observed lipid-containing cells in the liver of humans connecting nerve fibers and effector cells¹. Wake took an additional 20 years to discover that Ito cells and Kupffer or Stellate Cells were the same cells. It was Wake who put it all together, determining that these cells can be identified by the lipid droplet store of Vitamin A, which fluoresce. In addition, Wake found that the autofluorescence of Vitamin A caused the initial reaction to the Gold Chloride². Taken together these studies revealed the identity of what was initially thought to be connective tissue cells, then macrophages (phagocytes), and finally pericytes were all the same cells, to be stellate cells later called the hepatic stellate cell (HSC). The unique characteristic of morphology

and lipid droplet content allowed for the separation of these cells from other cells aiding originally in their discovery and later assisting in studying them *in vivo* and *in vitro*.

The discovery of the stellate cells by lipid droplet content identified their prominent role in lipid storage. Quiescent stellate cells activate in response to tissue damage. Activated stellate cells releasing lipid droplets that act as nutrients by engaging in translational pathways for cell survival and support the metabolic needs for proliferation³⁻⁵. In low nutrient or hypoxic conditions alternative pathways are upregulated for cell proliferation such as fatty acid metabolism resulting in increased uptake of lipid droplets. In addition to the storage of nutrients, evidence also supports that these cells act as support structures creating connections between cells that provide strength. The morphology of stellate cells with their long processes and their position between lobes connect cells and fill the gaps⁶. A smaller subset of stellate cells alluded to previously connected neurons to other liver cells. Moreover, another subset wraps around blood vessels much like pericytes supporting the vessels. While these stellate cells are seen near other cells, expression profiles indicate that they lack the contraction of pericytes that work to open and close vasculature¹. This suggests that while these cells act as supports, they may not directly impact blood flow regulation.

Further stellate cells express extracellular matrix (ECM) proteins such as collagen III and IV and laminins, fitting the role of support and possible signaling pathways indirectly through tension. The creation and the degradation of ECM as well as the regulation of ECM components, plays a significant role in signaling and tissue remodeling⁷. As such, stellate cells produce Matrix metalloproteinases (MMPs), including a disintegrin and metalloproteinase (ADAMs)⁸. Tissue staining of the liver

provided a clear indication of the role of stellate cells and the proteins they produce. Still, the isolation of these cells provides expression profiles to match the role, further elucidating the complexities of these star cells.

Undoubtedly, the discovery that the lipid droplet content of HSC causes the cells to float in a density gradient enabled further understanding of these cells *in vitro*⁹.

Digestion of the liver followed by density gradient centrifugation allowed for selective isolation of HSC when cultured on plastic. Additional staining with markers such as vimentin and desmin indicates mesenchymal origin. In addition, Kordes and colleagues identified that hepatic stellate cells are plastic in nature with potential to transition to adipocytes and osteocytes, concluding these cells to be mesenchymal stem cells¹⁰.

Studies looking at development through embryos to adults indicate the mesothelium to be the origin of hepatic stellate cells¹¹. There is currently much debate on the origin of stellate cells and their stem like character. This debate is further spurred by glial fibrillary acidic protein (GFAP) and nestin expression suggesting a possible neuronal source. This controversy, complicated expression, and plasticity confirms the difficulty of their discovery and study⁶. Within the liver, about 70-80% of HSC express GFAP, which overlaps with desmin combined to mark 90% of HSCs. Further complicating matters when grown in a dish, these cells activate to a fibroblast phenotype characterized by loss of lipid droplets, production of ECM proteins, and expression of cell specific markers α -Smooth muscle actin (α -SMA)¹². These cells store lipids and play a structural role in quiescent normal tissue or within the first 48 hours *in vitro*¹³. The resulting passage of time after roughly 48 hours causes activation resulting in signaling changes and the loss of lipid droplets. During activation as a result of injury the release of lipid droplets

including vitamin A provides a vital component for growth and regeneration and mediates immune responses¹⁴. Activation in vitro supports the role of HSC in liver fibrosis. Inflammatory signals after an injury result in the activation of HSC, which differentiate into myofibroblasts in the liver. Activated HSCs secrete extracellular matrix proteins, collagens, and metalloproteinases remodeling the injured tissue in response to a wound and inflammation. In addition, any indication of injury results in signaling cascades that lead to loss of vitamin A, the release of all lipid stores and changes in markers. In a wound healing environment, these lipid releases provide nutrients to repair, while the production of ECM remodel to repair the tissue, returning it to homeostasis. However, in a chronic wound, this process goes awry¹⁵⁻¹⁷. Markers including mesenchymal markers vimentin, desmin and vitamin A are a means to identify stellate cells, however these are all lost with activation creating challenges with tracking stellate cells through activation in a wound as well as the tumor microenvironment. Without a lasting marker, lineage tracing is made more difficult. A marker that lasts through activation is needed to understand the role of stellate cells in disease.

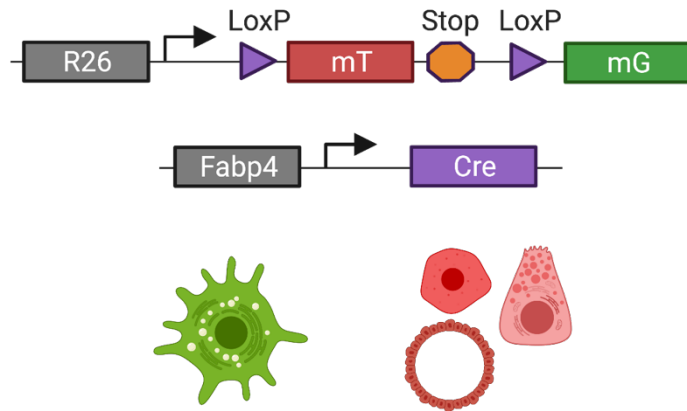
CRE (recombinase) models can be used to follow stellate cells through activation allowing for the determination of their role in a wound environment and pathologies. CRE systems are often combined with a reporter cassette. mTmG (Rosa26ACTB-tdTomato,-EGFP) is one such reporter cassette: when a cell does not express target protein, the promoter is inactive, CRE is not expressed, and tdtomato is expressed followed by a stop codon; however, when the promoter is active, CRE is produced excising out the tdtomato and stop codon resulting in GFP (green fluorescent protein) expression (Figure 1-1A). Mederacke and colleagues determined LRAT-CRE (Lecithin

retinol acyltransferase) could trace stellate cells in the liver due to the role of LRAT in lipid droplet formation a hallmark of stellate cells. This profound tracking mouse model demonstrated that it marked 99% of HSCs as defined by the coexpression of vitamin A by flow cytometry. LRAT-CRE also showed that 82-96% of hepatic myofibroblasts are of HSC origin, confirming HSC as the main source of fibroblasts in the liver¹⁸. These results showed that HSCs were progenitors for most of the myofibroblasts within the liver during fibrogenesis induced by CCl₄. CCl₄ induces liver inflammation, and stellate cell activation through oxidative stress, inflammatory responses and retinol metabolism among other pathways¹⁹. Following up on the HSCs activating to myofibroblasts, a depletion model was used where the active promoter causes the expression of CRE that excises a stop codon resulting in the expression of diphtheria toxin receptor (DTR) (Figure 1-1B). Administration of diphtheria toxin to mice results in the selective depletion of LRAT-expressing cells, including HSCs. Selective depletion of LRAT-expressing cells in a liver fibrosis model (CCl₄) results in the reduced matrix deposition and overall fibrosis phenotype compared to vector controls. The limitation of the LRAT model is that it does mark a rare population of vascular smooth muscle cells, which have been implicated in disease progression, and cannot be ruled out as a source of fibroblasts. In addition, the LRAT model marks other lipid droplet-containing cells, including hepatocytes; however, a majority of HSCs are marked, indicating the depletion of fibrosis is partly due to the HSCs²⁰. Additional CRE models have been used to study HSCs such as PDGFR β based on the expression of the gene by profiling HSCs. PDGFR β marks both quiescent and activated HSCs, and these studies found α v integrin production regulated

fibrosis. Depletion studies of α_v integrin protected against fibrosis induced by CCl₄, indicating a mechanism behind HSCs' role in fibrosis^{20,21}.

A

Cre⁻ host tissues: tdTomato
Cre⁺ cells and progeny: EGFP



B

Cre⁻ host tissues: tdTomato
Cre⁺ cells and progeny: EGFP & DTR

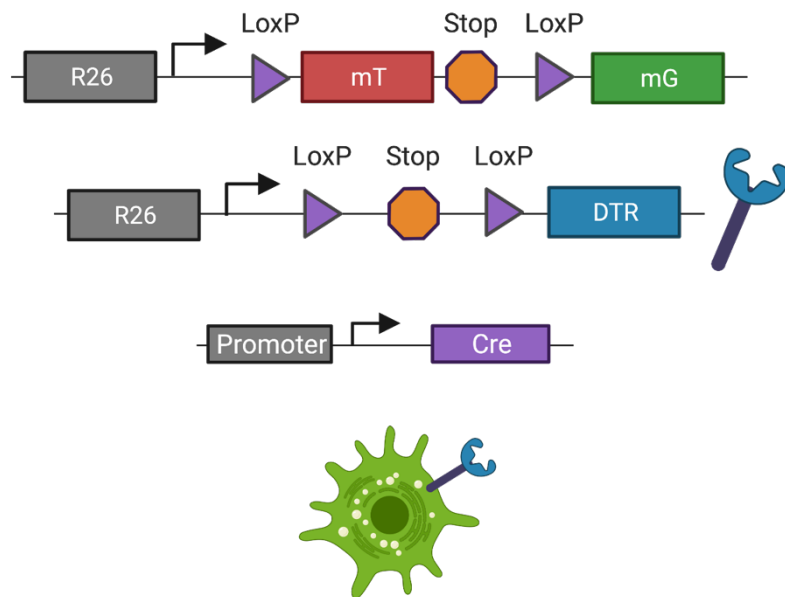


Figure 1-1. Genetically engineered mouse models to lineage trace and ablate specific cells based on promoter activity.

A schematic of the alleles used to label and track PSCs in vivo. Representative schematic to ablate the cells with activity of a specific promoter. Created with BioRender.com

Further CRE models include GFAP, which was found to be nonspecific²⁰. An additional model based on a collagen CRE marked 92.6% of α -SMA and 92% of vitamin A and desmin expressing cells; however, this model is complicated by collagen production by other cells and the deposition of collagen masking cells^{18,22–24}. While many limitations exist to the CRE models, likely the source of these limitations is heterogeneity of fibroblast origin impacting the role of the activated cells. These CRE models are likely marking fibroblasts of one cellular origin defining only the roles of these cells. Despite these limitations the results have shown that HSC activates to a myofibroblast phenotype that plays a role in fibrosis; however, heterogeneity seems to be present in function based on location as well as potential diversity in cell of origin²⁰.

It was once thought that pancreatic stellate cells (PSCs) were identical to HSC with the hope that the same markers and models could be used. However, many of the same challenges with loss of characteristics during activation, as seen by loss of markers and lipid storage in studies using isolation and cell culture²⁵. Incomplete marking with CRE models or nonspecific marking also plagued the pancreatic stellate cell field²⁶. Hepatic stellate cells are similar to pancreatic stellate cells, storing lipid droplets in quiescent form²⁷. A few markers translate from the liver to the pancreas, including desmin and vitamin A, vimentin, and nestin. PSC also activates with the induction of inflammation pathways. Markers of activated PSC are similar to HSC, including α -SMA, PDGFR α/β , and podoplanin (PDPN). With activation, PSCs also express ECM proteins and reduced lipid droplet stores²⁸. PSCs provide structure and nutrients in a wound healing environment, but this is exploited in a chronic wound, cancer^{29,30}. PSCs are implicated to be the source of CAFs based on HSC in liver cancer³¹. CAFs provide

nutrients in the form of lipids, signal to dysregulate the immune system, and provide scaffolding to support the tumor. In the case of PDAC, the stroma is made up of immune cells, CAFs, and the ECM produced in part by the CAFs; these stromal elements make up roughly 70% of the tumor^{32,33}. This abundance of fibroblasts and fibroblast material is indicated to be protumor and is thus a major target for developing enhanced therapeutics. In addition, the stroma is a physical barrier to drug delivery, demonstrating further the importance of targeting the stroma for PDAC treatment^{34,35}. Unfortunately, despite the similarities of HSC and PSC did not translate to CRE models that could be used to track the stellate cell through activation to fibroblast phenotype in the pancreas¹⁵. The lack of pancreatic CRE models has left a gap in knowledge and the assumption that PSCs give rise to a majority of CAFs in the pancreas to match the liver equivalent.

Ablation Models Identify Fibroblast Heterogeneity

Despite various cancer treatment options, PDAC remains a devastating disease with only 11% 5-year survival rates, one of the lowest among common tumor types³⁶. The 5-year survival rate has not moved significantly despite advances in treatment for other tumor types, including immunotherapies and combination chemotherapies³⁷. PDAC has remained an enigma. The dense stroma surrounding the tumor is thought to be part of the biology of PDAC preventing chemotherapy efficacy. This stroma comprises mainly regulatory immune cells, vasculature, CAFs, and the ECM. CAFs are the main producers of the ECM, making them a vital to the PDAC tumor microenvironment. These CAFs provide a physical barrier for therapies and provide a nutrient source in a nutrient-poor environment^{38,39}. The hypoxic nature of PDAC means resources are scarce; by definition,

this means a lack of oxygen causing glucose metabolism to be reduced and the necessity of nutrient scavenging⁴⁰. Stellate cells hold lipid stores and release them upon activation; this is corrupted by hypoxic and tumor signaling, providing a fat source to the tumor^{3,5}. While a lack of nutrients and oxygen generally halts the cell cycle preventing proliferation, survival signals are instead promoted through tumor cell signaling and inhibition of cytotoxic stress response via FAK (focal adhesion kinase)⁴¹. The ECM not only provides nutrients and contributes to the hypoxic environment, but it also provides scaffolding with which tumor cells can migrate for invasion and metastasis⁴². The CAFs that are the primary source of ECM also signal with tumor cells to regulate immune cells, creating immune dysregulation, increasing regulatory immune cells (mainly T-Reg cells), and sequestering cytotoxic immune cells to the periphery of the tumor^{32,43}. The dysregulation of the immune cells leads to a decrease in tumor cell killing by the exhaustion of cytotoxic cells and the physical barrier between immune and tumor cells⁴⁴. The ECM acts as a physical barrier to the immune system and therapeutics but is also a physical restraint to the tumor. This contradiction in roles is where homogeneous role of CAFs begins to crumble.

Within the pancreas, it was assumed that all fibroblasts resulted from resident pancreatic stellate cells following the model of hepatic stellate cell-derived fibroblasts. Unfortunately, recombinase mouse models developed to track hepatic stellate cells do not translate to the pancreas indicating a potential difference of the organs. Recombinase liver models that use Desmin to mark stellate cells show that stellate cells activate to a fibroblast phenotype. During the quiescent state, when the Desmin promoter is active the recombinase removes a stop codon on a reporter cassette resulting in a usually

fluorescent lasting mark even when the promoter is no longer on, or the cell is in an active state. In the liver, 90% of fibroblasts are marked with the Desmin CRE mice induced to develop tumors triggering the cell activation²⁰. These models did not translate to the pancreas even though pancreatic stellate cells are marked with Desmin. Due to the lack of a CRE model for the pancreatic stellate cells, it was assumed that stellate cells activated to CAFs in PDAC models. This assumption was based on isolated pancreatic stellate cells by density gradient and subsequent culture. In culture, these cells activate after two days. After two days, the morphology matches that of fibroblasts. In addition to the same morphology, the sequencing profiles of these cultured cells show expression of α -SMA and other fibroblast markers⁴⁵. Without a lasting marker, stellate cells giving rise to all CAFs in a pancreatic tumor could not be confirmed, so these cells' role was thought to be protumor.

Ablation models have been used to investigate the role of these CAFs in the pancreatic tumor. Rhim and colleagues based one model on canonical hedgehog signaling where Shh (sonic hedgehog) secreted by tumor cells results in downstream signaling to nearby stromal cells ending in CAF activation and increased desmoplasia. They conditionally knocked out Shh using an $\text{Shh}^{\text{fl/fl}}$; Pdx1-Cre ; $\text{p53}^{\text{fl/+}}$; $\text{Rosa}^{\text{LSL-YFP}}$, $\text{Kras}^{\text{LSL-G12D}}$ mouse, preventing hedgehog signaling of epithelial cells to the stroma, in essence stopping the activation, proliferation, and involvement of CAFs in tumor progression. While hedgehog signaling-inhibited mice were normal at birth, they developed tumors through Kras and p53 mutations; their survival was the opposite of expectation. As CAFs in the tumors were thought to support tumor progression, inhibition of these cells was expected to improve outcomes in mouse PDAC models. However, inhibition of hedgehog

signaling resulted in reduced desmoplasia but accelerated tumor growth, increased metastasis, and reduced survival⁴⁶. The same year Rhim and associates found that the stroma acts to restrain PDAC. Ozdemir and colleagues found similar results based on fibroblast ablation via α -SMA. A $Ptfla^{cre/+}; LSL-Kras^{G12D/+}; Tgfb2^{flox/flox}$ mouse crossed with α -SMA-tk transgene, activated by ganciclovir injections, depleted α -SMA⁺ CAFs resulting in significantly reduced survival, with tumors that were poorly differentiated. α -SMA depleted tumors also had dysregulated ECM and immune infiltration, reduced overall immune infiltration, and increased frequencies of Treg (T-regulatory) FoxP3⁺ cells. In addition to increased T-reg cells in α -SMA depleted samples, the remodeled immune systems had decreased effector cells, including F4/80⁴⁷. A third study led by Gunderson targeted fibroblasts using fibroblast activation protein and resulted in improved T cell infiltration and collagen deposition but did not enhance survival⁴⁸. In combination, these studies suggest that the stroma may physically restrain the tumor. Further supporting the stroma as a physical restraint are experiments with PDAC mouse models in which inhibition of lysyl oxidase like-2 manipulated the stromal matrix. Inhibition of lysyl oxidase like-2 resulted in low tissue stiffness and accelerated tumor growth, and reduced survival. Patients with a higher α -SMA content have increased overall survival compared to patients with low α -SMA tumor content⁴⁹. Overall, these studies identified that it wasn't as simple as CAFs and the stroma being protumor, but that CAFs and the ECM have anti-tumor roles. The pro and anti-tumor roles may be due to the same population of CAFs and high plasticity or CAF heterogeneity with separate functions for each subgroup, and potentially the highly sought after protumor CAF for specific targeting in combination with chemotherapies or immunotherapies in PDAC.

Diverging results of cancer-associated fibroblast ablation studies suggested a need for further studies to elucidate the functions of CAFs plasticity and heterogeneity. Due to evidence suggesting that fibroblasts in the typical wound healing environment have diverse roles, so might CAFs in the tumor microenvironment as tumors are often thought of as never healing wounds. However, evidence suggests that it is not just the plasticity of the CAF population but rather a heterogeneity of CAF populations, with heterogeneity resulting from either origin or signaling gradients. While the mechanisms of the tumor suppressive CAFs is hazy, there is increasing evidence of CAF heterogeneity. It is of utmost importance to identify what drives the heterogeneity of CAF populations to manipulate them for improved therapeutic outcomes, emphasizing increasing tumor-suppressive roles or reducing tumor supportive roles⁵⁰.

CAF Marker Expression Highlights Stromal Heterogeneity

Hepatic fibroblasts have common markers such that a majority are labeled with the use of LRAT (lecithin retinol transferase) and α -SMA which have led to an understanding that most liver CAFs originate from HSC; however, there is not a known pancreatic specific CAF marker. Prior to identifying liver fibroblast markers, fibroblasts were defined by the lack of CD45, and CD31 indicating they were not leukocytes or endothelial cells, respectively. The remaining cells of the pancreas can be identified as pericytes (NG2⁺), or cells of the islets of Langerhans, and the acinar and ductal cells of the exocrine pancreas (Figure 1-2). Fibroblasts in the pancreas were identified by the lack of markers for these pancreatic cell populations. Features that translate from the liver to the pancreas suggest distinct populations indicating heterogeneity across tissues. A few of

these translated markers are α -SMA/ACTA2, PDGFR- α in addition to FAP (fibroblast activation protein), FSP1, and PDPN markers used in the pancreas that vary in expression, indicating heterogeneity. α -SMA was thought to be a pan-CAF marker for both liver and pancreas but only for activated fibroblasts, not quiescent counterparts. However, there are activated fibroblasts that are α -SMA⁻ that express another standard marker, PDGFR- α ⁵¹. Both humans and mice have these α -SMA⁻ fibroblasts. In a tumor setting, most mouse CAFs are activated and in contact with the tumor though a minority, are spatially restricted and in pockets of stroma, lacking tumor interactions.

Kras^{+/LSLG12DV^{geo}}, Trp53^{lox/lox}, ElastTa/tetO-Cre, Rosa^{LSLEYFP} (KPeCY) mice have 75% of CAFs expressing α -SMA and 50% express PDGFR- α , while 9% are PDGFR- α and not α -SMA leaving a remaining 16% neither PDGFR- α nor α -SMA⁵². RNA sequencing results of KPC (Kras^{LSL-G12D/+}; Trp53^{LSL-R172H/+}; Pdx1-Cre) mice show 45% of CAFs have a low α -SMA/PDGFR- α overlap seen by ACTA2⁻ but PDGFR- α enriched cells^{51,53}.

While FSP1 can be a marker of fibroblasts, it also marks tumors cells that have transitioned from epithelial to mesenchymal and lost E-cadherin, resulting in FSP1⁺ as a nonspecific marker⁵⁴. In addition, there is minimal overlap between FSP1 and α -SMA in stromal cells separated from tumor cells by epithelial markers⁵⁵. While the role of FSP cells remains unknown, glycoprotein FAP on CAFs in human tumors is linked to PDAC growth escaping immune control, supported by subcutaneous depletion of FAP in KPC mice gaining immunological control of PDAC^{56,57}. These FAP⁺ stromal cells change through tumorigenesis starting as α -SMA⁻ to α -SMA⁺⁵⁷. While other markers are heterogeneous, PDPN appears to be a robust marker for pancreatic fibroblasts⁵³. RNA sequencing of KPC mouse CAFs show PDPN is a pan-CAF marker, further supported by

PDPN expression on human CAFs. Regarding PDPN in human tissues, high expression of PDPN is correlated with poor prognosis⁵⁸. PDPN is a cell surface marker, adding to its usage in pancreas fibroblasts studies in its ease of use by flow cytometry. Unfortunately, none of these markers are only for fibroblasts as PDPN also marks lymphatic endothelial cells. α -SMA marks pericytes and FAP mesenchymal cells in the skeletal muscle and bone. When designing models based on these markers, caution must be taken with respect to lack of specificity. CAF markers indicate heterogeneity; however, future studies are needed to identify pro-tumor verse anti-tumor subpopulations of CAFs.

Transcriptional Heterogeneity among PDAC CAFs

Fibroblast marker expression in combination with PDAC CAF ablation studies suggest heterogeneity in function. It has been identified by multiple mouse models of PDAC gene expression profiles and additional patient samples that there are numerous subpopulations of CAFs with distinct transcriptional programs. One such mouse model, single cell RNA sequencing of KPC tumors, suggests three CAF populations based on gene expression profiles; this coincides with patient sequencing expression profiles⁵³. The classic α -SMA population of CAFs is present as a subpopulation called myCAF seen in RNA sequencing profiles with gene expression of ECM components and contractility factors. A second population, first called iCAF or inflammatory CAF, does not express α -SMA but is marked by cytokine and chemokine expression, including IL-6 and CXCL12. An interesting third population is characterized by its presentation of MHCII (major histocompatibility complex class II) complex including CD74 but without costimulatory molecules classically needed by immune cells to present antigen to T cells.

This MHCII expressing population is termed apCAFs (antigen presenting CAFs) and a marker to separate it from the other two groups is Saa3. Studies of Saa3 null stroma correlate with increased levels of potentially tumoricidal CD11c⁺ CD206⁻ macrophages within the tumor space, indicating an immunomodulatory role that coincides with the antigen-presenting potential⁵².

Further single-cell sequencing studies of both mouse and human PDAC also show three subpopulations of CAFs with similar expression profiles though there are some distinct differences, perhaps due to analysis of different PDAC models. One study using KIC (Kras^{LSL-G12D/+}, Ink4a^{fl/fl}, Ptf1a^{Cre/+}) PDAC model termed the populations FB1, FB2, and FB3. FB1-3 is expressed in the normal pancreas and early-stage panINs (pancreatic intraepithelial neoplasia), while FB2 is not in advanced PDAC⁵⁹. FB3 appears to have a combination of myCAF features, including ACTA2 and Tagln (contractility) but features MHCII, a quality of apCAFs. FB1 has the expression of IL-6, CXCL12, and CCL12 cytokines and chemokines, which matches the iCAF (inflammatory CAF) population of Elyada et al. and Ohlund et al. Ohlund and colleagues found that signaling gradients are focal to the heterogeneity of CAFs, resulting in a difference of expression based on location within the tumor to be discussed in more detail in the signaling gradients section below. However, in studies of isolated human PDAC CAFs, the subtypes are present with similar transcripts suggesting that without signaling, heterogeneity is still present. Another study of 16 primary CAF samples found an additional subtype, totaling four subtypes⁶⁰. B&D subtypes in this study are myCAFs expressing ACTA2 and ECM elements. Subtype C is more similar to iCAF with an expression of inflammation mediators and complement components. Due in part to the bulk analysis of this study,

subtype A is likely heterogeneous and could hint at the variability of subtypes in expression. Subtype A features myCAF POSTN^{high} but iCAF ACTA2^{low}. Each of these studies supports the heterogeneity of CAFs, indicating three subgroups albeit with expression variability and non-discrete boundaries between groups (Figure 1-3)⁶⁰. It should be mentioned that there are limitations such as differences in techniques to isolate CAFs and sequencing depths that led to differences in expression profiles. Despite these potential limitations, there is support that biological and signaling differences may create a variety of biologically distinct milieus. These studies have many commonalities, but regarding the immune component, they differ in cytokine and chemokine identities that mark CAF populations, which suggests a potential avenue for study in treating PDAC or directing CAFs to remodel the immune microenvironment to be anti-tumor. Studies also need to be done to elucidate further the mechanisms underlying the transcriptional heterogeneity to guide treatment strategies.

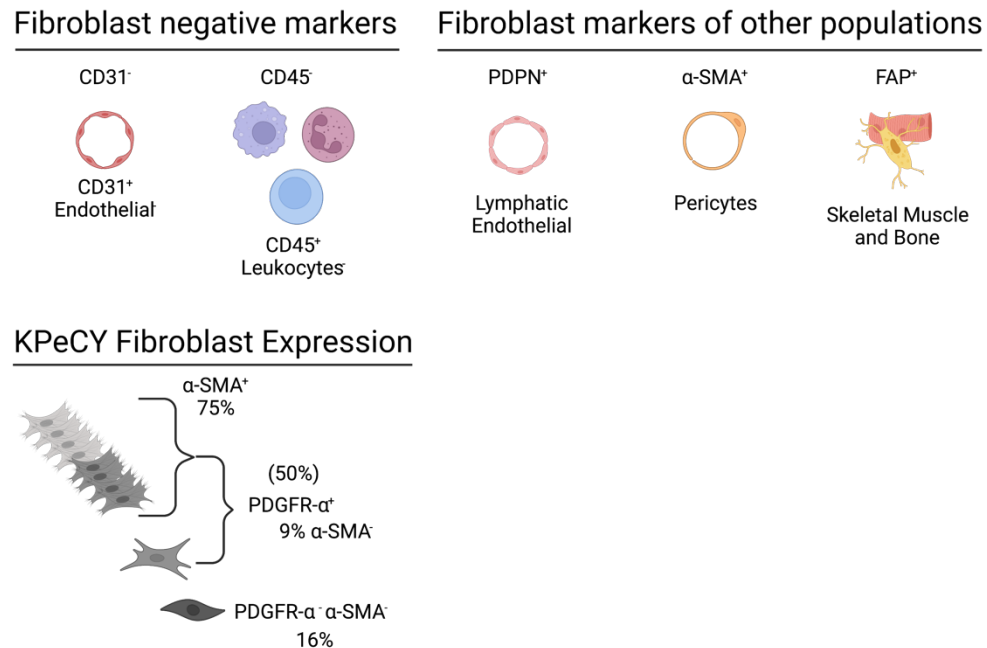


Figure 1-2. Fibroblast Marker Expression.

Prior to the identification of specific markers fibroblasts were identified by the lack of expression of common cell type markers CD31 (endothelial) and CD45 (leukocytes). Common fibroblast markers are not specific lymphatic endothelial cells also express PDPN, pericytes express α-SMA, and skeletal muscle and bone express FAP. There is heterogeneity of marker expression by fibroblasts in a KPeCY model flow cytometry revealed 75% of fibroblasts express α-SMA and 50% of those are α-SMA⁺ PDGFR-α⁺, 9% are PDGFR-α⁺ α-SMA⁻, 16% are α-SMA⁻ PDGFR-α⁻, Heterogeneity of marker expression suggests fibroblast heterogeneity. Created with BioRender.com

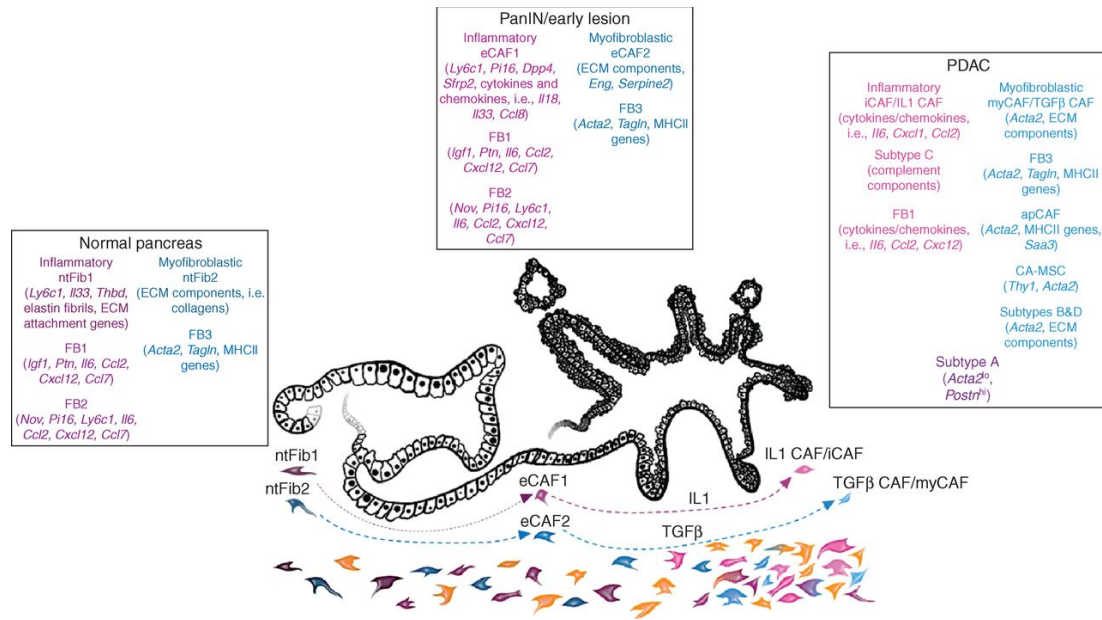


Figure 1-3. An overview of PDAC CAF subtypes identified from transcriptional profiling, including overlap of key markers for each population.

In normal pancreas tissue, early or PanIN lesions, and PDAC, fibroblast transcriptional programs generally fall into two categories: inflammatory signatures including cytokines and other immune modulatory molecules (indicated in shades of pink), and myofibroblastic signatures including classic markers of activated fibroblasts (*Acta2* and *Tagln*), ECM components and remodeling factors, and, in some myofibroblastic subpopulations, genes encoding the MHC II complex (indicated in shades of blue). Single-cell RNA sequencing has identified two (ntFib1 and ntFib2; ref. ⁶¹) or three (FB1, FB2, and FB3; ref. ⁵⁹) fibroblast populations in normal pancreas, and computational modeling suggests that these tissue-resident fibroblast populations likely give rise to CAFs⁶¹. On the basis of transcriptional similarities, it seems that these fibroblasts in normal pancreas tissue give rise to inflammatory (eCAF1 in ref. ⁶¹ and FB1/FB2 in ref. ⁵⁹) or myofibroblastic (eCAF2 in ref. ⁶¹ and FB3 in ref. ⁵⁹) fibroblasts in early lesions (per analysis in ref. 46 and indicated by the dashed arrows). Depending on analysis method, model used, and perhaps other factors, 2 to 4 CAF populations are found in established PDAC, although boundaries seem nondiscrete (e.g., subtype A in ref. ⁶⁰) and subpopulations exist within these CAF designations. An inflammatory population of CAFs (FB1 in ref. ⁵⁹, IL1 CAFs in ref. ⁶¹, iCAF in ref. ⁵¹, and subtype C in ref. ⁶⁰) seems to arise from tissue-resident inflammatory fibroblasts⁶¹ as a result of IL1 signaling^{61,62}. Myofibroblastic CAFs (FB3 in ref. ⁵⁹, TGFβ CAFs in ref. ⁶¹, myCAF in ref. ⁵¹, subtypes B and D in ref. ⁶⁰, and subpopulations of myofibroblastic CAFs including apCAFs in ref. ⁵³ and cancer-associated mesenchymal stem cells or CA-MSC in refs. ^{63,64}) seem to arise from tissue-resident myofibroblastic cells⁶¹ as a result of TGFβ signaling^{61,62}. Additional populations not fitted to these categories may also be present in some PDAC cases perhaps dependent on genotype or stage, indicated here in orange. Key genes expressed by these defined fibroblast or CAF subtypes are listed.

Signaling Gradients

There is evidence that tumor genotype and the downstream cascade of signaling gradients and cytokines shape the heterogeneity of the surrounding stroma, leading to variation in CAF subtypes present. PDAC cell-derived IL-1 and TGF- β regulate the transcriptional paracrine programs of CAFs⁶¹. The IL-1 produced by the tumor cells interacts with the IL1 receptor on CAFs to promote the transcriptional program linked to iCAF, through NF- κ B and LIF, resulting in autocrine JAK-STAT signaling in iCAFs. Opposite the IL-1 signaling driving an iCAF phenotype, TGF- β promotes a myCAF phenotype consistent with the fibrogenic profile^{65,66}. IL-1 and TGF- β act in opposing signaling gradients within the tumor. IL-1 expression is predominant in the stromal areas of the tumor relatively distant from cancer cells, opposing TGF- β in close proximity to the tumor cells or tumor center. TGF- β signaling appears has been shown to have dual fate controls, promoting myCAF phenotype while also suppressing iCAF fate⁶⁴. This dual fate control suggests some mutual exclusivity in CAFs subtypes seen by patient RNA sequencing results⁵³. This is reminiscent of transcription factor gradients determining cells fates in the developing embryo. Independent studies of IL-1 and separate analyses of TGF- β support myCAF and iCAF signaling gradient fate control. In addition to signaling gradients or spatial context, genetics or mutational state of the tumor also controls CAF phenotypes⁶⁷. While Kras is a primary driver of tumor formation, p53 plays a role in tumor formation and CAF heterogeneity^{67,68}.

P53 status determines paracrine signaling shaping nearby CAFs. A mutant KPC model with a gain of function mutation in p53 promotes a prometastatic and chemoresistant CAF profile compared to KPC mice in which tumors are p53 null. Cancer

cell-intrinsic p53 mutation results in higher NF- κ B and its target TNF α compared to p53 null tumors. Downstream TNF α stimulates the secretion of HSPG4 (gene encoding perlecan) by CAFs. Perlecan is an ECM component linked to the prometastatic and chemoresistant landscape of p53 mutant KPC models. p53 has also been correlated to the regulation of neutrophils both local and systemically and metastatic capacity in breast cancer. This regulation of neutrophils by p53 mutation may indirectly impact regulating PDAC CAFs adjacent to tumor cells, suggesting the immune microenvironment and CAF function may be regulated differently by different cancer mutational landscapes⁶⁹. Additional distinct tumor genetic aberrations can coexist within the TME (tumor microenvironment) and may be drivers of CAF heterogeneity.

Evidence suggests that it is not simply signaling gradients or tumor genotype that drive tumor heterogeneity but also CAF cell of origin. As mentioned, differentiation of PSC from quiescent in the normal pancreas was thought to give rise to a majority of activated myofibroblasts in the tumor, but additional mesenchymal origins for PDAC CAFs may indeed be relevant⁷⁰. Activated PSCs have substantial transcriptional plasticity, specifically in ligand-activated nuclear receptor RAR or VDR, which can suppress transcriptional profiles of activated CAFs. PSC transcriptional plasticity leads to a spectrum of activation states, including a fibroinflammatory potential for PSC CAFs^{71,72}. While it was thought that PSC gives rise to a majority of CAFs, a recent study identified two pancreas-resident fibroblast populations, one marked by GLI1 expression and another marked by HOXB6. The GLI1⁺ fibroblast population expands during carcinogenesis whereas HOXB6⁺ fibroblasts do not⁷³. In addition to a resident population of fibroblasts and PSC, mesenchymal stem cells give rise to a minor subset of CAFs.

These mesenchymal stem cell-derived CAFs were identified in primary patient samples and are a critical source of cytokine GM-CSF⁶³. More and more data suggest that CAF origin contributes to the heterogeneity of CAFs and may potentially coincide with the signaling gradients by which populations are activated. GM-CSF promotes PDAC proliferation and may play a broader role in the recruitment of the bone marrow niche to the PDAC CAF pool. Mesenchymal stem cells in murine PDAC regulate the promotion of macrophage function⁶⁴. In turn, studies have shown that macrophage OSM (Oncostatin M) stimulates inflammatory gene expression in CAFs through OSM receptors. These inflammatory gene-expressing CAFs promote tumor survival and migration signaling pathways⁷⁴. Further studies are needed to identify how origin impacts the role of CAFs to impact therapeutics more specifically via the alteration of CAFs.

Thesis Aims

The overall hypothesis of this dissertation is that pancreatic stellate cells activate to a fibroblast phenotype that plays a significant role in the ECM and tissue stiffness of PDAC. To evaluate this hypothesis we used mouse models, isolated cultured cells, and patient tumor samples as well as clinical RNA sequencing data from PDAC patients. The following chapters will address the following three aims.

The first aim was to investigate the use of an FABP4-CRE (fatty acid binding protein 4) mouse model for the tracking of pancreatic stellate cells within the normal pancreas as well as the pancreatic tumor microenvironment. This aim will address the unanswered question of do stellate cells give rise to a majority of CAFs in the pancreatic tumor, as well as provides a model to track them which was previously not available.

The second aim addresses the question of what role do stellate cell derived CAFs play in the tumor microenvironment. Answering this question would provide a basic understanding of what role cell of origin plays in CAF heterogeneity. Understanding to what extent stellate cells give rise to CAFs would provide critical information on targeting of stellate cells to provide improved combinatorial treatments in PDAC. While the role of signaling has been shown to play a role in heterogeneity. However it is not known what impact tumor genotype and downstream signaling gradients have on cell of origin and resulting CAF heterogeneity. Specific gene mutants of tumor cells may impact fibroblast origin cells differently driving certain subtypes activity and role in the TME. To investigate the role of stellate cells in the tumor environment we utilized a CRE-inducible diphtheria toxin receptor mouse with an AAV-FABP4-CRE ductal injection to selectively knockout stellate cells in an orthotopic tumor model.

A third aim was to explore the role of stellate cell derived CAFs in metastasis and survival. This study utilized cell lines derived from KPC mice previously identified to metastasize to the liver and lungs. In addition we developed of a genetically engineered mouse model that tracks stellate cells over the course of PDAC initiation and progression. We aimed to create a mouse that would allow us to answer previously unreachable questions about stroma in PDAC. Questions such as what role does CAF cell of origin play in metastasis? Do signaling of specific tumor cell mutation change the role of stellate cell derived CAFs? Is there a therapeutic target for stellate cells to improve survival? Understanding how signaling gradients impact cell of origin and further the role of the CAF, will provide important information regarding targeting the stroma to provide improved therapeutic options in PDAC.

The work herein supports the hypothesis that pancreatic stellate cells activate to a fibroblast phenotype that plays a significant role in the ECM and tissue stiffness of PDAC. Furthermore, this work suggests a potential avenue to target the protumor pancreatic stellate cell derived CAFs therapeutically in combination with current therapies to improve survival and reduce metastasis. Chapter 4 discusses future directions.

Chapter 2 : Mesenchymal Lineage Heterogeneity Underlies Non-Redundant Functions of Pancreatic Cancer-Associated Fibroblasts

Adapted from Peer Reviewed Published Manuscript⁷⁵

Helms EJ, Berry MW, Chaw RC, DuFort CC, Sun D, Onate MK, Oon C, Bhattacharyya S, Sanford-Crane H, Horton W, Finan JM, Sattler A, Makar R, Dawson DW, Xia Z, Hingorani SR, Sherman MH. Mesenchymal Lineage Heterogeneity Underlies Non-Redundant Functions of Pancreatic Cancer-Associated Fibroblasts. Cancer Discov. 2022 Feb;candisc.0601.2021. doi: 10.1158/2159-8290.CD-21-0601. PMID: 34548310.

Abstract

Cancer-associated fibroblast (CAF) are increasingly implicated in pancreatic cancer, but the origin of these cells and the function of distinct subsets remain inadequately understood. It is thought that pancreatic stellate cells are the source of all abundant and transcriptionally diverse CAFs in PDAC, implicating cytokines and growth factor gradients as the source of previously described CAF heterogeneity. Herein we investigated the function and differentiation of PSCs in vivo using a novel mouse model enabling PSC tracking in their host tissue. Contrary to current dogma, we found that PSCs gives rise to a numerically minor subset of PDAC CAFs. Through ablation of PSC-derived CAFs, we discovered the non-redundant function of these cells in shaping the PDAC microenvironment through the production of specific ECM components, mechanosensing, and tissue stiffness regulation. These findings collectively link stromal

evolution to the cell of origin, which in turn is connected to transcriptional heterogeneity and the unique functions based on the definition of the cell of origin.

Significance

Through specific tracking and ablation of PSCs, we discovered that they give rise to a numerically small subtype of CAFs that plays a significant role in the PDAC TME.

Along with the increasing studies of fibroblasts, it is clear that mesenchymal lineage heterogeneity along with signaling gradients shape the diversity of CAFs.

Introduction

PDAC is a devastating disease characterized by a dense stroma comprising up to 70% of the tumor mass. This exuberant stroma is comprised of mainly fibroblasts and the ECM they produce as well as vasculature and immune cells^{50,76–78}. Fibroblasts are thought to play a tumor-supportive role in providing metabolic nutrient transfer in a nutrient-poor hypoxic TME, thereby facilitating proliferation^{79–83}. CAFs also produce cytokines and chemokines that modulate the immune repertoire resulting in immune suppression. Ablation of CAFs in mouse models of PDAC results in the increased efficacy of immune checkpoint inhibitors^{47,53,57,64,84,85}. In the normal pancreas, activated fibroblasts play a role in wound healing by producing ECM and ECM remodeling enzymes. In the tumor, the wound healing properties of fibroblast are corrupted, leading to a dense network of dysregulated ECM. This dense dysregulated ECM impedes vasculature leading to hypoxia, and limits the delivery of intravenously administered therapeutics. Patients with high levels of ECM proteins such as TN-C (tenascin C) and

stiff fibrotic tumors have shortened survival^{86–88}. The identification of fibroblast tumor-supportive roles has motivated increased efforts to develop fibroblast-targeted therapies. These therapies include targeting CAFs through pathways that regulate phenotypes (hedgehog) or immune-modulatory pathways like CXCL12 and CXCR4, as well as targeting the ECM itself (hyaluronic acid)^{57,89–93}.

Thus far, however, targeting the stroma and the impact of CAFs on PDAC progression have become controversial. This controversy is due to results of genetic or pharmacologic ablation of CAFs in mice (through α -SMA or SHH), resulting in poorly differentiated tumors and worse so shortened survival, utterly contrary to expectations of a completely protumor stroma^{46,47,94}. Studies where the ECM is depleted rather than fibroblasts, such as collagen I, also accelerated mortality. Along the same lines, a study targeting collagen crosslinking by LOXL2 also found reduced survival as well as increased growth^{95,96}. On the other hand, in PDAC, patients with a higher stromal density (cellular and acellular) had a longer overall survival^{49,96}. While these ablation studies suggest a tumor-suppressive or homeostatic potential of CAFs, further investigation of this compartment is needed to elucidate the complexity with respect to disease progression.

While these findings are seemingly contradictory, it has been postulated that PDAC CAFs are heterogeneous with tumor growth suppressive roles from some subtypes and supportive functions of others. Single-cell RNA sequencing studies have further reconciled these opposing results, showing transcriptional heterogeneity in both PDAC mouse models and human PDAC^{51,53,59,60,64}. Essential consideration should be given to CAF subtype origin and increasingly important subtype functions. Thus far,

therapies to target the stroma to treat PDAC have been unsuccessful, which indicates that it is crucial to understand the potential roles of different CAF subtypes. It is of utmost importance to identify and specifically target tumor-promoting mechanisms by blocking the development or activation of tumor-supportive CAFs. Understanding tumor supportive mechanisms may increase the viability of combination therapeutic strategies. Currently, tracking models of PDAC CAFs are lacking, and it is vital to develop new models to track and manipulate CAFs within host tissues in a robust and specific way, based on the cell of origin.

Stellate cells are a resident mesenchymal cell type of the pancreas and thought to be the common origin of PDAC CAFs^{15,97}. Previously called ITO cells, and once thought to be lipocytes, stellate cells are found in the pancreas as well as the liver^{16,98}. Hepatic stellate cells have been traced by various Cre-based models that identified them to be the source of CAFs in liver cancers. These HSC Cre models have not been useful in the pancreas^{18,99}. Unfortunately, due to the lack of PSC Cre models, what is known about HSCs has been extrapolated to PSC biology. Cell culture models have also been used to study PSCs, but these lack the impact of other cells on PSC activation.

Stellate cells are quiescent in a healthy normal pancreas, storing lipid droplets containing vitamin A as retinyl esters in their cytoplasm. Quiescent stellate cells are implicated to play a role in tissue homeostasis by recycling the basement membrane. However, our understanding of PSC normal function is limited due to a lack of specific models¹⁰⁰. Isolated by density gradient centrifugation, stellate cells activate to a myofibroblast phenotype on stiff growth substrates such as standard tissue culture plastic. The lipids content of stellate cells is what allows for the density gradient isolation and

allows for the study of them in vitro. Transcriptional profiling of isolated, activated PSCs shows upregulation of ECM components and remodeling enzymes, growth factors other signatures consistent with CAFs, suggesting that PSCs are competent to give rise to CAFs during PDAC progression. However, these pancreatic stellate cells have not been tracked in vivo nor through activation in the context of tumorigenesis. Therefore, the contribution of PSCs to CAFs and the PDAC TME remains unknown. The function of PSC derived CAFs is also unknown in the context of PDAC. Recent work show signaling gradients of critical factors such as TGF- β and IL-1 can differentially program PSCs into inflammatory CAFs (iCAF) and myofibroblasts (myCAF) fates in vitro^{61,64}. To analyze PSCs' fates in vivo, we developed a mouse model to track and ablate PSC within the pancreas by leveraging their unique lipid storing capacity. We found that PSCs give rise to a numerically small subset of CAFs, suggesting that other unknown cell types give rise to a majority of CAFs. PSC CAFs, while a small subset, plays a significant non-redundant role in the modulation of the TME. PSCs CAFs modulate the TME by producing specific ECM components and tissue stiffness, suggesting these cells or their regulators may be therapeutic targets.

Results

Characterization of a Genetic Approach to Label and Track Stellate Cells in Normal Pancreas Tissue

RNA-seq data from isolated primary quiescent PSCs showed high expression of fatty acid binding protein 4 (Fabp4), a marker of adipocytes⁷². Originally adipocyte protein 2 (ap2) or fabp4 was thought to be a adipocyte specific marker in mice using ap2-

CRE. These ap2 mice were originally used for the study of PPAR- γ in insulin resistance, as ap2 is downstream of PPAR- γ . Fabp4 is a lipid chaperone with a role in regulating lipolysis in adipocytes¹⁰¹. Based on this high expression of Fabp4, a Cre-based approach to label and track PSCs was identified, previously used to study fat cells¹⁰². While quiescent PSCs express Fabp4, it is downregulated in the activated fibroblastic state. It is still reasoned that a lineage labeling approach driven by Fabp4 regulatory elements could label PSCs and CAFs that derive from them. To utilize Fabp4 to label and track PSCs, Fabp4-Cre mice were crossed to mTmG reporter mice. The mTmG report mouse contains an actab tdtomato egfp reporter allele (Figure 2-1A)^{102,103}. The Rosa26 promoter is followed by loxP sites on either side of tdtomato, and a stop codon, followed by GFP. When there is expression of Fabp4, the promoter is active, Cre is expressed, and the stop codon and tdtomato are excised, resulting in the expression of GFP. In a mouse with Fabp4-Cre and mTmG, only cells with Fabp4 activity and their progeny will be GFP positive, with the remaining cells tdtomato⁺. When we analyzed the pancreas of Fabp4-Cre; Rosa^{mTmG} mice, we noted rare GFP cells in the peri acinar spaces. These GFP cells' morphology and frequency match that of published PSC characteristics in addition to electron microscopy analysis (Figure 2-1B)^{25,104}. To examine the specificity of the GFP cells, we analyzed the GFP cells for markers of other pancreatic cell types that PSCs lack. Expression of markers CD31 for endothelial cells, CD45 for leukocytes, and NG2 for pericytes was restricted to the Tdtomato⁺ population and lacked the GFP population lineage label (Figure 2-1C,1D,1E; supplementary Fig. S1A). While expression of CD31⁺ was restricted to Tdtomato⁺ cells, there was perivascular localization of a subset of PSC GFP⁺ cells as reported for HSCs (Figure 2-1C)¹⁸. To further assess specificity, we

isolated $tdtomato^+$ and GFP^+ from mouse pancreas by FACs and measured by qPCR expression of markers of various pancreatic cell types (supplementary Fig. 2-S1B, Figure 2-1F). CSPG4 encoded NG2; a pericyte marker was not expressed in the GFP fraction. This gene expression data suggests that the numerically small population of pericytes is within the larger $tdtomato$ population (liver was a positive control). Acinar, ductal, and β cell markers were restricted to the $tdtomato$ fraction, while mesenchymal marker Vim and putative stellate cell/mesenchymal marker Des were enriched in the GFP fraction. Our results suggest that GFP marks PSCs in $Fabp4-Cre; Rosa26^{mTmG}$ mice pancreata.

Next, we assessed how pervasively GFP marks PSCs. We analyzed this in two ways. First, we isolated PSCs from $Fabp4-Cre; Rosa26^{mTmG}$ mice pancreata by density gradient centrifugation, as the lipid contents of quiescent PSCs cause them to float. As this density gradient isolation is an enrichment, not a purification, we plated the cells isolated at the interface and stained for Desmin to increase our confidence that we did not have contamination by cell types of similar density. Nearly all cells in this isolation were GFP PSCs (Figure 2-1G, Supplementary Fig. 2-S1C). As a second analysis of pervasiveness, we took advantage of the best-known function of stellate cells to store vitamin A in their cytoplasmic lipid droplets as retinyl esters. These vitamin A retinyl esters cause stellate cells to emit a blue-green autofluorescence that can be analyzed by flow cytometry, previously shown in HSCs¹⁰⁵. Flow cytometry of single-cell suspensions from $Fabp4-Cre; Rosa26^{mTmG}$ mice normal pancreas revealed expected vitamin A⁺ PSC frequencies (Supplementary Fig. 2-S1D). Our results indicate that nearly all these vitamin A⁺ cells were GFP^+ (Figure 2-1H). In effect, these results suggest $Fabp4-Cre;$

Rosa26^{mTmG} mice feature specific and pervasive GFP labeling of stellate cells in the pancreas.

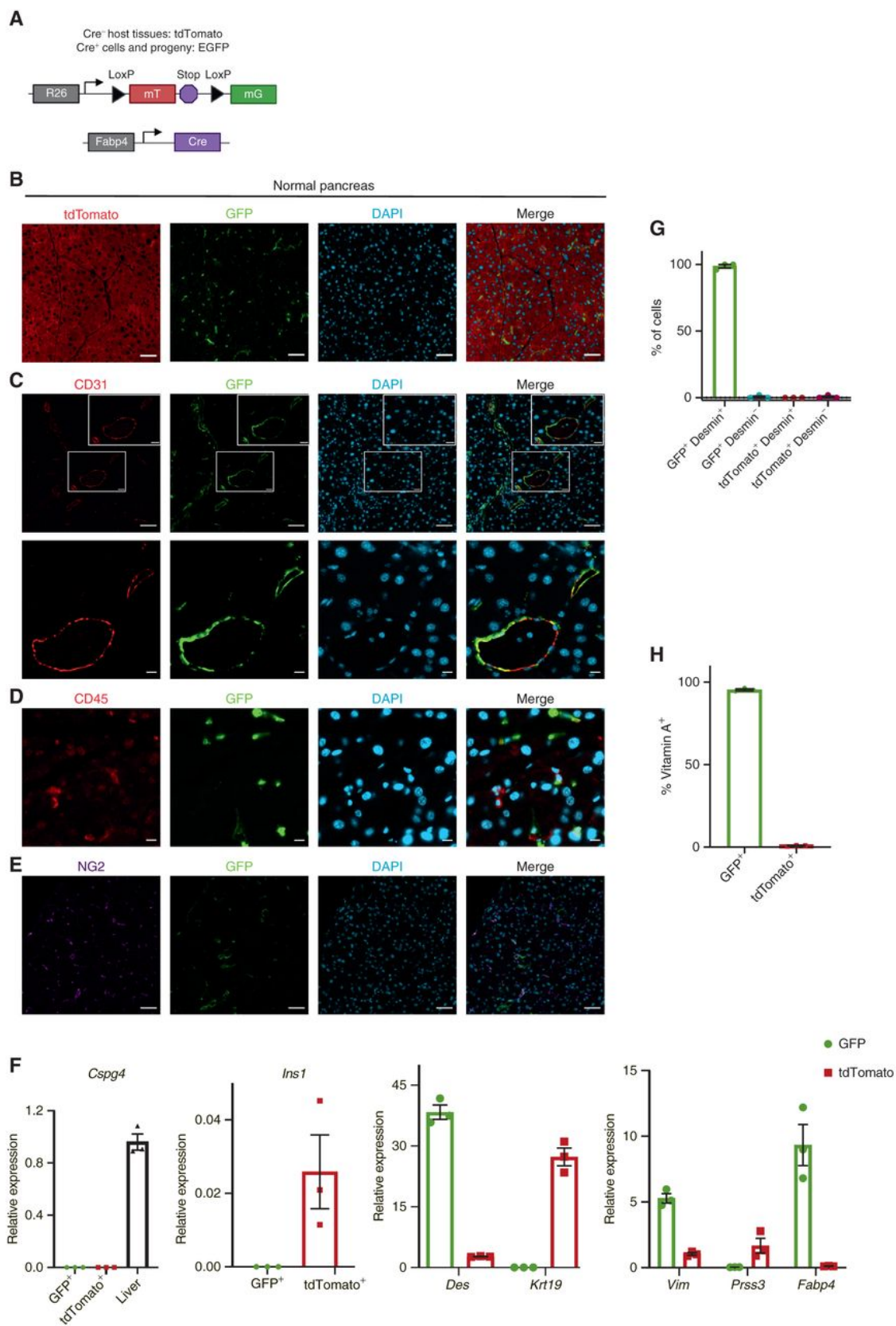


Figure 0-1. Fabp4-Cre marks stellate cells specifically and pervasively within normal pancreas tissue.

(A) Schematic of the alleles used to label and track PSCs in vivo. Created with BioRender.com (B) Representative image of normal pancreas tissue from Fabp4-Cre;Rosa26^{mTmG} mice (n = 7) showing rare GFP⁺ cells within a predominantly tdTomato⁺ tissue. Scale bar, 50 μ m. (C) Representative image of normal pancreas tissue from Fabp4-Cre;Rosa26^{mTmG} mice (n = 3) showing CD31⁺ endothelial cells and GFP⁺ cells, some of which are adjacent to vessels. Top images: scale bar, 50 μ m; bottom images: scale bar, 10 μ m. (D) Representative image of normal pancreas tissue from Fabp4-Cre;Rosa26^{mTmG} mice (n = 3) showing CD45⁺ leukocytes and GFP⁺ cells. Scale bar, 10 μ m. (E) Representative image of normal pancreas tissue from Fabp4-Cre;Rosa26^{mTmG} mice (n = 3) showing NG2⁺ pericytes and GFP⁺ cells. Scale bar, 50 μ m. (F) qPCR for the indicated genes in GFP⁺ and tdTomato⁺ cells isolated from normal pancreas tissue from Fabp4-Cre;Rosa26^{mTmG} mice by FACS (n = 3, with each replicate pooled from two mice), including markers of pericytes (Cspg4; liver is a positive control), stellate cells and potentially other mesenchymal cells (Des), ductal cells (Krt19), mesenchymal cells (Vim), acinar cells (Prss3), and β cells (Ins1); Fabp4 was included as a control. Data were normalized to 36b4 and are presented as mean $\pm$ SEM. (G) Quantification of GFP⁺ cells and tdTomato⁺ cells out of total, desmin⁺ PSCs isolated by density centrifugation from normal pancreas tissue in Fabp4-Cre;Rosa26^{mTmG} mice (n = 3) and analyzed by immunofluorescence microscopy. Data are presented as mean $\pm$ SEM. (H) Flow cytometry results depicting GFP⁺ and tdTomato⁺ cells among all vitamin A⁺ PSCs in normal pancreas tissue from Fabp4-Cre;Rosa26^{mTmG} mice (n = 3). Data are presented as mean $\pm$ SEM.

Analysis of Stellate Cell Contribution to the CAF Pool in the PDAC

Microenvironment

Our Fabp4-Cre; Rosa26^{mTmG} model allows us to track the fate of PSCs during pancreatic tumorigenesis and formally address PSCs' contribution to the PDAC CAF population. When we orthotopically implanted Kras^{LSL-G12D/+}; Trp53^{LSL-R172H/+}; Pdx1-Cre (KPC)¹⁰⁶ PDAC cells into Fabp4-Cre; Rosa26^{mTmG} mice, we consistently saw the expansion of GFP⁺ cells in the tumor microenvironment with a CAF-like morphology (Figure 2-2A). To characterize the GFP⁺ cells in the tumor, we stained for CAF markers α -SMA and pan-CAF marker podoplanin (PDPN)^{51,53}. GFP⁺ cells expressed both CAF markers confirming that PSCs give rise to PDAC CAFs; however, it demonstrates that

they yield a subset of CAFs within the myCAF population (Figure 2-2B, C). We acknowledge that transplantation models have limitations; however, our model has similar total PDPN positive cell frequencies to those reported in autochthonous PDAC (Figure 2-2D)⁶⁴. Contrary to our expectations across PDAC models, PSCs gave rise to a minority of CAFs, raising the possibility that transcriptional heterogeneity among CAFs is in part due to cells of origin. We confirmed our findings by flow cytometry in two different KPC-derived models and with two different CAF cell-markers (PDPN or PDGFR α). In these two models, PSC-derived CAFs give rise to approximately 10-15% of total PDPN⁺ CAFs (Figure 2-2E,F,G). These results suggest PSCs give rise to a numerically minor subset of CAFs, which prompted us to examine if PSC-derived CAF populations play unique roles in the tumor microenvironment or if these CAFs harbor similar transcriptional profiles and functions to CAFs of other origins.

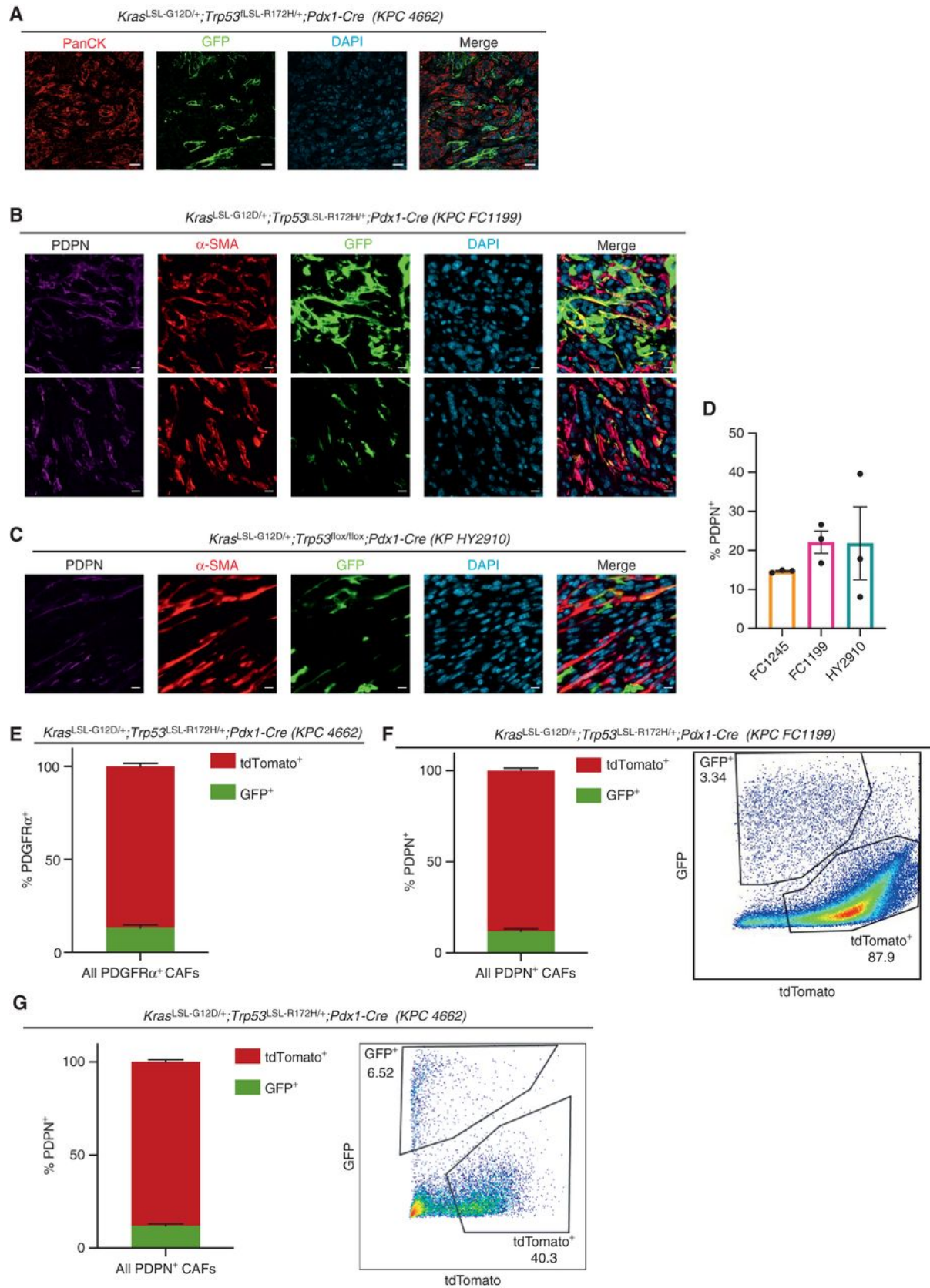


Figure 0-2. Stellate cells give rise to a numerically minor subset of PDAC CAFs.

(A) IHC staining of PDAC (KPC 4662) in Fabp4-Cre;Rosa26^{mTmG} hosts (n = 5), with GFP in green and panCK (tumor cells) in red. Scale bar, 20 μ m. (B) IHC staining of PDAC (KPC FC1199) in Fabp4-Cre;Rosa26^{mTmG} hosts (n = 3), stained for GFP, PDPN, and α -SMA. Scale bar, 10 μ m. (C) IHC staining of PDAC (KP^{flox/+}C HY2910) in Fabp4-Cre;Rosa26^{mTmG} hosts (n = 3), stained for GFP, PDPN, and α -SMA. Scale bar, 10 μ m. (D) Quantification of IHC staining for PDPN in the indicated transplantable PDAC models. (E) Flow cytometry analysis of PDGFR α , GFP, and tdTomato in KPC 4662 tumors in Fabp4-Cre;Rosa26^{mTmG} hosts (n = 5). Data are presented as mean $\pm$ SEM. For E–G, CAF frequency calculations excluded the tdTomato[−]GFP[−] fraction constituting transplanted PDAC cells. (F) Flow cytometry analysis of PDPN, GFP, and tdTomato in KPC FC1199 tumors in Fabp4-Cre;Rosa26^{mTmG} hosts (n = 8). Data are presented as mean $\pm$ SEM. (G) Flow cytometry analysis of PDPN, GFP, and tdTomato in KPC 4662 tumors in Fabp4-Cre;Rosa26^{mTmG} hosts (n = 3). Data are presented as mean $\pm$ SEM.

Distinct transcriptional profiles of CAFs from a stellate cell or non-stellate-cell origin

Our next step was to determine the role of PSC-derived CAFs by analyzing transcriptional profiles to find if they are redundant in function to CAFs of other origins. To assess redundancy, we implanted established KPC cell lines into the pancreas of Fabp4-Cre; Rosa26^{mTmG} mice. From these orthotopic tumors, we isolated GFP PDPN⁺ cells (PSC origin) as well as Tdtomato PDPN⁺ cells (non PSC origin) by FACs and performed RNAseq (supplementary Table 2-1). Both GFP and Tdtomato populations expressed broad pan-CAF markers FAP, PDPN, ACTA2, Lrrc15, and similar levels of most collagen genes⁶⁴. However, we identified vital transcriptional differences in CAF populations suggesting transcriptional heterogeneity among PDAC CAFs is influenced by cytokines and growth factor gradients in the TME but also mesenchymal lineage (log2 fold change >1, upregulated in tdTomato+: 1,163 genes; log2 fold change <−1, upregulated in GFP+: 427 genes) (Figure 2-3A, B). Gene ontology analysis of our RNAseq revealed PSC CAFs had enrichment of genes involved in cell adhesion, ECM-

receptor interaction, and axon guidance (Figure 2-3C). Gene enrichment analysis of non-PSC CAFs had significant enrichment for immune-modulatory processes, “inflammatory response,” and “complement,” as well as metabolic processes including hypoxia (Figure 2-3D). PSC CAFs expression enrichment showed cell adhesion molecules that facilitate leukocyte trafficking and/or cancer cell spatial patterning that has not been previously characterized to CAFs, including Tie1 (tyrosine kinase With Immunoglobulin Like And EGF Like Domains 1) ; however, Tek/Tie2 was not enriched in PSC CAFs (Figure 3E). In addition to cell adhesion molecules, PSC CAFs had unique enrichment for ECM components implicated in tissue stiffness and PDAC aggressiveness, including TNC and Hspg4 (encodes perlecan) (Figure 2-3E)⁸⁸. Differential expression of Hspg4 gained significance light of a recent in study comparing CAFs from genetically engineered mouse models of PDAC with mutant KRAS and p53 mutant (R172H) compared to KRAS with p53 loss⁶⁷. Stromal expression of perlecan drives mutant p53 (R172H) PDAC to have significantly more pro-metastatic behavior and chemoresistance when compared to p53 null tumors. Axon guidance cues were also significantly enriched in PSC CAFs, including those of the Slit/Robo family with potential implications of CAF regulation of tumor innervation. Enrichment of immune-modulatory cytokines and chemokines and genes encoding the major histocompatibility complex class II in the tdtomato CAF fraction suggests that iCAFs and apCAFs are not of PSC origin^{51,53}. Our data of differentially expressed genes are supported by comparison to single-cell RNA-seq data from KPC mice which revealed that genes differentially expressed in PSC-derived CAFs vs. non-PSC-derived CAFs are significantly enriched in iCAFs and apCAFs compared to myCAFs (Figure 2-3F; see Supplementary Methods). iCAF and

myCAF fate specification receptors for secreted ligands such as TGF β and IL1 are expressed at similar levels in tdtomato CAFs vs. GFP CAF populations, indicating that epigenetic state or downstream signaling components may promote PSCs myCAF transcriptional state.

Next, to validate our mouse model findings concerning PSC-derived CAF frequencies, we selected marker combination α -SMA and Tie1 from our RNAseq datasets. While Tie1 is highly expressed on endothelial cells, it is unique to PSC-derived CAFs among total CAFs; combined with α -SMA, we could identify PSC-derived CAF frequencies in human patient samples (supplementary Fig. 2-S2B and 2-S2C). Consistent with our mouse models, a cohort of PDAC patient tumor sections showed a minor population of Tie1⁺ α SMA⁺ CAFs in a more expansive α -SMA⁺ population (Figure 2-3G). To analyze frequencies of PSC-derived CAFs more extensively as well as heterogeneity across human PDAC, we used a tumor microarray containing four spatially distinct punches for each of 153 patients. We co-stained this array for Tie1 and α -SMA and quantified the double-positive cells out of total α -SMA and found evidence of heterogeneity across patients. Some patients have high numbers of PSC CAFs and others low levels (Figure 2-3H). Two conclusions can be drawn from these CAF frequencies (Figure 2-3I). First, almost all samples harbored some putative PSC-derived CAFs at frequencies similar to our mouse models. Second, all patient samples showed putative PSC-derived CAFs were a minority of CAFs in the tumor microenvironment, highlighting the mesenchymal lineage results in heterogeneity. We validated our patient findings with an independent cohort of 43 patient samples; while this is a smaller set, they were whole tissue sections compared to small punches. This smaller cohort showed

similar frequencies of PSC-derived CAFs to those we observed in the tissue microarray as well as our mouse model (supplementary Figure 2-S2D and Figure 2-3J). All these data sets indicate a minority of CAFs are of PSC origin. In essence, our results suggest mesenchymal lineage heterogeneity underlies transcriptional heterogeneity among PDAC CAFs, which is relevant to PDAC.

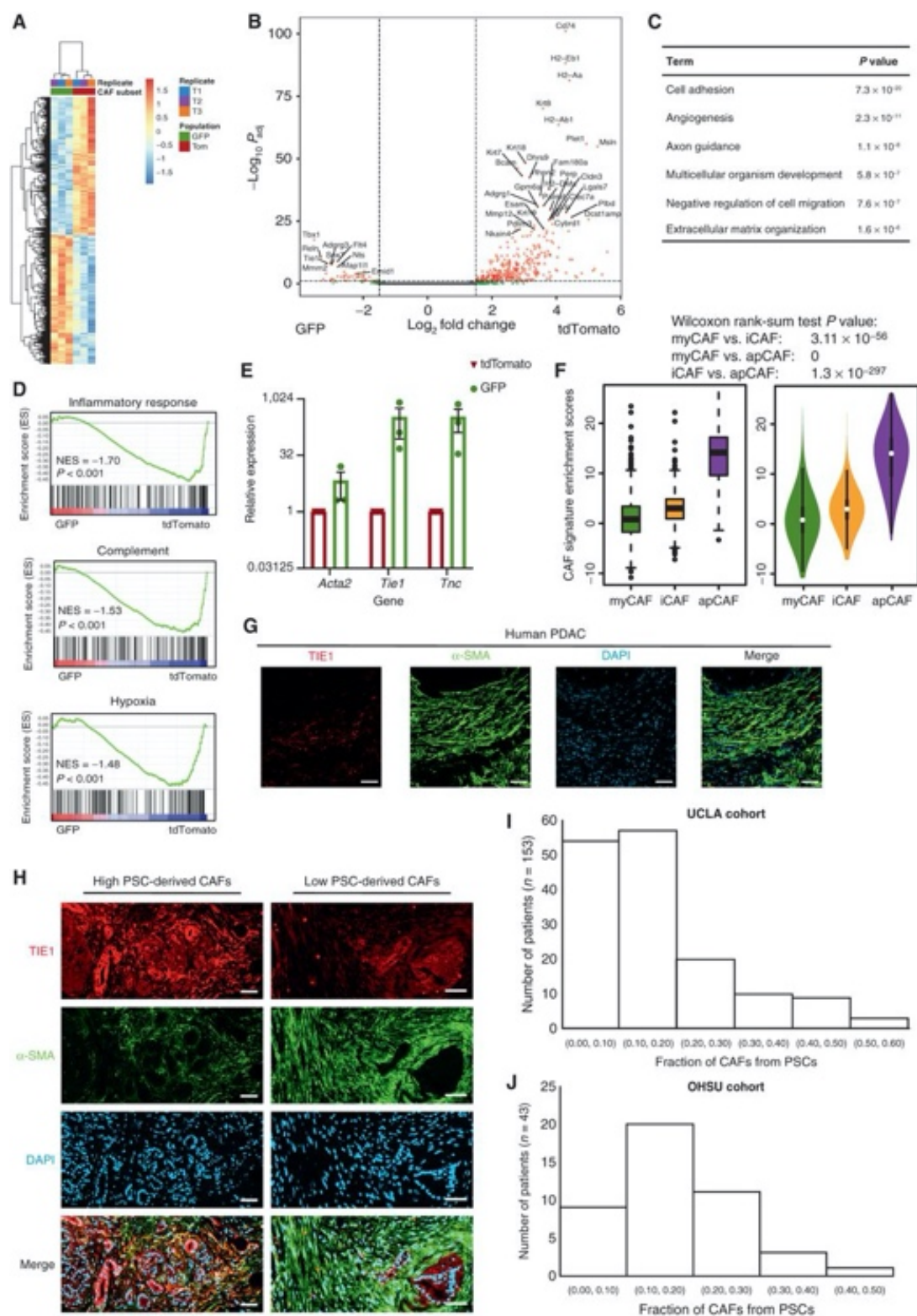


Figure 0-3. Mesenchymal lineage heterogeneity gives rise to transcriptional heterogeneity among PDAC CAFs.

Heat map (A) and volcano plot (B) depicting differentially expressed genes in PSC-derived (GFP^+) versus non-PSC-derived (tdTomato^+) CAFs from KPC FC1199 PDAC in $\text{Fabp4-Cre;Rosa26}^{\text{mTmG}}$ hosts ($n = 3$), identified by RNA-seq. In B, gray circles indicate genes not different between the populations, green circles indicate genes with $\log_2$ fold change >1 or <-1 , and pink circles indicate genes with the same $\log_2$ fold change criteria and $P < 0.05$. (C) Gene ontology analysis identifying the top terms enriched in association with genes upregulated at least twofold in PSC-derived CAFs compared with non-PSC-derived CAFs. (D) Gene set enrichment analysis showing pathways or processes with transcriptional signatures enriched in the tdTomato^+ CAF population. NES, normalized enrichment score. (E) qPCR for the indicated genes on PSC-derived and non-PSC-derived CAFs sorted from KPC FC1199 PDAC in $\text{Fabp4-Cre;Rosa26}^{\text{mTmG}}$ hosts ($n = 3$) by FACS. Data were normalized to 36b4 and are presented as mean $\pm$ SEM. (F) Box (left) and violin (right) plots indicating enrichment scores for differentially expressed genes between GFP^+ and tdTomato^+ CAFs among myCAF-, iCAF-, and apCAF-associated genes per previously published small conditional RNA-seq results. (G) Representative IHC staining of human PDAC for TIE1 and α -SMA ($n = 5$). Scale bar, 50 μm . (H) Representative images from a human PDAC microarray after IHC staining for TIE1 and α -SMA ($n = 153$). Scale bar, 50 μm . (I) Quantification of $\text{TIE1}^+\alpha\text{-SMA}^+$ area out of total $\alpha\text{-SMA}^+$ area on each patient sample from the array. Different regions from the same patient were averaged together to yield one frequency per patient sample (four punches per patient, 612 total tumor regions analyzed, 153 plotted here after averaging for each patient). (J) Quantification of $\text{TIE1}^+\alpha\text{-SMA}^+$ area out of total $\alpha\text{-SMA}^+$ area using whole PDAC tissue sections ($n = 43$) from an independent patient cohort.

Targeted ablation of PSC-derived CAFs via retrograde ductal injection of viral CRE to unveil functional significance

We next interrogated if PSC-derived CAFs' unique transcriptional profile translated to a non-redundant role in PDAC. Ablation of PSC-derived CAFs allowed us to analyze their impact on the PDAC TME. In order to ablate PSC-derived CAFs, we crossed Cre-inducible DTR mice to $\text{Fabp4-Cre; Rosa26}^{\text{mTmG}}$ mice. Treatment of Diphtheria Toxin (DT) to the resulting offspring would lead to PSC-derived CAF depletion¹⁰⁷. Two limitations of this model needed to be overcome. Systemic ablation of Fabp4 -expressing cells would lead to ablation of adipocytes as they have high expression of the Cre, which would confound our interpretation of results. The second obstacle was

determined while characterizing the model where we noted a small population of CD45⁺ GFP⁺ cells comprising about 2% of leukocytes that would also be targeted for ablation with the DTR strategy. On this second point, we did not observe CD45⁺ GFP⁺ in normal pancreas, suggesting that Fabp4-expressing leukocytes are not resident but rather are recruited during tumor progression. An adapted retrograde ductal injection of viral particles directly to the pancreas addresses both limitations and enables specific targeting of PSC-derived CAFs^{108,109}. Viral transduction of Fabp4-Cre allows for spatial control to the pancreas as well as negates the adipose, hematopoietic, and any other cells or tissues that have Fabp4 activity. This viral transduction also enables a somatic introduction of Fabp4-Cre into an adult mouse. A minimal Fabp4 promoter and enhancer element were needed to fit upstream of the Cre in the AAV vector but needed to be substantial enough to activate in primary PSCs. Pilot experiments revealed that KP1 was an optimal serotype to transduce PSCs. In vivo ductal injection of AAV-KP1 Fabp4-Cre resulted in PSC labeling to a similar extent in mice with a Fabp4-Cre allele (Figure 2-4A).

After overcoming these obstacles, we moved to tumor modeling; we took our Rosa26^{mTmG/iDTR}, subjecting them to a single surgery which included a ductal injection of AAVKP1-Fabp4-Cre as well as an orthotopic injection of KPC PDAC cells (Figure 2-4B). Established tumors had expected frequencies of GFP⁺ cells while lacking CD45⁺ GFP⁺ cells. As PDPN is acquired upon activation to the CAF phenotype, it is suspected that GFP cells that lack PDPN are in a quiescent or not fully activated state. To assess the function of PSC-derived CAFs directly instead of secondary impacts of other cells, we established a tumor-host with AAV. We enrolled mice when tumors reached 5-6 mm in diameter by ultrasound measurement. We enrolled size-matched tumors with either

vehicle or DT over the course of 5 days leading to acute ablation of PSC-derived CAFs. After 5 days of dosing, we harvested tumors for analysis. This short-term PSC ablation did not visibly disrupt histology of the normal pancreas nor PDAC. Consistent with PSCs giving rise to a minor set of CAFs, selective targeting of a numerically minor population resulted in DT tumors with high levels of CAFs expressing α -SMA (Figure 2-4C). Based on transcriptional profiling analysis indicating enrichment of ECM components in the GFP or PSC-derived CAF subset, we found TNC was reduced with PSC ablation (Figure 2-4D). We next analyzed total collagen abundance and found it unchanged, suggesting PSC-derived CAFs regulate specific ECM components. Further analysis of collagen by second-harmonic generation revealed fibrillar collagen abundance modestly decreased with PSC-derived CAF ablation, but it was not significant (Figure 2-4E,F). Looking into the broader role of ECM regulation and mechanosignaling, p-MLC2 (phosphor-myosin, light chain 2) was reduced with PSC-derived CAF ablation consistent with decreased tension (Figure 2-4G). In order to assess tumor stiffness directly, we measured Young's modulus in control and PSC-depleted PDAC by atomic force microscopy. PSC-derived CAF ablation tumors were significantly softer compared to controls (Figure 2-4H,I) (Young's modulus for control = 1.02 ± 0.02 kPa, PSC- depleted = 0.74 ± 0.04 kPa; normal murine pancreas ≈ 0.50 kPa)¹¹⁰. FAK signaling has been shown to facilitate tissue stiffness and tumor-promoting mechanisms in PDAC^{88,111}. We measured pFAK (Y397) and found that these signaling events were markedly reduced in PSC-derived CAF ablated tumors (Figure 2-4J). In addition, we assessed proliferation and apoptosis but found no significant difference with PSC-derived CAF ablation in tumors. The abundance of immune populations is linked to CAF function, including CD8⁺ T cells and

myeloid cells. We found no difference in CD8⁺ T cell abundance with PSC-derived CAF ablation, whereas CD68⁺ myeloid cells abundance was significantly albeit modestly reduced with ablation (Supplementary Figures 2-S3F-S3I). We do not exclude a functional relationship between PSC-derived CAF and these features over the course of the tumor development. These results suggest that PSC-derived CAFs drive establishment of tumor-promoting desmoplastic milieu. We, therefore, developed an ECM signature enriched among PSC-derived CAFs from our RNA-seq datasets and tested this signature for its prognostic value. Although bulk stromal density and collagen are associated with a better prognosis in PDAC, this PSC-associated ECM signature was linked to PDAC patients with a worse prognosis (Figure 2-4K; Supplementary Table 2-2)^{49,95}. Overall, these results suggest that PSC-derived CAFs regulate specific stromal microenvironment features associated with PDAC aggressiveness.

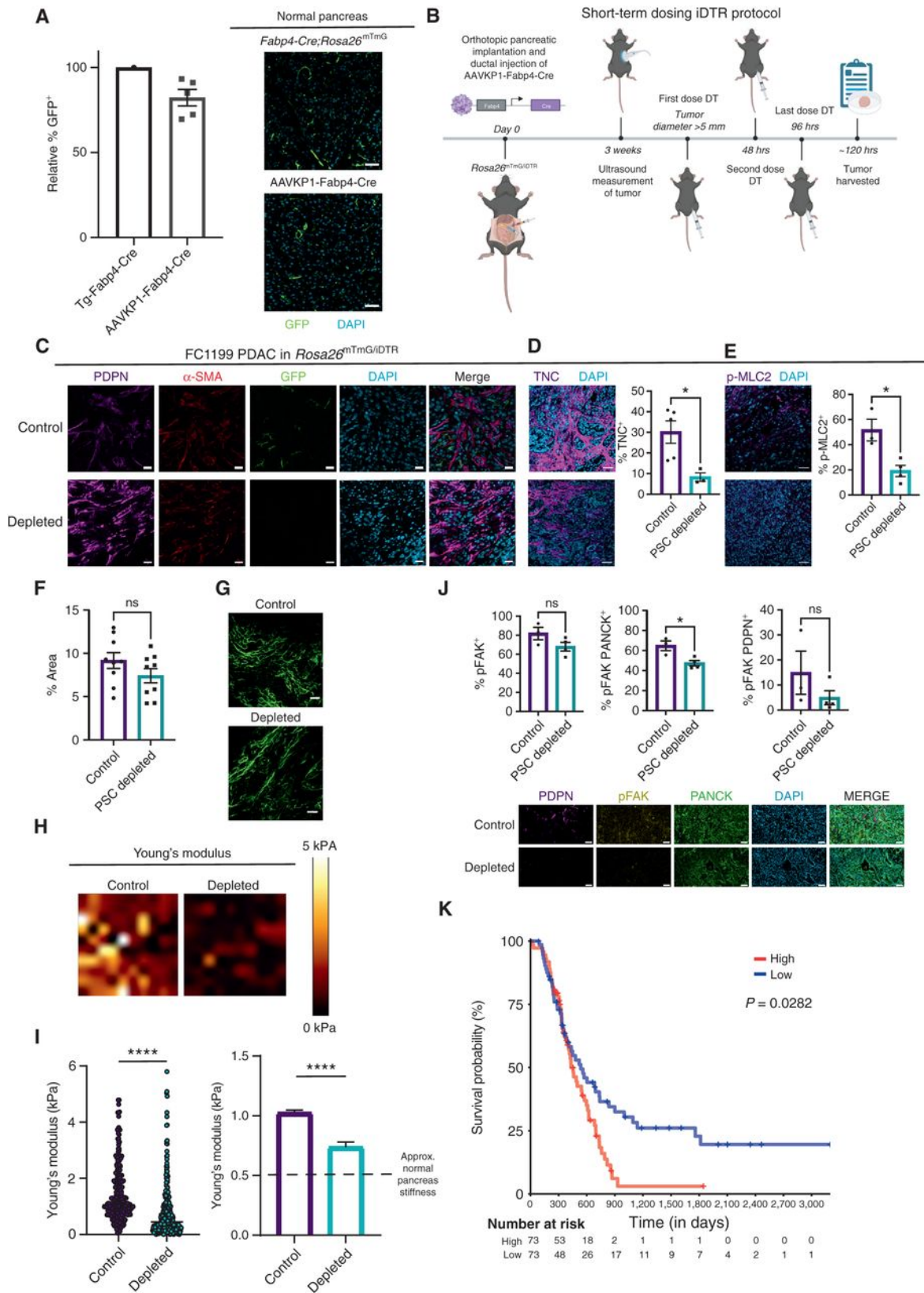


Figure 0-4. Targeted ablation reveals unique roles for PSC-derived CAFs in regulation of the ECM and mechanosignaling.

(A) IHC staining and quantification of GFP⁺ cells in normal pancreas tissue from Fabp4-Cre;Rosa26^{mTmG} mice and from Rosa26^{mTmG/iDTR} mice 7 days after intraductal injection with AAVKP1-Fabp4-Cre (n = 5). Data are presented as mean ± SEM. Scale bar, 50 μm. (B) Schematic of tumor modeling using intraductal injection of AAVKP1-Fabp4-Cre and orthotopic transplantation of KPC PDAC cells into Rosa26^{mTmG/iDTR} hosts. Created with BioRender.com (C) IHC staining for GFP, PDPN, and α-SMA of KPC FC1199 PDAC in Rosa26^{mTmG/iDTR} hosts with intraductal injection of AAVKP1-Fabp4-Cre, enrolled when tumors reached 5 to 6 mm in diameter and treated with PBS or DT for 5 days (n = 4). Scale bar, 20 μm. (D) IHC staining for TNC of KPC FC1199 PDAC in AAVKP1-Fabp4-Cre-injected Rosa26^{mTmG/iDTR} hosts, enrolled at 5 to 6 mm in tumor diameter and treated with PBS or DT for 5 days (n = 3). Scale bar, 50 μm. Data are presented as mean ± SEM. (E) IHC staining for p-MLC2 of PDAC samples as described in D. Scale bar, 50 μm. Data are presented as mean ± SEM. Quantification (F) and images (G) of fibrillar collagen content analyzed by second harmonic generation with normalized intensity as a quantification of concentration in control and PSC-depleted PDAC (n = 3 per group). Scale bar, 42.5 μm. (H) Force maps generated by atomic force microscopy (AFM) on KPC FC1199 PDAC in AAVKP1-Fabp4-Cre-injected Rosa26^{mTmG/iDTR} hosts (n = 3 per treatment group, control: 1,063 data points, depleted: 717 data points), excised after 5 days of treatment with PBS or DT. (I) Quantification of Young's modulus per AFM measurements on control and PSC-depleted PDAC as described in F. The dashed line on the graph to the right denotes the approximate stiffness of normal murine pancreas tissue. (J) IHC staining for p-FAK (Y397) of control and PSC-depleted PDAC harvested after 5 days of depletion (n = 3). Scale bar, 50 μm. Data are presented as mean ± SEM. K, Kaplan–Meier plot depicting overall survival of patients with PDAC with high versus low expression of a PSC-derived CAF ECM gene signature comprising 99 genes (see Methods), plotting the upper versus lower quartile (n = 73 per arm). *, P < 0.05; ****, P < 0.0001 by unpaired t test; ns, not significant.

Relationship between Tumor Genotype and Stromal Evolutionary Routes with Respect to CAF Origins

Our results in PDAC patient samples suggest heterogeneity in PSC-derived CAF frequencies, which led us to question whether tumor genotype regulates stromal evolution from distinct cells of origin. Supporting this notion, we observed significantly elevated Hspg4 (perlecan) expression in PSC-derived CAFs compared to CAFs of other origins. This difference in expression between CAFs of different origins leaves the possibility that previously reported distinctions between CAFs associated with p53-mutant versus p53-

null PDAC reflect differential recruitment of PSCs into the CAF pool⁶⁷. We began to test the relationship between cancer cell-intrinsic p53 status and stromal evolution by comparing two PDAC models from the same genetic background (C57BL/6J) and the same driver mutation Kras (G12D), but with one featuring a p53 mutant (R172H) and the other featuring p53 loss. From our Fabp4-Cre; Rosa26^{mTmG} mice, we harvested sized-matched tumors and found that total PDPN⁺ CAF frequencies were not different, but PDPN⁺ GFP⁺ cells were fewer or that PSC-derived CAFs made a significantly lower contribution to the CAF populations of p53 null PDAC (Figure 2-5B,C). Next, we assessed the causal role of p53 in CAF recruitment by generating an isogenic model by Cas9 and two different single-guide RNAs targeting Trp53. We knocked out p53 in a p53-mutant (R172H) parental line to produce p53 null cells (Figure 2-5D). We transplanted these lines as well as control empty vectors in pancreata of Fabp4-Cre; Rosa26^{mTmG} mice, and harvested size-matched tumors, followed by the analysis of CAF lineages by flow cytometry (Figure 2-5E). Loss of p53 led to a reduction in PSC CAF frequencies out of PDPN CAFs, though it is of note that one p53 null line was a trend and not significant (Figure 2-5F). p53 null tumors harbored a substantial CAF population as expected. In addition, p53 null tumors had reduced p-MLC2 compared to mutants consistent with a previous study, showing reduced PSC-derived CAF frequencies (Figure 2-5G)⁶⁷. To identify candidate p53-regulated factors that promote or suppress PSC activation, we probed RNA-seq data from a recent study comparing KPC PDAC cells in which p53 was knocked down with short hairpin RNA to parental p53-mutant controls¹¹². From this RNA seq dataset, we identified the top 40 up- or downregulated genes in the p53 knockdown cells. Of these genes, we found 17 upregulated genes in p53 mutants'

controls and 21 upregulated in p53 knockdown cells that encode secreted factors (soluble or membrane-associated) that may participate in paracrine interactions with the stroma (Figure 2-5H). In combination, these results suggest cancer cell-derived factors stimulate stromal evolution and that distinct PDAC genotypes may be differentially responsive to stroma-targeted therapies independent of stromal density because of distinct mesenchymal cells of origin.

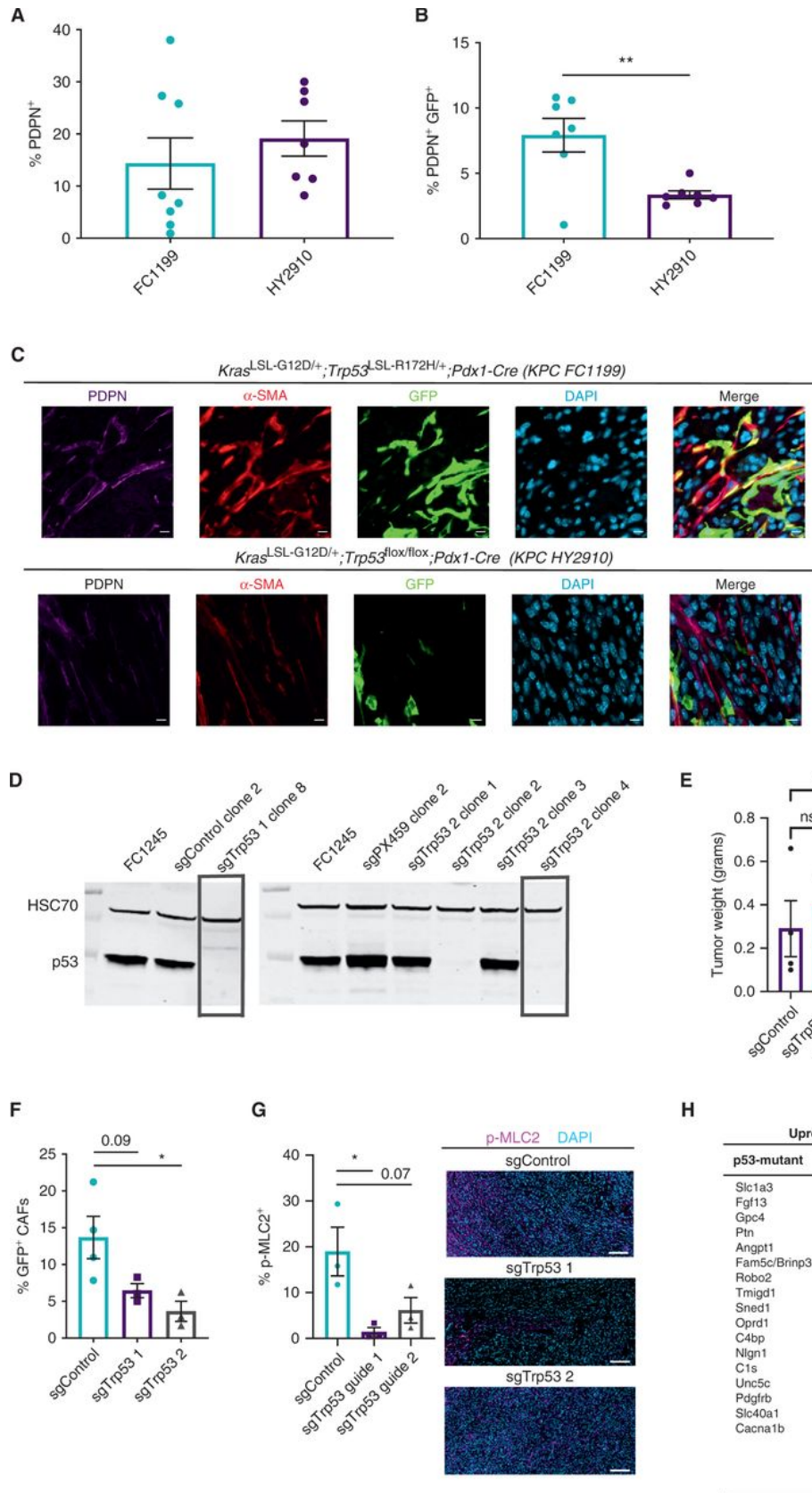


Figure 0-5. Tumor genotype with respect to p53 status influences stromal evolutionary routes.

(A) Flow cytometry analysis of PDPN⁺ cells in size-matched KPC FC1199 (p53 R172H, n = 8) and HY2910 (p53-null, n = 7) PDAC in Fabp4-Cre;Rosa26^{mTmG} hosts. Data are presented as mean $\pm$ SEM. (B) Flow cytometry analysis of PDPN, GFP, and tdTomato in the tumors described in A to quantify the percentage of CAFs derived from PSCs. Data are presented as mean $\pm$ SEM. (C) IHC staining for GFP and PDPN on KPC FC1199 and HY2910 PDAC in Fabp4-Cre;Rosa26^{mTmG} hosts (n = 3). Scale bar, 10 μ m. (D) Western blots for p53 and HSC70 (loading control) using whole-cell lysates from parental KPC FC1245 (p53 R172H) cells or derivative lines transfected with control plasmid or one of two sgTrp53 sequences. Boxes indicate clones selected for experimentation. (E) Tumor weights at experimental endpoint from control and sgTrp53 PDAC in Fabp4-Cre;Rosa26^{mTmG} hosts. (F) Flow cytometry analysis of PDPN, GFP, and tdTomato in size-matched control (n = 4) and sgTrp53 (n = 3 per line) PDAC in Fabp4-Cre;Rosa26^{mTmG} hosts. Data are presented as mean $\pm$ SEM. (G) IHC staining for p-MLC2 in size-matched control and sgTrp53 PDAC in Fabp4-Cre;Rosa26^{mTmG} hosts (n = 3). Scale bar, 100 μ m. (H) List of candidate secreted factors differentially expressed in p53-mutant versus p53-null PDAC cells. *, P < 0.05; **, P < 0.01 by one-way ANOVA; ns, not significant.

Discussion

Discovery of hepatic stellate cells as the source of liver fibrosis followed by the discovery of the pancreatic stellate cell in 1998 provided a crucial basis for understanding the cellular source of pancreatic fibrosis^{18,104}. Hepatic stellate cells as the source of fibrosis in the liver after injury and identification of similar fibrogenic potential of PSC lead to reasonable speculation that PSCs are the cellular source of pancreatic fibrosis and the extensive desmoplastic reaction in PDAC^{15,18,97}. Activation of PSCs in culture reveals a transcriptional program consistent with CAF features, including expression of α -SMA⁷². PSC cultures also showed their ability to give rise to the previously defined iCAF and myCAF subtypes dependent on soluble cues and growth substrates⁶¹. Considering the plasticity of PSCs and their presumed dominant role as contributors to the PDAC stroma, we expected that our genetic means to track and ablate PSC-derived CAFs would target most if not all CAFs in the PDAC microenvironment. It was surprising to find that PSCs

give rise to a numerically small minority of CAFs in PDAC with frequencies in part dependent on tumor genotype. As we continue to explore the functions of PSC-derived CAFs, we will further analyze clinical cohorts, leveraging heterogeneity in these cell frequencies among patient samples to investigate potential stratification strategies to tailor therapies informed by CAF origin. We acknowledge the lack of mechanistic data in our studies; however, there is importance in our observation of pancreatic stromal evolution that may serve as a starting point for upcoming mechanistic studies aimed to identify specific tumor-promoting functions of this CAF subset. PSC-derived CAF transcriptional profiles are distinct from CAFs of another origin; our results put into the context of recent literature suggest that CAF heterogeneity results from at least two sources: signaling gradients regulating common cells of origin and mesenchymal lineage heterogeneity. Our experiments ablating PSC-derived CAFs suggest functional heterogeneity underlies transcriptional heterogeneity. Transcriptional and phenotypic plasticity among CAFs poses limitations in the feasibility of targeting specific subsets therapeutically. However, our model enables targeted ablation of a defined subset of CAFs, raising the possibility that CAF subsets have sufficient functional distinctions that suggest targeting subsets therapeutically may indeed be possible. The notion that targeting specific protumor function is feasible is supported by recent study revealing that myCAFs are differentially dependent on the hedgehog pathway such that hedgehog inhibition skews CAFs in PDAC, increasing iCAFs while reducing myCAFs⁹⁰. In addition, this study suggests that in PDAC, iCAFs have a potential immune-suppressive function due to their impact on tumor-infiltrating T cells. Due to the unique functions of distinct CAFs, targeting specific CAFs emerges as a viable strategy in combination with

current therapies to improve efficacy. It is of utmost importance to extend these functional analyses to additional CAF populations. Still, it would require the development of relevant models to understand distinct roles in the TME and inform us about potential targets for therapeutic intervention.

The PSC-derived CAFs' high expression of α -SMA and ECM components suggest that they are of the myCAF or TGF- β subset^{51,53,64}. However, due to total collagen remaining unchanged and the remaining abundance of α -SMA CAFs after PSC-derived CAF ablation, PSC-derived CAFs are only a subset of myCAFs. CAFs can be divided into subgroups based on FAP and α -SMA expression, resulting in functionally distinct populations in several tumor types. FAP^{HI} CAFs predominantly synthesize and remodel ECM, whereas α -SMA^{HI} CAFs mediate contraction¹¹³. This CAF heterogeneity is governed by substratum and ECM composition, which is regulated by CAF phenotype, creating a feedback loop. Our results suggest that PSCs give rise to CAFs in both functional categories, indicating PSCs may give rise to multiple small subsets with defined roles. Single-cell sequencing approaches will be helpful to address the contribution of PSC-derived CAFs to each of these categories. PSC-derived CAFs regulate specific ECM components as well as tissue stiffness and other biophysical properties that are all implicated in tumor aggressiveness and metastasis^{67,88}. Based on PSC-derived CAFs regulating features of tumor progression and aggressiveness, we speculate that PSCs give rise to a tumor-promoting myCAF subset that needs to be investigated in depth in future studies. Studies highlighting the dependence of myCAFs on hedgehog signaling and the detrimental outcomes upon long-term or genetic SHH inhibition, it seems likely that a subset of myCAFs not derived from PSCs is tumor

suppressive^{46,94}. The non-PSC-derived myCAF subset is also expected to promote a less aggressive, more differentiated PDAC. Prior studies give correlative evidence that other myCAFs are tumor-suppressive by showing immune suppression upon reduction of overall myCAF frequencies in the TME^{85,90}. It is crucial to identify tumor suppressive CAFs and their homeostatic or beneficial functions in order to maintain these functions. It should be a goal to maintain tumor-suppressive CAFs or functions in future stroma-targeted therapies to improve efficacy.

Our data address the critical question of the contributions of PSC to CAFs, and demonstrate that PSCs give rise to a numerically minor CAF population in PDAC. Our data, however, leaves another pressing question that needs to be addressed of where a majority of PDAC CAFs originate. Murine PDAC single-cell RNA-seq data has partially addressed this question identifying the origin of apCAFs as mesothelial cells⁶⁴. The remaining CAFs, including iCAFs and non-PSC-derived myCAFs origins, remains unknown but may come from pancreas resident fibroblast populations or the bone marrow. For other cancer types, bone marrow-derived mesenchymal stromal cells are essential contributors to the CAF populations, such as in breast cancer¹¹⁴. However, the contribution of bone marrow progenitors to PDAC CAFs remains to be determined. Regarding fibroblasts in the normal pancreas, recent lineage tracing studies have identified two distinct fibroblasts populations, one marked by hedgehog responsive transcription factor *Gli1* and the other by tissue-restricted mesenchymal transcription factor *Hoxb6*⁷³. These pancreas fibroblast populations are of similar frequencies; however, in the early stages of pancreatic carcinogenesis, the *Gli1*⁺ population expands while the *Hoxb6*⁺ CAF population does not. The expansion of *Gli1*⁺ fibroblasts suggests

that this population contributes to the stroma associated with precursor lesions. Due to our study being limited to transplantable PDAC models, analysis of stromal evolution in autochthonous models is essential to identify alterations in stromal composition throughout stepwise tumorigenesis. Future studies are needed to define the mechanisms that shape PDAC CAFs and discover subsets' unique functions. These future studies will improve our understanding of the impact of various subsets on PDAC progression and identify stromal targets for therapeutic intervention.

Materials and Methods

Animals

All experiments performed in mice were reviewed and overseen by the institutional animal care and use committee at Oregon Health & Science University in accordance with NIH guidelines for the humane treatment of animals. Fabp4-Cre (005069) and Rosa26^{mTmG} (007676) mice from Jackson Laboratory were used for PSC analyses and orthotopic transplantation experiments at 8-12 weeks of age, including male and female mice. Rosa26^{mTmG} and iDTR (007900) mice from Jackson Laboratory were used for retrograde ductal injection and orthotopic transplantation for PSC ablation experiments at 8-12 weeks of age, including male and female mice.

Human tissue samples

Human patient PDAC tissue samples donated to the Oregon Pancreas Tissue Registry program (OPTR) with informed written patient consent (IRB approved, IRB00003609) in accordance with full ethical approval by the Oregon Health & Science University

Institutional Review Board were provided by the OHSU Brenden-Colson Center for Pancreatic Care and the Knight BioLibrary, upon pathology review by Dr. Rosemary Makar and secondary review by Dr. Christopher Corless.

The PDAC tumor microarray was described previously¹¹⁵. Human PDAC specimens were obtained from patients who underwent surgical resection of primary PDAC under IRB-approved protocol IRB11-000512. This study was conducted under strict compliance with institutional ethical regulations. The study had minimal risk per the IRB protocol, and thus informed consent was not necessary.

PSC isolation and analysis

PSCs were isolated from healthy pancreas tissue from 8 week old Fabp4- Cre; Rosa26^{mTmG} mice as previously described⁷². Upon retrieval from the density gradient interface, PSCs were plated on glass coverslips in DMEM containing 10% fetal bovine serum. On day 2 of culture, cells were fixed in 4% paraformaldehyde for 15 mins at room temperature, washed three times with PBS, and permeabilized with 0.1% Triton X-100 for 10 mins at room temperature. Following permeabilization, coverslips were blocked for 1 hr at room temperature in blocking solution (8% BSA), then transferred to a carrier solution (1% BSA) containing diluted antibodies against Desmin and GFP (Desmin: Cell Signaling Technology D93F5, GFP: Cell Signaling Technology 4B10). Coverslips were incubated with the primary antibody solution for 3 hr at room temperature, then washed five times for 5 mins each in PBS. Secondary Alex-fluor conjugated secondary antibodies were diluted in the same carrier solution (1:400) and added to the coverslips for 1 hr at room temperature. Coverslips were then washed five times for 5 mins each in PBS and

mounted with Vectashield mounting medium containing DAPI. Cells were imaged using the LSM 880 confocal microscope described above, and co-stained cells out of total Desmin-positive cells were scored manually.

Cell culture

FC1199 and FC1245 (provided by Dr. David Tuveson), 4662 (provided by Dr. Robert Vonderheide), and HY2910 (provided by Dr. Haoqiang Ying) PDAC cell lines were derived from primary murine PDAC. Cell lines were routinely passaged in DMEM (Thermo Fisher) containing 10% FBS (HyClone) for no more than 25-30 passages. Primary PSCs were also cultured in DMEM containing 10% FBS after isolation from mouse pancreas and until fixation and analysis. Cell lines were routinely tested for Mycoplasma at least monthly (MycoAlert Detection Kit, Lonza). Cell line authentication was not performed.

Immunohistochemistry

For mouse tissue harvest, mice were anesthetized and euthanized according to institutional guidelines. Pancreas tissue or tumors were excised carefully and fixed overnight in 10% neutral buffered formalin or embedded in OCT and frozen at -80°C. Fixed tissues were paraffin-embedded, sectioned, deparaffinized, and rehydrated through an ethanol series and ultimately in PBS. Following antigen retrieval, tissue samples were blocked for two hours at room temperature in blocking solution (8% BSA) and transferred to a carrier solution (1% BSA) containing diluted antibodies. Sections were incubated overnight at room temperature and then washed five times for 5 minutes each

in PBS. Secondary Alexa-fluor conjugated antibodies diluted in the same carrier solution (1:200) were added to the sections for two hours at room temperature. Sections were then washed five times for five minutes each in PBS, autofluorescence quenched with the TrueVIEW reagent (Vector Laboratories), stained with DAPI, and mounted with Vectashield mounting medium. Fresh-frozen tissues were sectioned, fixed, and used to stain for CD45 in FC1199 tumors and for NG2 in normal pancreas tissue, then counterstained with DAPI-containing Vectashield mounting medium for fluorescence microscopy. Antibodies used for immunohistochemistry were as follows: CD31: Abcam ab28364, GFP: Cell Signaling Technology 4B10 (mouse) or D5.1 (rabbit), CD45: Abcam ab25386, NG2: EMD Millipore AB5320, a-SMA: Thermo Fisher MA511547, PDPN: Thermo Fisher 14-5381-81, TIE1: Abcam ab111547 (mouse tissues, Supplementary Figure S2B), TIE1: Biorbyt orb11476 (mouse tissues, Supplementary Figure S2C), TIE1: Thermo Fisher PA527903 (human tissues), TNC: Abcam ab108930, pMLC2: Cell Signaling Technology 3674, pFAK: Thermo Fisher 700255, Ki67: Cell Signaling Technology 12202, CC3: Cell Signaling Technology 9664, CD8: Abcam ab203035, CD68: Abcam ab125047. Stained tissues were imaged using a laser-scanning confocal inverted microscope (LSM 880, Carl Zeiss, Inc.), and a 40x/1.1 NA water objective or 63x/1.4 NA oil objective was used to image the samples. Slides were scanned using a Zeiss Axio Scan.Z1 and quantified using QuPath or Aperio software. For quantification of TIE1/a-SMA colocalization on human PDAC sections, images were acquired on an AxioScan.Z1 using a 10x 0.45 NA plan-apochromat lens. Fluorochromes were excited with a Colibri 7 light source (Carl Zeiss), and excitation and emission light were passed through the following Zeiss filter sets for the appropriate channel: DAPI- 96 HE; Alexa-

fluor 488- HE 38; Alexa-fluor 594- 71 HcRed. Images were analyzed for colocalization in ZEN v2.3 (Carl Zeiss). Thresholds for the AF594 and AF488 channels were set by eye for each slide (three slides total) for the TMA analysis. Within each slide, the same thresholds were used across all tissues. Dynamic range was set to a 14-bit image (16384 maximum intensity).

Fluorescence activated cell sorting and flow cytometry

For flow cytometry analysis of normal pancreas tissue, pancreata were harvested, briefly minced with scissors, and digested with 0.02% Pronase (Sigma-Aldrich), 0.05% Collagenase P (Sigma-Aldrich), and 0.1% DNase I (Sigma-Aldrich) in Gey's balanced salt solution (GBSS, Sigma-Aldrich) at 37°C for 20 mins. After dissociation, tissue was triturated until large pieces were no longer visible, and the resulting cell suspension was filtered through a 100-µm nylon mesh. Cells were then washed with GBSS, pelleted, subject to red blood cell lysis in ACK lysis buffer (Thermo Fisher) for 3 mins at room temperature, washed in FACS buffer (PBS containing 2% FBS), pelleted, and resuspended in FACS buffer for flow cytometry to analyze vitamin A positivity based on autofluorescence using the 405 nm laser on a BD Fortessa flow cytometer. To analyze endothelial cells, following red blood cell lysis, cells were incubated with CD16/CD32 antibody (BD Biosciences 553141) to block Fc receptors for 2 mins at room temperature, then stained with a PerCP/Cy5.5-conjugated CD31 antibody (BioLegend 102522) for 30 mins on ice. Stained cells were washed with cold FACS buffer, pelleted, and resuspended in cold FACS buffer for flow cytometry analysis.

For analytical flow cytometry or FACS on PDAC tissues, tumors were harvested, minced with scissors, and dissociated in DMEM containing 1 mg/ml Collagenase IV (Thermo Fisher), 0.1% Soybean Trypsin Inhibitor (Thermo Fisher), 50 U/ml DNase I (Sigma-Aldrich), and 0.125 mg/ml Dispase II (Thermo Fisher) at 37°C for 1 hr. Digested tissues were pelleted, resuspended in 0.25% Trypsin in DMEM, and incubated at 37°C for 10 mins, then washed with cold DMEM containing 10% FBS and pelleted. Resuspended cells were filtered through a 100 µm cell strainer, pelleted, washed with DMEM + 10% FBS, and pelleted again. Cells were resuspended in ACK lysis buffer as described above, washed with FACS buffer, pelleted, and resuspended in FACS buffer. Fc block was performed as described above, then cells were stained with antibodies for 30 mins on ice: biotinylated anti-PDPN (BioLegend 127404), biotinylated anti-PDGFRa (anti-CD140a, BioLegend 135910), PerCP/Cy5.5-conjugated anti-CD31 (BioLegend 102522), and/or PE/Cy7-conjugated anti-CD45 (BioLegend 103113). Cells were then washed with FACS buffer and pelleted. When biotinylated antibodies were used, incubated with APC Streptavidin (BD 554067) for 30 mins on ice, washed with FACS buffer, pelleted, and resuspended in FACS buffer. After staining for the purpose of CAF sorting and subsequent RNA-seq, RNase inhibitor (New England Biolabs M0314, 1:40) was added to the cell suspension, and CAFs were sorted into TRIzol LS (Thermo Fisher). Flow cytometry analysis was performed on a BD Fortessa, while CAF sorting was performed on a BD FACSAria Fusion. Flow cytometry data were analyzed with FlowJo software. GFP⁺ or tdTomato⁺ CAF frequencies were calculated out of the total GFP⁺ plus tdTomato⁺ population, excluding any cells lacking either lineage label which represent tumor cells.

Orthotopic PDAC experiments

Male and female mice at 8-12 weeks of age were used as hosts for PDAC orthotopic transplantation, using genotypes described in the Results section and figure legends. Mice were anesthetized with ketamine and xylazine, and pancreata were injected with 5×10^3 FC1199 or FC1245 cells (lines provided by Dr. David Tuveson), 1×10^5 4662 cells (provided by Dr. Robert Vonderheide), or 1×10^4 HY2910 cells (provided by Dr. Haoqiang Ying), all derived from primary PDAC in $Kras^{LSL-G12D/+};Trp53^{LSL-R172H/+};Pdx1-Cre$ mice (FC1199, FC1245, 4662) or $Kras^{LSL-G12D/+};Trp53^{flox/flox};Pdx1-Cre$ mice (HY2910) of a C57BL/6J genetic background. Orthotopic transplantation was performed as previously described³. For PSC-derived CAF ablation experiments, pancreata were imaged beginning 14 days after transplantation by high-resolution ultrasound using the Vevo 770 imaging system; mice were enrolled on study when tumors reached 5-6 mm in diameter. Enrolled mice were treated with sterile PBS or 25 ng/g diphtheria toxin (List Biological Laboratories) by intraperitoneal injection every 2 days and tumors were harvested on day 5 post-enrollment for analysis.

Retrograde ductal AAV delivery

A promoter and enhancer element upstream of mouse *Fabp4* (from Addgene #8858) was cloned into pAAV-iCre-WPRE (Vector Biosystems) upstream of Cre. KP1-serotyped AAV- *Fabp4*-Cre was generated and tittered by the OHSU Molecular Virology Core. AAVKP1- *Fabp4*-Cre viral stock was diluted to 1×10^{10} viral genomes/ml in 10 μ g/ml DEAE-Dextran (Sigma-Aldrich) in PBS, incubated for 30 mins at room temperature, then placed on ice. Retrograde ductal injections were performed as previously described¹⁰⁸.

Mice were anesthetized with ketamine and xylazine until non-responsive to toe pinch. Feet were taped down with surgical tape on a sterile mat. Entire abdomen was shaved, and the shaved area cleaned with 70% ethanol and betadine. A midline incision was made about 1 inch long, and incision edges were secured with hemostatic forceps. Non-weave sterile gauze was moistened with sterile PBS. Intestines were gently extracted using circle forceps, and gently laid onto damp gauze. Intestines were slowly extracted until duct was visible. Exposed intestines were covered with damp gauze to keep moist, and sterile PBS was continually added throughout the procedure as necessary to keep gauze and tissue from drying out. One microvascular clip was placed on the cystic duct near the gallbladder. The sphincter of Oddi was located; a 30G insulin syringe was loaded with 100 μ l viral solution (to inject 1×10^9 viral genomes per mouse), and the needle inserted through the sphincter of Oddi into the common bile duct up to its convergence with the cystic duct, about halfway to the clip. Viral solution was slowly injected over the course of 2 mins. The needle was left in place for 30 sec after completion of the injection, then slowly and gently removed. The clip was then removed. Intestines were carefully returned to the abdomen, muscle layer closed with vicryl sutures, and skin closed with sterile suture clips. When performed together with orthotopic transplantation of PDAC cells, cells were injected after removal of the syringe and clip.

Atomic force microscopy

Fresh tumor tissues were placed in the middle of a disposable cryomold and embedded in Optical Cutting Temperature (OCT) compound. Tissues were sectioned with a cryostat at a thickness of 50 μ m and adhered to positively charged microscope slides. Prior to

measurements, samples were thawed at room temperature in DMEM/F-12 supplemented with 10% FBS and a protease inhibitor cocktail (Sigma Aldrich 11836170001). Atomic force microscopy (AFM) measurements were performed with a NanoWizard 4 XP BioScience with HybridStage (Bruker) mounted on a Zeiss Axio Observer inverted optical microscope. Dull triangular silicon nitride ScanAsyst-Fluid probes were used ($k = 0.7 \text{ N/m}$; Bruker) at a maximum applied force of 4 nN and approach speed of 5 $\mu\text{m/s}$. Each probe was calibrated with the thermal oscillation method prior to measurements on each tissue. Force curves were analyzed to obtain the Young's modulus using the JPK Data Processing Software package with the Hertz/Sneddon model assuming an incompressible tissue and a Poisson's ratio of 0.5 with tip parameters provided by the manufacturer's specifications. Three independent samples were imaged from each treatment group at three locations at varying tissue depths with a scan size of 100 μm^2 with force measurements spaced every 10 μm .

Second harmonic generation imaging and analysis

Unfixed, hydrated control ($n = 3$) and PSC-depleted ($n = 3$) tumors embedded in OCT were sectioned at 15 μm thickness. The SHG images were acquired with a Zeiss LSM 780 multi-photon microscope mounted on a Zeiss Observer Z1 inverted microscope stand. Tissues were imaged with a Coherent Chameleon Vision II ultrafast pulsed infrared laser (680-1080 nm) tuned to 800 nm. A Zeiss 20x air objective ($\text{NA} = 0.8$) focused the beam onto the sample and SHG imaging was detected from the back-scattered signal with a Gallium arsenide phosphide (GaAsP) non-descanned detector. For image analysis three representative fields of view were analyzed per sample. A minimal

threshold in the SHG signal was set and maintained across all images with ImageJ (NIH) and was used to obtain the percent area and normalized intensity.

Survival analysis of a PSC ECM gene signature

The PSC-derived CAF ECM signature genes (Supplementary Table 2-2) were defined as the overlap of the genes annotated in the Reactome Extracellular Matrix Organization pathway and the upregulated genes in GFP⁺ CAFs (FDR < 0.01 and fold change > 1.25) per our RNA-seq analysis. In the signature survival analysis, 297 samples were collected with survival time from the OHSU Brenden-Colson Center for Pancreatic Care Tempus dataset. The gene set variation analysis (GSVA) algorithm with the default settings, as implemented in the GSVA R package (version 1.34.0), was applied to calculate the gene signature score for each sample. Next, the samples were stratified into two groups based on the quantile values of the signature scores (upper quartile versus lower quartile).

Survival curves of these two groups of patients were analyzed by Kaplan-Meier method with statistical significance calculated using the log-rank test. The Kaplan-Meier estimator and log-rank test were calculated in the survival R package (version 3.2-3).

Statistical analysis

Statistical analyses were performed using GraphPad PRISM software. Student t test was used to compare two groups to each other. One-way ANOVA was performed when multiple conditions were compared for one variable. Tukey post hoc tests were used after ANOVA analyses to perform multiple group comparison. Analysis with a p-value < 0.05 was considered statistically significant.

Data availability

All sequence data from this study have been deposited in the publicly available Gene Expression Omnibus under accession number GSE143805.

[Supplementary Material](#)

Supplementary Figure 1

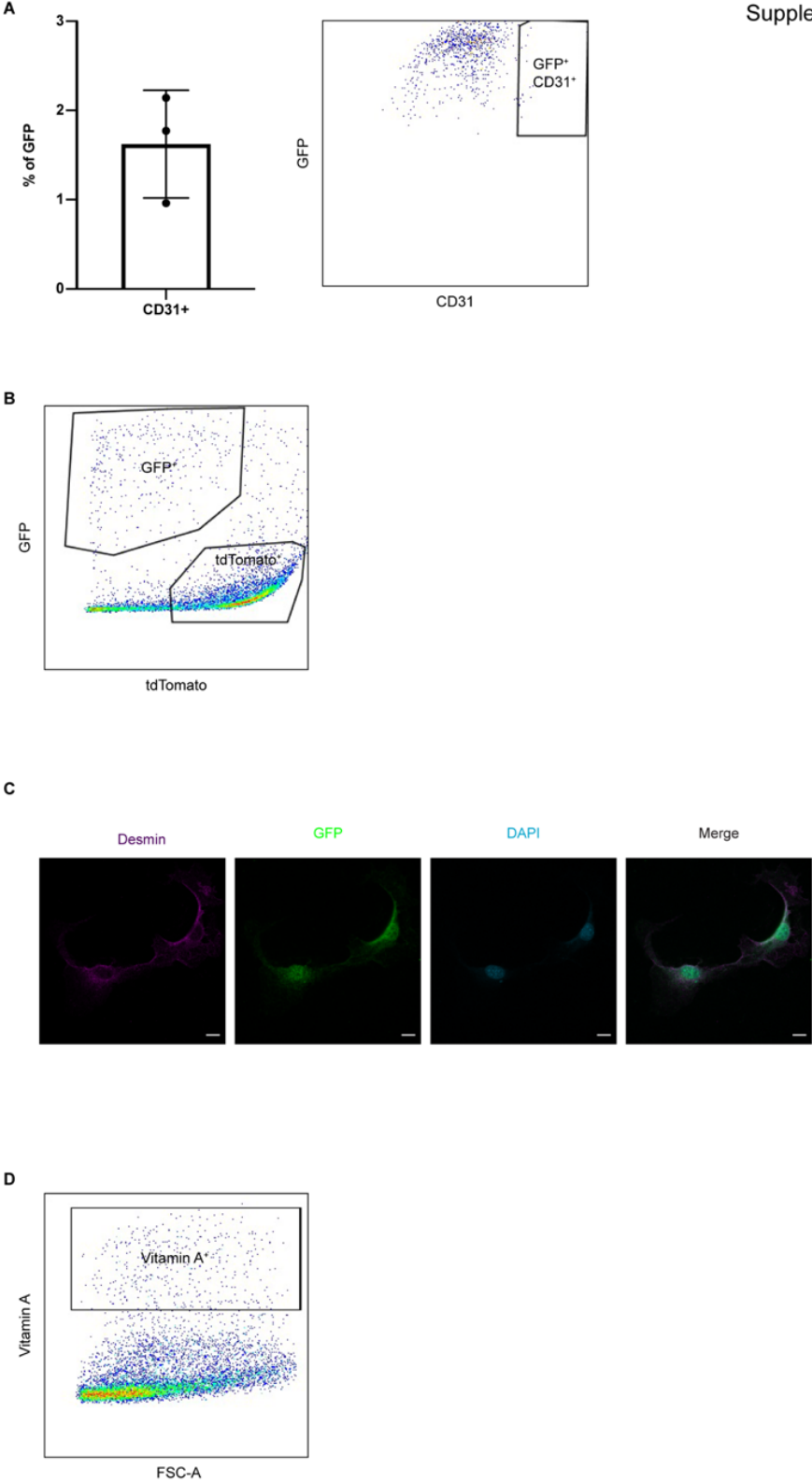


Figure 2-S 1 related to Figure 1.

(A) Flow cytometry analysis of CD31, GFP, and tdTomato on normal pancreas tissue from *Fabp4-Cre;Rosa26mTmG* mice (n = 3). Data are presented as mean $\pm$ SEM. (B) Representative flow cytometry plot showing GFP⁺ and tdTomato⁺ populations in normal pancreas from *Fabp4-Cre;Rosa26mTmG* mice used for FACS. (C) Immunofluorescence staining for Desmin and GFP on PSCs isolated from *Fabp4-Cre;Rosa26mTmG* mice by density centrifugation. Scale bar = 10 μ m. (D) Representative flow cytometry plot showing vitamin A⁺ cells in normal pancreas from *Fabp4-Cre;Rosa26^{mTmG}* mice.

Table 1

https://aacr.silverchair-cdn.com/aacr/content_public/journal/cancerdiscovery/12/2/10.1158_2159-8290.cd-21-0601/1/21598290cd210601-sup-265398_2_supp_7388515_qz8vyy.xlsx?Expires=1648249786&Signature=pCt6JFgkcOLNSu4kxj5sW7BrwKu0gF~DX3zc5IJ0~3UmbePI260QTGYEodKEudwE-OGne5-3F0NDIO6RXCXgvH5fIJ2vaAHnp6fT8oEqSkzB6xe3e0y7j7twN2542NbPvUJk2z42GJ3NnqFC-scjRsTuh-Eux9WexK~ornieVTX4BMGJEWuKYqLgg54r6U5OlgOYniIsj~-mXaE~TtHmMKxP8WOsValg32-mVL2ko7VrAyhYDb17f5XfNqta87WV33mi5MvQqJ6x8JQGP~CpZQC1SuY8QD80FMgJF5nFoaBMSgkFGL6eMTzAKqgdWuzWvpC20UiTy5F~55YOgRO9nA__&Key-Pair-Id=APKAIE5G5CRDK6RD3PGA

Supplementary Figure 2

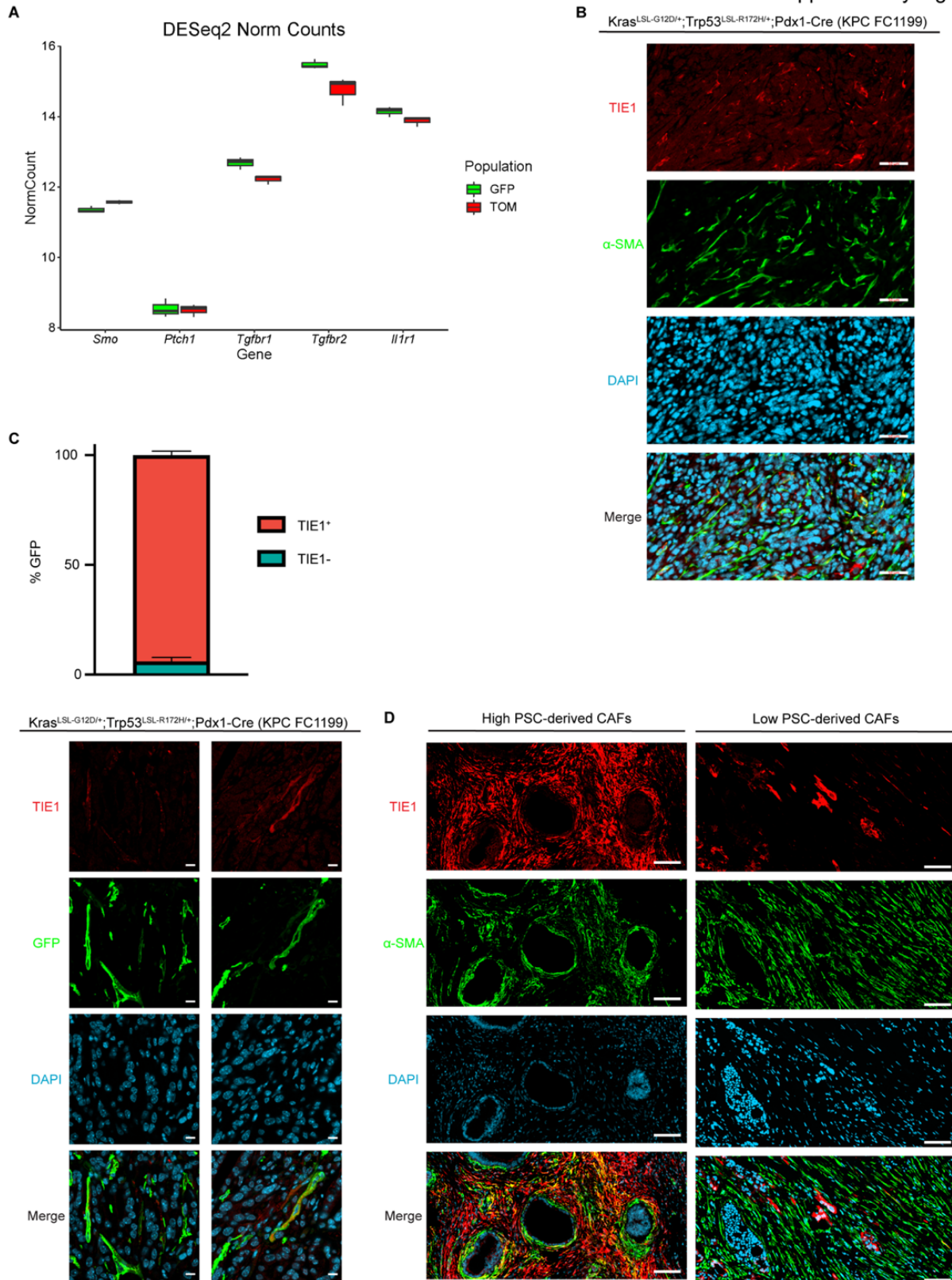


Figure 2-S 2 related to Figure 3.

(A) Plot of transcript abundance of the indicated ligand receptors from RNA-seq results comparing GFP⁺ and tdTomato⁺ CAFs. (B) Immunohistochemical staining for TIE1 and α -SMA in KPC FC1199 PDAC in *Fabp4-Cre; Rosa26^{mTmG}* mice (n = 3). Scale bar = 50 μ m. (C) Immunohistochemical staining for TIE1 and GFP in KPC FC1199 PDAC in *Fabp4-Cre; Rosa26^{mTmG}* mice (n = 3). Scale bar = 10 μ m. (D) Representative immunohistochemical staining of human PDAC tissue sections (n = 43) for TIE1 and α -SMA. Scale bar = 100 μ m.

Table 2

https://aacr.silverchair-cdn.com/aacr/content_public/journal/cancerdiscovery/12/2/10.1158_2159-8290.cd-21-0601/1/21598290cd210601-sup-265398_2_supp_7388516_qz8v88.xlsx?Expires=1648249786&Signature=Cvp-zM1jh8xCXLD4budXcwzT0~tGIABgt4pxy250P6FoB0d4ZVi~NEIsUnGcbCI2mN63Di9VlpYjPJ~aoMjeNoJaHn9NQZBzz9yBwLtC4WUoUrkQ1tJ5FagPRIRgfbbGBelmdSr328MUCt4o22q-uCbQXDnHtILNvkg5NETV0kGBAnCP-Ygbx~zsDrwCPQcC88IpmDg8d79KsBm6e6WW4eTG5jU1X5v76Hztokze1aAqnnkU637~Dl5yQWrbjeJ8qTA7Cr24z5zQKQ-n0ryQgNb9EnibYweT0cCNizCB33oFlCb1IYgfBpADugzcAlXaUujAXCWEHP~zAlXYOiHgBg__&Key-Pair-Id=APKAIE5G5CRDK6RD3PGA

Supplementary Figure 3

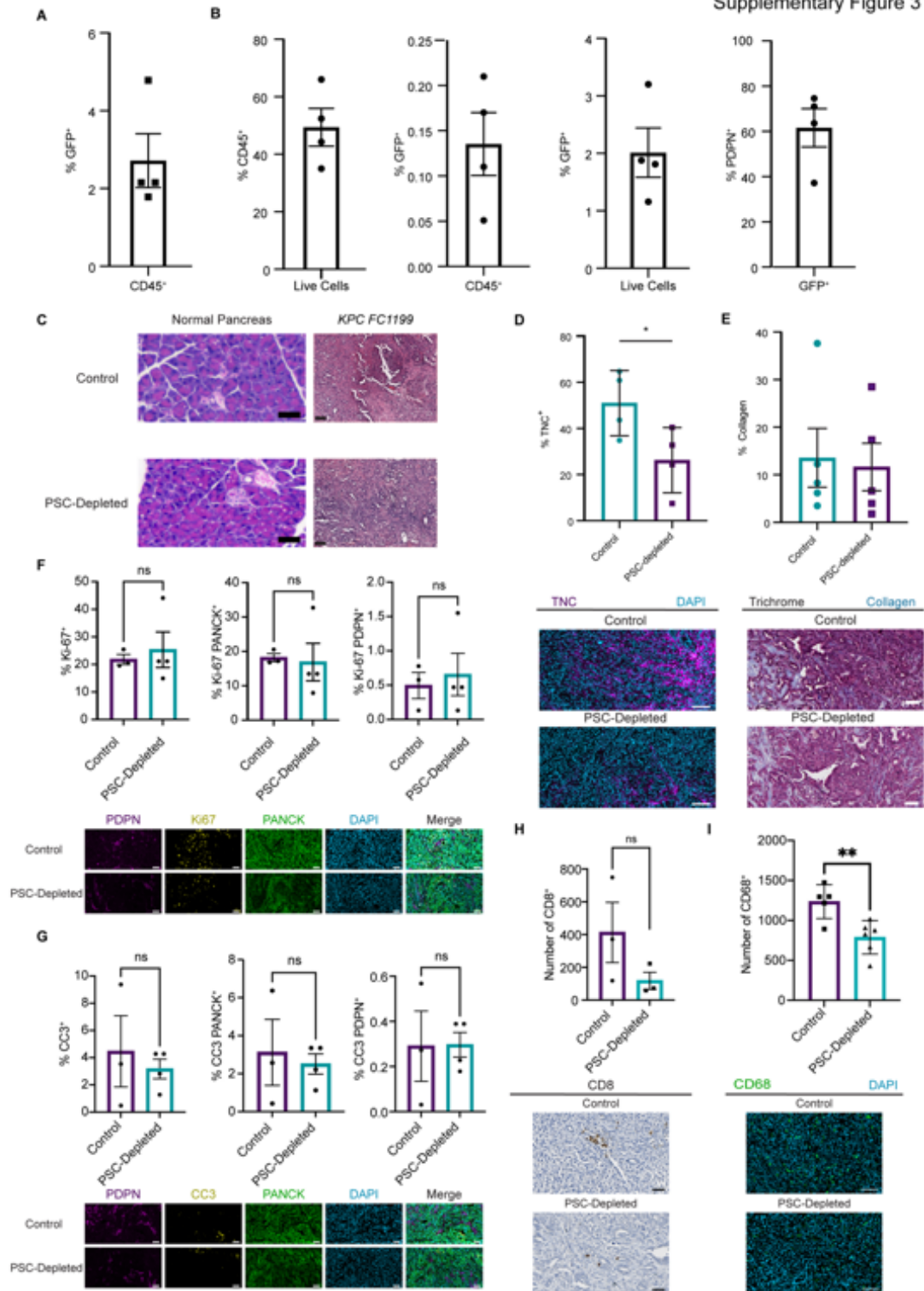


Figure 2-S 3 related to Figure 4.

(A) Flow cytometry analysis of CD45 and GFP in KPC FC1199 PDAC in *Fabp4-Cre; Rosa26^{mTmG}* mice (n = 4). Data are presented as mean $\pm$ SEM. (B) Flow cytometry analysis of CD45, GFP, and tdTomato in KPC FC1199 PDAC in *Rosa26^{mTmG/iDTR}* mice transduced with intraductal AAVKP1-Fabp4-Cre (n = 3). Data are presented as mean $\pm$ SEM. (C) Representative H&E images of normal pancreas or PDAC in *Rosa26^{mTmG/iDTR}* mice after 5 days of PSC depletion. Normal, scale bar = 50 μ m; PDAC, scale bar = 100 μ m. (D) Immunohistochemical staining for TNC in KPC FC1245 PDAC in *Rosa26^{mTmG/iDTR}* mice transduced with intraductal AAVKP1-Fabp4-Cre and treated with PBS or DT for 5 days (n = 3-4). Scale bar = 10 μ m. Data are presented as mean $\pm$ SEM. *p < 0.05 by unpaired t-test. (E) Trichrome staining and quantification of aniline blue signal (collagens) of KPC FC1199 PDAC in AAVKP1-Fabp4-Cre-injected *Rosa26^{mTmG/iDTR}* hosts, enrolled at 5-6 mm in tumor diameter and treated with PBS or DT for 5 days (n = 5). Scale bar = 50 μ m. (F) Immunohistochemical staining for Ki67, PDPN, and panCK in KPC FC1199 PDAC in *Rosa26^{mTmG/iDTR}* mice transduced with intraductal AAVKP1-Fabp4-Cre and treated with PBS or DT for 5 days (n = 3-4). Scale bar = 50 μ m. Data are presented as mean $\pm$ SEM. (G) Immunohistochemical staining for CC3, PDPN, and panCK in KPC FC1199 PDAC in *Rosa26^{mTmG/iDTR}* mice transduced with intraductal AAVKP1-Fabp4-Cre and treated with PBS or DT for 5 days (n = 3-4). Scale bar = 50 μ m. Data are presented as mean $\pm$ SEM. (H) Immunohistochemical staining for CD8 in KPC FC1199 PDAC in *Rosa26^{mTmG/iDTR}* mice transduced with intraductal AAVKP1-Fabp4-Cre and treated with PBS or DT for 5 days (n = 3). Scale bar = 50 μ m. Data are presented as mean $\pm$ SEM. (I) Immunohistochemical staining for CD68 in KPC FC1199 PDAC in *Rosa26^{mTmG/iDTR}* mice transduced with intraductal AAVKP1-Fabp4-Cre and treated with PBS or DT for 5 days (n = 5). Scale bar = 50 μ m. Data are presented as mean $\pm$ SEM. **p < 0.01 by unpaired t-test.

Supplementary Methods

Gene expression analysis by qPCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher) per manufacturer's instructions. The isolated total RNA (1 μ g) was reverse-transcribed to produce cDNA using iScript Reverse Transcription Supermix kit (Bio-Rad). Real-time PCR was performed using SYBR Green supermix (Bio-Rad). The cDNA sequences for genes of interest were obtained from the mouse genome assembly (<http://genome.ucsc.edu>) and gene-specific primers were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3/primer3_code.html). Relative gene expression was

normalized using the 36B4 housekeeping gene. The following primer sequences were used:

36B4 (*Rplp0*): F: 5'-GTGCTGATGGGCAAGAAC-3', R: 5'-AGGTCCTCCTTGGTGAAC-3'; *Ins1*: F: 5'-TATAAAGCTGGTGGGCATCC-3', R: 5'-GGGACCACAAAGATGCTGTT-3'; *Fabp4*: F: 5'-GATGGTGACAAGCTGGTGGT-3', R: 5'-AATTTCCATCCAGGCCTCTT-3'; *Des*: F: 5'-ACTTGACTCAGGCAGCCAAT-3', R: 5'-ATCCTCCAGCTCCCTCATCT-3'; *Krt19*: F: 5'-TGACCTGGAGATGCAGATTG-3', R: 5'-AATCCACCTCCACACTGACC-3'; *Vim*: F: 5'-ACGGTTGAGACCAGAGATGG-3', R: 5'-TCTTGCGCTCCTGAAAAACT-3'; *Cspg4*: F: 5'-TTACCTTGGCCTTGTTGGTC-3', R: 5'-GAGCTGGAGCAAGAGATGGT-3'; *Prss3*: F: 5'-TCTGTCCCCTACCAGGTGTC-3', R: 5'-GTTGGGGTGCTTGATGATCT-3'; *Tie1*: F: 5'-TGCCCTTTTAGCCTTGGTGT-3', R: 5'-AGGATGGTCTCTTCACCCGA-3'; *Tnc*: F: 5'-CCTGGACGGCATCGGAGAAT-3', R: 5'-TTGTTTGGTGCCCTTGAGTGA-3'; *Acta2*: F: 5'-TCAAGGAGAAGCTGTGCTATGT-3', R: 5'-TTCGTGGATGCCCCGCTGA-3'

RNA-seq

RNA was isolated from sorted GFP⁺ and tdTomato⁺ CAFs (n = 3) using TRIzol and the Qiagen RNeasy Mini Kit with on-column DNase digestion for clean-up. Libraries were created using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio USA, Inc.). The input was 10 ng and 8 PCR cycles were used for the prep. Libraries were quantified using KAPA qPCR and pooled in an equimolar ratio. Libraries were sequences

on the Illumina HiSeq 2500 on a Single Read Flow Cell using Illumina HiSeq SR cBot Cluster Kit v4 and HiSeq SBS v4 reagents.

RNA-seq reads were aligned to the human reference genome (GRCh38, release 84) using STAR (version 2.5.2b) with default parameters (2). The STAR “GeneCounts” module was used to quantify the number of reads mapping to each gene. Gene expression quantified by read counts from STAR were used as input into DESeq2 for differential expression gene (DEG) analysis (3). Subsequent DEG analyses were performed using the 12,749 genes with counts per million (cpm) greater than 1 in at least four samples. The DEGs were called with a false discovery rate (FDR) less than 0.05. In the DESeq2 package, counts were normalized using the variance stabilizing transformation (VST) module in DESeq2 for downstream analyses. For heatmap generation, differentially expressed genes were clustered using Pearson correlation distance and the complete clustering method from the pheatmap R package.

Generation of p53-null PDAC cell lines

The pSpCas9(BB)-2A-Puro(PX459) v2.0 plasmid (Addgene #62988) was used to clone guide sequences targeting *Trp53* per supplier’s protocol: sgRNA #1: CACCGACCCTGTCACCGAGACCCC, sgRNA #2: CACCGGGAGCTCCTGACACTCGGA. The FC1245 PDAC cell line was transfected with control plasmid or plasmid containing either of the sg*Trp53* sequences and subject to selection with 2 µg/ml puromycin for 4 days. Single-cell clones were expanded and screened for p53 expression by Western blot, and clones with loss of p53 protein were selected for *in vivo* experiments.

Western blotting

For all Western blots, cells were harvested in ice-cold PBS and lysed in RIPA buffer containing phosphatase and protease inhibitors. Proteins were resolved on a 4-12% Bis-Tris NuPAGE gel (Invitrogen), then transferred onto a PVDF membrane. Membranes were probed with primary antibodies and infrared secondary antibodies: p53 (Cell Signaling Technology 2524), HSC70 (Santa Cruz sc-7298), anti-rabbit Alexa Fluor Plus 680 (Thermo Fisher A32734) and anti-mouse Alexa Fluor Plus 800 (Invitrogen A32730). Protein bands were detected using the Odyssey CLx infrared imaging system (LICOR Biosciences).

PSC-derived versus non-PSC-derived CAF signature comparison to sc-RNA-seq profiles

We validated the performance of our differentially expressed gene signature from GFP⁺ versus tdTomato⁺ CAFs related to 3 CAF populations (iCAF, myCAF, and apCAF) defined in a previous study (4). The signature contains both significantly up-regulated and down-regulated differential expression genes in GFP (FDR < 0.05 without any foldchange cutoff). We built a pseudo-regulon based on the direction of the signature genes, i.e., the up-regulated and down-regulated genes were served as the positive and negative target genes of the constructed pseudo-regulon, respectively. The single-sample VIPER (function name: viper) in the viper R package (5) was employed to infer the activity of the constructed pseudo-regulon, which was used as the signature enrichment score for each cell. The statistical p-values were determined using the Wilcoxon rank-sum test.

Supplementary references

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Chapter 3 : Characterization of PSC derived CAFs

Preamble: Overarching problem and approach

The survival rate of PDAC has stayed largely consistent, barely moving percentage points over the last decades, currently sitting at the bottom of common tumor types¹¹⁶. Commonly, PDAC is found in advanced stages where the tumors have metastasized^{117,118}. Despite advancements in treatments, including chemotherapies and immunotherapies that work in other cancers, PDAC has remained resistant^{119,120}. These therapeutic options are thought to be thwarted by the stroma in multiple ways. One of these ways is through the physical barrier the ECM and fibroblasts create that blocks drug delivery. PDAC tumors are composed of more stroma than tumor cells³⁸. The stroma comprised up to 80% of the tumor¹²¹. Attempts have been made to study depleting this stroma to improve therapeutic penetration of the tumor and efficacy. Ablation of the stroma by various methods, including direct and indirect, did not result in improved outcomes of mice with orthotopic tumors. Loss of stroma through CAF ablation in these models resulted in disorganized ECM, poorly differentiated tumor histology, and worsened survival^{46,47,94}. These results indicated heterogeneity in the CAF populations, with not all CAFs being protumor⁵¹. Our recent study identified a population of protumor CAFs that derive from pancreatic stellate cells. PSC-derived CAFs appear to be responsible for specific ECM components (including TN-C), mechanosensing molecules (p-MLC2), and tissue stiffness⁷⁵. Each of these components is associated with tumor progression and invasion^{122–124}. The outcome of our study of PSC-derived CAFs indicates that they may be a target to deplete the protumor aspect of the stroma. Depleting the protumor compartment of the stroma in combination with standard of care therapy

chemotherapeutics or previously inefficacious therapies (immunotherapies) could improve patient outcomes, moving that survival rate. However, there is much still to understand about these PSCs for accurate and efficient targeting. Important steps include identifying what other roles PSC-derived CAFs play in the tumor, including primary tumor growth and metastasis, as well as the identification of factors produced by PSC-derived CAFs that may promote PDAC progression and thus serve as potential therapeutic targets. In line with these goals, temporal analysis of PSC-derived CAF abundance during PDAC initiation through pre-malignant lesion progression to invasive cancer is important to understand the potential, relative importance of PSC-derived CAFs versus CAFs of other origins at distinct steps of PDAC evolution.

We first looked at the impact of PSC-derived CAFs in metastasis. To study metastasis, we utilized our AAV-Fabp4-Cre ductal injections in Rosa26^{mTmG/iDTR} mice for PSC-derived CAF depletion. Commonly, orthotopically implanted KPC cell lines do not metastasize; however, the Stanger lab has characterized cells isolated from KPC mice that do metastasize¹²⁵. They provided these lines for us to use in PSC-derived CAF ablations. Secondly, we sought to characterize PSC-derived CAFs throughout PDAC initiation and progression. In order to study this population across the tumor development, we had to cross our Fabp4-Cre; Rosa26^{mTmG} mice into a genetically engineered mouse model that develops PDAC via premalignant lesions. Due to the use of Cre to drive Fabp4, we obtained Pdx1-FlpO mice to drive frt-flanked Kras mutations and p53 loss. Lastly, we investigated the impact of tumor genotype and downstream signaling on CAF heterogeneity. This study followed up on our previous results showing that p53

gain of function mutation, particularly R172H, results in increased PSC-derived CAFs compared to p53 null tumors⁶⁷.

Identifying the roles of PSC-derived CAFs and the impact of signaling on these cells will help us identify ways to target them. Targeting the protumor cells of PDAC could result in improved therapeutics.

Abstract

Recently the drivers of fibroblast heterogeneity have become an essential topic of discussion for improved therapeutic efficacy in PDAC. The cell of origin has been determined to be a driver of heterogeneity, with stellate cells giving rise to a minor but tenacious subset of fibroblasts. However, it is still clear that signaling gradients determined by the cancer cells' unique genetics drive heterogeneity of the fibroblasts, including regulating the cells of origin activation resulting in varying levels of heterogeneity. Here we analyzed the contribution of the subset of fibroblasts derived from stellate cells to the metastatic progression. We also investigated the impact of tumor cells' gain of function p53 mutations on the pancreatic stellate cell-derived CAF population and how this impacted overall heterogeneity. It has been postulated that PSC-derived CAFs play a role in liver and lung metastasis, enabling cancer cell migration and invasion or potentially setting up the tumor cells for survival at distant sites. Targeted ablation of PSC-derived CAFs showed an overall decrease in metastasis and an overall increase in survival in a metastatic mouse model. Our results also hint at the need to look further at perlecan production by PSC-derived CAFs driving the metastatic nature of p53 gain of function mutation. Together, these findings indicate that tumor genotype regulates

PSC-derived CAFs involvement in the tumor compartment and that these PSC-derived CAFs are essential to the metastatic process as well.

Introduction

PDAC is a dense tumor with a poor survival rate due in part to its stromal reaction preventing effective therapeutics access to the tumor¹²⁶. The stroma is densely packed with fibroblasts, and these fibroblasts primarily produce the ECM¹²⁷. Recent ablation studies have determined that this stroma is heterogeneous, as loss of fibroblasts indistinctly resulted in disorganized ECM and worsened survival^{46,47,94}. Follow-up studies have determined that both cell of origin and signaling gradients determined by tumor genotype play a significant role in the heterogeneity of CAFs in the tumor microenvironment^{62,67}. It is now understood that in order to target the stroma to improve tumor treatment outcomes, we must first identify the protumor CAF subsets and what roles they play to exploit these unique pathways.

Since the discovery of CAF heterogeneity through ablation studies, the race has begun to identify the role of each subset. Single-cell sequencing studies have determined that fibroblast heterogeneity changes over the course of the developing tumor⁵³. These studies took snapshots from the normal pancreas, early panINs, and full-on PDAC. From the normal pancreas, it was determined that at least two populations of fibroblasts precursors reside in healthy tissue. These populations give rise to inflammatory and myofibroblast populations that set up the microenvironment of the developing tumor at the panIN stage⁵⁰. Once the tumor reaches invasive PDAC, three distinct populations of CAF emerge: iCAFs, myCAFs, and apCAFs (antigen-presenting CAFs)^{59,60,64}. Recently,

we discovered that PSC gives rise to but a subset of CAFs, that have a unique role and appear to be among the myCAF's though certainly not the entire myCAF subset. While more studies are needed to determine if individual subsets of PSC-derived CAFs have distinct roles, to be determined by single-cell analysis, studies have begun to investigate how significant this small population is on the growing tumor. We have identified that PSC-derived CAFs are responsible for important protumor components, including TN-C and p-MLC2. In addition, PSC-derived CAFs play an essential role in tissue stiffness, possibly through FAK signaling⁷⁵. ECM and stiffness are linked to tumor progression and metastasis, leading us to investigate the impact of PSC-derived CAFs on metastasis.

Snapshots of the PDAC tumor stroma over the course of tumor development have highlighted various populations and hinted at the role of each of these and how they change. Normal pancreas stellate cells whose function is in structure, fat storage, and wound healing give rise to a subset of CAFs where these roles are exploited^{29,128}. PSC-derived CAFs are one set responsible for ECM components and tissue stiffness in PDAC and are indicated to have some impact on immune cell infiltration through this mechanism remains unknown. It also remains unknown how PSC-derived CAFs evolve with the tumor through normal pancreas with inflammation to PanIN stages to PDAC. Until recently, it was not possible to track PSC-derived CAFs. Now utilizing a lineage trace Fabp4-Cre with a Pdx-FlpO driven mutant Kras and loss of p53, we can trace PSC-derived CAFs through the developing tumor to determine how they set the environment along with the progression of PDAC from start to expansion.

Until recently, it was thought that PSCs gave rise to all of the CAFs of PDAC, and signaling gradients were identified as the drivers of heterogeneity and CAF subsets

or various roles^{61,74}. While cell of origin has been identified as a source at least, in part, of CAF heterogeneity, evidence suggests that signaling gradients are essential as well. Critical signaling gradients produced in the tumor environment are crucial to the CAF heterogeneity, deriving subsets with distinct roles. Identified through PSC isolation, TGF- β signaling favors a myCAF phenotype playing a crucial role in ECM, while IL-1 is implicated in driving an iCAF phenotype regulating immune cells. IL-1 induces JAK/STAT signaling within the tumor that antagonizes TGF- β resulting in spatial implications. IL-1-induced iCAFs reside more so in the stroma farther from the tumor cells, whereas TGF- β regulates myCAFs often adjacent to tumor cells⁶¹. A recent study suggests that tumor cell genotype and tumor cell proximities control CAF heterogeneity. Specifically, p53 status is implicated in determining the outcome of paracrine signaling on neighboring CAFs. Studies comparing CAFs isolated from KPC tumors (p53 mutant) versus Kras^{LSL-G12D/+}; Trp53^{flox/+}; Pdx1-Cre tumors (p53 null) found CAFs close to PDAC cells harboring this p53 mutation are shaped to promote metastasis and chemoresistance. Various signaling pathways downstream of this mutant p53 are implicated in driving the protumor phenotype of nearby CAFs, mainly perlecan. Perlecan is thought to be a part of the signaling pathway regulated by CAFs as p53 mutant tumor cells secrete high levels of TNF α which is a target of NF- κ B, which stimulates CAFs to secrete HSPG2 or perlecan an ECM component linked to heightened prometastatic and chemoresistant phenotypes⁶⁹. Besides perlecan, in a study comparing the secreted factors of p53 mutant PDAC cells compared to p53 null PDAC cells implicated PDGFR- β to be expressed more highly in the presence of mutant p53. A 2015 study implicated PDGFR- β to induce pancreatic cancer cell invasion and metastasis, finding that inhibition of PDGFR- β prevented the

formation of metastases¹¹². It remains to be determined if PDGFR- β contributes to PSC derived CAFs frequencies and whether or not p53 mutation leads to this PDGFR- β signaling. It is also unknown if the PDGFR- β signaling drives PSC-derived CAFs to cause the increased metastasis, whether or not CAFs are the culprit downstream of p53 and PDGFR- β . Now that it is possible to trace PSC-derived CAFs within orthotopic tumors with differing p53 statuses, we are one step closer to answering this question. It should be noted that PDGFR- β is a marker used to identify CAFs indicating that CAFs have the expression of this receptor but not necessarily the downstream mechanism linked to metastatic progression. However, the role of tumor genotype and its resulting downstream signaling on CAF cell of origin and resulting heterogeneity pathways are still incompletely understood. Our recent results indicate that mutant p53 tumors have increased contribution of PSCs to the CAF pool when compared to p53 null⁷⁵. It is still to be determined if perlecan is elevated as a consequence of increased frequency of PSC-derived CAFs. As PSC-derived CAFs are pro-tumor and prometastatic, identifying perlecan as a potential link among p53 status, CAF cell of origin, and metastatic spread could be an avenue to target the stroma to prevent the blocking of chemotherapeutics and reduce metastatic potential. While tumor cells and p53 mutations exploit the PSC-derived fibroblast's role in wound healing and normal tissue structure, it may be possible to control the signaling pathways that drive PSC-derived CAFs or mechanisms derived from them, to increase chemotherapies access to the tumor and reduce metastasis with the potential to improve outcomes.

We found that PSC-derived CAF ablation reduced metastasis and increased survival, indicating these cells to be prometastatic. Importantly, we have developed a

method to track these PSC-derived CAFs through tumor progression to identify pathways to exploit to prevent chemotherapy resistance derived from these cells. In addition, we found that perlecan production is of interest as a potential pro-metastatic function of these cells; however, it appears that other pathways may be of more importance or in conjunction with perlecan as a consequence PSC-derived CAF levels.

Results

Targeted ablation of PSC-derived CAFs divulges function in regulation of metastasis.

To identify the impact of PSC-derived CAFs on PDAC metastasis, we orthotopically implanted 2838 cells isolated from KPC mice in the Stanger lab. The Stanger lab graciously provided these cells to us and had shown them to metastasize to the liver and lung when implanted in the pancreas¹²⁵. 2838 cells were implanted in mice 9-12 weeks of age in the same surgery as intraductal transduction of AAVKP1-Fabp4-Cre in our Rosa^{iDTR/mTmG} ablation model as previously described in Chapter 2. Mice were monitored by ultrasound. Once tumors reached 3 mm in diameter, they were enrolled and treated with control or DT over 5 days. From there, tumor growth was monitored once a week by ultrasound, and mice were monitored for morbidity. Once mice reached endpoint (morbidity: hunched posture), they were euthanized, and primary tumors weighed. To identify metastasis, lungs and livers were harvested (Figure 3-1A). There was no significant difference in primary tumor weights at the endpoint between control and PSC-derived CAF-depleted mice (Figure 3-1B). As primary tumor size was not different, it can be implicated that the difference in metastasis is not the result of differences in the growth of the primary tumor. The leading sites of metastasis of the PDAC are the liver and the lung, with local invasion to the spleen common as well¹²⁹.

Liver and lungs were sectioned for H&E staining followed by hand counts of one section per mouse to prevent counting sections of the same met more than once. H&E analysis of lungs that were PSC-derived CAF depleted compared to controls showed a trend of fewer metastases; however, it did not reach statistical significance, potentially due to low n (Figure 3-1C). On the other hand, liver metastasis had a trend towards a reduction with PSC-derived CAF depletion compared to control tissues further samples would further reveal any significance (Figure 3-1D). As these were survival studies, analysis of PSC-derived CAF depleted mice showed improved survival (with one mouse currently still on study at the time of this writing) compared to PSC-derived CAF intact mice (Figure 3-1E). Together these results suggest that PSC-derived CAFs play a role in the metastatic niche, impacting survival.

Development of a model to characterize PSC Derived CAFs through PDAC initiation and progression.

KPC mice develop pancreatic tumors through Pdx1-Cre activation of Kras^{LSL-G12D} mutation and p53^{LSL-R172H/+} loss. These tumors develop through inflammation to panIN to PDAC tumor formation¹⁰⁶. However, this model is not usable in conjunction with PSC-derived CAF tracking due to the need for the Fabp4-Cre to label the PSC lineage. To enable analysis of stromal evolution in a genetically engineered mouse model that progresses through all stages of PDAC development, we used a dual recombinase approach with a Pdx1-FlpO driver of Kras^{FSF-G12D} activation and p53^{flr/+} loss, allowing for the additional driver Cre under the Fabp4 promoter to track PSC-derived CAFs^{130,131}. Breeding of hemizygous PdxFlpO^{ki} and Fabp4-Cre mice to homozygous mice Rosa^{mTmG} p53^{flr/+} with heterozygous Kras^{FSF-G12D} resulted in pups heterozygous for all alleles at a ratio of 1/16 (Figure 3-2). Three pups were enrolled for the initial study, monitored by palpation of the pancreas starting at two months of age, followed by ultra-sounding beginning at 4 months of age. Tumors began to form around 6 months of age, and once they reached 30mm² in area by ultrasound, mice were euthanized. At the time of euthanasia, primary tumors and pancreas will be harvested and fixed to examine, PSC-derived CAFs from the adjacent normal pancreas, PanINs, and PDAC tumors as early in tumor development; the tumor does not take up the complete pancreas. At the time of this writing, three mouse have developed tumors. These results will identify if PSC-derived CAFs play a role at all stages of the developing tumor, starting at early PanIN lesions.

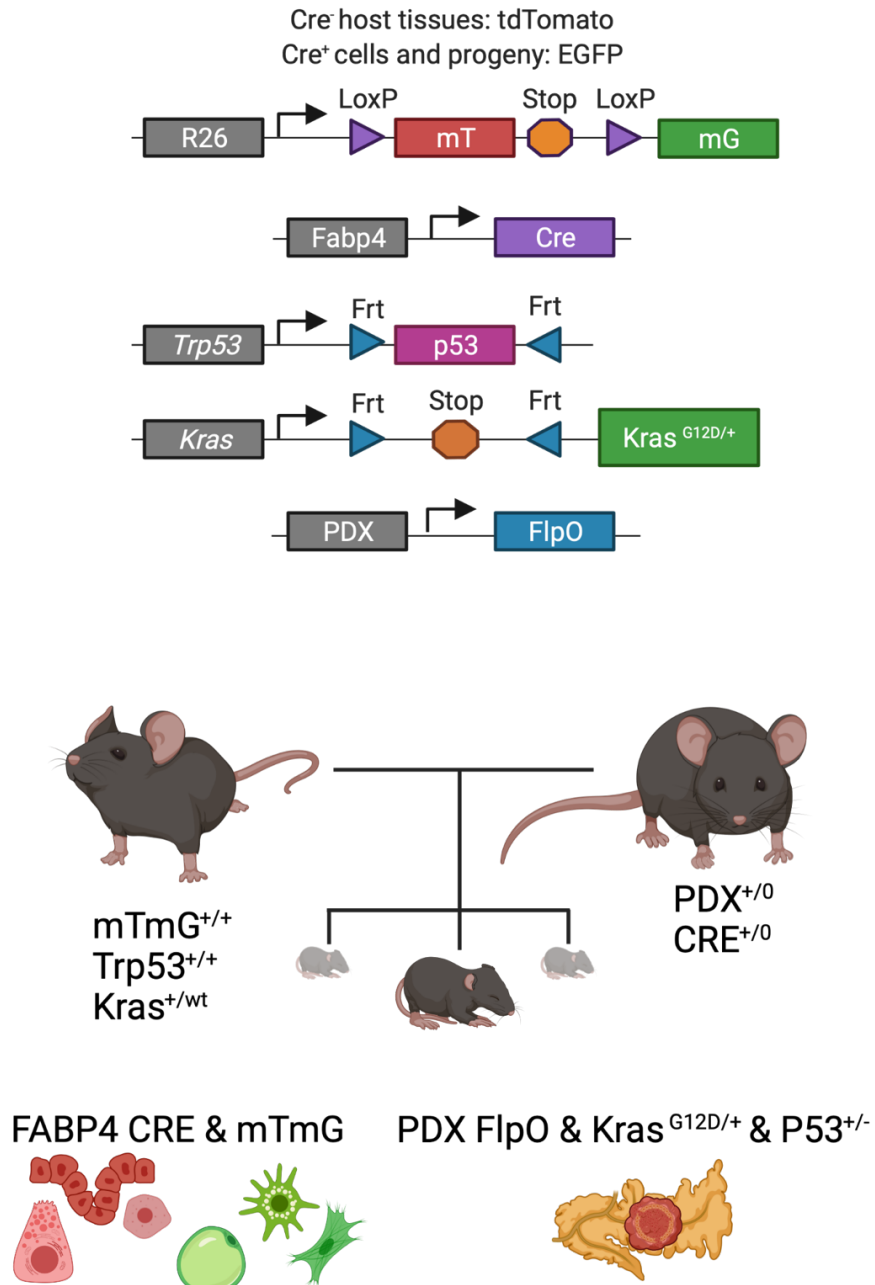


Figure 0-2. Development of a model to characterize PSC Derived CAFs through PDAC initiation and progression.

Schematic outline of mouse breeding strategy to produce Fabp4-Cre, Rosa26^{mTmG} PdxFlpO^{ki} p53^{frt/+} Kras^{FSF-G12D/wt} mice. F2 mice Fabp4-Cre and Rosa26^{mTmG} (reporter), to trace PSC-derived CAFs; PdxFlpO^{ki} p53^{frt/+} Kras^{FSF-G12D/wt} to develop PDAC tumors.
Created with BioRender.com

Identifying the mechanism of tumor genotype on stromal regulation.

Our results suggest CAF heterogeneity results from the cell of origin and an impact of tumor genotype on stroma role in respect to the cell of origin. We previously compared two tumor lines from the same background with the same driver mutation in Kras but differed concerning p53 status. In these tumors, p53 gain of function mutation R17H was compared to tumors with p53 loss. Size matched tumors from these models showed a reduction in PSC-derived CAFs with loss of p53. Following up, we identified through Trp53 knockout that while only significant in one p53 null line, a trend occurred with a reduction of PSC-derived CAFs compared to the parental mutant p53 tumor line. The resulting trends in reducing proteins linked to PSC-derived CAFs indicated that tumor genotype drives PSC-derived CAF contribution in the tumor and resulting heterogeneity. As this particular p53 mutation is gains function and results in increased metastasis in the human equivalent tumors, it is vital to identify the driver and signaling pathway to find targets to prevent protumor PSC-derived CAFs. PDGFR- β has been indicated as a mediator in p53 mutant tumors for tumor invasion and metastasis. Identified through sequencing of shRNA p53-knockdown compared to p53 controls showed upregulation of PDGFR- β . This study indicates that PDGFR- β (a marker for fibroblasts) could regulate CAF heterogeneity downstream¹¹². In our depletion model of PSC-derived CAFs, depleted tumors slightly reduced PDGFR- β however, this was not significant, and there was high heterogeneity across samples (Figure 3-3A). However, in our p53 knockout lines compared to the parental control, both knockouts had no difference in PDGFR- β expression with no trend (Figure 3-3B). Together these results indicate that PDGFR- β is unlikely to be the regulator of PSC-derived CAFs regarding

p53 tumor genotype. However, it does not exclude that tumor derived PDGFR- β signaling does not by some downstream protein impact the PSC-derived CAF involvement in p53 tumors. Complicating this matter PDGFR- β is a marker of CAFs but is also expressed on the surface of p53-mutant cancer cells. On the surface of p53-mutant cancer cells PDGFR- β is implicated in driving metastasis but this is likely to be mechanistically distinct from its role on PSC-derived CAFs. From our previous publication through sequencing, we found high expression of HSPG4 (encodes perlecan) in our PSC-derived CAF subset. Perlecan is linked to increased metastasis in p53 mutant tumor lines, and is produced by PSC-derived CAFs linking p53, stroma, and perlecan production to invasion and metastasis⁶⁷. We found an no difference in expression of perlecan in our p53 mutant tumors (Figure 3-3C). However, our data also suggest that follow-ups on perlecan for depleted tissues are warranted to determine if p53 mutation regulates PSC-derived CAF production of perlecan leads to changes in invasion and metastasis. While we have a p53 mutant line from a KPC mouse 2838, we would need to knockout p53 from this line to look directly at p53s impact on PSC-derived CAF perlecan on metastasis. It should be noted that the appearance of the perlecan in deplete sample's structure was more disconnected and less continuous compared to controls. Further analysis would be needed to quantify this difference. Though this difference in structure could be due to tissue artifacts it was consistently occurring in depleted samples and did not really appear across controls.

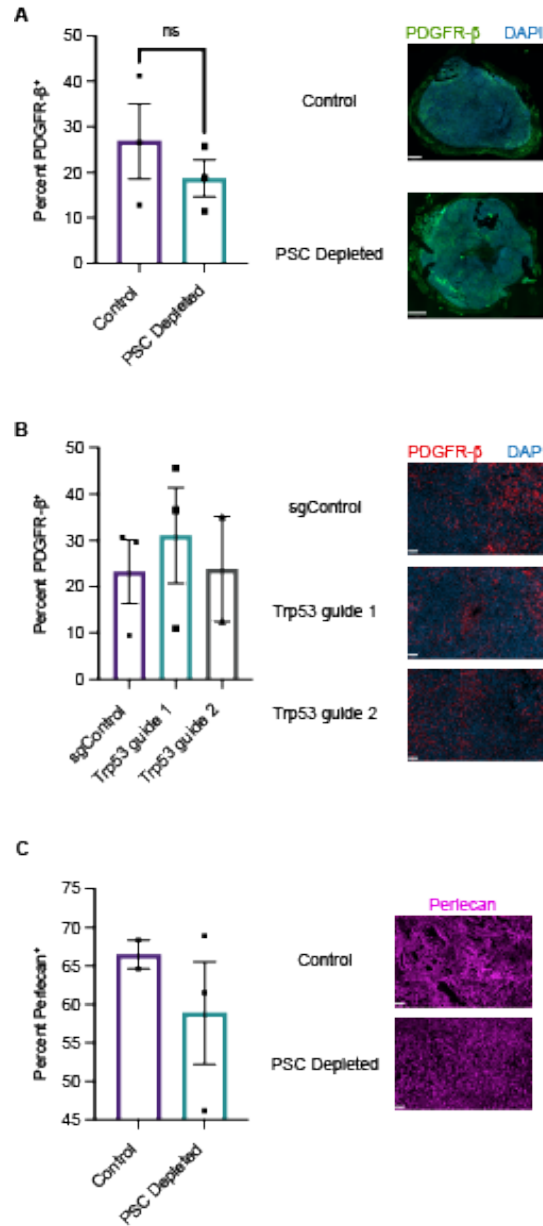


Figure 0-3. Identifying the mechanism of tumor genotype on stromal regulation.

(A) Immunohistochemical staining for PDGFR-β of KPC FC1199 PDAC in AAV-KP1-Fabp4-Cre-injected Rosa26^{mTmG/iDTR} hosts, enrolled at 5-6 mm in tumor diameter and treated with PBS or DT for 5 day (n=3). Scale bar = 500 μm, 1 mm. Data are presented as mean ± SEM. ns by unpaired t-test. (B) Immunohistochemical staining for PDGFR-β size-matched control and sgTrp53 PDAC in Fabp4-Cre;Rosa26^{mTmG} hosts (n=3,3,2). Scale bar = 100 μm. Data are presented as mean ± SEM. (C) Immunohistochemical staining for Perlecan of KPC FC1199 PDAC in AAV-KP1-Fabp4-Cre-injected Rosa26^{mTmG/iDTR} hosts, enrolled at 5-6 mm in tumor diameter and treated with PBS or DT for 5 days (n=2,3). Scale bar = 100 μm. Data are presented as mean ± SEM.

Discussion

The shocking results of CAF ablation revealed a subset of CAFs that played different roles in the tumor^{46,47,94}. Protumor roles have been identified, from supporting metabolism to providing a network for cells to invade and metastasize^{40,56,132}. Anti-tumor roles of CAF subtypes are less understood but have been linked to immune cell activation, BMPs, and physical restraint to the tumor since ECM was dysregulated in ablation studies^{47,49,133}. As PSC-derived CAFs are producers of specific matrix components, mechanosensing molecules, and tissue stiffness which are linked to aggressive tumor features: invasion and metastasis, it is reasonable to speculate that this subtype may be protumor and promote progression. Indeed, we have shown that PSC-derived CAFs are linked to lung and liver metastasis. Not only did we identify that these cells were involved in metastasis, but when metastasis was added to the model, these cells were linked to a reduction in survival. These data contrast with results from a non-metastatic model, where ablation of PSC-derived CAFs had no impact on overall survival (unpublished data). As tumor genotype specifically, a gain of function mutation to p53 has been shown to increase metastasis, as have PSC-derived CAFs^{67,75,112}. It was fair to speculate that PSC-derived CAFs may be downstream of this p53 mutation, as they are enriched in these tumors, which could result in the metastatic phenotype of this tumor. In order to follow this pathway and connect them, identifying the recruiters of PSC-derived CAFs in p53 mutant tumors would indicate mechanisms. Comparison of PSC-derived CAF depleted tumors and controls resulted in no difference to perlecan however these tumors were consistent for the gain of function p53 mutation which could be driving perlecan production across CAFs subtypes. Further studies are warranted to see the

impact of p53 loss on PSC-derived CAF perlecan production and could indicate compensation cell types. Due to CAF plasticity, it is fair to speculate that loss of one CAF subtype could drive other populations to compensate if signaling is present from the tumor. This data is impactful to the development of treatment strategies aimed at CAF subtypes as others could compensate rendering the treatment ineffective, however this line of thinking leads to targeting the protein directly in this case targeting either p53 or perlecan. Perlecan appears to be unaffected with the PSC-derived CAF ablation indicating other subtypes could compensate or that p53 mutations drive multiple subtypes production of perlecan and that perlecan is advantageous to the tumors progression and spread. Further study is needed to linking p53 studies of increased perlecan to p53 mutations resulting in increased PSC-derived CAF involvement in the tumors⁶⁷. These mechanistic studies are in progress to link tumor genotype, CAF cell of origin, and outcomes of invasion and metastasis.

Common mutations in PDAC tumors include Kras, p53, and Smad4 but various other mutations are obtained over the progression of the tumor, starting at inflammation ending in PDAC^{134–136}. Initially, inflammation recruits wound healing cells, including CAFs and immune cells, which further signal cell survival and proliferation as seen in a wound healing process¹³⁷. The tumor corrupts CAFs to exploit the wound healing properties that aid in tumor cell survival, such as dysregulation of the immune system, the scaffolding of ECM proteins, and nutrient production¹³⁸. It is reasonable to speculate that fibroblasts in the inflamed pancreas change over the course of the tumor progression. In order to identify how subtypes of fibroblasts change on their journey to CAFs, we utilized our lineage tracing Fabp4 model in conjunction with a KPF tumor development

model. Signaling gradients of these tumors are likely to shape the specific cell of origin PSC-derived CAFs. We have identified that this genetically engineered mouse model mouse survives and produces tumors around 6 months of age. Ongoing studies will investigate signaling changes, downstream metastasis, overall survival, and how PSC-derived CAF are involved with or impacted by each of these.

It is increasingly clear that signaling gradients and cells of origin shape the CAF heterogeneity of the tumor based on tumor genotype^{62,67,135}. PSC-derived CAFs are a numerically minor subset in the tumor, albeit with a distinct role; however, this leaves a remaining majority of CAFs to be of a different origin. Single-cell RNA-seq data suggest that apCAF are of a mesothelial cell origin based on transcriptional profiles⁶⁴. The remaining iCAF and myCAF (non-PSC derived) are likely from resident fibroblast populations or the bone marrow^{139,140}. However, these fibroblast populations will require additional tracing models to identify their roles or contributions to the tumor. Further RNA sequencing profiles have identified that normal pancreas stellate cells or fibroblasts do not contribute equally to the tumor and that heterogeneity is lost over the progressing neoplasia. For example, Gli1+ fibroblasts expand in early carcinogenesis while Hoxb6+ fibroblasts do not⁷³. It will be essential to identify alterations of stromal composition and the role of each component to identify better which populations are protumor and target for deactivation based on studies of these populations through tumorigenesis. Identifying and tracing anti-tumor populations may reveal ways to shift protumor CAFs to anti-tumor phenotypes or methods to increase these populations through signaling gradients that favor them. Already, hedgehog signaling has been identified as necessary to the activation of myCAF more so than iCAF. Inhibiting this hedgehog pathway reduced

myCAFs and increased iCAFs, which led to immunosuppression and growth suppression⁹⁰. Meanwhile, depletion of type I collagen in myCAFs resulted in immune suppression and tumor progression⁹⁵. Therefore, future studies to define the mechanisms shaping CAF heterogeneity and the functions of CAF subsets will improve our understanding of CAFs as well as give future direction to perhaps steer towards stromal targets for therapeutic intervention to allow chemotherapies or even perhaps immunotherapies to be efficacious in PDAC.

Materials and Methods

Animals

All experiments performed in mice were reviewed and overseen by the institutional animal care and use committee at Oregon Health & Science University in accordance with NIH guidelines for the humane treatment of animals. Fabp4-Cre (005069) and Rosa26^{mTmG} (007676) mice from Jackson Laboratory were used for PSC analyses and orthotopic transplantation experiments at 8-12 weeks of age, including male and female mice. Rosa26^{mTmG} and iDTR (007900) mice from Jackson Laboratory were used for retrograde ductal injection and orthotopic transplantation for PSC ablation experiments at 8-12 weeks of age, including male and female mice.

PdxFlpO^{ki} mice were (provided by Dr. Michael Ostrowski) crossed with the previously described Fabp4-CRE mice to create a drive breeder mouse. Kras^{FSF-G12D} mice were crossed with p53^{frt/+}, Rosa^{mTmG} mice to obtain a breeder mouse. These breeder mice were crossed to obtain a GEMM mouse where the Kras oncogene is activated, and p53 is

deleted resulting in PDAC mice (KPF) with a reporter cassette to track PSC-derived CAFs through GFP expression when the Fabp4 promoter is active. PCR and gel electrophoresis identified mice carrying all five alleles PdxFlpO^{ki}, p53^{flt/+}, Kras^{FSF-G12D}, Fabp4-CRE, Rosa^{mTmG}. Additional control mice were identified lacking the driver PdxFlpO^{ki}. Mice were monitored by palpation beginning at 2 months followed by ultrasounds once a week at 3 months of age. Once tumors were identified by ultrasound to be 3 mm in diameter mice were euthanized, tumors harvested along with lungs and livers. Overall health was monitored by laboratory and veterinary staff regularly.

Cell culture

FC1199 and FC1245 (provided by Dr. David Tuveson), 2838 (provided by Dr. Ben Stanger) PDAC cell lines were derived from primary murine PDAC. Cell lines were routinely passaged in DMEM (Thermo Fisher) containing 10% FBS (HyClone) for no more than 25-30 passages. Cell lines were routinely tested for Mycoplasma at least monthly (MycoAlert Detection Kit, Lonza). Cell line authentication was not performed.

Immunohistochemistry

For mouse tissue harvest, mice were anesthetized and euthanized according to institutional guidelines. Pancreas tissue or tumors were excised carefully and fixed overnight in 10% neutral buffered formalin or embedded in OCT and frozen at -80°C. Fixed tissues were paraffin-embedded, sectioned, deparaffinized, and rehydrated through an ethanol series and ultimately in PBS. Following antigen retrieval, tissue samples were blocked for two hours at room temperature in blocking solution (8% BSA) and

transferred to a carrier solution (1% BSA) containing diluted antibodies. Sections were incubated overnight at room temperature and then washed five times for 5 minutes each in PBS. Secondary Alexa-fluor conjugated antibodies diluted in the same carrier solution (1:200) were added to the sections for two hours at room temperature. Sections were then washed five times for five minutes each in PBS, autofluorescence quenched with the TrueVIEW reagent (Vector Laboratories), stained with DAPI, and mounted with Vectashield mounting medium. Fresh-frozen tissues were sectioned, fixed, and used to stain for perlecan in FC1199 tumors then counterstained with DAPI-containing Vectashield mounting medium for fluorescence microscopy. Antibodies used for immunohistochemistry were as follows: GFP: Cell Signaling Technology 4B10 (mouse) or D5.1 (rabbit), α -SMA: Thermo Fisher MA511547, PDGFR- β Ms mAB: Abcam ab69506, PDGF Receptor β Rb: Cell Signaling Technology 3169S PDPN: Thermo Fisher 14-5381-81, Perlecan: EMD Millipore MAB1948P, TIE1: Abcam ab111547 (mouse tissues, Supplementary Figure S2B), TIE1: Biorbyt orb11476 (mouse tissues, Supplementary Figure S2C), TIE1: Thermo Fisher PA527903 (human tissues). Stained tissues were imaged using a laser-scanning confocal inverted microscope (LSM 880, Carl Zeiss, Inc.), and a 40x/1.1 NA water objective or 63x/1.4 NA oil objective was used to image the samples. Slides were scanned using a Zeiss Axio Scan.Z1 and quantified using QuPath or Aperio software. For quantification of TIE1/ α -SMA colocalization on human PDAC sections, images were acquired on an AxioScan.Z1 using a 10x 0.45 NA plan-apochromat lens. Fluorochromes were excited with a Colibri 7 light source (Carl Zeiss), and excitation and emission light were passed through the following Zeiss filter sets for the appropriate channel: DAPI- 96 HE; Alexa-fluor 488- HE 38; Alexa-fluor 594- 71

HcRed. Images were analyzed for colocalization in ZEN v2.3 (Carl Zeiss). Thresholds for the AF594 and AF488 channels were set by eye for each slide (three slides total) for the TMA analysis. Within each slide, the same thresholds were used across all tissues. Dynamic range was set to a 14-bit image (16384 maximum intensity).

Orthotopic PDAC experiments

Male and female mice at 8-12 weeks of age were used as hosts for PDAC orthotopic transplantation, using genotypes described in the Results section and figure legends. Mice were anesthetized with ketamine and xylazine, and pancreata were injected with 5×10^3 FC1199 or FC1245 cells (lines provided by Dr. David Tuveson), 1×10^5 4662 cells (provided by Dr. Robert Vonderheide), or 2×10^4 HY2910 cells (provided by Dr. Ben Stanger), all derived from primary PDAC in $Kras^{LSL-G12D/+}; Trp53^{LSL-R172H/+}; Pdx1-Cre$ mice (FC1199, FC1245, 2838) of a C57BL/6J genetic background. Orthotopic transplantation was performed as previously described³. For PSC-derived CAF ablation experiments, pancreata were imaged beginning 14 days after transplantation by high-resolution ultrasound using the Vevo 770 imaging system; mice were enrolled on study when tumors reached 5-6 mm in diameter. Enrolled mice were treated with sterile PBS or 25 ng/g diphtheria toxin (List Biological Laboratories) by intraperitoneal injection every 2 days and tumors were harvested on day 5 post-enrollment for analysis.

Retrograde ductal AAV delivery

A promoter and enhancer element upstream of mouse Fabp4 (from Addgene #8858) was cloned into pAAV-iCre-WPRE (Vector Biosystems) upstream of Cre. KP1-serotyped

AAV- Fabp4-Cre was generated and tittered by the OHSU Molecular Virology Core. AAVKP1- Fabp4-Cre viral stock was diluted to 1×10^{10} viral genomes/ml in 10 μ g/ml DEAE-Dextran (Sigma-Aldrich) in PBS, incubated for 30 mins at room temperature, then placed on ice. Retrograde ductal injections were performed as previously described ¹⁰⁸. Mice were anesthetized with ketamine and xylazine until non-responsive to toe pinch. Feet were taped down with surgical tape on a sterile mat. Entire abdomen was shaved, and the shaved area cleaned with 70% ethanol and betadine. A midline incision was made about 1 inch long, and incision edges were secured with hemostatic forceps. Non-weave sterile gauze was moistened with sterile PBS. Intestines were gently extracted using circle forceps, and gently laid onto damp gauze. Intestines were slowly extracted until duct was visible. Exposed intestines were covered with damp gauze to keep moist, and sterile PBS was continually added throughout the procedure as necessary to keep gauze and tissue from drying out. One microvascular clip was placed on the cystic duct near the gallbladder. The sphincter of Oddi was located; a 30G insulin syringe was loaded with 100 μ l viral solution (to inject 1×10^9 viral genomes per mouse), and the needle inserted through the sphincter of Oddi into the common bile duct up to its convergence with the cystic duct, about halfway to the clip. Viral solution was slowly injected over the course of 2 mins. The needle was left in place for 30 sec after completion of the injection, then slowly and gently removed. The clip was then removed. Intestines were carefully returned to the abdomen, muscle layer closed with vicryl sutures, and skin closed with sterile suture clips. When performed together with orthotopic transplantation of PDAC cells, cells were injected after removal of the syringe and clip.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM software. Student t-test was used to compare two groups to each other. One-way ANOVA was performed when multiple conditions were compared for one variable. Tukey post hoc tests were used after ANOVA analyses to perform multiple group comparison. Analysis with a p-value < 0.05 was considered statistically significant.

Next Steps

Each of these three figures is a separate ongoing project. For the first figure, current work investigates the signaling gradients that result from PSC-derived CAFs that may lead to changes in metastasis. As for figure 2, current and future members will continue this work. Identifying how PSC-derived CAFs shape the tumor microenvironment through its formation will give insights into potential weaknesses that can be exploited to prevent these cells' pro-metastatic and invasive roles for future treatments. In conjunction, following up on Figure 3 to identify the mechanism for PSC-derived CAFs involvement in the tumor based on tumor genotype will aid in identifying targets. In addition, tumor genotypes enrollment of specific cells of origin CAFs may determine which patients may benefit most from combination therapies of chemo or immune therapy with anti-stromal treatments.

Chapter 4 : Overcoming the Struggle of Treating Pancreatic

Cancer

Abstract

Pancreatic cancer is a dismal disease with a five-year survival rate of roughly 9% that has not improved much in recent years^{116,141}. PDAC (Pancreatic Ductal Adenocarcinoma) is difficult to treat with few drug options. The current standards of treatment are the antineoplastics gemcitabine for all stages of PDAC and FOLFIRINOX for metastasized PDAC^{142,143}. The lack of therapeutic options for PDAC is due to dense desmoplasia, a notable characteristic of the disease that prevents drug delivery, contributing to chemo resistance^{144,145}. Here, I will explore the current therapies' discovery, function, and clinical trials, as well as dive deeper into the limitations of these treatments with an emphasis on potential treatments targeting the stroma.

Introduction

PDAC is a relatively uncommon cancer; however, its incidence is increasing, and it is on track to become the second leading cause of cancer-related deaths by 2030¹⁴⁶.

Data from 2020 puts the incidence of PDAC almost even with mortality, 495,773 cases worldwide with 466,003 deaths¹⁴⁷. Unless caught in an early stage, PDAC is a generally incurable disease with few treatment options. Surgical resection is the most impactful on survival, but the rate at which PDAC is resectable is low at 20%. The remaining 80% of PDAC patients are either borderline resectable/locally advanced 25-30% or 50-55% have metastatic disease. Borderline resectable, locally advanced, and metastatic disease still

remain at less than 2 years of survival past diagnosis¹⁴⁸. For those patients with resectable disease, adjuvant therapy gives an overall survival of 54.4 months FOLFIRINOX or 35 months with gemcitabine¹⁴⁹. These two-remaining standards of care chemotherapeutics stand against pancreatic cancer; however, they are not without often severe side effects. FOLFIRINOX is already a four-drug regimen, but gemcitabine with nab-paclitaxel marginally improves survival, overall similar in effectiveness¹⁵⁰. Various other combinations and treatments have come and gone finding no hold and no impact on this devastating disease. These various combination therapies have failed due to toxic side effects, drug resistance, or both. Targeted therapies to genetic mutations common in PDAC were hopeful, but the heterogeneity of cancer cell mutations and stromal impact caused most to fail. One targeted therapy showed an improvement with survival: erlotinib targeting epidermal growth factor receptor with gemcitabine resulted in a two-week extended survival. Common mutations in PDAC are also the objective for targeted therapies, the most common being Kras; however, mutant Kras binds GTP with such high affinity it renders therapies ineffective. There is also a trial of evaluating the effectiveness of anti-p53 in combination with nab-paclitaxel/gemcitabine, allowing for continued hope¹⁵¹. Chemotherapies have had some impact on PDAC, but the survival rate has not shifted as drastically as it has for other tumor types despite immense progress. Chemotherapies target the cancer cell to impede proliferation or cause so much DNA damage the cell cannot continue; a second means of targeting cancer cells is using the body's own defense system. Immunotherapies enable the body's defenses to fight the tumor cloaked within. While the first immunotherapy was FDA-approved in 1986 and was a cytokine called IFN- α 2 concept that the immune system could be turned against

cancer had been detected decades earlier. Built on the therapy of inoculation in the 18th century, first used to prevent severe infections of smallpox, Coley's toxin caused an erysipelas infection that, when resolved, also resulted in tumor ablation¹⁵².

Immunotherapies have improved survival for many tumor types, including anti-PD-L1/PD-L1 use in lymphoma, and lung cancer, and anti-CTLA-4 in melanoma¹⁵³. The recalcitrant nature of PDAC has extended to immunotherapies; however, trials of combinations, including those with anti-PD-1/PD-L1 and anti-CTLA-4, rarely result in a durable response^{154,155}. Chemotherapies and immunotherapies in PDAC are plagued with the same key struggles. The stromal dilemma, as a key regulator of ECM, immune cells, and cell survival (through metabolism support and cytotoxic stress pathways), the stroma defends the tumor against therapies in both a physical manner and a biologically functional barrier. The PDAC stroma is a physical barrier to chemotherapies and immune cells rendering the tumor isolated from treatment breaching attempts. Stiffness of the tumor created by the density of the stroma also generates interstitial pressure resulting in compressed vasculature, in turn reducing the chemotherapies' access to the tumor. Not only a physical barrier, the cells of the stroma, mainly fibroblasts and activated stellate cells, secrete growth factors and cytokines that dysregulate the immune cells. Cytotoxic T cells are infrequent in the PDAC microenvironment while regulatory cells like M2 macrophages, neutrophils, and T-regulatory cells are relatively abundant. This immunosuppressive microenvironment impedes the effectiveness of immunotherapies as PDAC is a cold tumor (low immune cells)³³. While it could be indicated that this is the stromal dilemma, it stretches farther as CAF ablation studies resulted in unexpected consequences. While it was expected that CAF ablation in mouse models would result in

improved chemotherapeutic efficacy, it ended in reduced survival and dysregulated ECM instead^{46,47,52}. Parallel to this CAFs are implicated in impeding immunotherapies, a study in 2013 found that ablation of FAP⁺ cells including CAFs, though FAP is not specific to CAFs, fostered efficacy of anti-PD-L1, immune checkpoint blockade, in mouse models of PDAC⁵⁷. This is the stromal dilemma. The stroma is no longer thought to be a one-sided, pro-tumor, homogenous population but is rather a heterogeneous diverse aspect of the tumor microenvironment. The race was on to find the protumor stroma components, CAFs, for combination therapies and therapies to increase the anti-tumor aspects of the tumor. Many current combination therapies targeting the stroma alongside the tumor are not targeted and have not been as successful as predicted. However, these stroma targeting strategies are not pro, or anti-tumor CAF targeted, as the individual populations of cells responsible for these roles had not been identified until much more recently^{75,156–158}. Anti-stromal therapies have ranged from targeting ECM proteins, vascular normalization, and the CAFs but not subpopulations. Direct targeting of subpopulations of fibroblasts has not made it to the clinic. Still, there is promise in mouse models indicating IRAK4/IL-1R could target iCAFs, leading to increased sensitivity of the tumor to gemcitabine in vivo. JAK/STAT is similarly suggested to be used against iCAFs. myCAFs, on the other hand, lack "straightforward" therapeutics³⁸. Current therapies for PDAC do not sustainably increase survival and come with a cost: side effects that diminish the quality of life. PDAC has a high recurrence rate and a high rate of metastasis, making this tumor type deadly with few options³³. Stromal therapies continue to be a topic of discussion for joint therapies. Therapies targeted at subtypes have risen to the surface as the next step. Herein I will discuss the current standard of care treatments,

the failures of immunotherapies, and the potential most promising anti-stromal therapies. I will also look at the potential future direction for targeting the PSC-derived CAFs, myCAFs, identified as the tenacious sidekick of PDAC.

Standard of Care Treatments

Gemcitabine

Prior to 1980, scientists thought PDAC to be a chemoresistant disease, in part due to the stroma¹⁴². Gemcitabine was initially discovered in the 1980s by Larry Hertel's lab as an antiviral therapy as it would inhibit viral DNA synthesis. Prior to synthesizing gemcitabine, arabinonucleosides, most notably Ara-C, were used to treat acute leukemias. Unfortunately, Ara-C is quickly inactivated by deamination in the body, whereas gemcitabine is prevented from deamination inactivation by 2' fluorines. Hertel and colleagues discovered gemcitabine has a narrow therapeutic index (slight dose differences could lead to therapy failure or drug reactions) in treating viral infections; however, its impact on RNA and DNA made it a candidate for cancer treatment¹⁵⁹. In 1995, gemcitabine clinical trials began in PDAC via the Investigational New Drug treatment program that allowed promising drugs to be used in advanced disease before FDA approval. Gemcitabine is an antineoplastic agent that works as an anti-metabolite; as such is a deoxycytidine analog (Figure 4-1)¹⁶⁰. This deoxycytidine analog is converted to active metabolites difluorodeoxycytidine triphosphate and difluorodeoxycytidine diphosphate, which incorporate into the cytosine residue position in DNA. Competition between gemcitabine metabolites and deoxycytidine is further lessened by the metabolite's inhibition of the ribonucleotide reductase, preventing the reduction of DNA

precursors' oxygen leading to fewer deoxycytidine. Further, the metabolites' inhibition of the ribonucleotide reductase results in an increased need for deoxycytidine residues. With the analog now a commodity, its two fluorine groups on the base result in a negative charge in place of the hydrogen positive charge, preventing the polymerase from replicating. Only one more base can be incorporated past the analog resulting in a termination of the DNA strand. The faulty gemcitabine base cannot be excised by repair mechanisms, mainly base excision repair, due to the normal base beside it. This event is called masked chain termination. Thus, the mechanism of gemcitabine is to halt cell division by preventing DNA synthesis. A beneficial side effect of gemcitabine treatment is the inhibition of the ribonucleotide reductase, which results in a need to recruit bases extracellularly, which increases the uptake of the drug itself as a base analog¹⁶¹. As a nucleoside analog and a hydrophobic negatively charged molecule, it requires a transporter to get into the cell, mainly ENT1. Gemcitabine resistance occurs with reduced expression of the ENT1 transporter. Bioavailability of gemcitabine hydrochloride in the acidic form favors Lipinski's rule of five with a ClogP of -1.47, a molecular weight of 263.2, 3 hydrogen bond donors, and 8 hydrogen bond acceptors; however, it is not administered orally¹⁶². While bioavailability of gemcitabine favors Lipinski's rule of five, the tumor microenvironment of PDAC alters the uptake of gemcitabine limiting its impact. Dalin and colleagues found that PSCs release deoxycytidine in PDAC directly competing and thereby reducing the uptake of the cytidine analog, gemcitabine, by tumor cells¹⁶³. Similarly, Macrophages were also found to release pyrimidines, which compete for uptake and metabolism with gemcitabine¹⁶⁴. While gemcitabine molecular weight and acidic form are favored and result in bioavailability, featured cells of PDAC cause

competition which prevents gemcitabine uptake into the tumor cells reducing effectiveness. While gemcitabine makes it to PDAC cells they do not uptake it as well as in other tumors causing it to be ineffective and giving rise to resistance.

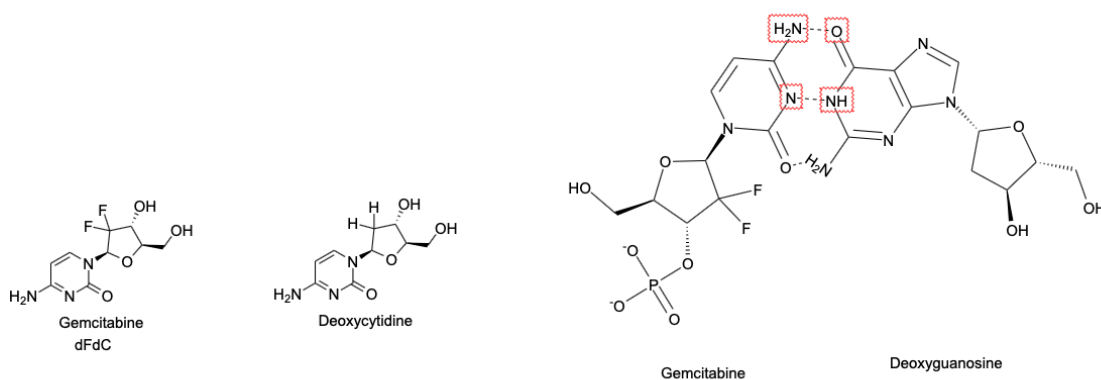


Figure 0-1. Gemcitabine first-line drug treatment for PDAC.

(A) Gemcitabine mimicking Deoxycytidine. Structures

Patients with PDAC are administered Gemcitabine via intravenous, as oral administration has just 10% bioavailability due to liver metabolism to dFdU (2'2'-difluorodeoxyuridine), a gemcitabine metabolite. This metabolite has a long half-life and is excreted in the urine¹⁶⁵. There is potential that adding hydrophobic groups to the amine groups may provide protection from deamination and allow for oral administration. However, more research needs to be done to evaluate if these additions would increase the bioavailability and cytotoxicity overall¹⁶⁶. Gemcitabine clinical trials began in 1995, but since then have expanded out to many combinatorial trials in PDAC in hopes of improved survival¹⁴². Prior to gemcitabine, 5-fluorouracil was the standard of treatment in PDAC¹⁶⁷. Phase II trials compared the two, with gemcitabine at 1000 mg/m² weekly

for 3 weeks on and one week off (for 4 cycles) resulted in median 3-year survival of 20.5 months or 31%. This was compared to 4 cycles of 5-fluorouracil at 250mg/m² daily for one 3-week cycle followed by 4-week cycles and 2 weeks breaks, resulting in 16.9 months or 22%¹⁶⁸. Phase III clinical combinatorial treatments found that gemcitabine and cisplatin had a longer time to progression and a better response rate compared to gemcitabine alone. However, combining 5-FU or metalloproteinase inhibitors with gemcitabine was not promising^{169,170}. Unfortunately, chemoresistance due to the aforementioned uptake reduction, due to reduced ENT1 expression, occurs within weeks, inhibiting the promise of the first chemotherapy for PDAC and only moving the survival rate marginally¹⁶².

FOLFIRINOX

Often, PDAC is discovered in late stages resulting in low survival rates. While gemcitabine is the standard of care for all stages of PDAC, combinatorial FOLFIRINOX is approved for advanced stages of PDAC¹⁷¹. FOLFIRINOX contains a pyrimidine analog fluorouracil, which acts similar to gemcitabine, inhibiting replication and preventing cell division¹⁷². The combination also contains Folinic acid, which aids the Fluorouracil as they both inhibit TS (Thymidylate synthase), which is responsible for converting deoxyuridine-monophosphate (a component of RNA) to deoxythymidine (a component of DNA). Folinic acid and fluorouracil work in conjunction to inhibit the production of the pyrimidine base, thymidine, that fluorouracil mimics. It is inhibiting the production that results in an increased requirement for thymidine, which in turn increases the uptake of fluorouracil in replicating cells¹⁷³. In addition to the two TS inhibitors in

FOLFIRINOX: Irinotecan is a topoisomerase inhibitor that prevents DNA unwinding. Rounding out FOLFIRINOX is Oxaliplatin, which causes DNA crosslinking. All four work in permutation to target DNA replication and prevent cell division (Figure 4-2)¹⁴³.

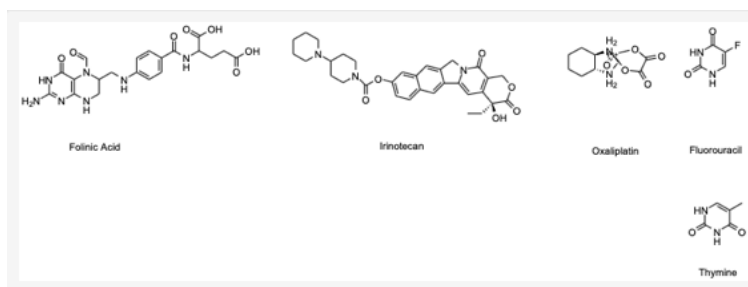


Figure 0-2. The components of FOLFIRINOX.

(A) Fluorouracil mimics thymine structure comparison. FOLFIRINOX components structures

Fluorouracil is applied topically to treat skin cancer; however, it can be used intravenously for the treatment of other solid internal tumors like metastatic PDAC¹⁷⁴. The components of FOLFIRINOX are given by intravenous in a specific order: oxaliplatin, Folinic acid, oxaliplatin again, irinotecan, and finally fluorouracil. Phase I trials of the four-drug combination in multiple tumor types determined the maximum tolerated dose and the recommended dose. After enrolling 34 patients with escalating doses, 85/200 mg/m² was the maximum tolerated dose, and 85/180 mg/m² was set as the recommended dose. The Phase 1 FOLFIRINOX study regimen found that it was most effective in digestive cancers, including two pancreatic tumors¹⁷⁵. Phase II clinical trials of FOLFIRINOX were initiated to determine efficacy and safety in comparison to gemcitabine. Patients treated with FOLFIRINOX had a median survival of 11.1 months compared to 6.8 months with gemcitabine. Although these results appeared promising,

toxicity was increased with the FOLFIRINOX^{176–178}. Advanced phase III clinical trials mirrored Phase II trials, as the administration of FOLFIRINOX increased median survival time and toxicity side effects¹⁷⁹. Despite Phase III trials occurring in 2014, PD/PK/tox (pharmacokinetics/pharmacodynamics) were not determined for the polychemotherapy until just last year (abstract)¹⁸⁰. Previously the 5FU PK was used for the combination, as it was thought that there was no PK interaction of the components. 5FU has a half-life of 8-14 minutes and a non-linear PK curve due to saturable hepatic degradation^{180,181}. FOLFIRINOX is now commonly used to treat advanced-stage PDAC tumors in order to shrink them before resection¹⁷⁹. One toxicity of FOLFIRINOX, neutropenia, is likely the result of oxaliplatin-dependent antibodies that target neutrophils. Thrombocytopenia is another common side effect caused by both 5FU and oxaliplatin injuring the liver. These toxicities result in dose decreases and discontinuation^{180,182}. A study in 2018 found a reduction in side effects like neutropenia without a 5FU bolus and a reduction in irinotecan¹⁸³. These tumors are frequently found in a progressed state (stages 3-4) when they cannot be resected; at this point, treatments like FOLFIRINOX, despite the toxicities, are commonly used to reduce the tumor in hopes of resection^{141,184}.

Immunotherapies in PDAC and why they were unsuccessful

Treatment options for metastatic PDAC are limited, this is the most common stage of diagnosis, and it is unresectable. Radiation and chemotherapy are the standard options for metastatic disease, but there was hope that immunotherapies might progress the survival of patients at this stage of disease¹⁵¹. Immune checkpoint inhibitors are one

type of immunotherapy that works by stimulating or inhibiting the immune regulators to enhance the already existing anti-cancer immune response. Inhibition of PD-L1/PD-1 is one such modulator of the immune system to destroy melanoma and lung cancer cells. PD-L1 from cancer cells binds to PD-1 on cytotoxic T-cells to signal that they are normal cells, when in fact, this is a corruption of self-tolerance as cancer cells are mutated imposters of normal cells. Inhibition of PD-1/PD-L1 allows the cytotoxic T-cells to recognize the unhealthy proliferating tumor cells and signal for their demise¹⁸⁵. PD-L1 binding to T-cell PD-1 reduces proliferation and cytokine release as well as T-Cell survival. PD-1 inhibitors pembrolizumab and nivolumab are FDA approved for use in metastatic melanoma and non-small cell lung cancer. CTLA-4 is an additional pathway that immunotherapies were tested against and functioned in other tumor types, but not PDAC. While PD-1 inhibits the immune cells of later immune responses in the peripheral tissues, CTLA-4 works at an earlier time point when T-cells are in the lymph node. It also differs from PD-1 as it competitively inhibits the stimulatory molecule CD28, which binds typically to b7 to produce T-cell proliferation and activation. Ipilimumab, anti-CTLA-4 immunotherapy, is approved for use in metastatic melanoma¹⁸⁶. Ipilimumab, nivolumab, and pembrolizumab are all in clinical trials involving advanced PDAC; however, preliminary data of these alone and in combination with standard of care chemotherapies have not had the same significant impact on the disease as seen in other solid tumor types¹⁵¹. This resistance to immunotherapies in PDAC could be due to the low mutational burden of this tumor. In contrast, melanoma and lung cancer have a much higher mutational burden resulting in less immune escape as more mutations make tumor cells more recognizable as non-self cells. While the physical barrier of the stroma may

not be the barrier to these immunotherapies, the dysregulation of the immune system driven by stromal cells might be the culprit. PDAC is a "cold" tumor or rather has low immune cells in the tumor space with active to destructive immune cells sequestered outside the stroma and tumor space¹⁸⁷. Cytokines released by CAFs signal to inhibit cytotoxic cells like T-cells and M1 macrophages, while other cytokines release upregulates M2 macrophage, neutrophils, and T reg cells; in other words, the cells that decrease active immune cells for self-tolerance¹⁸⁸. PDAC immunotherapy trials are still ongoing, with remaining hope. The main culprit preventing both chemotherapies and immunotherapies effectiveness in PDAC must be addressed for future progress, the stroma.

Early-Stage Stromal Treatments

The stromal cells of PDAC form a network for the tumor cells to invade and metastasize, which prevents resection. Together, the PSCs (Pancreatic Stellate Cells) and fibroblasts provide growth factors and barriers to therapeutics. It has been found that the density of the collagen and extracellular matrix proteins creates a physical barrier for drug treatments, especially in PDAC, which is commonly made up of >75% of the tumor space^{140,189}. The focus of drug development has turned to anti-stromal treatments in hopes of future combinatorial therapies where the anti-stromal drug allows for entry and sensitivity to chemotherapeutics. Unfortunately, while there are encouraging anti-stroma treatments, they often have off-target and unwanted side effects. Several of the potential drugs aim to target the source of the ECM by impacting the pancreatic stellate cells. These PSCs are quiescent in normal tissue, but upon injury or disease, they activate to a

fibroblast phenotype and begin the process of ECM production. Vitamin D analogs, such as calcipotriol, have been found to inactivate PSCs. In a mouse model of PDAC, combinatorial calcipotriol and gemcitabine resulted in increased uptake of gemcitabine, further reducing tumors and improved overall survival compared to gemcitabine alone. Various vitamin A metabolites can revert PSCs to a quiescent state. Vitamin D is also a means to deactivate PSC, which activate to protumor CAFs. One such vitamin D analog therapy is in phase I clinical trials in combination with gemcitabine and nab-paclitaxel or separately with pembrolizumab⁷². FAK has been seen to regulate stiffness and is potentially the paracrine signaling target of PSC-derived CAFs that results in PDAC stiffness⁷⁵. FAK inhibition decreases CAF proliferation and is implicated in rendering PDAC sensitive to immune and chemotherapies; as such, defactinib, a FAK inhibitor, is in phase I clinic trials combined with pembrolizumab and gemcitabine¹¹¹. Directly targeting PSC-derived CAFs is one anti-stromal method; however, another avenue is targeting proteins secreted by CAFs such as Olaptese (anti-CXCL12), Siltuximab (anti-IL-6), MSC-1 (anti-LIF), and AP12009 (antisense oligonucleotide aimed at TGF- β). All are in stage I trials with either chemotherapies or immunotherapies as combination drugs. Pamrevlumab (anti-CTGF) and PEGPH20 (Pegvorhyaluronidase alfa), depletes hyaluronic acid, are also aimed at CAF-produced proteins, the latter in phase 3 clinical trials with promising preliminary results. Additional therapies have been directed at the cross-talk between CAFs and other cells through cell surface receptors, including CXCL12, TGF- β , and JAK/STAT pathways, all in various phases of combination trials¹⁹⁰. Candesartan and Olmesartan also act on PSCs, reducing cell proliferation and downstream inflammation¹⁹¹. Complete ablation of fibroblasts via sonic hedgehog

inhibition has been attempted in mice further to elucidate the role of fibroblasts in the tumor microenvironment; however, it resulted in more gemcitabine resistant tumors and reduced survival^{46,47}. Further investigations aimed at understanding the role of the PSC and fibroblast are needed before clinical studies. Targeting the stromal ECM components is another avenue being investigated. Primarily, the focus is on reducing collagen or collagen linking enzymes. In preclinical studies, losartan, primarily used to treat high blood pressure, was determined to impact collagen and hyaluronic acid synthesis, thereby targeting both the proteins and linking enzymes, respectively (Figure 4-3B)¹⁹². Still, in its early stages, anti-CSF-1 was seen to inhibit macrophage production of collagen crosslinking enzymes, which suggests CSF-1 has the potential to target a source of the crosslinking enzymes¹⁹³. PEGPH20, which targets hyaluronic acid, was combined with gemcitabine and nab-paclitaxel with promising disease-free survival. Still, another study was closed that combined PEGPH20 and FOLFIRINOX due to detrimental impacts on the overall survival of patients⁴¹. While there is optimism in drug therapeutics, PDAC is a complex ECM heterogeneous disease, which will likely need several options for combinatorial treatments. The focus on anti-stroma therapeutics still provides hope of breaking through the barrier to cure PDAC¹⁹⁴.

Still, in the fundamental science realm, studies identify new stromal targets, including those impacting a subtype of CAFs or one origin of a subset of CAFs known to be protumor. For example, a study inhibiting heat shock protein-90 in mouse models of PDAC altered the stroma and allowed anti-PD-1 therapy to increase CD8⁺ and CD4⁺ T-cell infiltration. In particular, it was identified that heat shock protein 90 inhibited inflammatory signals resulting in deactivated PSC-derived CAFs growth, further

signaling to reduce JAK/STAT and MAPK signaling, and α -SMA, a component on CAFs¹⁹⁵. CD200 promotes immunosuppression and could be a potential target for use with immunotherapies, though more studies are needed to determine the efficacy of this¹⁹⁶. Much in the same way, in a mouse model of PDAC Halofuginone directly inhibits stellate cells which results in a decrease in ECM components and increases immune infiltration indicating that it might be used with immunotherapies³³. More hopeful methods to target various aspects of CAFs are ongoing in early preclinical trial research, including β ig-h3, HSP47, IL-33, NRG1, cadherin11, and Netrin G1, with more unpublished still¹⁹⁰.

Chapter 5 : Future Directions

Introduction: All the Possibilities of a PSC-CAF Depletion model

Identifying Fabp4-Cre as a means to lineage label and track PSC-derived CAFs, along with the use of an intraductal injection of virus containing this Cre in a diphtheria toxin receptor-based ablation mouse model, has opened up a variety of avenues to test the importance of PSC-derived CAFs. In Chapter 2, the preliminary role of PSC-derived CAFs involvement in ECM and tissue stiffness was determined within the context of the tumor. From the RNA sequencing screen mentioned within that chapter, many more potential pathways and roles were postulated based on the significant differences in expression between PSC-derived CAFs compared to non-PSC-derived CAFs cells.

Looking into the Basic Biology of PSCs

Prior to identifying a means to track PSC-derived CAFs, assumptions were made due to the loss of specific markers with activation of these cells. Preliminary implications show that PSCs are found in multiple locations within the pancreas, including interlobular, intralobular, and around the vessels, similarly to the liver equivalent HSCs¹⁹⁷. It's a running joke that looking at stellate cells will cause them to activate, with relevant signals or insults including inflammation. Fabp-4 allows for tracing PSC-derived cells within the pancreas, meaning that we can trace these cells during and in inflammation. Chronic inflammation results in many changes to immune cells and blood vessels and can accelerate or support to tumor formation, as is seen in chronic pancreatitis patients having increased risk of PDAC¹⁹⁸. PSC has been implicated as the

primary source of fibrosis, but tracing them has been difficult as inflammation begins the processes that results in loss of stellate cell markers¹⁹⁹. Future projects looking at PSC in the normal pancreas to identify their role, followed by chronic inflammation induction, could elucidate the role of PSC in inflammation. Inflammation can be caused in the pancreas of mice by cerulean injections, ethanol, and surgical means²⁰⁰. Comparisons between the normal pancreas PSC and the PSC-derived CAFs found in inflammation can then be made. Profiles by RNA sequencing could be obtained from normal pancreas PSC and inflammation PSC-derived activated fibroblasts following isolation by flow sorting for GFP+ cells. Likely enrichment of PSC-derived fibroblasts through the use of PDPN with GFP in the sorting process will be needed due to some CD45+ cells being a small but albeit GFP+ cell type. These CD45+ GFP+ cells appear to be recruited and not a resident population; inflammation pathways could recruit them; this would need to be seen in the inflammation model by IHC (immunohistochemistry) on tissues for CD45. Following RNA sequencing or, meanwhile, IHC and RNA isolation/qPCR could be used to follow up on genes identified as changing following inflammation. Critical genes of interest could be looked at in the depletion model of PSC-derived CAFs using the iDTR intraductal injection model as inflammation could be induced rather than tumor cells implanted. PSC-derived CAFs could be depleted before inflammation induction to allow healing after the transduction by AAV-Fabp4-Cre. Harvesting the tissue and staining for proteins of interest could identify if other cells compensate for the loss of PSC-derived CAFs or if PSC-derived CAFs regulate these genes. These genes could be looked at, at the protein level by IHC or RNA by qPCR from flow sorting. Through tracking, inflammation induction, and depletion models, the critical PSC-derived CAF mediators

of inflammation could be identified, and ways to silence them more specifically developed for those with chronic conditions at risk of PDAC.

RNA sequencing revealed an abundance of pathways regulated by PSC derived CAFs, chance of hope in identifying a treatment target

While our paper to trace PSC-derived CAFs looked at one aspect identified in the RNA sequencing screen, ECM/ tissue stiffness, gene ontology also identified several other pathways unique to this subset. We began to look at one additional gene expression cluster, immune regulatory components. CAFs and the stroma, while a physical block to immune responses, also are implicated in releasing cytokines and chemokines that reduce cytotoxic immune cell abundance and activate regulatory or immune-suppressive cell types¹⁹⁸. We looked at CD8+ T cells by IHC and the myeloid marker CD68 in the depletion of PSC-derived CAF. CD68 myeloid cells were significantly decreased with PSC depletion; there was no difference in T-cells, however. Flow panels on control vs. depleted tissues are one possible future direction to identify immune cells that PSC-derived CAFs impact. It would also be possible to look at these cells by IHC stains. A whole project could be based on immune changes driven by PSC-derived CAFs by looking at the abundance of immune cells in the tumor space and the location of these cells, and their exhaustion or activity. Flow panels could identify immune cell abundance changes, and the use of exhaustion markers and activation markers, co-stimulatory molecules could be seen by IHC as well. Cytokine arrays would elucidate the levels produced by isolated PSC-derived CAF compared to those isolated in quiescent form by

cell sorting to identify what profiles may be acting on immune cells that PSC-derived CAFs produce.

Exploring the prospects of PSCs involvement with angiogenesis

PSCs are found near the vessels of the pancreas, where they are thought to support the vasculature. PSCs in normal tissues are thought to play a structural role²⁰¹. RNA sequencing of GFP PDPN cells identifies a cluster of highly expressed genes involved in angiogenesis. It was believed that PSCs could not cause contraction of vessels in the way that pericytes do¹. This lack of contraction would mean that PSCs would not regulate the flow of vasculature; however, in the context of a tumor, CAFs are thought to regulate vessels preventing chemotherapeutics from reaching the tumor cells via the production of extracellular matrix^{104,202}. PSC-derived CAFs enrichment of genes related to angiogenesis could indicate an alternative pathway not structurally altering the vessel flow through the contraction but potentially preventing vessel expansion or growth into the tumor. This reduction in vessels growth into tumors could also explain the hypoxic nature of PDAC tumors. As the tissues are already available from PSC-depleted tumors, a starting place to follow up on this project would be to stain vessels (CD31) and identify the number of vessels/ vessel lengths and widths. Hypoxic areas could be counted and measured from these tissues to determine the impacts of PSC-derived CAFs on vasculature outcomes. As hypoxia and poor tumor perfusion reduce the effectiveness of therapies, identifying if PSC-derived CAFs regulate this could be a starting point for targeting this protumor CAF contribution to increase therapeutic efficacy. Losartan influences microvasculature and reduces desmoplasia, which could imply an impact on

PSC-derived CAFs. Administering losartan in the PSC-derived CAF tracing model would show any effect of losartan on these cell types seen by staining for activity markers of PSCs such as vitamin a, desmin, α -SMA, vimentin in conjunction with GFP. Identifying the location of PSC-derived CAFs could also have implications of changes due to losartan if more or fewer PSCs are around vessels^{203,204}. It is reasonable to speculate that PSC-derived CAFs could be the middle man of losartan's impact on desmoplasia and increase chemotherapeutic efficacy seen by preliminary clinical study results.

One avenue for angiogenic regulation is Tie1. PSC-derived CAFs express Tie1 identified in the RNAseq screen, and it is also implicated in regulating angiogenesis. Tie1 is associated with inflammation but is an orphan receptor with no known ligand. Tie1 may modulate Tie2 activity through dimerizing with Tie2, which is implicated in increased inflammation. Tie1 is also thought to stabilize vessels while Tie2 has the opposite effect destabilizing them, with results that are context dependent^{205,206}. Tie1 is found at vessel tips, but the role of this protein is not entirely understood²⁰⁷. A neutralizing antibody against Tie1 has shown reduced metastasis to the lung, and the complete mechanism is to be determined²⁰⁸. This antibody could be used in PSC depletion studies to determine if the angiogenic regulation of PSC-derived CAFs could be inhibited. This antibody has been used in mouse models of breast cancer and lung cancers but not PDAC^{209,210}. As there is plasticity in PSCs and CAFs, the roles may not be as strict as is assumed; regulating the function or specific protein made by a subtype may be a better, more directed pathway to target the protumor roles of PSC derived CAFs²¹¹.

Proposed investigation of PSC-Derived CAFs impacts on chemotherapeutics

CAFs are key producers of the ECM found to prevent chemotherapeutic infiltration into the tumor³⁸. With the stroma making up to 80% of the tumor space, including CAFs and ECM, access to the tumor is limited²¹². PDAC tumors are also hypoxic, further limiting vasculature that otherwise would be a pathway for chemotherapeutics into the tumor space. The hypoxic nature of PDAC tumors also results in pH gradients that change the activity pockets of chemotherapeutics inactivating them^{34,212,213}. It remains to be studied precisely how PSC-derived CAFs participate in these pathways of chemotherapeutic prevention. Intraductal injections of Fabp4-Cre and orthotopic injection of tumor cells into the pancreas of iDTR mice with gemcitabine or gemcitabine and nab-paclitaxel would be a four-arm study to identify the overall impact of PSC-derived CAF depletion on chemotherapeutics. The four-arm study would include control arms of vehicle and gemcitabine or gemcitabine and nab-paclitaxel, and experimental arms of the same with diphtheria toxin administration. Tumors would be grown to a standard size (area of 10 mm²) as measured by ultrasound, followed by diphtheria toxin or control, and finally, chemotherapeutics delivered. The primary endpoint will be tumor growth to a 30 mm² area. The secondary endpoint will be when greater than 20% of one arm is morbid (hunch posture, 20% weight loss, ascites). Stanger cell line 2838 could be used to monitor metastasize, or other lines that do not metastasize could be used to look at primary tumors. Harvested tumors could be stained for cell proliferation marker Ki67 and PANCK (tumor cell marker) to see if the proliferation of the tumor changes with chemotherapeutics and loss of PSC-derived CAFs²¹⁴. Changes to apoptosis or necrosis could also be identified by CC3 and either ALT or miR-122,

respectively²¹⁵. For metastatic studies, liver and lungs would be harvested in addition to primary tumors, and H&E staining done to measure the size and number of metastases. Long-term studies could also be done similarly but with the endpoint being survival. Primary tumors harvested from all arms would be weighed and tumors measured over time by ultrasound to detect changes in tumor growth with PSC-depletion and chemotherapeutics. Radiolabeling drugs could also be an option to look at chemotherapeutics access to the tumor by seeing if more labeled drugs are within the tumor space with PSC depletion. Additional studies with the same outline could be done with FOLFIRINOX, another standard of care therapy for PDAC. Thus PSC-derived CAFs may be relevant to entry into the tumor or otherwise impact chemotherapeutic efficacy.

Proposed investigation of PSC-derived CAFs impacts on immunotherapies

The physical barrier of the stroma is not only an inhibitor of chemotherapies but also immunotherapies, as it promotes immune cell exclusion and immune-suppressive phenotypes of the immune cells present in the tumor microenvironment. A combination of PSC-derived CAF ablation and tumor implantation with immunotherapies such as anti-PD-1 and anti-CTLA-4 could fill this knowledge gap^{154,155}. Survival studies, metastasis studies, and impacts on proliferation, necrosis, and apoptosis would proceed resembling those in the chemotherapy section but replacing chemotherapies with immune therapies. Flow studies on cells isolated from these tumors could look at the immune milieu and its changes along with panels to look at the activation markers and exhaustion profiles. Staining of tissues could confirm changes and identify the location of immune cells in the

stroma and tumor space. These studies would identify the effect of loss of the PSC-derived CAF barrier on immunotherapies and, in turn, show any changes to immune activity. If loss of PSC-derived CAFs results in efficacy changes for immunotherapies, these impacts would be seen by tumor size differences between arms, metastatic numbers/sizes, and immune milieu changes.

Investigating the likelihood of PSC-inhibitors

From the implication that CAF ablation studies unexpectedly reduced survival and ECM dysregulation came investigations of heterogeneity in the CAF compartment^{46,47,50}. PSCs give rise to a numerically small subset of CAFs, specifically a myCAF subset⁷⁵. This PSC-derived CAF subset is consistent with a protumor phenotype by producing specific ECMs, tissue stiffness, and recruitment or in situ expansion of myeloid cells. Preliminary data also suggest that PSC-derived CAFs are protumor in promoting metastasis. The ablation of PSC-derived CAFs has trends of reduced lung and liver metastases and trends for increased survival (unpublished data). Targeting protumor CAFs in combination with therapies is now the focus of hope. Vitamin A/D analogs could be used to inactivate CAFs, specifically PSC-derived CAFs, which could be administered to Fabp4-Cre mice with orthotopic tumor implantation²¹⁶. Staining the tumor with desmin or vimentin could identify if vitamin analogs inactivate PSC-derived CAFs in vivo. Ultrasound or endpoint tumor weights could also determine impacts on tumor growth. Stains for CAF markers could also be used to see if there is no impact of vitamin analogs on CAF activity, as these markers would be lost through deactivation.

TN-C is made by PSC-derived CAFs, as is p-MLC2, which are connected to tissue stiffness, tumor progression, invasion, and metastasis^{122,123,217,218}. Antibodies or molecules designed to degrade TN-C could be tested on PDAC tumors. Antibodies or competitive inhibitors could be developed for p-MLC2 or its downstream effectors and tested in mouse models of PDAC. FAK is also a regulator of tissue stiffness and its downstream impacts. There is currently a drug targeting FAK available which could be tested mechanism in the PSC-derived CAF depletion model to identify if pFAK inhibition works through PSC-derived CAFs^{111,219}.

The odds that PSC-derived CAFs can stratify patients

There is a PSC-derived CAF ECM signature that is correlated with decreased survival. This signature could be used to identify patients that would benefit perhaps most from anti-stromal therapies. These anti-stromal therapies could be inhibiting PSC activity or explicitly directed at the ECM components made by PSC-derived CAFs. Patient tumors would be a means to look at how each patient responds to different treatment options based on low or high expression of this ECM signature. Tie1 plus α -SMA can be a markers of PSC-derived CAFs in human patient tumors⁷⁵.

Concluding Remarks

PDAC is a destructive disease that has been difficult to treat, contributing to its progress towards being the 2nd leading cause of cancer-related deaths²²⁰. The current standard of treatment, gemcitabine, works by mimicking a DNA base, while the standard for advanced disease FOLFIRINOX combines different DNA replication inhibitors

preventing proliferation^{142,143}. These current treatments have intense side effects and offer short-lived survival benefits. Often, the treatments are used with the hopes of shrinking the tumor before surgical resection. However, once the disease has progressed to advanced unresectable disease, it has already metastasized, resulting in reduced benefits of the treatments⁴¹. In addition, the stroma blocks drug delivery while also supporting the tumors' growth through metabolites and providing a network of ECM for tumor cells to metastasize^{140,141}. A few clinical trials suggest that anti-ECM drugs can work in combination with the standards of treatment, though some of these trials failed^{191,192}. This failure of combining anti-ECM drugs may result from anti-tumor aspects of the stroma that are the focus of current research that began when the anti-SHH targeting of fibroblasts resulted in reduced survival in mouse studies^{46,47}. Vitamin A and retinoic acids and losartan and CSF-1 show some targeting of PSC and ECM^{191–193}. More research is needed for specific targeting of the stromal components before clinical trials due to the potential of tumor-suppressive roles of CAFs, compensatory mechanisms of other cells, and off-target impacts¹⁹⁴. Current research has identified PSC-derived CAFs to be protumor. Further research on this subset of cells could fill the gap and develop stromal treatments. Theoretical links suggest a few existing drugs may target PSC-derived CAFs, but further research is required to determine mechanisms and impacts on chemotherapeutics and survival (Figure 5-1 A & B). Lastly A better understanding of the diverse stromal cells and the ECM they produce may fill the gaps and allow for more precise targeting of the stroma for better PDAC treatments.

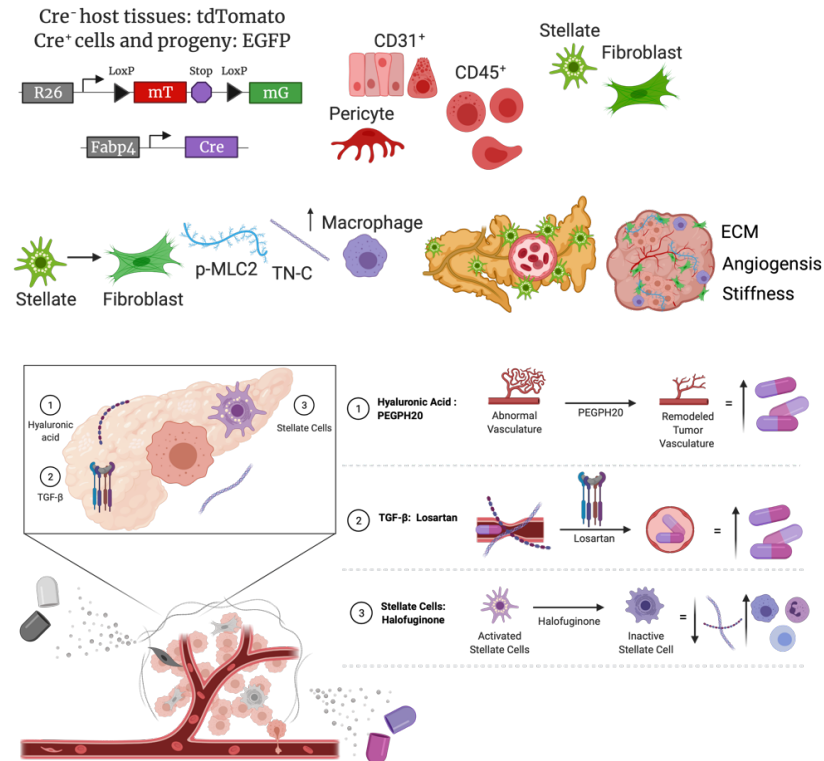


Figure 0-1. Schematic representation of Fabp4-CRE mouse model and potential therapeutic links

A. Fabp4-Cre mouse model tracks PSC-derived CAFs role in the PDAC TME marking PSC and a subset of CAFs in the TME specifically by GFP expression using a mTmG reporter. Remaining cells of the pancreas including CD31⁺, CD45⁺, and pericytes are tdtomato⁺ indicated by lack of fabo4 expression. PSC derived CAFs in the PDAC TME are responsible for p-MLC2, TN-C and signaling to macrophages as seen through PSC-derived CAF depletion model using DTR and retrograde transduction of AAV-Fabp4-Cre ductal injection with orthotopic implantation of KPC tumor cell lines. PSC derived CAF depletion results in specific ECM component reductions, and reduced stiffness, as well as implications towards involvement in angiogenesis. B. schematic representation of potential therapeutics aimed at the stroma that have theoretical links to PSC-derived CAFs. PEGPH20 targets hyaluronic acid and remodels the vasculature, which could link to the PSC-derived CAF angiogenic potential that requires further study⁸⁷. PEGPH20 has shown modest promise in clinical trials⁹². Losartan works as an antifibrotic by inhibiting TGF-β and the downstream activation of CAFs. Losartan also acts on vasculature through angiotensin II receptor blockade. The complete mechanism behind this two-pronged approach is unknown but speculation links to the PSC-derived CAFs as a possible component that could be clarified through further studies utilizing the Fabp4-Cre mouse. Losartan is currently in combination trials with FOLFIRINOX²⁰⁴. Halofuginone inactivates stellate cells causing downstream reductions in ECM and increases in immune infiltration. PSC derived CAFs are one subset of CAFs in the TME and it stands to reason halofuginone is likely to be a potential inhibitor of this protumor subset. Halofuginone is in in vitro studies¹⁹². Created with BioRender.com

Chapter 6 : References

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