Differential Contribution of Pancreatic Fibroblast Subsets to the Pancreatic Cancer Stroma

by

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Dedication

For my grandparents

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a deadly cancer that is projected to become the second leading cause of cancer-related death by 2030. The 5-year survival rate for PDAC is only 10% which can be attributed to the aggressive and chemo-resistant nature of the cancer. PDAC, starting from its precursor lesions, is characterized by an extensive fibroinflammatory stroma. Cancer-associated fibroblasts (CAFs) are a prominent cellular component of the stroma, but their role during carcinogenesis remains controversial, with both tumor-supporting and tumor-restraining functions reported in different studies. One explanation for these contradictory findings is the heterogeneous nature of the fibroblast populations and the functionally distinct roles each subset might play in carcinogenesis. However, the origin of CAFs and their respective contribution to carcinogenesis has yet to be explored. Here, we show that Gli1 and Hoxb6 label distinct fibroblast populations in the healthy mouse pancreas. We developed a dual-recombinase approach that allowed us to induce pancreatic cancer formation through FlpO-driven epithelial recombination of Kras while labelling $Gli1^+$ or $Hoxb6^+$ fibroblasts in an inducible manner. Using these models, we followed the fate of these two fibroblast populations during the process of carcinogenesis. While $Gli1^+$ fibroblasts and $Hoxb6^+$ fibroblasts are present in similar numbers in the healthy pancreas, they contribute differently to the stroma in carcinogenesis. Namely, $Gli1^+$ fibroblasts proliferate and contribute to the fibrotic reaction, while $Hoxb6^+$ cells do not. Our findings suggest that not all resident fibroblast populations expand and transition into cancer-associated fibroblasts within the pancreas, and that fibroblasts are heterogenous with potentially distinct functional roles even at the healthy stage. These findings advance our understanding of how the pancreatic fibroinflammatory environment is established during carcinogenesis.

Chapter 1 Introduction to Pancreatic Fibroblast Heterogeneity

In the pancreas, the mesenchyme surrounds and supports epithelium development though epithelial-mesenchymal crosstalk [1] and differentiates to form cell types such as fibroblasts, pancreatic stellate cells, and pericytes [2,3]. Fibroblasts are spindle-shaped cells which proliferate, express smooth muscle actin (SMA), and deposit extracellular matrix (ECM) when activated by local signals released from surrounding cells. Activated fibroblasts, also known as myofibroblasts, help regulate wound repair and recovery before dramatically reducing in number through apoptosis when the wound is healed, resolving the associated fibrosis [4,5]. In the context of certain pancreatic diseases, such as chronic pancreatitis and pancreatic cancer, the fibrosis is not resolved, and it progressively impairs normal tissue function.

Cancer-associated fibroblasts (CAFs), a term which refers to all fibroblastic cells in the tumor microenvironment, are a prominent cellular component of pancreatic ductal adenocarcinoma (PDAC). PDAC is a deadly disease and is the currently the third leading cause of cancer deaths in the USA; over 56,000 adults are diagnosed each year and almost all are expected to die from the disease [NIH SEER Database]. The characteristic fibroinflammatory stroma of PDAC starts to accumulate at the onset of disease progression and can contribute to over 70% of the tumor volume [6,7]. Given the abundance of fibroblasts in these pancreatic diseases, there has been great research interest into the role of fibroblasts in pancreatic diseases over the past 20 years. Despite this extensive

effort, the role of fibroblasts still remains controversial. Numerous studies have identified tumor-supporting roles for cancer-associated fibroblasts (CAFs), such as recruiting immunosuppressive cells [8–10], supporting tumor metabolism [11], and creating a barrier to drug delivery by depositing extracellular matrix (ECM) [12–16]. However, recent data has also identified tumor-suppressive functions within the stroma [17–20]. One explanation of these seemingly contradictory findings could be that CAFs are more heterogeneous than previously thought. To investigate this possibility, researchers are utilizing a combination of animal models, human samples, and novel bioinformatic techniques to study the tumor microenvironment (TME) in greater depth.

Genetically engineered mouse models are a useful tool to study the *in vivo* complexity of pancreatic tumor microenvironment. Several mouse models utilize mutations that are common to PDAC and faithfully recapitulate the histology of human disease. Most of these models feature an oncogenic mutation of KRAS, a gene that is almost universally disrupted in PDAC [21,22]. Mice that express oncogenic KRAS (*K*: *LSL-KRAS^{G12D}*) in the pancreatic epithelium (*C: Pdx1-Cre* or *Ptf1a-Cre*) are predisposed to forming pre-cancerous lesions, and this process can be synchronized and accelerated by inducing acute pancreatitis [23–25]. These "*KC"* mice are a useful tool for studying the early stages of pancreatic neoplasia. To study metastatic pancreatic cancer *in vivo*, *KC* mice have been crossed with loss-of-function tumor suppressor lines, such as mutant p53 (*KPC)* [26], p16-Ink4a/p19-Arf (*KIC or KPP*) [27]. In addition to these *Cre*-driven models of PDAC, researchers have recently generated a *FlpO*-driven mouse model (*KF, K*: F*SF-KRAS^{G12D}; F: Ptf1a-FlpO*) [28,29]. This model can then be crossed with other cell type-targeting *Cre* lines, enabling genetic manipulation of multiple cell types within the TME.

This is of particular interest to the study of stromal cells in PDAC, whose origin, fate, and function has been poorly understood. This combined genetic system allows for cell-type specific lineage tracing, in which a *Cre*-expressing cell lineage can be identified throughout different stages of PDAC progression [30]. This model can also be used to ablate a *Cre*-targeted cell lineage by introducing an inducible and transgenic lethality to diphtheria toxin [31]. Together, the combination of disease modeling with a diverse genetic tool kit makes the mouse a useful system for investigating the role of fibroblasts in pancreatic disease.

These models become increasingly powerful when paired with large-scale genomic, proteomic, and transcriptomic analysis. These bioinformatic techniques have enabled researchers to characterize diverse cell populations within the TME to a much greater depth than previously possible. In particular, single-cell RNA sequencing has been used to identify distinct transcriptional subpopulations in breast [32], colon [33], and lung cancer [34]. Now, similar techniques are being used to characterize different cell populations in the pancreas, at different stages of development and disease [35–38]. By analyzing fibroblast populations in both human patients and mouse models, researchers are starting to identify conserved patterns of fibroblast heterogeneity.

Here, I explore what is known about fibroblast populations in the pancreas, with a focus on heterogeneity. I discuss the different populations that have been identified in the developing, adult, and diseased pancreas, and discuss some of the current challenges facing the field.

Mesenchyme function and heterogeneity during pancreas development

During embryogenesis, the pancreatic buds emerge from the gut endoderm, and receive key signaling cues from the mesoderm-derived mesenchyme (for review see [39]). The essential role of the mesenchyme in pancreas development was proposed in the 1960s, when pancreas cultures lacking mesenchyme failed to form primitive acinar structures [40–42]. Almost 50 years later, this idea was tested *in vivo* by conditionally depleting the developing pancreatic mesenchyme. Researchers utilized an Nkx3.2-Cre mouse line in combination with a Cre-dependent diptheria toxin receptor (DTR) to ablate a broad mesenchymal population in the developing pancreas, leading to a severe reduction in pancreas growth [43]. Thus, the mesenchyme plays an essential role during pancreas development. Further, proper pancreas development requires communication between the mesenchyme and the epithelium through an array of inter-connected signaling pathways.

During pancreas development, the repression of specific epithelial signals is important. One classic example is the requirement for repression of epithelial *Sonic Hedgehog* (*Shh*) expression for proper pancreas lineage specification [44–46]. Ectopic *Shh* expression in the pancreatic epithelium drives intestinal differentiation at the expense of pancreas [44], while antibody-mediated inhibition of SHH is sufficient to induce the expression of pancreas differentiation markers in endoderm explants [46]. Further, mesenchyme-specific activation of the HH pathway restricts epithelial growth, while stimulating mesenchymal hyperplasia [47]. Following pancreatic lineage specification, there is a continuing requirement for epithelial-mesenchymal cross-talk for growth and

maturation of the pancreas. Mesenchymal *Hox6* plays a key role in this process, as loss of *Hox6* paralogs in the mesenchyme impairs endocrine cell differentiation [48].

Historically, the pancreatic mesenchyme has been viewed as a homogenous population, equally competent to respond to different developmental cues. However, recent data indicate that specific mesenchymal subpopulations have enriched activity for particular signaling pathways [35], suggesting a previously under-appreciated level of heterogeneity within the mesenchyme. Research efforts in the past few years have sought to characterize this mesenchymal heterogeneity in greater detail. One such effort utilized Nkx3.2-Cre as a lineage trace, allowing researchers to study this mesenchyme-derived population throughout development and adulthood. Proteomic analysis of the Nkx3.2 lineage revealed that this population changes its expression profile from embryonic to postnatal stages, giving rise to a pericyte population in adults that supports endocrine differentiation [49,50]. Further, depleting these cells in the adult reduces B-cell function [51], demonstrating that the functional role of mesenchymal populations can change from development to adulthood.

Novel technologies have expanded our concept of heterogeneity within the developing pancreatic mesenchyme by identifying additional distinct populations of fibroblasts. Single cell RNA sequencing of the pancreatic mesenchyme at different stages of embryonic development revealed seventeen transcriptionally distinct clusters [35]. While some of these populations were clearly identifiable (e.g., mesothelium, vascular smooth muscle, etc.), many of these clusters represented previously undefined groups of mesenchymal cells within the developing pancreas. This transcriptional analysis also identified candidate markers for these novel groups, which can identify distinct stromal

populations *in vivo*. By linking related transcriptional groups across development, the authors determined that mesenchymal populations undergo dramatic transcriptional changes throughout development, and that mesothelial cells may give rise to multiple mesenchymal subtypes. While this research suggests lineage relationships and unique functions between these different mesenchymal subpopulations, this has yet to be directly tested. Fortunately, the existence of commercially available Cre lines (WT1-GFPCre and WT1-CreERT2 [52] provides an opportunity to investigate a subset of these mesenchymal populations during pancreas development. The future generation of Cre lines for other mesenchymal subpopulations (e.g., *Stmn2*, *Barx1*) will allow even greater functional characterization of these proposed lineages throughout pancreas development.

Beyond the open questions within the context of development, the relationship between mesenchymal heterogeneity in the embryo and the adult organ is still poorly understood. Many of the recently identified mesenchymal subpopulations have not been followed into adulthood, and as a result it is unknown which populations persist in the adult organ, and how their functional role may change. The work by Landsman et al., Harari et al., and Sasson et al. demonstrates how genetic lineage tracing tools can be used to determine the contribution of different mesenchymal populations to adult tissues, and evaluate the function of these populations at developmental and postnatal timepoints [43,49,51].

Fibroblast heterogeneity in the healthy pancreas

The bulk of the healthy pancreas is comprised of epithelial tissue, predominantly lobular acinar cells which secrete digestive enzymes into the small intestine via a ductal

system. Mesenchymal cells, conversely, occupy less than 10% of the mature organ [53]. Despite their relatively low abundance, pancreatic fibroblasts are surprisingly diverse. Recent data has started to define these different populations in new levels of detail and suggest novel functions in the healthy pancreas.

One group of fibroblasts with a relatively well-defined origin and adult function are the pancreatic pericytes. Derived (at least in part) from the *Nkx3.2*-expressing embryonic mesenchyme [49], these cells are found adjacent to endothelial cells throughout the adult pancreas [54]. Importantly, these cells can be distinguished from other fibroblast populations *in vivo* by the expression of *NG2* [3,55]. Despite their presence throughout the pancreas, pericytes have primarily been studied in association with pancreatic islets. Ablating the Nkx3.2 lineage of pericytes via DT reduced the insulin response to a glucose load in mice [51]. Further, live imaging data from pancreatic slice cultures suggest that pericytes respond to neural and islet-derived signals to regulate blood flow to the islets [56]. While these data suggest that pericytes act as an essential mediator for the endocrine pancreas, their impact on the exocrine pancreas still remains poorly understood. Utilizing existing genetic tools to target pericytes *in vivo* (e.g. Nkx3.2-Cre, NG2-Cre [43,57]), could determine how this cell type impacts other pancreatic functions.

In contrast to the endothelial association of pericytes, mesenchymal stem cells (MSCs) have been found in close association with pancreatic exocrine tissue [58,59]. While no single marker has been found to conclusively mark MSCs, they can be identified through flow cytometry as $CD45^{-};CD44^{+};CD49a^{+};CD73^{+};CD90^{+}$ [60,61]. These isolated pancreatic MSCs retain the ability to differentiate into adipocytes, chondrocytes, and osteoblasts [58–61]. However, it is unknown whether MSCs contribute to multiple

mesenchymal cell types *in vivo,* or whether they impact exocrine or endocrine function in the healthy pancreas. In the absence of direct lineage tracing tools, isolated MSCs could be compared to other fibroblast populations through RNA sequencing analysis, in order to determine: 1) whether MSCs have a unique interactome that may impact other cell types, and 2) whether MSCs share a transcriptional "lineage" with other mesenchymal populations [35], suggestive of MSC differentiation in the pancreas.

In addition to MSCs, the exocrine portion of the pancreas is also the reported niche for pancreatic stellate cells (PSCs). PSCs have received substantial attention for their suggested contribution to fibrosis in the context of pancreatic disease [62–66]. PSCs have been defined by the presence of lipid droplets and their ability to "activate" aSMA expression and deposit ECM when isolated in 2D culture [53,67]. Additional PSC markers have been suggested that can be identified through staining, including desmin and GFAP[53]. It is worth noting, however, that many of the markers used to identify PSCs historically have either been inaccurate (such as the neuron-detecting GFAP) or overlap with general fibroblast populations (e.g., Desmin, aSMA) [68]. Further, no lineage tracing of these populations *in vivo* has been done, making their developmental origin as well as their direct contribution to pancreatic fibrosis unclear. It is therefore worth considering whether the cells we refer to as "PSCs" are not a single population, but rather a heterogeneous group of different fibroblast cell types that are independently capable of contributing to pancreatic fibrosis.

Much like the development field, researchers studying heterogeneity within adult pancreatic fibroblasts have utilized large-scale transcriptome analysis to describe these populations on a molecular level. One research group performed single cell RNA

sequencing on a combination of healthy adult mouse and human samples, in order to identify transcriptional patterns consistent between species [69]. The researchers identified two distinct clusters of PSCs in both mice and humans: which they defined as “activated” (enriched for ECM-associated genes, including COL1A1 and FN1) and “quiescent” (enriched for adipogenic genes, including ADIRF and FABP4). Within the human samples, the researchers also found a subgroup within the “activated” PSCs that was enriched for cytokines, suggesting a possible immune-modulating population in the healthy organ [69]. Although these data identify PSC populations with unique transcriptional profiles, it still remains unclear whether these populations have independent functions in the healthy pancreas. Further, the authors do not differentiate the “PSCs” analyzed from other fibroblast populations, such as pericytes or MSCs. It is therefore unknown whether these subcategories are unique to PSCs or shared by multiple fibroblast populations within the adult pancreas.

Fibroblast heterogeneity in pancreatic injury and disease

Beyond their role in the healthy pancreas, there has been great interest in the role of fibroblasts in pancreatic disease. In particular, fibroblasts have been studied extensively for their role in fibrosis, the accumulation of dense, ECM-rich stroma following chronic pancreatic injury [70]. Fibrosis is considered a hallmark of chronic pancreatitis and pancreatic cancer, and has been suggested as a barrier to therapies and an impediment to tissue recovery [71]. However, new data has revealed that the role of this fibrotic stroma is more complex. In the following sections, we discuss the known roles of fibroblasts within

the context of these fibrotic pancreatic diseases, the growing evidence for fibroblast heterogeneity, and present some of the open questions that still remain in the field.

Chronic pancreatitis is a painful disease with limited therapy options, and patients face an increased likelihood of developing pancreatic cancer [72,73]. Histologically, chronic pancreatitis is identified by a loss of mature acinar tissue, increased inflammation, and an abundance of fibrotic tissue [74]. Multiple rodent models have been generated in order to recapitulate this chronic human disease. These models utilize exposure to exogenous compounds (e.g., Trinitrobenzene sulfonic acid [TNBS], caerulein), surgical intervention (e.g., pancreatic duct ligation) or a combination of both to induce pancreatic damage, acinar necrosis, and eventually fibrosis [75–77]. Early studies stained for fibroblast markers in a combination of chronic pancreatitis patient samples and TNBS-treated rats, and found that PSCs were abundant in ECM-rich fibrotic areas [75]. *In vitro* studies further indicated that isolated PSCs could activate in response to a number of pancreatitis-associated cues, including TGFβ, TNFα, and ethanol [63,64,78,79]. More recent data has indicated that fibroblasts may affect chronic pancreatitis development through interactions with the immune system. In a repeated caerulein mouse model of chronic pancreatitis, researchers found that isolated PSCs are able to alternatively activate macrophages via IL-4 and IL-13, and disrupting this interaction reduced fibrosis *in vivo* [77]. A different group utilized a combined caerulein and pancreatic duct ligation model, and found that PSCs can activate in response to immune complement signal C5a [80]. Antagonizing this signaling pharmacologically minimized the accumulation of fibrotic stroma [80]. Although these studies indicate this fibro-inflammatory cross-talk can promote pancreatitis, recent evidence has also suggested that these two populations also

communicate during tissue recovery. Disruptions of stromal HH signaling in mice, particularly through the loss of a single *Gli1* allele, delays recovery from pancreatitis and alters cytokine production in pancreatic fibroblasts [17]. Loss of GLI1 function has also been linked to inflammatory bowel diseases, suggesting that this HH-mediated immune modulation may be a common feature of chronic inflammatory diseases [81].

Although these studies have enhanced our understanding of fibroblasts in chronic pancreatitis, they have predominantly focused on PSCs. Given the evidence for distinct fibroblast populations in both development and the healthy adult pancreas, one current area of interest is how different populations of fibroblasts contribute to chronic pancreatitis. A group of researchers collected human chronic pancreatitis samples and analyzed the expression of general fibroblast markers (e.g., aSMA, Desmin) as well as proposed stellate cell markers (e.g., CD34, NGFR, Tenascin C) through immunohistochemistry [82]. Interestingly, several of the proposed stellate cell markers, occupied distinct niches (e.g., periacinar vs. periductal) within the fibrotic microenvironment. Although this non-uniform expression is suggestive of fibroblast heterogeneity, unbiased single cell RNA sequencing efforts of broad fibroblast populations would provide better resolution of the stromal diversity in chronic pancreatitis. Further, lineage labeling of healthy fibroblast populations (described above) prior to inducing chronic pancreatitis would directly determine the relative contribution of different populations to fibrosis. Such genetic tools could also be paired with DT-mediated modes of targeted ablation [51], allowing researchers to determine the functional contribution of these different populations to fibrosis development and maintenance.

Fibroblast heterogeneity in pancreatic cancer

PDAC is believed to arise from pre-cursor lesions, most commonly pancreatic intraepithelial neoplasias (PanINs) [83]. Oncogenic mutations in KRAS are believed to be a key part of this PanIN progression process[22,84]. As the epithelium progresses through neoplasia and eventually carcinogenesis, the stroma also undergoes dramatic changes. Fibroblasts activate and transition into CAFs, a process that involves the production of chemokines, growth factors, and extracellular matrix [85–87]. Interestingly, fibroblast activation is dependent on oncogenic KRAS within the epithelium, and is reversed when oncogenic KRAS is inactivated in the epithelium [88].

CAFs were previously considered to be a homogenous cellular component of the tumor, all derived from a single population of pancreatic stellate cells (PSCs). However, a growing body of research supports the idea that CAFs are heterogenous, both in their phenotype and their function within the microenvironment. This heterogeneity can exist across multiple axes: physical location within the tumor microenvironment, ability to respond to different intercellular signals, and transcriptional profiles.

Positional heterogeneity – Heterogeneity based on localization

Evidence of cancer-associated fibroblast heterogeneity began to accumulate with descriptive reports of nonuniform staining of fibroblast markers. In human and mouse PDAC, researchers found that common fibroblast markers such as αSMA, podoplanin, PDGFRα/β, fibroblast specific protein 1 (FSP1), and desmin varied in their staining intensity, distribution, and overlap throughout the tumor tissue [28,82,89–94]. Interestingly, researchers have identified marker expression differences between the stroma

immediately adjacent to tumor tissue compared to more distant stroma. In both KPC mice and human PDAC, tumor-adjacent stroma expressed higher levels of αSMA, while more distant fibroblasts expressed IL6 [93,95]. The differing spatial distribution between these CAF subtypes suggests that different populations occupy unique niches within the carcinogenic stroma.

Despite progress in identifying these different CAF populations, the clinical significance of stromal markers has remained controversial. The expression of broad CAF markers has been correlated with worse prognosis in patients; including PDGFRβ [89], αSMA [82,96], and podoplanin [92]. However, clinical efforts to target broad stromal populations have shown minimal or inconsistent benefits to patient survival (reviewed in [97]), making the clinical utility of these correlative studies unclear. Further, these correlations are likely confounded by heterogeneity within these broad populations. To gain a better sense of the heterogeneity within CAFs, techniques such as multiplex immunohistochemistry would allow researchers to determine the spatial distribution of different subtypes in relation to other elements of the TME. This would provide better clarity of the exact niches of these different sub populations.

Interactive heterogeneity – Heterogeneity based on cell signaling

Throughout the process of pancreatic cancer progression, the developing tumor epithelium produces a vast array of secreted proteins. This dramatically changes the nature of cell signaling within the tumor microenvironment, notably within the stroma. However, there is heterogeneity in cell signaling activity within stromal populations, and

different CAF populations respond to different signaling molecules. A selection of key stromal cell signaling pathways are highlighted below:

Hedgehog Signaling

Hedgehog (HH) signaling is a core signaling pathway in human pancreatic cancer, first described over a decade ago when sonic hedgehog ligand (SHH) was found to be expressed in a majority of pancreatic tumor cell lines and human patients [84,98,99]. Later, Hedgehog signaling was shown to function in a paracrine manner in pancreatic cancer. The tumor cells secrete HH ligands, which bind to the transmembrane receptor Patched 1 (PTCH1) on the surrounding fibroblasts [100–102]. Binding of the ligand to PTCH1 releases the inhibition of Smoothened (SMO), which in turn activates the GLI family of HH transcription factors (for review see [103]). In the adult pancreas, the activity of the HH pathway is generally low unless activated in the context of injury or disease.

Although canonical HH signaling has been identified in PDAC, the exact role of HH is complex. Disrupting the HH pathway by SMO inhibition reduced tumor growth in a subcutaneous tumor model [100], and slightly prolonged survival when combined with chemotherapy in tumor-bearing KPC mice [12]. These data supported a model in which HH promoted tumor growth and could potentially be targeted to improve patient survival. This led to a clinical trial of the small molecular SMO inhibitor, IPI-926, also called saridegib, (Infinity Pharmaceuticals), and a parallel trial of a different SMO inhibitor, GDC-0449, or vismodegib (Genentech) in patients with pancreatic cancer. Unfortunately, the Infinity trial had to be terminated early due to decreased survival in the experimental group [104] and

the Genentech trial provided little to no benefit to patients [105,106]. To explain the trial findings, researchers re-evaluated the role of HH signaling in pancreatic cancer.

After the clinical trial results became public, the question of HH signaling in pancreatic cancer was revisited in two studies that inactivated the expression of *Shh* in the KPC models of pancreatic cancer. In both studies, investigators observed faster development of invasive tumors and shortened lifespan for the mice [18,107]. However, the tumors observed were quite different: Rhim et al. observed tumors devoid of fibrosis; conversely, Lee and coauthors observed tumors that retained the fibrotic stroma (albeit with a slight reduction on quantitation) yet progressed faster. Targeting the HH pathway results in a complex phenotype that underscores the need to more thoroughly examine the role of CAFs in tumor growth and survival. A caveat of both studies is that SHH is only one of three HH ligands, and at least two of them, SHH and IHH, are expressed in pancreatic cancer. Thus, it is likely that inactivation of SHH led to a reduction, but not ablation of HH activity. HH signaling has profoundly different effects in a dosage-dependent manner in embryonic development (for review see [108]; whether the same paradigm applies to cancer is less clear. Taking a different approach, our group has inactivated the HH coreceptors, GAS1, BOC, and CDON, which are HH pathway activators and are expressed in CAFs. We showed that fibroblasts with decreased levels of HH signaling promote tumor growth, while HH-unresponsive fibroblasts do not [109]. It will therefore be important to consider how levels of HH-response vary throughout the tumor microenvironment, and how these different HH-responding populations are influencing pancreatic tumor development.

TGF-β Signaling

Another signaling pathway that is highly active within the tumor microenvironment is the TGF-β pathway. Some of the first studies into pancreatic fibroblasts identified TGF-β signaling as a pro-fibrotic pathway [63,67]. However, dissecting the TGF-β interaction network within the diseased pancreas is complex, as multiple cell types (including fibroblasts) within the microenvironment express TGF-β ligands, allowing for both autocrine and paracrine modes of signaling [64,66]. Within the context of PDA progression, the role of TGF-β signaling has been controversial, as there have been conflicting reports over whether disruption of TGF-β signaling is protective [110,111] or deleterious [112–115]. However, it has been suggested that both effects are possible, and that the role of TGF-β may depend on the stage of disease and the cell populations involved [116,117]. Recent work in support of this idea has demonstrated that while global disruption of TGF-β signaling increased immune evasion, targeting TGF-β signaling specifically in CD8 T-cells slowed PanIN progression [118]. Since another study found genetic ablation of CD4+ T cells is sufficient to block PanIN formation and reduce the quantity of SMA^+ CAFs [119], these data highlight the complex cross talk between the immune cells, epithelium, and CAFs, and the importance of detailed characterization of these different signaling pathways within the tumor microenvironment to better understand how heterogeneous stromal populations may influence cell signaling in PDA progression.

Transcriptional heterogeneity – Heterogeneity based on gene expression

The transcriptional profile of CAFs can help differentiate distinct CAF populations, especially with the increasing availability of single-cell RNA sequencing as a tool to

examine rare and heterogenous populations in an unbiased manner. A series of papers classified pancreatic CAFs from a KPC mouse model and human PDAC into simple replicable subsets with distinct transcriptional profiles and activation pathways. Pancreatic CAFs may generally be characterized by either a myofibroblast "myCAF" or inflammatory "iCAF" phenotype [38,93,95]. The myCAF population is directly adjacent to tumor cells, expresses high levels of αSMA, and is maintained by the TGFβ pathway [93,95]. These αSMA-expressing myCAFs are believed to be tumor restricting, as depletion of αSMA-expressing fibroblasts promoted PDAC progression [20]. A separate population, the distant iCAF population, expresses IL6 among other inflammatory chemokines and cytokines, and relies on Il1/Jak-Stat signaling [93,95]. A third antigen-presenting "apCAF" population has recently been described after performing single-cell RNA-seq on ten treatment-naïve PDAC patients [38], though another group described the antigen-presenting population as part of the healthy-associated mesothelium when analyzing the same data set [37]. This second group was, however, able to independently identify an IL1-driven iCAF-like subset and a TGFβ-driven myCAF-like subset from *KPP* mice [37].

Another recent single cell RNA-seq study analyzed the fibroblasts from healthy and $Kras^{LSL\text{-}G12D}$;$Ink4a^{fl/fl}$;$Ptf1a^{Cre/+}$ (KIC) mice throughout lesion development [91]. The researchers identified three fibroblast populations in the healthy and early lesion stage, and only two in the advanced PDAC. The transcriptional profiles of the CAF populations were described as inflammatory and myofibroblastic, consistent with the previously defined iCAF and myCAF subtypes. Notably, for sequencing analysis done on genetically engineered mouse models, the exact profile of the fibroblast groups is significantly

impacted by the specific model used [37,38,91,120,121], highlighting the importance of stroma – epithelial cross talk in tumor microenvironment formation and model selection [122].

These new transcriptional data sets of PDAC converge on the concept that there are at least two transcriptionally, and likely functionally, distinct subgroups of CAFs – with either an inflammatory or myofibroblast dominant phenotype. Notably, these CAF subset designations appear to be interconvertible *in vitro* [38,95] and share a common base fibroblast program [37,95]. For example, inflammatory CAFs can also contribute to ECM deposition by expressing hyaluronan and collagens [37,38]. The boundaries between CAF populations are likely fluid and context-dependent. It is therefore worth tracing these different subtypes *in vivo* throughout different stages of PDAC progression, to see how different CAF populations respond to a changing TME.

Challenges in studying fibroblast heterogeneity

Although the research described above has provided novel insight into fibroblast heterogeneity, the field is currently challenged by conflicting data. It has been particularly difficult to come to a consensus regarding the specificity of fibroblast population markers and their expression patterns. For example, commonly used fibroblast markers such as PDGFRα, αSMA, PDPN have each been described as a pan-stromal marker by some groups, and a subtype specific marker by others [37,38,93,123,124]. Further, αSMA+ and PDGFRα+ fibroblasts were reported to be nearly mutually exclusive in mouse models of PDAC [91], while other research reported that these populations overlap [90]. Such inconsistencies in the field have made it difficult to generate a unified model of stromal

heterogeneity. As such, a number of considerations must be accounted for when evaluating fibroblast populations in PDAC.

The first consideration is the source of the tissue. Although human patients and mouse models have many similarities within the tumor microenvironment [15,37,38], the progression of the disease differs fundamentally between these two groups; the former typically acquiring mutations and experiencing histological changes over the course of years, while the latter experiences accelerated disease progression driven by oncogenic mutations as early as embryonic development. Obtaining data from human patients is also complicated by natural variability in patient cohorts (age, sex, lifestyle, stage of disease, etc.), as well as the relative scarcity of patient tissue. Data from mouse models can also be difficult to compare, considering the multitude of models (e.g. KPC, KIC, KPP) utilizing different oncogenes to drive carcinogenesis. An analysis of the effect of mutant tumor suppressor gene p53 versus loss of p53 observed key differences in the stiffness of the ECM matrix and the susceptibility to chemotherapy [121], which suggests the slight genetic variations of modeling can impact the stromal microenvironment. In addition, using an *in vitro* cell system introduces environmental cues, either by tumor conditioned media [123], signaling molecules [95], or dimensionality of the culture conditions (2D vs 3D) [38,121] that impact the cell state and phenotype. Since CAF subtypes may interconvert with one another given the appropriate cue, care must be made to not overgeneralize findings [95].

Another major source of data discrepancy may be due to technical variability while obtaining and processing samples. Different studies have utilized very different protocols to isolate, digest, sort, sequence, and analyze stromal populations, and as a result there

are dramatic differences in cell types captured and abundance of fibroblasts. This can have very real impact on sequencing efforts, as low or biased yields will limit the ability to fully capture all of the fibroblast populations within the tumor microenvironment. To resolve some of these conflicting findings moving forward, there needs to be enhanced transparency and collaboration regarding the processing of both mouse and human samples. In particular, developing optimized protocols to enrich for stromal populations will be necessary in order to capture the diversity of fibroblasts that exist within the tumor microenvironment. There also needs to be more specific vocabulary to acknowledge the origin, profile, and function of different fibroblast populations. Simply referring to all fibroblasts throughout pancreatic cancer progression as “CAFs” fails to capture the diversity and complexity of this critical cell population.

Origin of cancer-associated fibroblasts

Pancreatic CAFs have long been assumed to derive from a resident population of pancreatic stellate cells (PSCs). However, growing data supports the possibility that multiple fibroblast populations in the healthy pancreas may contribute to the heterogeneity of CAFs. Lineage-tracing experiments in fibrosis models in other organs such as the liver, kidney, heart, skin, and lung, consistently demonstrate that various resident fibroblast populations proliferate in response to injury to contribute to fibrosis [125–131]. Beyond PSCs, MSCs have been identified healthy tissue and are expanded in neoplastic tissue, raising the possibility that MSCs can also contribute to neoplastic stroma [60,132]. Beyond these resident progenitors, it has been suggested that some CAFs may arise in part from bone marrow derived cells [133] or transdifferentiate from pericytes and endothelial cells (for

review see [134]). Single-cell RNA sequencing data suggests that CAFs may arise in part from cancer cells that have transformed through EMT, though they account for a relatively small portion of fibroblasts [32,37]. Although these populations have been suggested to give rise to CAFs, researchers have been largely limited by a lack of effective lineage-tracing tools for these populations *in vivo*. It is therefore difficult to conclusively determine the relative contributions of these different populations to the PDAC stroma.

Instead, gene ontogeny analysis has been used to track similar transcriptional programs throughout lesion development and to parse the origin of pancreatic CAFs. As mentioned earlier, Dominguez *et al* utilized single cell RNA sequencing analysis to define transcriptionally distinct fibroblast populations in the KPP mouse model [37]. The authors similarly sequenced healthy and adjacent-to-tumor samples to construct a hypothetical evolutionary roadmap. The transcriptional profiles of the two healthy fibroblast populations ultimately mapped closer to either a tumor-associated myCAF or iCAF population than themselves, suggesting CAF evolution evolves from healthy fibroblast heterogeneity [37]. However, since these populations were linked *in silico*, this data set can only suggest a lineage relationship between healthy resident populations and CAFs.

In a smaller study by Bernard *et al*, transcriptomic changes were examined by single cell RNA sequencing on surgically resected samples that progressed from low-grade to high-grade noninvasive intraductal papillary mucinous (IPMN) and PDAC samples [120]. Similar to the studies described above, the researchers were able to identify two distinct CAF clusters that corresponded to myCAF and iCAF phenotypes. However, they found that these populations emerged at different stages of PDAC progression. While the myCAF phenotype was detected at all three stages of PDA

progression (low-grade IPMN, high-grade IPMN, PDAC), the iCAF phenotype was only observed in PDAC [120]. Together, these data support the idea that distinct fibroblast populations in the healthy pancreas evolve over time to give rise to unique CAF subtypes [37,120]. However, in the absence of direct lineage tracing of healthy populations throughout PDAC progression, the true origin of different CAF populations will remain uncertain.

Targeting cancer-associated fibroblasts in the clinic

While our understanding of CAFs in PDAC is still evolving, interactions between CAFs and the tumor microenvironment have been leveraged for potential therapies. CAFs have many reported tumor-promoting functions, including metabolic support, recruitment of immunosuppressive cells, and creating a physical barrier to drugs through ECM deposition and remodeling (Reviewed by [11,122]). Many clinical trials have hoped to improve patient survival by targeting these CAF-associated elements of the tumor microenvironment (thoroughly summarized in [135]). However, recent data demonstrates that CAFs have also tumor-suppressive roles [18,20,107,109], making the clinical targeting of the stroma all the more complex. I have included a brief summary of some of these strategies and potential next steps below.

A popular clinical strategy has been to target pro-tumor intercellular signaling pathways, due to the extracellular accessibility of pathway ligands and receptors. A notable attempt to target CAF signaling involved inhibiting the HH signaling pathway. Although HH inhibitor IPI-926 with chemotherapeutic gemcitabine was reported to improve survival in mouse models of PDAC [12], neither IPI-926 nor Vismodegib (GDC-

0449) significantly improved survival in human patients (described in detail above) [105,106,136]. In addition to HH, vascular endothelial growth factor (VEGF) signaling has been targeted as a potential anti-stromal therapy. VEGF is produced by both tumor cells as well as stromal cells [137], and VEGF expression has been correlated with decreased survival in human patients [138]. Clinical trials tested anti-VEGFa monoclonal antibodies (bevacizumab) and small-molecule inhibitors of VEGF receptors (Axitinib) in combination with chemotherapy, but neither strategy improved survival in human patients [139,140]. Although it is difficult to pinpoint the exact reasons these trials failed, one underappreciated factor could be the heterogeneity of the stroma, in which distinct populations respond differently to treatment.

As an alternative to targeting CAF cells directly, several clinical trials have tried to target the ECM-rich physical stromal environment. One particular component of the ECM, hyaluronic acid (HA), is frequently overexpressed in PDAC patients [13,14], and can promote tumor growth and facilitate drug resistance [141]. Depleting HA with a stabilized version of hyaluronidase (PEGPH2O) in *KPC* mice decreased interstitial pressure and increased drug delivery to the site of the tumor, and improved survival in combination with gemcitabine [13,14]. A phase II clinical trial found a minor improvement (1 month) in progression-free survival for all patients receiving PEGPH2O alongside dual chemotherapy (Nab-paclitaxel and Gemcitabine), and a moderate benefit (4 months) for patients with high HA expression [142]. This suggested that this might be a viable strategy specifically for HA-high patients. However, a phase III clinical trial that specifically enrolled HA-high PDAC patients failed to improve overall survival, leading to the cancellation of the clinical trial [143]. Although the full data for this most recent trial has not been publicly

released, it appears that targeting the dense ECM is not sufficient to significantly improve patient survival.

A potential complication with the strategies described above could be that these inhibitors may be disrupting the stroma too broadly, resulting in the disruption of tumor-restricting fibroblast populations. Thus, it is worth considering whether more targeted approaches against specific pro-tumor CAF populations would be a more effective therapy option. Recent work (summarized above) has identified three CAF populations in PDAC: myCAFs, iCAFs, and apCAFs [38,93,144]. Of these three, iCAFs in particular have been shown to have pro-tumor activity via IL1/JAK/STAT signaling [144]. Inhibition of JAK-STAT signaling reduced the expression of inflammatory cytokines in PSCs and reduced tumor growth in KPC mice [144]. Two phase I clinical trials (NCT02550327, NCT02021422) will be testing the efficacy of the IL1-receptor antagonist (Anakinra) in PDAC, which may restrict the pro-tumor activity of iCAFs. Although this trial acknowledges the heterogeneity of CAF populations, several concerns still remain. Although JAK-STAT inhibition reduced tumor growth over the course of 10 days [144], it is unclear how prolonged treatment would affect the survival of KPC mice. It is also unclear whether this timeline would effectively suppress the iCAF phenotype over extended periods of time, or whether CAF populations would change to fill the functional niche of iCAFs. More fundamentally, the *in vivo* role of these different populations throughout pancreatic cancer progression is still poorly understood, as most of the inferred function so far has been based on *in vitro* experiments or correlative data following systemic drug treatments. In the absence of targeted *in vivo* manipulations of these different populations, it is difficult to fully understand how these different populations interact with each other and within the dynamic tumor

microenvironment. Fortunately, single cell sequencing efforts by the same group have identified a suite of potential markers that correspond to iCAFs (e.g., *Clec3B*, *Ly6C*, *Col14a1*), myCAFs (e.g., *Acta2*, *Thy1*, *Col12a1*), and apCAFs (e.g., *H2-Ab1*, *Saa3*, *Slpi*) [38], opening the possibility for CAF-subtype genetic tools to be developed. Inducible systems such as CreER would avoid disrupting other populations expressing these same markers during embryonic development, and could be paired with FlpO-based (*Ptf1a-FlpO*, *Pdx1-FlpO*) modes of pancreatic carcinogenesis (i.e. *KF*, *KPF* [28,29]*)* to manipulate of CAF populations throughout carcinogenesis *in vivo*. Without closely characterizing the dynamic stromal responses to proposed anti-CAF treatments, future clinical trials will likely face the same disappointing results.

Conclusions and future directions

Fibroblasts are a dynamic and complex component of the pancreatic stroma, from development to adulthood to cancer. Advances in transcriptomic sequencing tools in both animal models and humans have helped define distinct populations of fibroblasts, throughout many stages of development and disease (**Figure 1-1**). Many questions still remain, however, and several coordinated efforts are necessary in order to advance the study of fibroblast heterogeneity. First, the latest advances in large scale transcriptomics need to be paired with spatial information *in vivo*. This is key in order to better understand how these diverse populations are organized and what cell types they physically interact with. Pairing transcriptional analysis with multiplex immunohistochemistry may enhance our ability to comprehensively map out the interactions of the microenvironment. Further, the generation and utilization of stromal genetic lineage tracing in animal models will allow

us to follow these diverse populations throughout different stages of pancreas development and disease. With the advances in dual recombination models [28,29] it is possible to identify and manipulate multiple populations within the *in vivo* microenvironment, to see how these cell types evolve, function, and interact. As we gain deeper insight into the complex nature of fibroblast heterogeneity, we will be better poised to leverage these populations clinically in the years to come.

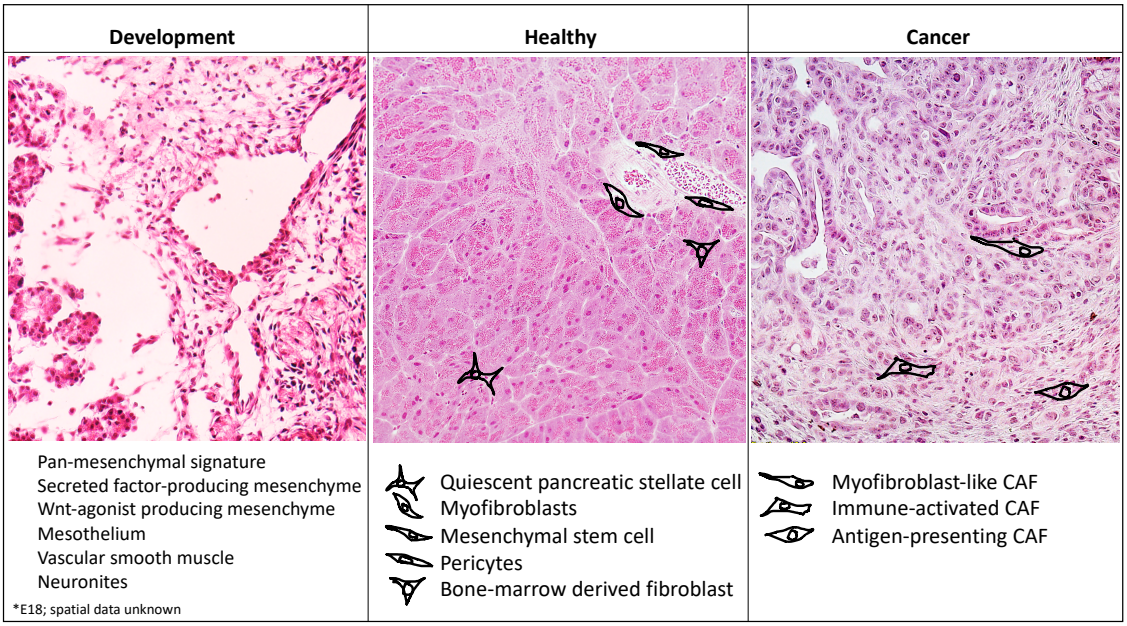

Figure 1-1 Schematic of fibroblast heterogeneity from development to cancer. A cartoon representation of the location and diversity of healthy mesenchymal cells and cancer-associated fibroblasts overlaid on H&E stains from mouse pancreata.

Chapter 2 Differential Contribution of Pancreatic Fibroblast Subsets to the Pancreatic Cancer Stroma

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a deadly cancer that is projected to become the second leading cause of cancer-related death by 2030[145]. The 5-year survival rate for PDAC is only 9% which can be attributed to the aggressive and chemoresistant nature of the cancer, in addition to an often late diagnosis[146]. In PDAC, more so than other solid tumors, a high percentage of the tumor volume is occupied by non-malignant cells that form the tumor "stroma"[7]. The cellular component of the stroma includes fibroblasts, infiltrating immune cells, vascular elements, and nerves[122]. This fibroinflammatory environment develops during the early steps of tumorigenesis and supports tumorigenesis and metastasis, although the roles of the individual components are still not fully understood[122].

Fibroblasts are a prominent and active cellular component of the stroma; they contribute to the secretion of extracellular matrix, and produce chemokines and growth factors that affect immune, endothelial, and cancer cell growth and function [122,134,147]. Initially, research into pancreatic cancer-associated fibroblasts (CAFs) supported a unilateral pro-tumorigenesis role[8,12,13,148–150]. However, other results suggested that fibroblasts could restrain tumor formation and that depleting them might accelerate carcinogenesis[20]. This paradox might be explained by the observation that fibroblasts are not a homogenous population, but exists in subsets with different roles [60,61,93,95].

Recently, several studies have highlighted the heterogeneity of the CAF subpopulations. This concept has made considerable progress thanks to the advent of single-cell sequencing to tease apart populations in an unbiased manner [20,60,93–95,132,151–153]. In human and mouse PDAC tissue, researchers observe several fibroblast subpopulations, characterized by distinct patterns of gene expression[38,91,123]. However, a remaining open question in the field is the origin of the CAFs, and whether their heterogeneity is driven by different progenitors. While pancreatic CAFS have long been assumed to derive from the resident pancreatic stellate cells, no formal lineage tracing has been conducted to support this idea. The healthy pancreas contains mesenchymal cells in periacinar, perivascular, and periductal regions of the exocrine pancreas. The potential of these resident cells in populating the fibroinflammatory environment and shaping the heterogeneity of the pancreatic cancer-associated stroma is unknown.

Here, we describe two fibroblast populations that are present in the healthy pancreas, marked by the expression of Gli1 or Hoxb6. Gli1 is a zinc-finger transcription factor and downstream effector and target gene of the Hedgehog signaling pathway, which is upregulated in human and mouse pancreatic cancer[17,154,155]. We have previously described a small population of $Gli1^+$ fibroblasts in the healthy pancreas[17,109]. In a number of other organs, tissue-resident $Gli1^+$ cells are mesenchymal progenitors that associate closely with the vasculature until activation into proliferative myofibroblasts by injury[131,156–159]. We sought to determine whether $Gli1^+$ fibroblasts similarly proliferate in the pancreas in response to neoplasia. In contrast, Hoxb6 labels the entirety of the mesenchyme during pancreas development[160], but based on our current data, is restricted to a subset of fibroblasts in the adult pancreas. Importantly,

Gli1 and Hoxb6 are expressed in separate populations in the healthy pancreas. We used lineage tracing and dual recombinase approaches to follow the fate of each cell population during carcinogenesis and determined that Gli1^{+} cells proliferated and contributed to the fibrotic reaction in pancreatic cancer, while Hoxb6^{+} cells do not.

Methods

Mouse strains

All mouse protocols were conducted with approval from the University Committee on Use and Care of Animals (UCACA). Gli1$^{eGFP/+}$ mice[75] were crossed with Ptf1a$^{FlpO/+}$;Kras$^{FSF\text{-}G12D/+}$ (KF) or Ptf1a$^{FlpO/+}$;Kras$^{FSF\text{-}G12D/+}$;Trp53$^{FSF/+}$ (KPF) mouse models donated by Dr. Crawford[44] to generate KF;Gli1$^{eGFP/+}$ and KPF;Gli1$^{eGFP/+}$ crosses. Gli1$^{CreERT/+}$ (JAX, #007913) or Hoxb6$^{CreERT/+}$ (contributed by Dr. Wellik[76]) mice were crossed into Rosa26$^{YFP/+}$ (JAX, #006148) or Rosa26$^{Tom/+}$ (JAX, #007909) reporter mice. These mice were then further crossed with the KF and KPF mice. Experimental and control animals were treated in parallel. Mice were housed in specific pathogen-free facilities at the University of Michigan Rogel Cancer Center.

In vivo experiments

All experiments were initiated in adult mice aged between 5-8 weeks of age. Acute pancreatitis was induced over 48 hours by two 8-hour serial intraperitoneal injections of caerulein (Sigma) as previously described[163,164]. To activate the CreER^{T} transgene, mice received 4mg of tamoxifen in corn oil/3% ethanol (Sigma) per day via oral gavage for 5 days. Where mentioned, mice received an additional three weeks of tamoxifen chow (400 tamoxifen citrate mg/kg diet, Envigo).

Immunohistochemistry and immunofluorescence

All immunohistochemical (IHC) and immunofluorescent (IF) stains were performed as previously described[88]. Primary antibodies used were YFP (1:200, Abcam), PDGFRβ (1:100, Abcam), CK19 (1:50, Iowa Developmental Hybridoma Bank), SMA (1:1000, Sigma), Amylase (1:100, Sigma), EPCAM (1:300, Cell Signaling), PDPN (1:300), CD31 (1:50, Cell Signaling), Vimentin (1:100, Cell Signaling), NG2 (1:100, Abcam), Lyve-1 (1:100, Abcam). Images were taken with an Olympus BX-51 microscope, and CellSens (Olympus) Standard software. For IF, Alexa Fluor-conjugated (Invitrogen) secondary antibodies were used. Prolong Gold-DAPI (Invitrogen) was used to counterstain the cell nuclei. The images were acquired using a Nikon A1 inverted confocal microscope and NIS-Elements software. At least five separate 60x fields (>180 cells) for IF or 20x fields for IHC from more than one experimental sample were quantified using HALO software (Perkin Elmer).

Transmission electron microscopy (TEM)

Tissues for transmission electron microscope analysis were prepared by University of Michigan Microscopy Core and images were acquired using Philips CM-100 transmission electron microscope.

Flow Cytometry

Pancreatic single cell suspensions were prepared as described[119] by mincing the tissue and then digesting in 1mg/mL collagenase IV (Sigma-Aldrich) at 37°C for 15-20 minutes. Digested samples were filtered through a 40μm strainer and subjected to a red blood cell lysis step. Samples were submitted for flow analysis in HBSS with 2% FBS. Antibodies were used in combinations of the following: CD45-Pacific Orange (1:50, BD

Pharm), PDGFRα-PE (1:50 BD Pharm), PDGFRα-PECy7 (1:50 BD Pharm). Fluorescence activated cell sorting (FACS) was performed on a MoFlo Astrios (Beckman Coulter) and data analyzed using Summit 6.1 Software and Flow Jo 10.6. The gating strategy was as follows: cells were first selected on forward (FSC-A) and side (SSC-A) scatters to exclude debris. Then single cells were gated based on SSC-H versus SSC-W and FSC-H versus FSC-W parameters. Alive cells were selected from an SSC-H versus DAPI plot. Non-immune cells were selected from an SSC-H versus CD45-Pacific Orange plot. At least $1x10^5$ events that pass these selection parameters were recorded for each sample. Further gating based on PDGFR, YFP, and Tomato parameters was used to analyze and sort the cells.

Statistical Analysis

The student t-test with Welch's correction or non-parametric Brown-Forsythe and Welch ANOVA using Dunnett's T3 multiple comparisons tests were performed by the Graphpad Prism 8 software to analyze the statistical differences between experimental cohorts. Significance was established for p-values < 0.05. All data presented as mean +/- standard error (SEM).

Results

Gli1$^+$ fibroblasts are present in the healthy and neoplastic pancreas

A well-established model for pancreatic carcinogenesis involves the targeted expression of mutant *Kras* in the murine pancreas. Oncogenic mutations in *Kras* are a near universal feature of human pancreatic cancer[165] and occur early during disease progression[166,167]. Expression of mutant *Kras* in genetically engineered mice leads to the formation of pancreatic intraepithelial neoplasia, PanIN, that with time, or additional

genetic manipulation, progress to cancer. Using a Gli1$^{EGFP/+}$ reporter, we conducted a detailed analysis of Gli1 expression in the normal pancreas, in PanINs, and in pancreatic cancer.

First, we examined the pancreata of healthy young adult mice between 4-8 weeks of age that are heterozygous for Gli1$^{eGFP/+}$. Gli1$^{eGFP/+}$ is a knock-in allele that faithfully recapitulates the expression of the endogenous locus (**Figure 2-1**A). The mice lack one functional allele of Gli1 but are nevertheless viable and fertile[168]. Since the natural fluorescence of EGFP does not persist following fixation and paraffin embedding, we used an anti-GFP antibody to visualize the reporter expression in immunohistochemistry (IHC) or immunofluorescence (IF). We observed Gli1^{+} cells in a perivascular location in the healthy pancreas, in a similar position previously described in the kidney, liver, lung, heart, and bone [131,157–159] (**Figure 2-1**B). IF staining with alpha smooth muscle actin (αSMA) and PDGFRβ, both fibroblast markers, indicated that, as previously described for the Hedgehog target gene Ptch1[100], Gli1 is expressed only in pancreatic fibroblasts (**Figure 2-1**C).

Next, to evaluate Gli1 in PanIN lesions, we crossed Ptf1a$^{FlpO/+}$;Kras$^{FSF\text{-}G12D/+}$ (KF) mice with the Gli1$^{EGFP/+}$ reporter, generating KF;Gli1$^{eGFP/+}$ mice (**Figure 2-1**A). KF mice[44], similar to the Cre-based KC mice[41], can recapitulate the progression of pancreatic cancer, though they rarely spontaneously develop neoplastic lesions[41]. PanIN lesions were accelerated in KF mice by inducing acute pancreatitis in mice aged 5-8 weeks as previously described[45–47]. As expected, we observed widespread PanIN development in KF;Gli1$^{EGFP/+}$ mice 3 weeks following acute pancreatitis. Immunostaining for EGFP revealed conspicuous positive staining limited to the stroma (**Figure 2-1**B). As in the

healthy pancreas, staining was limited to the fibroblasts (SMA$^+$ and PDGFRβ^+ cells) and not detected in epithelial cells (**Figure 2-1**C). Finally, to examine Gli1 in high-grade PanINs and pancreatic cancer, we generated KF;Trp53$^{FSF/+}$;Gli1$^{eGFP/+}$ mice (**Figure 2-1**C). In these animals, FlpO recombinase activates the oncogenic Kras allele while also deleting one copy of the tumor suppressor Trp53, thus essentially mimicking the commonly used KPC model[48]. These mice were aged for approximately 20 weeks until becoming moribund. In advanced disease progression, Gli1 can become expressed in epithelial cells[30]. We observed that the expression of the EGFP reporter in this cohort resembled that of low-grade lesions, with prevalent stromal expression (**Figure 2-1**B, C). Thus, Gli1 expression is present in a small subset of fibroblasts in the normal pancreas and becomes more prevalent in the neoplastic pancreas, and in pancreatic cancer. We observed no expression of the EGFP reporter in amylase-expressing acinar cells (**Figure 2-1**C).

Then, we used flow cytometry to obtain a quantitative measure of Gli1$^+$ fibroblasts in the healthy and neoplastic pancreas using PDGFRα, as a cell surface marker for fibroblasts. The expression of EGFP was limited to PDGFRα^+ cells in healthy and neoplastic pancreata alike (**Figure 2-1**D), as we observed minimal expression of EGFP in CD45$^+$ immune cells (data not shown). Among PDGFRα^+ cells, the mean EGFP positivity was 21% in the healthy pancreas, 62% in low-grade lesions, and 39% in high-grade lesions/cancer (**Figure 2-1**D). Together our data show that Gli1 expression is limited to a subset of fibroblasts in the healthy pancreas. In the neoplastic pancreas, Gli1 expression expands to a larger proportion of fibroblasts, a finding that might be explained by either *de novo* expression of Gli1 or expansion of Gli1$^+$ cells.

Pancreatic stellate cells (PSCs) have long been presumed to be the predominant mesenchymal population[62]. PSCs, similar to the hepatic stellate cells, store vitamin A in lipid droplets in the cytoplasm[53], which are visible as electron-dense inclusions in TEM images. Intrigued by the mesenchymal populations of the healthy pancreas, we used Transmission electron microscope (TEM) to visualize and confirm the existence of both PSCs and fibroblasts in healthy mouse pancreata (**Figure 2-1**E).

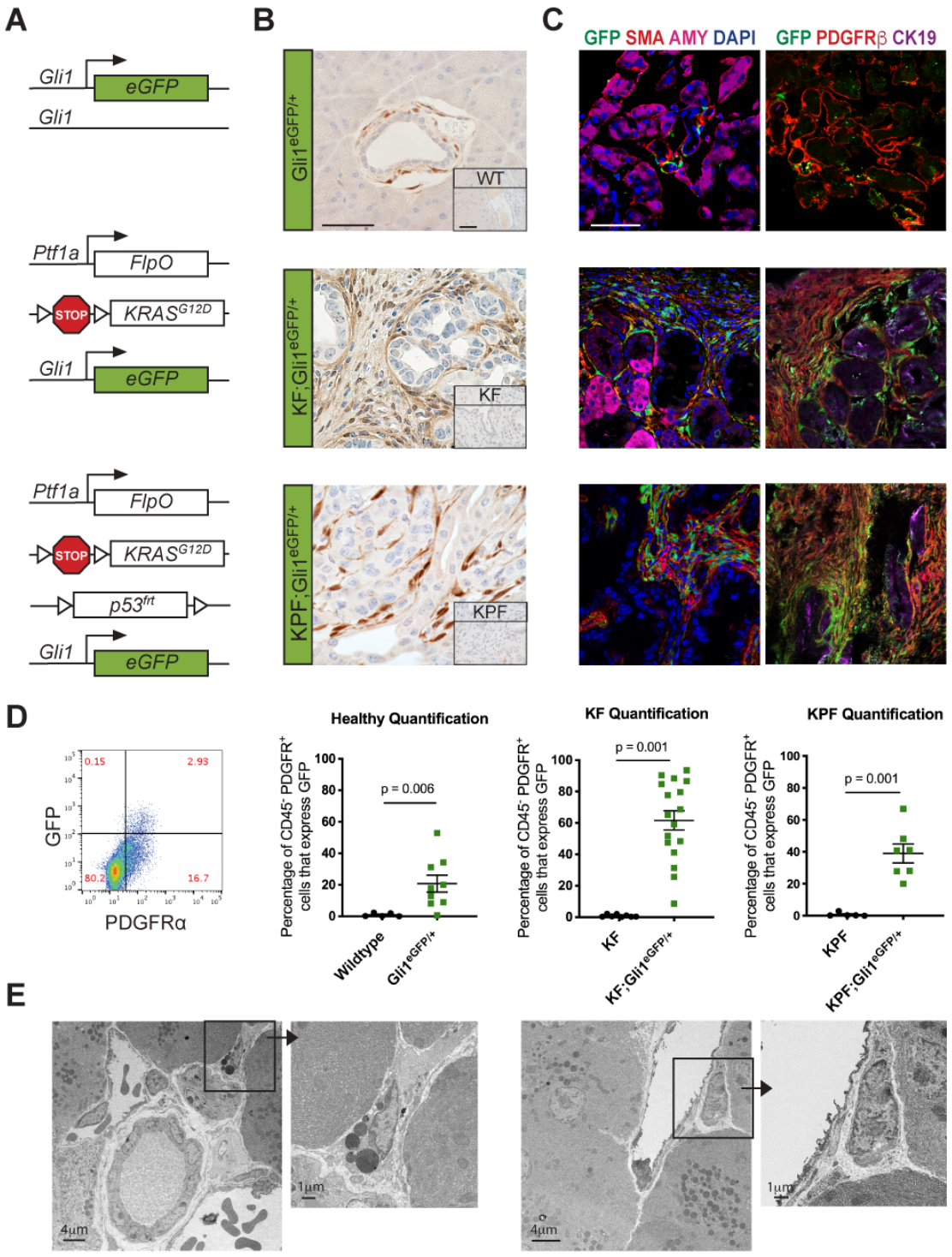

Figure 2-1 Gli1^{+} fibroblasts are present in healthy and neoplastic pancreas. (A) Genetic schemes for the knock-in Gli1$^{eGFP/+}$ reporter mouse models. (B) Immunohistochemical (IHC) staining for YFP. Insets have no Gli1$^{eGFP/+}$ reporter. (C) Immunofluorescence (IF) staining of GFP(green), SMA(red), and AMY(pink); and GFP(green), PDGFR(red), and CK19(pink). (D) Representative flow cytometry plot of GFP against PDGFRα, gated on DAPI^{-} CD45^{-} cells, and quantification of the percentage of GFP^{+} PDGFRα^{+} cells at the different disease stages (n≥5). All data expressed as mean +/- SEM. (E) TEM images of a healthy mouse pancreas. Scale bar = 50um

Establishing a lineage tracing approach for Gli1$^+$ fibroblasts

We established a lineage tracing approach to follow the fate of Gli1$^+$ fibroblasts. For this purpose, we bred Gli1$^{CreERT/+}$ knock-in mice with mice conditionally expressing YFP or tdTomato from the Rosa6 locus, Rosa26$^{LSL\text{-}YFP/LSLYFP}$ hereby RYFP or RTom (**Figure 2-2**A). In the resulting dual transgene progeny, administration of tamoxifen induces Cre recombination, permanently labelling those cells expressing Gli1 and their progeny.

To examine Gli1-expressing cells in the healthy pancreas, mice aged 5-8 weeks old were administered tamoxifen daily for 5 consecutive days by oral gavage, and then analyzed a week later (**Figure 2-2**B). As expected, immunostaining for YFP in Gli1$^{CreERT/+}$;RYFP mice showed an expression pattern similar to the Gli1^{eGFP} mouse (**Figure 2-2**C). Labeled Gli1 cells often colocalized with fibroblast markers such as SMA and PDGFRβ (**Figure 2-2**D). Gli1$^+$ cells did not colocalize with Lyve-1$^+$ lymphatic endothelial cells, though were closely associated, but not overlapping, with NG2$^+$ pericytes (**Figure 2-2**D).

We sought to validate that our lineage tracing system sufficiently labeled the healthy Gli1 population. We crossed our Gli1$^{CreERT/+}$;RTom mice into Gli1$^{EGFP/+}$ mice to generate Gli1$^{CreERT/+}$;Rtom;Gli1$^{EGFP/+}$ transgenic animals that allowed us to compare real-time expression of Gli1 with short-term lineage-tracing (**Figure 2-2**E). A week after tamoxifen administration, we harvested the healthy tissue and looked at the expression of the two fluorescent reporter genes. In this model, we found that the recombination-induced tdTomato expression was closely associated, if not colocalized, with EGFP expression (**Figure 2-2**F).

To obtain a quantitative measure of the YFP in our healthy Gli1$^{CreERT/+}$;RYFP mice, we performed flow cytometry as previously described. On average, 3% of the PDGFRα positive cells were labelled by YFP (**Figure 2-2**G). Consistent with our direct reporter, immune cells (CD45^{+}) did not express YFP (**Figure 2-2**H). By gating exclusively on live YFP^{+} cells, we conclude that fewer than 10% of the Gli1$^{CreERT/+}$;RYFP cells are CD45 positive. Almost all are PDGFRα positive (82%), and about 30% of the YFP^{+} cells co-express mesenchymal stem cell marker, CD105 (**Figure 2-2**I). Overall, our analysis revealed that the Gli1CreERT allele recombined the expected cell population, and that about a sixth of the Gli1-expressing cells in the pancreas were successfully recombined. Some discrepancy from the expression of a gene and CreER-mediated recombination is expected, and likely exacerbated in this case by the low level of expression of Gli1. However, the recombination efficiency was sufficient to track these cells during carcinogenesis.

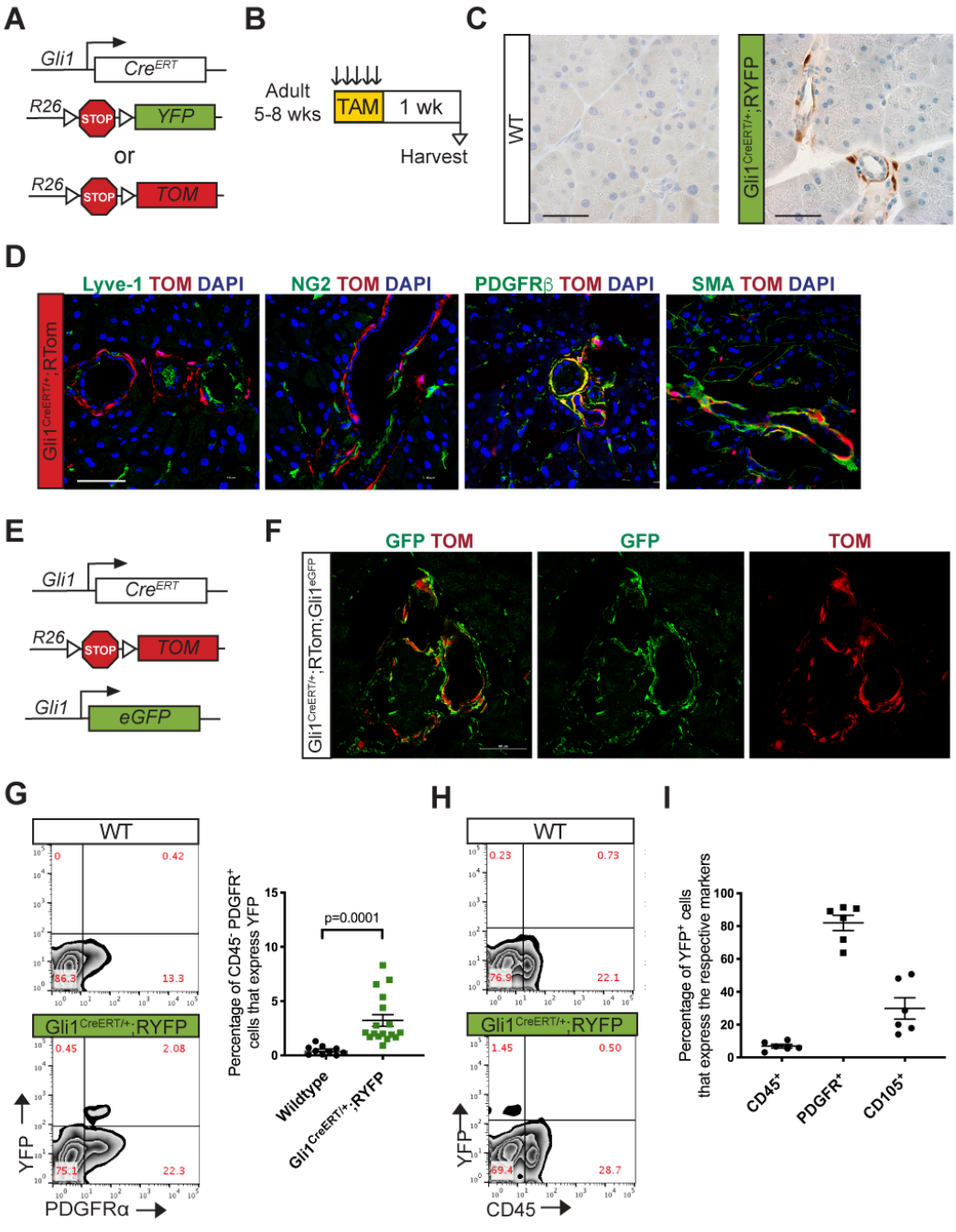

Figure 2-2 CreERT model labels healthy adult Gli1-expressing fibroblasts. (A) Genetic scheme for Gli1$^{CreERT/+}$ crossed with either a Rosa26$^{YFP/+}$ or a Rosa26$^{tdTomato/+}$ reporter. (B) Experimental design for examining healthy adult mice expressing Gli1 in the pancreas. Adult mice aged 5-8 weeks were given tamoxifen gavages (4mg/mouse/day) for 5 days. Tissue was examined one week after completing gavages. (C) IHC staining for YFP, and (D) IF staining of Lyve-1(green), Tomato(red), DAPI(blue); NG2(green), Tomato(red); DAPI(blue); PDGFR(green), Tomato(red), DAPI(blue), and SMA(green), Tomato(red), DAPI (blue) in Gli1$^{CreERT/+}$;RTom samples. (E) Genetic scheme for a Gli1$^{CreER/+}$;Gli1$^{EGFP/+}$,RTom mouse model. (F) IF staining of GFP and tomato in the Gli1$^{CreERT/+}$;Gli1$^{EGFP/+}$;RTom mouse. (G) Representative YFP vs PDGFRα, flow cytometry plots of DAPI^{-} cells in a healthy Gli1$^{CreERT/+}$;RYFP mouse and wildtype control. Flow cytometry quantification of the percentage of YFP^{+} PDGFRα^{+} cells in Gli1$^{CreERT/+}$;RYFP mouse and wildtype control (n≥10), p = 0.0001. (H) Representative YFP vs CD45 flow cytometry plot. (I) Flow cytometry quantification of the percentage of YFP^{+} cells that express CD45, PDGFRα, or CD105. All data expressed as mean +/- SEM. Scale bar = 50um.

Gli1$^+$ fibroblasts expand during the formation of neoplastic lesions in the pancreas

While the healthy pancreas consists mostly of epithelial cells, pancreatic cancer and the precursor lesions known as PanINs, are characterized by extensive accumulation of fibroinflammatory stroma[122]. KF mice[29], like their counterpart KC[166], are born and reach adulthood with a normal pancreas, notwithstanding the expression of oncogenic Kras. Lesions occur spontaneously and stochastically, but can be accelerated by the induction of acute pancreatitis[169]. We crossed our Gli1$^{CreERT/+}$;RYFP mice with the KF model to generate KF;Gli1$^{CreERT/+}$;R26YFP mice (**Figure 2-3**A). To determine the contribution of Gli1$^+$ fibroblasts to the fibrotic reaction, we administered tamoxifen to experimental and control mice when they reached 5-8 weeks of age, prior to the occurrence of spontaneous lesions. Then we induced acute pancreatitis and harvested the tissue 3 weeks later (**Figure 2-3**B). The 3-week time point was chosen because we and others have shown that this is when the pancreas parenchyma is almost completely replaced with low-grade PanIN lesions surrounded by an extensive fibroinflammatory reaction. We performed IHC for YFP, and found Gli1$^+$ progeny were significantly more abundant in the neoplastic tissue than in the healthy tissue (**Figure 2-3**C,D). As in the healthy pancreas, Gli1 expression was confined to the fibroblasts (PDGFRβ) and excluded from ductal or acinar epithelial cells (**Figure 2-3**E). Furthermore, Gli1$^{CreERT/+}$;RYFP mice without KF alleles exposed to the same treatment fully recovered from pancreatitis (**Figure 2-3**F), and in these tissues YFP expression resembled that of untreated healthy mice.

To determine whether Gli1$^+$ progeny persist through cancer progression and without an acute inflammation induction event, we generated KPF;Gli1$^{CreERT/+}$;RYFP mice (**Figure 2-4**A). We administered tamoxifen as these animals reached early adulthood (5-

8 weeks), and then let them develop lesions with time. We harvested the pancreata when the mice were expected to harbor both high-grade PanIN lesions and malignant disease, approximately 18 weeks after labelling, and found $Gli1^+$ fibroblasts still present in the stroma (**Figure 2-4**B, C, D).

Unlike the normal pancreas, the neoplastic tissue contains a myofibroblast-like population characterized by the expression of smooth muscle actin (SMA). We observed partial co-localization of YFP and SMA in both KF and KPF lineage models (**Figure 2-4**E). By quantifying the dual IF stain we observed lineage-traced $Gli1^+$ cells on average contributed to less than half of the myofibroblast population (**Figure 2-4**F). Meanwhile, the majority of our lineage-traced $Gli1^+$ cells are myofibroblastic: an average of 76% of the YFP^+ cells in the KF model co-expressed SMA and an average of 70% of the YFP^+ cells in both models co-expressed SMA (**Figure 2-4**F, lower panel). Healthy resident $Gli1^+$ fibroblasts expand and contribute to the SMA^+ myofibroblast population during carcinogenesis.

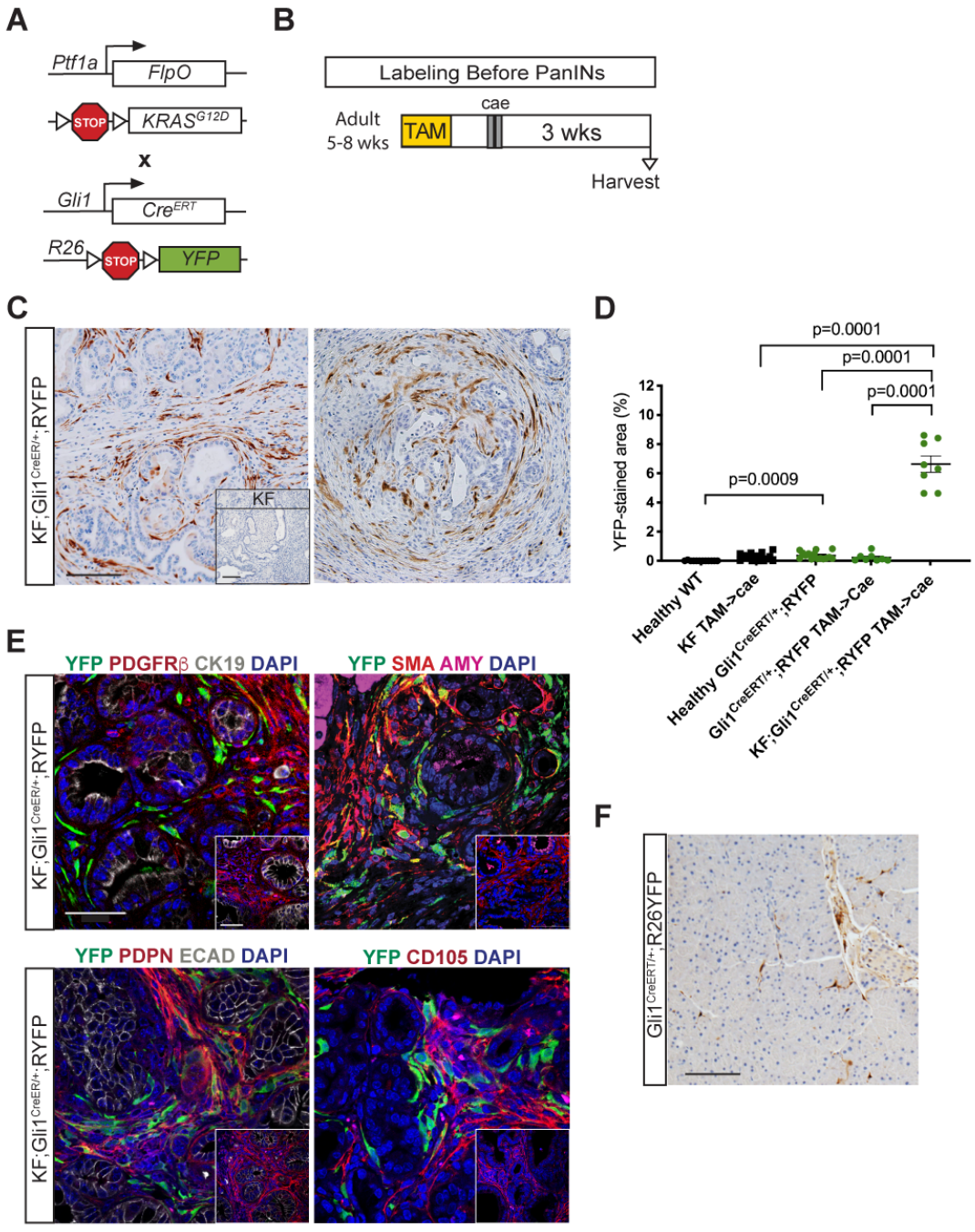

Figure 2-3 Gli1^{+} fibroblasts lineage-traced before pancreatitis-induced PanIN lesion formation contribute to the stroma. (A) Genetic scheme for a KF;Gli1$^{CreERT/+}$;RYFP mouse. (B) Experimental design for labelling adult (5-8wks old) mice before PanIN generation. Adult mice 5-8 weeks old were given 5 tamoxifen gavages and rested a week before two days of caurelein injections to induce pancreatitis. After 3 weeks, mice were harvested. (C) IHC staining for YFP in KF;Gli1$^{CreERT/+}$;RYFP and KF control tissue (inset) labelled before PanIN generation. Scale bar = 100um. (D) Quantification of YFP positive staining area from IHC (n≥8) (E) IF staining on KF;Gli1$^{CreERT/+}$;RYFP and KF control (inset) samples of YFP(green), PDGFR(red), CK19(white), DAPI (blue); YFP(green), SMA(red), Amylase(pink), DAPI(blue); YFP(green), PDPN(red), ECAD(white), DAPI(blue); and YFP(green), CD105(red), and DAPI(blue). Scale bar = 50um. (F) A representative YFP IHC image of a Gli1$^{CreERT/+}$;RYFP mouse following the same acute pancreatitis labelling scheme. Scale bar = 100um

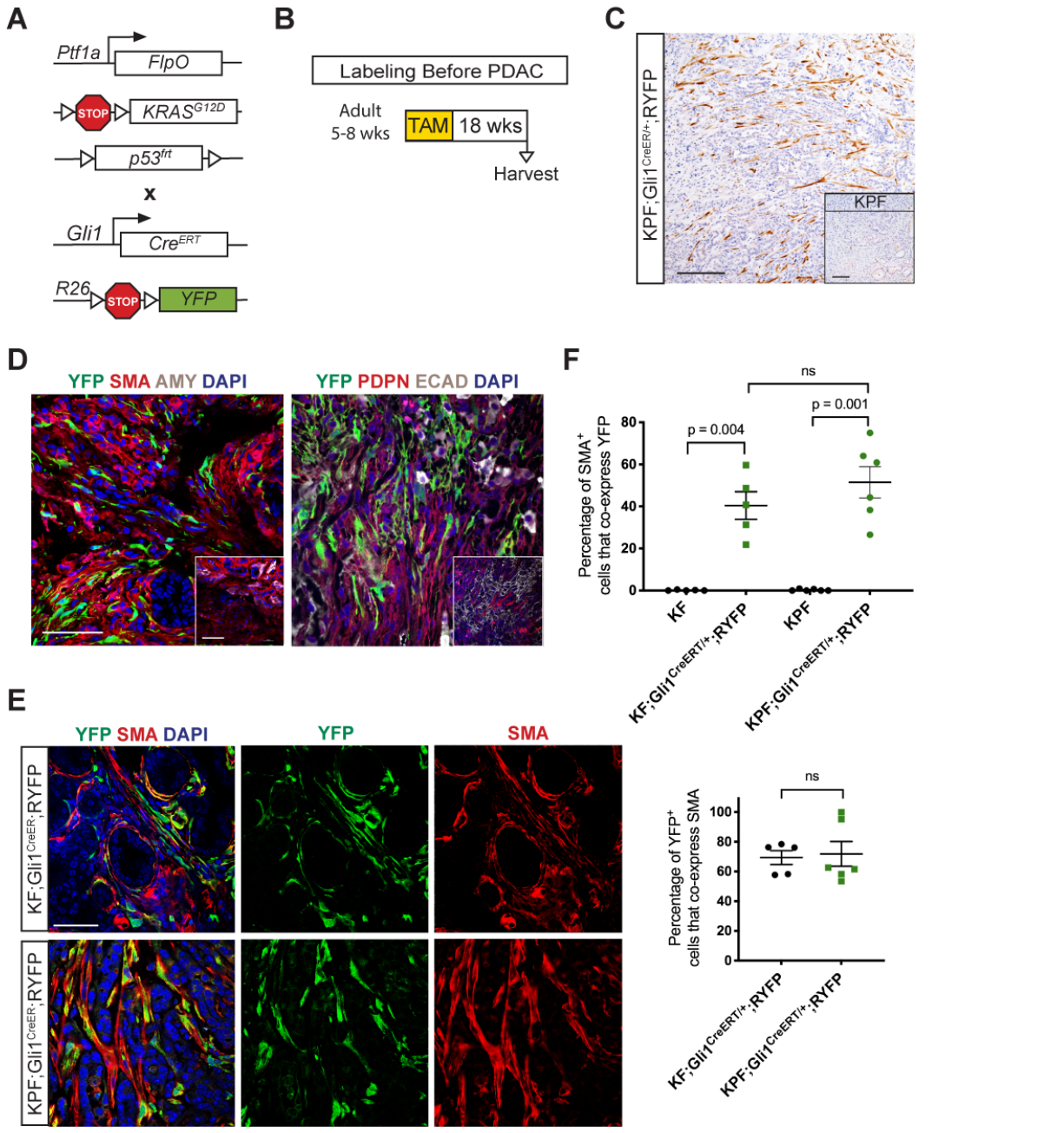

Figure 2-4 Gli1^{+} fibroblasts contribute to the stroma when lineage-traced in a spontaneous pancreatic carcinogenesis model. (A) Genetic scheme for a KPF;Gli1$^{CreERT/+}$;RYFP mouse. (B) Experimental design for labelling adult mice (5-8wks old) before aging. Adult mice 5-8 weeks old were given 5 tamoxifen gavages and were harvested upon evidence of disease burden. (C) IHC staining for YFP in KPF;Gli1$^{CreERT/+}$;RYFP and KPF control (inset) mice. Scale bar = 100um. (D) IF staining for YFP(green), SMA(red), Amylase(gray), and DAPI(blue); and YFP(green), PDPN(red), ECAD(white), DAPI(blue) in KPF;Gli1$^{CreERT/+}$;RYFP and KPF control (inset) samples. Scale bar = 50um. (E) Merged and single channel IF images for YFP(green), SMA(red), and DAPI(blue) in KF and KPF Gli1^{CreER};RYFP samples. Scale bar = 50um. (F) IF quantification of the percentage of SMA^{+} cells that co-express YFP in lineage-traced KF and KPF samples (n≥5). (F) IF quantification of the percentage of YFP^{+} cells that co-express SMA in lineage-traced KF and KPF samples (n≥8). All data expressed as mean +/- SEM.

The pancreas is home to heterogenous fibroblast populations that differentially contribute to the neoplastic stroma

We then sought to determine whether all pancreatic fibroblast populations expand during carcinogenesis. For this purpose, we sought to track a different population of fibroblasts, namely Hoxb6^{+} cells. The homeobox factor Hoxb6 is widely expressed in the mesenchyme of the developing pancreas, and regulates the embryonic development of the organ[160]. To assess the expression of Hoxb6 in the adult pancreas, we followed a similar strategy to that described before for Gli1. We generated Hoxb6$^{CreERT/+}$;RYFP or Hoxb6$^{CreERT/+}$;RTom, administered tamoxifen once they reached adulthood, and harvested the tissues one week later (**Figure 2-5**A, B). Interestingly, the distribution of Hoxb6^{+} fibroblasts appeared different than that of Gli1^{+} fibroblasts, with the former being interspersed in the pancreas parenchyma, rather than concentrated around blood vessels and ducts (**Figure 2-5**C). Though like Gli1, Hoxb6 labels a small subset of cells in the adult pancreas and is limited to fibroblasts, with no expression in other cell compartments, including endothelial cells (**Figure 2-5**D-G).

To determine whether Gli1^{+} and Hoxb6^{+} fibroblasts indeed represented different cell populations, we generated Hoxb6$^{CreERT/+}$;RTom;Gli1$^{eGFP/+}$ mice to dual label the populations (**Figure 2-6**A). To maximize labeling of Hoxb6^{+} cells, we first administered tamoxifen by oral gavage, as previously described, and then placed the mice on tamoxifen chow for three weeks before harvesting the tissue (**Figure 2-6**B). Although the low frequency of each cell population complicated the analysis, we detected mainly single labeled tdTomato^{+} or EGFP^{+} cells, and, less frequently, cells expressing both reporters (**Figure 2-6**C). To obtain a quantitative measure, we performed flow cytometry on the

pancreata from mice expressing one or both reporters. In Hoxb6$^{CreERT/+}$;RYFP mice, an average of 6% of the PDGFRα^+ fibroblasts were labeled with the reporter (**Figure 2-5**E). In the dual labelled Hoxb6$^{CreERT/+}$;RTom;Gli1$^{eGFP/+}$ mice, we observed closer to 20% PDGFRα^+ cells that only expressed tdTomato, the Hoxb6 marker (**Figure 2-6**D). The higher incidence of Hoxb6 in the later experiment can be attributed to the additional three weeks of tamoxifen chow, allowing a more complete recombination, and a stronger fluorophore to detect in flow analysis. In the Hoxb6$^{CreERT/+}$;RTom;Gli1$^{eGFP/+}$ mice, about 10% of PDGFRα^+ cells express both Hoxb6 and Gli1 (**Figure 2-6**D). We conclude that Hoxb6 expression sufficiently captures a healthy mesenchymal cell population within the healthy pancreas, distinct from those that express Gli1, and with only occasional overlap.

Now, with these separate fibroblast markers, we could finally address whether healthy resident fibroblasts expand equally during carcinogenesis. Thus, we generated KF;Hoxb6$^{CreERT/+}$;RYFP mice (**Figure 2-7**A). We labelled Hoxb6$^+$ expressing cells prior to lesion formation, and harvested the tissue 3 weeks after inducing pancreatitis, as described above for Gli1$^+$ traced animals (**Figure 2-7**B). As expected, the pancreas parenchyma in these animals was replaced by widespread PanIN lesions and surrounding stroma, and mice without KF alleles fully recovered from pancreatitis (**Figure 2-7**C, D). Within the stroma, labelled fibroblasts were rare (**Figure 2-7**D). Flow cytometry analysis confirmed these observations: while Gli1$^+$ cells give rise to an average of 14% of the fibroblasts within the stroma, Hoxb6$^+$ cells give rise to only 2% (**Figure 2-7**E, F).

Finally, we altered our experimental design, inducing pancreatitis in adult mice, and then inducing Cre recombination two weeks later to examine the overall expression of Gli1 and Hoxb6 in PanINs, and to determine the extent of *de novo* expression of either

marker. The pancreata were harvested 3 weeks after the induction of pancreatitits in this experiment (**Figure 2-8**A). IHC and flow cytometry analysis showed a slightly higher prevalence of Gli1$^+$ fibroblasts (an average of 20% of PDGFRα^+ cells) than in the samples labeled prior to PanIN formation, indicating that Gli1 may be activated *de novo* in cells that were previously not expressing it (**Figure 2-8**B, C). In contrast, the expression of Hoxb6 was still rather minimal and observed in only about 2% of the PDGFRα^+ fibroblasts (**Figure 2-8**B, C).

To expand on an observation that our Gli1$^+$ traced cells often remained near neoplastic epithelial cells, we revisited our KF;Gli1$^{CreERT/+}$;RYFP samples and examined areas of lesser lesion burden. In those areas, we noticed that our lineage-traced Gli1$^+$ cells surround the nascent areas of acinar to ductal metaplasia and PanINs (**Figure 2-9**A). A subset of pancreatic fibroblasts in PDAC are described as SMA$^+$ myofibroblast-CAFs (myCAFs) and are positioned adjacent to the epithelial cancer cells[93]. We co-stained our lineage-traced Gli1$^+$ cells with αSMA to further examine if our Gli1$^+$ were myofibroblast-like myCAFs. The resulting staining around these early lesions suggested not all YFP$^+$ cells were SMA$^+$, and not all SMA$^+$ cells were YFP$^+$ (**Figure 2-9**B). Gli1$^+$ fibroblasts may play a myCAF-like role in nascent lesion development, however even at this earliest stage fibroblast heterogeneity is present. In summary, our data show that the healthy pancreas is home to heterogenous fibroblast populations, which have a differential potential to expand during the process of carcinogenesis. While we were able to lineage trace two of these populations, our data also indicates that other progenitors for the fibrotic stroma must exist, to contribute to the remaining fibroblast populations.

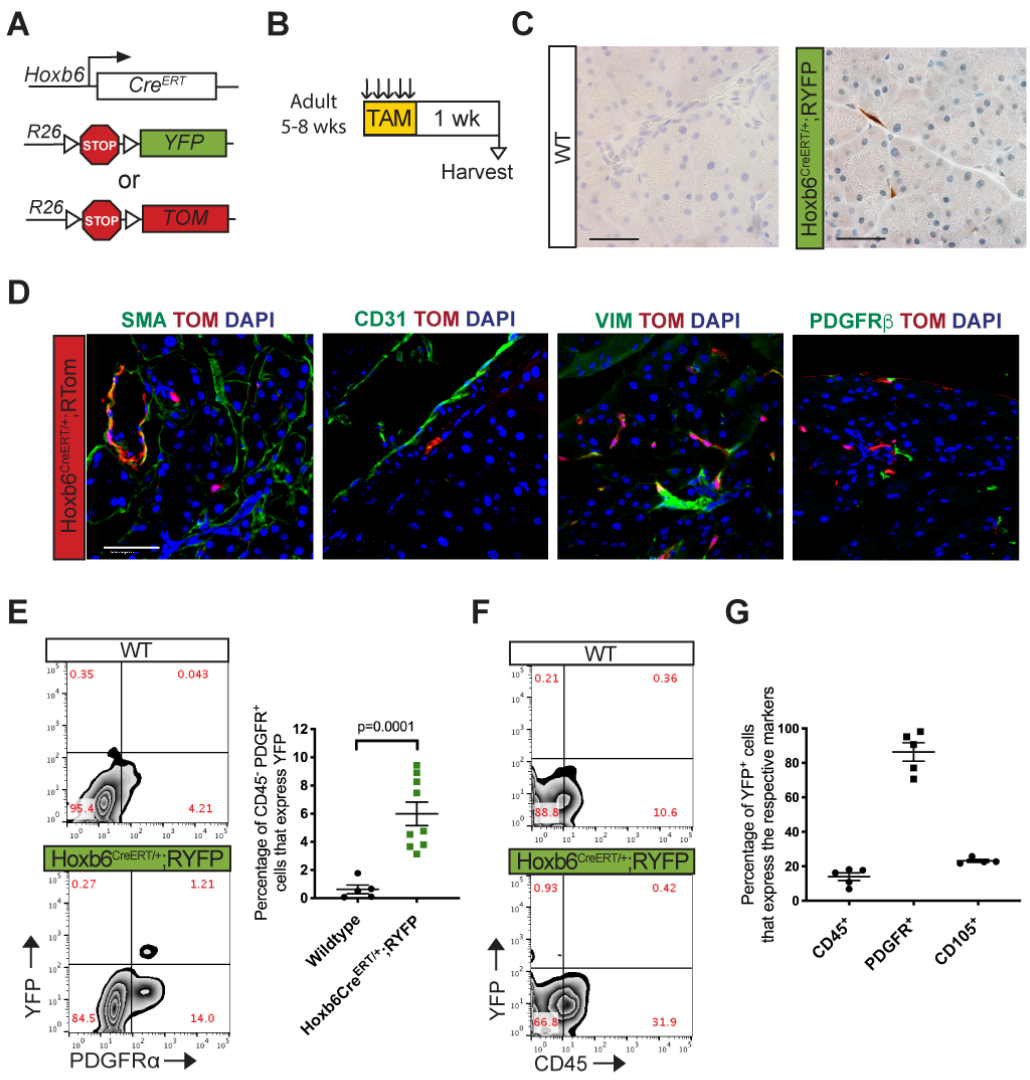

Figure 2-5 Hoxb6 labels a subset of mesenchymal cells in the healthy pancreas. (A) Genetic scheme for Hoxb6$^{CreERT/+}$ crossed with either a RYFP reporter or RTom reporter. (B) Experimental protocol for examining healthy adult mice expressing Hoxb6 in the pancreas. Adult mice aged 5-8 weeks were given tamoxifen gavages (4mg/mouse/day) for 5 days. Tissue was examined one week after completing gavages. (C) IHC staining for YFP. (D) IF staining of SMA(green), Tomato(red), DAPI (blue); CD31 (green), Tomato (red), DAPI (blue); Vimentin (green), Tomato (red), DAPI (blue); and PDGFR (green), Tomato(red), DAPI (blue) in Hoxb6$^{CreERT/+}$;RTom tissue. Scale bar = 50um. (E) Representative PDGFRα$^+$ flow cytometry plots of DAPI$^-$ cells in a Hoxb6$^{CreERT/+}$;RYFP mouse and wildtype control, and quantification of the percentage of YFP$^+$ PDGFRα$^+$ cells (n≥5), p = 0.0001. (F) Representative YFP vs CD45 flow cytometry plot. (G) Flow cytometry quantification of the percentage of YFP$^+$ cells that express CD45, PDGFRα, or CD105. All data expressed as mean +/- SEM.

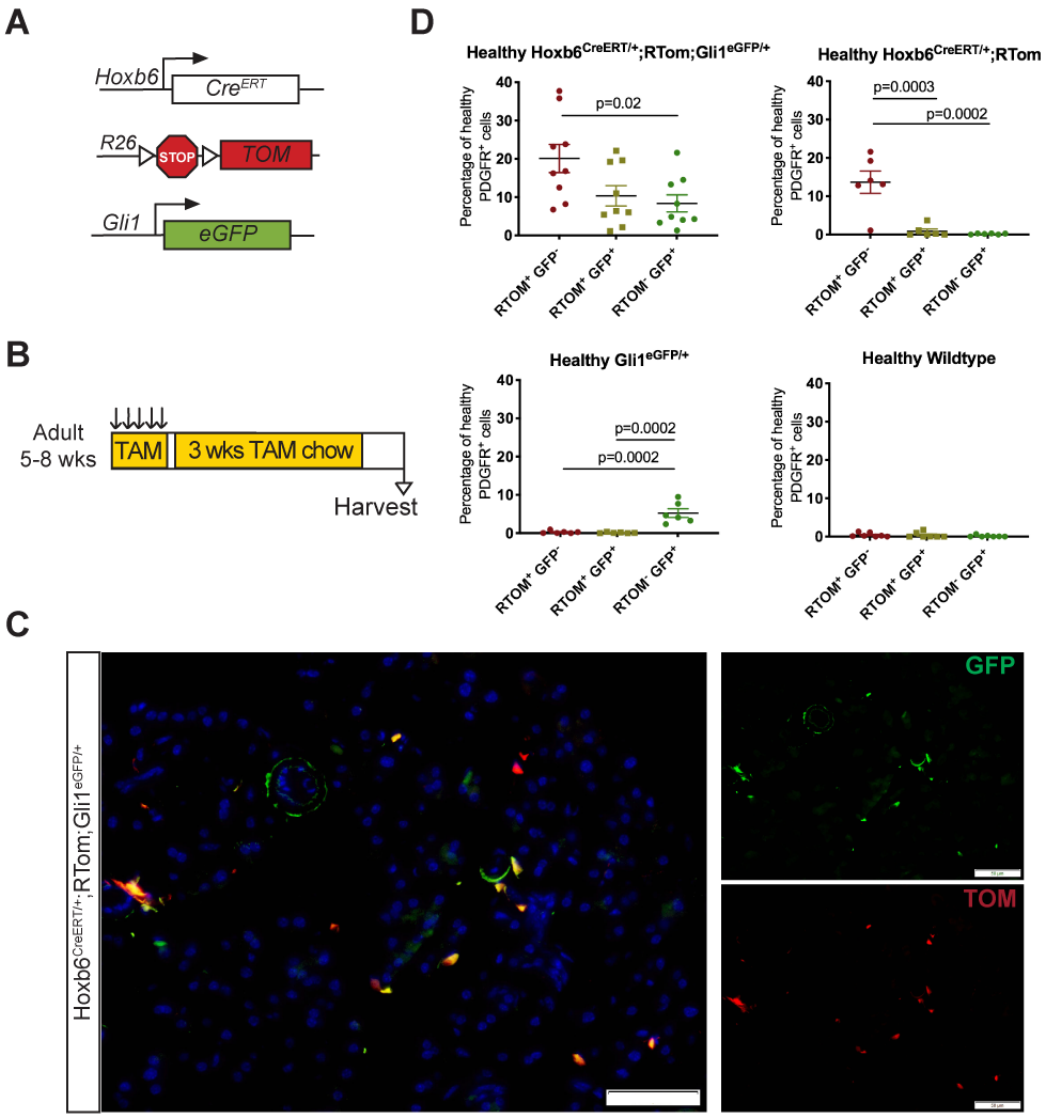

Figure 2-6 Hoxb6 and Gli1 are expressed in different populations. (A) Genetic scheme for a Hoxb6$^{CreERT/+}$;RTom;Gli1$^{EGFP/+}$ mouse model. (B) Experimental design for examining healthy Hoxb6$^{CreERT/+}$;RTom;Gli1$^{EGFP/+}$ mice. Adult mice aged 5-8 weeks were given tamoxifen gavages (4mg/mouse/day) for 5 days. Mice were then placed on tamoxifen chow for 3 weeks. Tissue was examined one week after completing chow regimen (C) IF staining of GFP and Tomato in the Hoxb6$^{CreERT/+}$;RTom;Gli1$^{EGFP/+}$ mouse. Scale bar = 50um. (D) Flow cytometry quantification of the percentage of healthy CD45$^-$ PDGFRα$^+$ cells that expressed either Rtom$^+$GFP$^-$, RTom$^+$GFP$^+$ or Rtom$^-$GFP$^+$ in Hoxb6$^{CreERT/+}$;Rtom;Gli1$^{EGFP/+}$ or control mice (n≥6). All data expressed as mean +/- SEM.

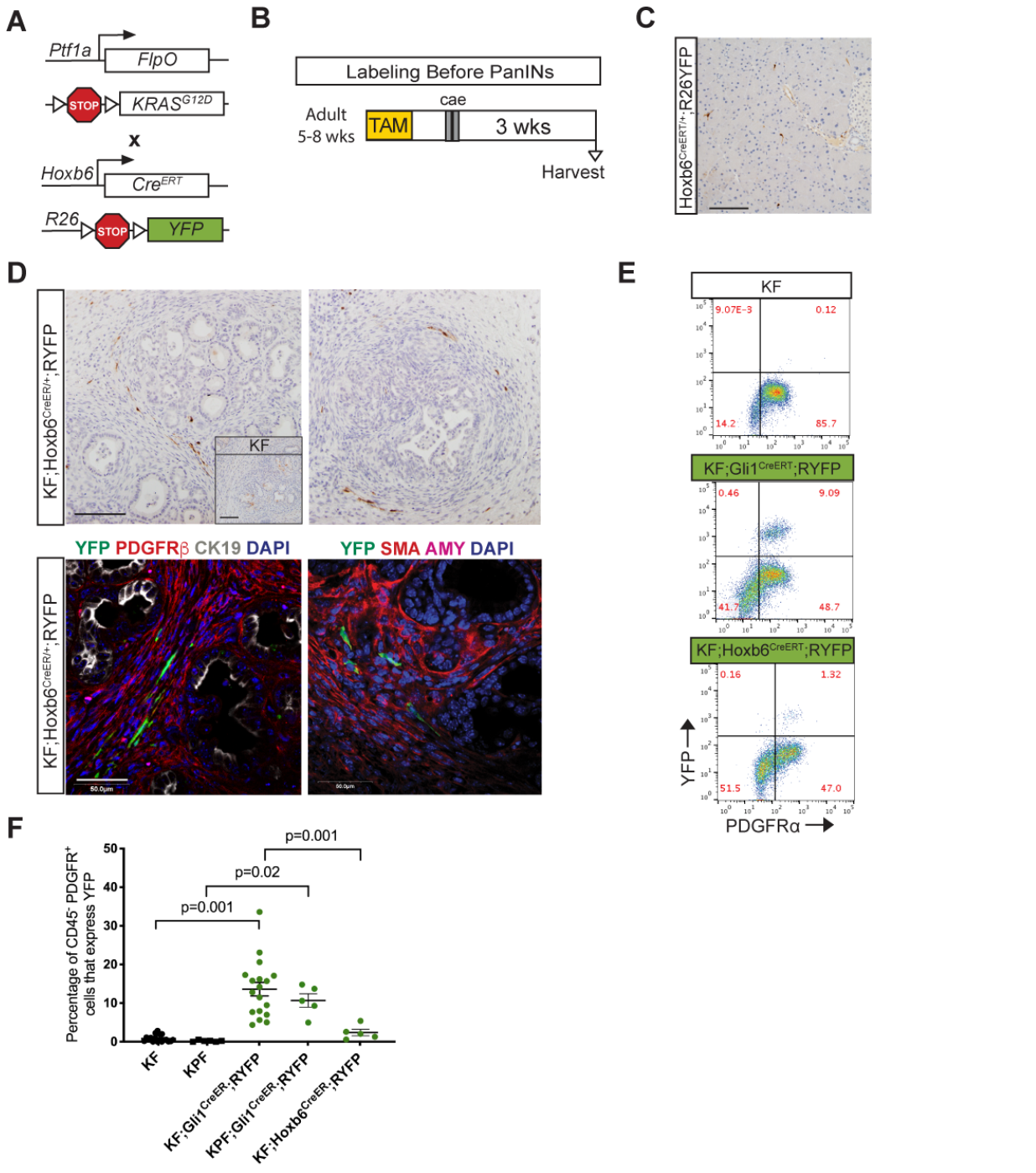

Figure 2-7 Hoxb6^{+} fibroblasts lineage-traced before pancreatitis-induced PanIN lesion formation do not contribute to the stroma. (A) Genetic scheme for the KF;Hoxb6$^{CreERT/+}$;RYFP model. (B) Experimental design to labelling adult (5-8wks old) mice before PanIN generation. Adult mice 5-8 weeks old were given 5 tamoxifen gavages and rested a week before two days of caurelein injections to induce pancreatitis. After 3 weeks, mice were harvested. (C) A representative Hoxb6$^{CreERT/+}$;RYFP mouse labelled before PanIN generation. Scale bar = 100um (D) IHC staining for YFP (scale bar = 100um) and IF of YFP(green), PDGFR(red), CK19(white), DAPI (blue) and YFP(green), SMA(red), Amylase (pink), DAPI (blue) (scale bar = 50um) in KF;Hoxb6$^{CreERT/+}$;RYFP and KF control (inset) mice labelled before PanIN generation. (E) Representative YFP vs PDGFRα flow cytometry plot of DAPI^{-}, CD45^{-} cells in a KF control, KF;Gli1$^{CreERT/+}$;RYFP, and KF;Hoxb6$^{CreERT/+}$;RYFP mouse (F) Flow cytometry quantification of the percentage of CD45^{-} PDGFR^{+} cells that express YFP in lineage-traced Gli1 and Hoxb6 mice and their controls (n≥5).

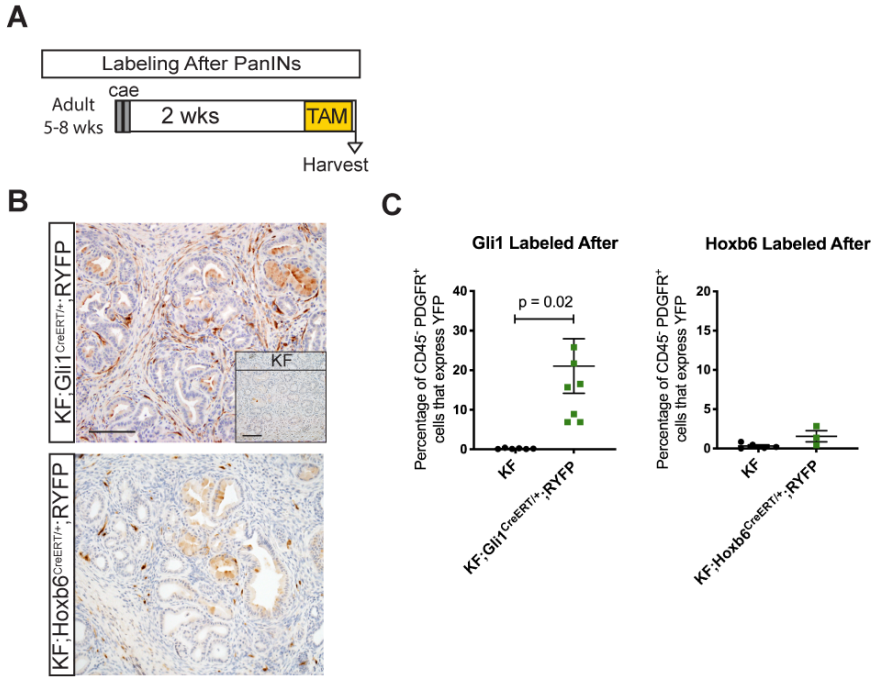

Figure 2-8 Gli1^{+} healthy cells still contribute more than Hoxb6 to PanIN lesion stroma when labelled after lesion generation. (A) Experimental design for labelling after PanIN generation. Adult mice 5-8 weeks old were given caerulein injections, rested for 2 weeks, and then given five gavages of tamoxifen before tissue collection. (B) IHC staining for YFP in KF;Gli1$^{CreERT/+}$;RYFP and KF;Hoxb6$^{CreERT/+}$;RYFP mice labelled after PanIN generation. KF control mice in insets. Scale bar = 100um. (C) Flow cytometry quantification of the percentage of CD45^{-}PDGFR^{+} cells that express YFP in KF;Gli1$^{CreERT/+}$;RYFP and KF;Hoxb6$^{CreERT/+}$;RYFP mice labelled after PanIN generation (n≥2). All data expressed as mean +/- SEM.

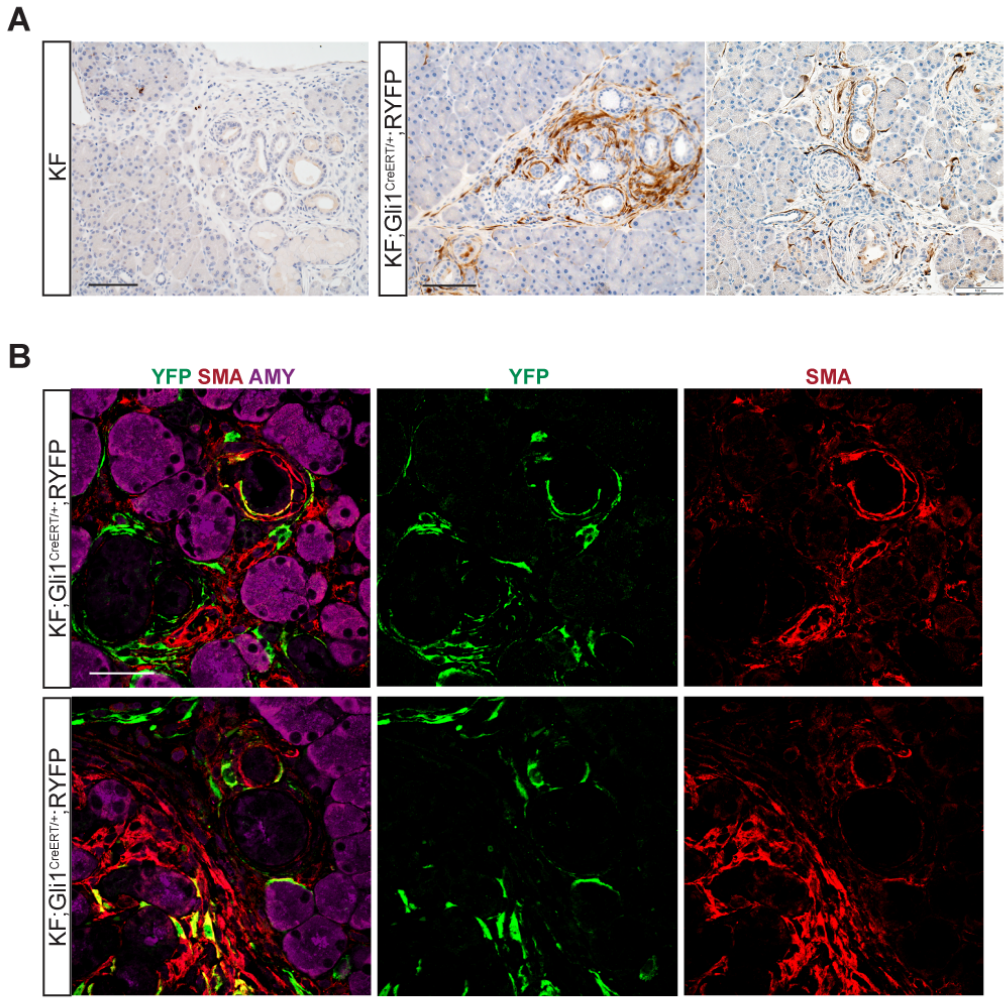

Figure 2-9 Lineage-traced Gli1 fibroblasts are present around the earliest lesion development. (A) IHC staining for YFP in KF;Gli1$^{CreERT/+}$;RYFP mice labelled before PanIN generation. Scale bar = 100um. (B) IF merged and single channel images for YFP (green), SMA (red), and amylase (pink) in KF;Gli1$^{CreERT/+}$;RYFP mice. Scale bar = 50um.

Discussion

In the pancreas, the mesenchyme surrounds and supports epithelium development though epithelial-mesenchymal crosstalk [51] and differentiates to form cell types such as fibroblasts, pancreatic stellate cells, and pericytes [52,53]. Activated fibroblasts, also known as myofibroblasts, help regulate wound repair and recovery[54,55], but in the context of certain pancreatic diseases, such as chronic pancreatitis and pancreatic cancer, the fibrosis is not resolved. Pancreatic adenocarcinoma is associated with an extensive fibroinflammatory stromal reaction. Fibroblasts are an abundant, active, and heterogeneous player within the tumor microenvironment. Currently, functionally distinct subclasses of CAFs have been defined in other cancers such as breast carcinoma[21,56], colorectal carcinoma[57], and lung adenocarcinoma[22]. In pancreatic cancer, a putative mesenchymal stem cell population has been identified in mouse and human samples[16,17], and CAFs have been classified into myCAF, iCAF, and apCAF populations with potentially distinct functions and activation pathways[14,15,23].

Here, we sought to determine which fibroblasts in the healthy pancreas give rise to CAFs during the progression of carcinogenesis. A population of pancreatic stellate cells, containing lipid droplets with vitamin A deposits, similar to the liver hepatic stellate cells, has been described[49,58,59] and is traditionally considered the source of the fibrotic reaction in pancreatic cancer. However, no experimental validation supports the notion that stellate cells lead to CAFs. Recent single-cell transcriptomic profiles of healthy or low-grade lesion fibroblast populations have been mapped *in silico* to CAFs, which suggests that resident pancreatic fibroblasts do contribute to CAFs[26,28]. In order to directly

trace resident fibroblast populations, we first needed to define markers of distinct populations of fibroblasts.

We characterized two different populations of pancreatic fibroblasts present in the healthy organ by the expression of the transcription factors Gli1 and Hoxb6. Gli1 is a target gene and transcriptional effector of the Hedgehog signaling pathway (for review see [31,60]). Hedgehog signaling is active throughout embryonic development of the gastrointestinal tract but needs to be suppressed to allow for the formation of the pancreas anlage [60–62]. However, some low level of Hedgehog signaling persists[63] to maintain homeostasis and assist in injury recovery [35,64,65]. Thus, we found that a subset of fibroblasts in the healthy pancreas consistently express Gli1 and do not have characteristics of stellate cells. Meanwhile, Hoxb6 is expressed throughout the mesenchyme of the developing pancreas[66], and in a portion of fibroblasts in the adult organ. While there is partial overlap in Gli1 and Hoxb6 expressing cells, largely they consist of separate populations. We thus set out to lineage trace these populations with the goal to answer the following questions: 1) whether either fibroblast population gives rise to CAFs; 2) whether all fibroblast populations expand during carcinogenesis; and 3) whether different functional CAF subpopulations derive from different progenitors in the healthy pancreas.

Gli1$^+$ fibroblasts have been lineage-traced in several organ fibrosis models. Following injury, the majority of Gli1$^+$ cells acquire SMA expression and contribute to fibrosis[131]. Approximately 40% of myofibroblasts in the kidney, liver, and lung, and 60% myofibroblasts in the heart originated from tissue-resident Gli1$^+$ progenitors after a fibrosis-inducing injury[131,156]. In addition, Gli1 progenitors contribute about half of the

myofibroblasts in instances of endochondral heterotopic ossification and bone marrow fibrosis[156,159]. Gli1$^+$ fibroblasts have not been characterized in the pancreas, and their contribution to tumor-associated fibroblasts has not yet been studied. We found Gli1 to be expressed in 3-20% of healthy PDGFRα^+ fibroblasts, adjacent to pericytes and localized in a perivascular position. When we lineage-traced the progeny of healthy Gli+ fibroblasts, we found they expanded in quantity and contributed to CAFs.

Hoxb6 is a developmental transcription factor prominently expressed in the pancreas mesoderm until E16.5[160]. We show that Hoxb6 expression is present in the healthy mouse pancreas in 7-20% of PDGFRα^+ fibroblasts and not in immune cells. We had hypothesized that this fibroblast subpopulation would expand and contribute to the fibrotic stroma in a similar manner as the Gli1$^+$ population due to the relative prevalence of the cells and due to the occasional overlap between Hoxb6 and Gli1 expression. Interestingly, as we lineage-traced Gli1 and Hoxb6 cells from the healthy pancreas into carcinogenesis, we found that they contributed in a very different manner to the stroma. While Gli1+ fibroblasts expanded during carcinogenesis, Hoxb6$^+$ cells failed to do so. Further, the expression of Gli1 was extensive in lesion and up to advanced disease, while Hoxb6 expression did not increase (**Figure 2-10**).

Our findings suggest that not all resident fibroblast populations expand and transition into cancer-associated fibroblasts within the pancreas, and that fibroblasts are heterogenous with separate functional roles even at the healthy stage. Gli1$^+$ cells likely are contributing to the tumor-associated myCAF population, though it is worth noting that even within the Gli1$^+$ cells of the healthy pancreas, there is heterogeneity. For instance, a subset of Gli1$^+$ cells also express Hoxb6. This dual positive population does not appear

to proliferate since we observed minimal Hoxb6^{+} fibroblasts among the PanIN stroma when we lineage-traced Hoxb6^{+} cells. However, Hoxb6^{+} fibroblasts could conceivably be proliferating and dying before our analysis time points. We hypothesize that a Gli1^{+} Hoxb6^{-} subset is proliferating, but unfortunately due to the fact that Hoxb6 has close homology with other homeobox genes, further antibody or RNA -based investigation is hindered. Instead, an investigation into the fate, function, and drivers of other fibroblast populations is needed to best comprehend the complexity of the fibrotic reaction. Different CAF subpopulations may derive from different progenitors, as suggested in an experiment where the single-cell transcriptional profiles of two healthy fibroblast populations mapped closer to the tumor-associated myCAF or iCAF populations than to themselves[26].

Understanding the complexity of fibroblasts in healthy organs and disease has been hampered by the limited availability of specific markers. Pancreatic cancer fibroblasts have long been assumed to derive from resident stellate cells[49], with no *in vivo* experimental support. Here, we show that non-stellate cell fibroblast populations exist in the pancreas and they have the ability to expand during carcinogenesis. In addition, we determined that the ability to contribute to the cancer-associated stroma during carcinogenesis is unique to only some fibroblast subsets in the pancreas. It is important to note that Gli1^{+} fibroblasts give rise to less than half of the total fibroblast population during carcinogenesis, conservatively measured by our Cre-recombinase lineage model, indicating that other subsets with the ability to expand must exist in the healthy organ. Alternative sources of the CAFs may arise from other resident fibroblast populations, circulating progenitor cells from the bone marrow, or pericytes (reviewed in [6,67]). Overall,

these findings are consistent with fate-mapping experiments performed in the liver[34,68], kidney[34,69], heart[34,70], lung[34,71,72], spinal cord[73], bone[33], and skin[74], in which resident fibroblasts proliferate in response to injury to contribute, at least partially, to the organ fibrosis. Our findings advance our understanding of how the pancreatic fibroinflammatory environment is established during carcinogenesis and offer new directions with which to examine the main stromal regulators of carcinogenesis, and eventually, improve patient outcome.

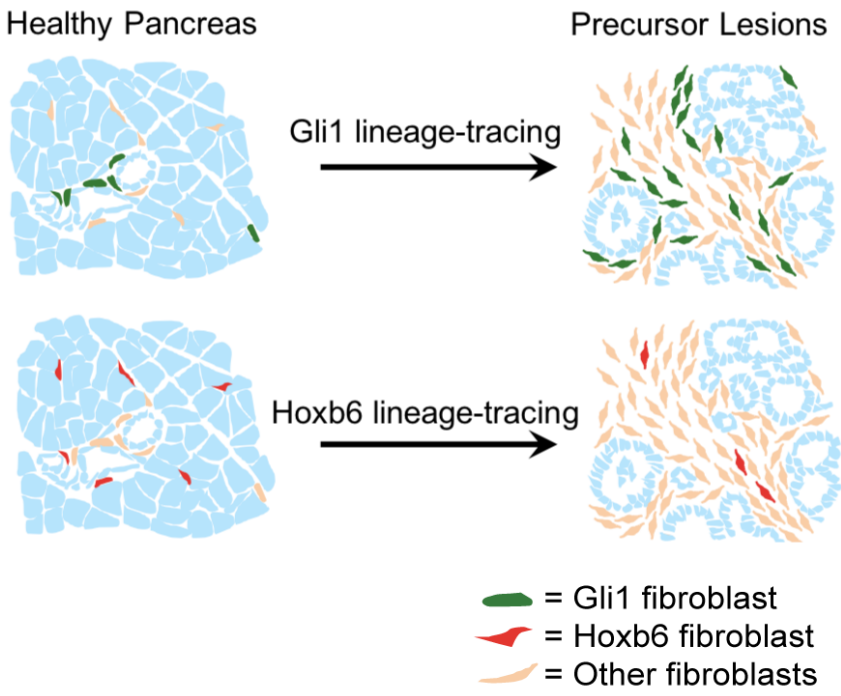

Figure 2-10 Graphical Abstract. The healthy pancreas contains a heterogenous mix of fibroblasts. Lineage-tracing Gli1-expressing fibroblasts results in expansion and contribution to cancer-associated fibroblasts. In contrast, lineage-tracing Hoxb6-expressing fibroblasts results in no expansion.

Chapter 3 Conclusions and Future Directions

Summary and Conclusions

Pancreatic cancer is characterized by a highly fibroinflammatory stroma. Cancer-associated fibroblasts (CAFs) are a dominant, dynamic, and controversial component of the stroma. With developments in transcriptomic sequencing and animal modeling, researchers are now beginning to grasp the depth of the transcriptional and functional diversity of CAFs. Fibroblast heterogeneity is becoming an increasingly likely explanation for the seemingly paradoxical tumor-promoting and tumor-repressive qualities of CAFs[93]. My thesis work sought to examine the origins of pancreatic CAFs and their heterogeneity, and to determine whether fibroblasts in the healthy pancreas give rise to CAFs during carcinogenesis.

The healthy pancreas contains mesenchymal cells such as fibroblasts, pancreatic stellate cells (PSCs), pericytes, and mesenchymal stem cells[37,53,55,60]. The potential of these resident cells in populating the fibroinflammory environment is unknown partially due to a lack of appropriate markers and a fate-tracking model. We first identified two markers of healthy mouse fibroblast populations. Thanks to previous research done by our lab, we knew Gli1 was expressed in a subset of fibroblasts in the healthy and fibrotic pancreas[17]. Hoxb6 is a transcription factor active in the developing pancreas mesenchyme, but had not yet been examined in the context of the adult organ[160]. I found Gli1 and Hoxb6 expressing fibroblasts in the healthy pancreas to be in mostly spatially

distinct populations. Of interest, Gli1 fibroblasts are adjacent to pericytes and do not have the characteristics of pancreatic stellate cells (PSCs). Since PSCs have long been assumed to be the source of CAFs[65], we were eager to track a non-PSC population through carcinogenesis.

I labeled Gli1 and Hoxb6 cells respectively before lineage-tracing these populations during KRAS-driven carcinogenesis. We observed an expansion of $Gli1^+$ cells, but not $Hoxb6^+$ cells in the precursor and cancer- associated stroma. My findings suggest that some, but not all, resident fibroblasts can expand to contribute to the stroma. The majority of the $Gli1^+$ traced CAFs express SMA, which is a marker for the myofibroblast phenotype currently explored in CAF heterogeneity[37,38,93], though only about a third of SMA^+ CAFs were $Gli1^+$ derived. This is consistent with Gli1 lineage-tracing efforts in several organ fibrosis models. Approximately 40% of myofibroblasts in the kidney, liver, and lung, and 60% myofibroblasts in the heart originated from tissue-resident $Gli1^+$ progenitors after a fibrosis-inducing injury[131,156]. While $Gli1^+$ cells contribute to myofibroblast CAFs, alternative sources of CAFs must exist.

Taken together this work sheds light on the evolving fibroblast heterogeneity associated with pancreatic carcinogenesis. We are the first to show that a resident fibroblast population contributes to the fibroinflammatory environment of cancer in any organ, outside of fibrosis-associated wound repair. Several limitations of our model system do exist. One is that we may not be achieving complete recombination and labelling all of our cells of interest. We are likely underrepresenting the contribution of $Gli1^+$ and $Hoxb6^+$ cells to the stroma. Another limitation of our work is that we chose very few time points to examine the lineage-tracing progression. $Hoxb6^+$ and $Gli1^+$ may be

dynamically contributing to the stroma at an earlier time point prior to our notice, though we were limited by the complicated genetic modeling to do a more thorough time course analysis. Overall, a number of exciting avenues of exploration still remain to be investigated, such as whether the developmental origin of CAFs has any functional significance, how plastic or context-dependent these fibroblast populations are, and how this research translates to developing new therapeutic strategies for patients.

Future Directions

Investigating the functional significance of Gli1-derived cancer-associated fibroblasts

Our findings show that Gli1^{+} fibroblasts expand to contribute to the CAF population. We have not addressed how this correlates with fibroblast function, and that should be the immediate next step for this project. As we are accumulating single-cell RNA sequencing data sets, we can perform a gene differential analysis of Gli1 or Hoxb6 expressing cells in the healthy or cancerous pancreas. A GO enrichment analysis could elucidate any particular pathways our Gli1 and Hoxb6 cells upregulate as a way to investigate their function. The limitations to this are that we do not currently have any healthy pancreas data sets with enough fibroblasts or read depth to detect Hoxb6 or Gli1 transcription levels. And while we can search for the genes in pancreatic cancer data sets, the data fail to provide information on cells that have derived from Gli1^{+} or Hoxb6^{+} progenitors.

Another strategy to examine fibroblast population function is to ablate the population and observe the effects in tumor generation and stroma formation. The Diptheria toxin receptor (DTR) transgene is a popular strategy to kill cells of interest when

exposed to the toxin. Unfortunately, this strategy failed to ablate Gli1$^+$ or Hoxb6$^+$ cells due to a failure to localize the DT receptor to the membrane (**Figure 3-1**). Since we cannot easily target our cells of interest for ablation, an alternative strategy we explored utilized sorted fibroblast populations. We tested the ability of healthy Gli1 and lineage-traced KF;Gli1 cell cultures to impact tumor weight or growth over time when coinjected with tumor cells in nude mice. We observed no significant differences in tumor weight or growth between healthy Gli1 positive/negative populations or between lineage-traced KF;Gli1 positive/negative populations (**Figure 3-2**). A limitation is that the immune microenvironment is compromised in nude mice. Further experimentation using a syngeneic model or organoid co-culture may elucidate further functional differences between our fibroblast populations.

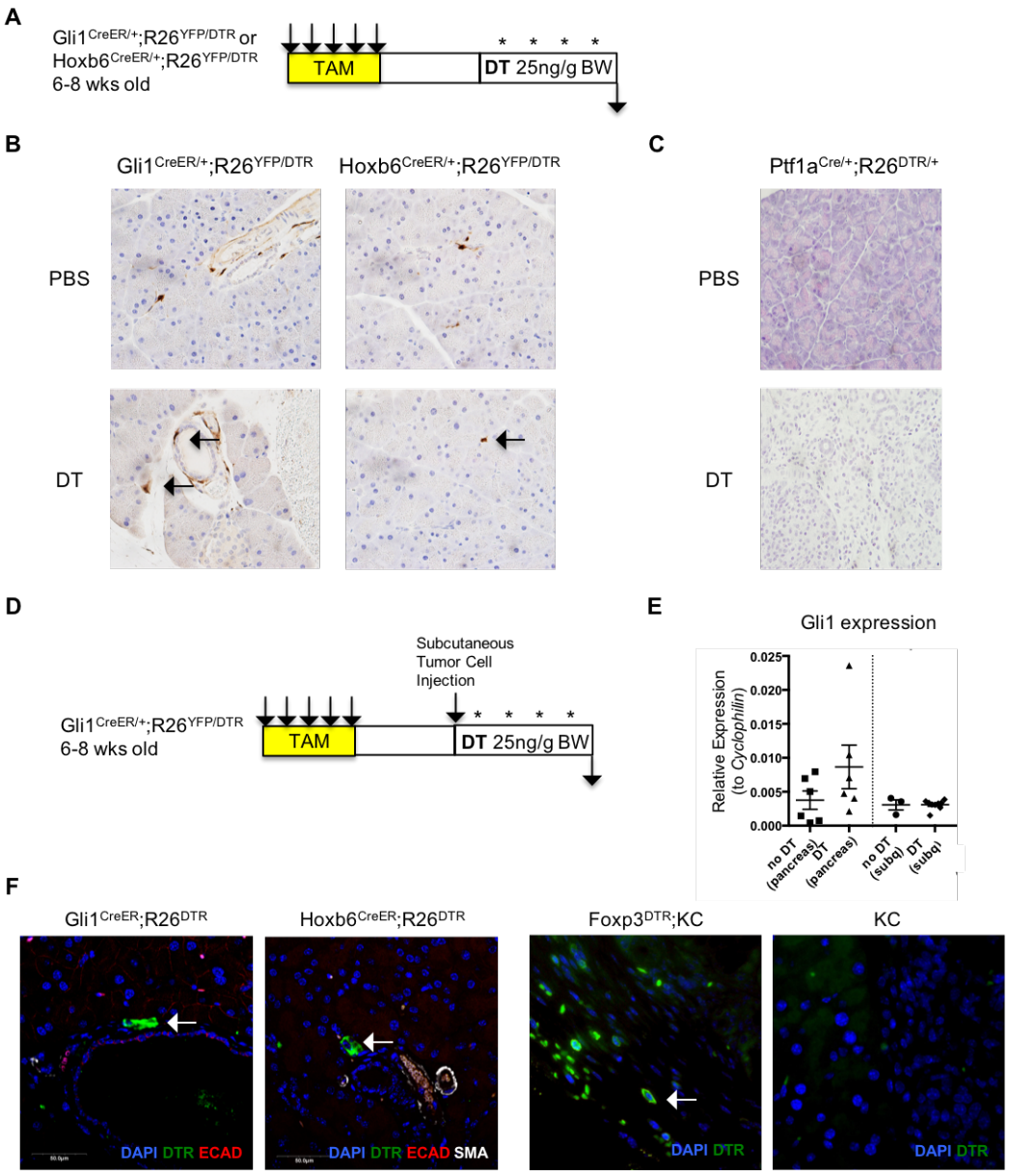

Figure 3-1 Diptheria toxin receptor (DTR)-assisted ablation of fibroblast subsets failed due to improper localization of receptor to the cellular membrane. A) Adult mice with Cre-inducible DTR and YFP expression received five tamoxifen gavages and then four DT injections (25ng/g BW) to conditionally ablate Gli1 or Hoxb6 cells. (B) Immunohistochemical staining for YFP revealed lack of ablation of Gli1 or Hoxb6 cells with DT treatment. (C) The protocol repeated with a pancreatic-specific Cre driver, *Ptf1a*-Cre, showed successful widespread acinar depletion via HE staining. (D) Next, adult Gli1^{CreER};R26$^{DTR/YFP}$ mice were treated with tamoxifen and then an injection of subcutaneous tumor cells before DT ablation. (E) Gli1 expression, as determined by qPCR, was not reduced in the tumors or pancreata of DT-treated mice. (F) Co-IF staining of DTR in Gli1^{CreER} or Hoxb6^{CreER};R26^{DTR} sections showed the receptor localized to the cytoplasm of the cell, and not the membrane. In contrast, in a Foxp3^{DTR} model, the receptor is membrane-localized.

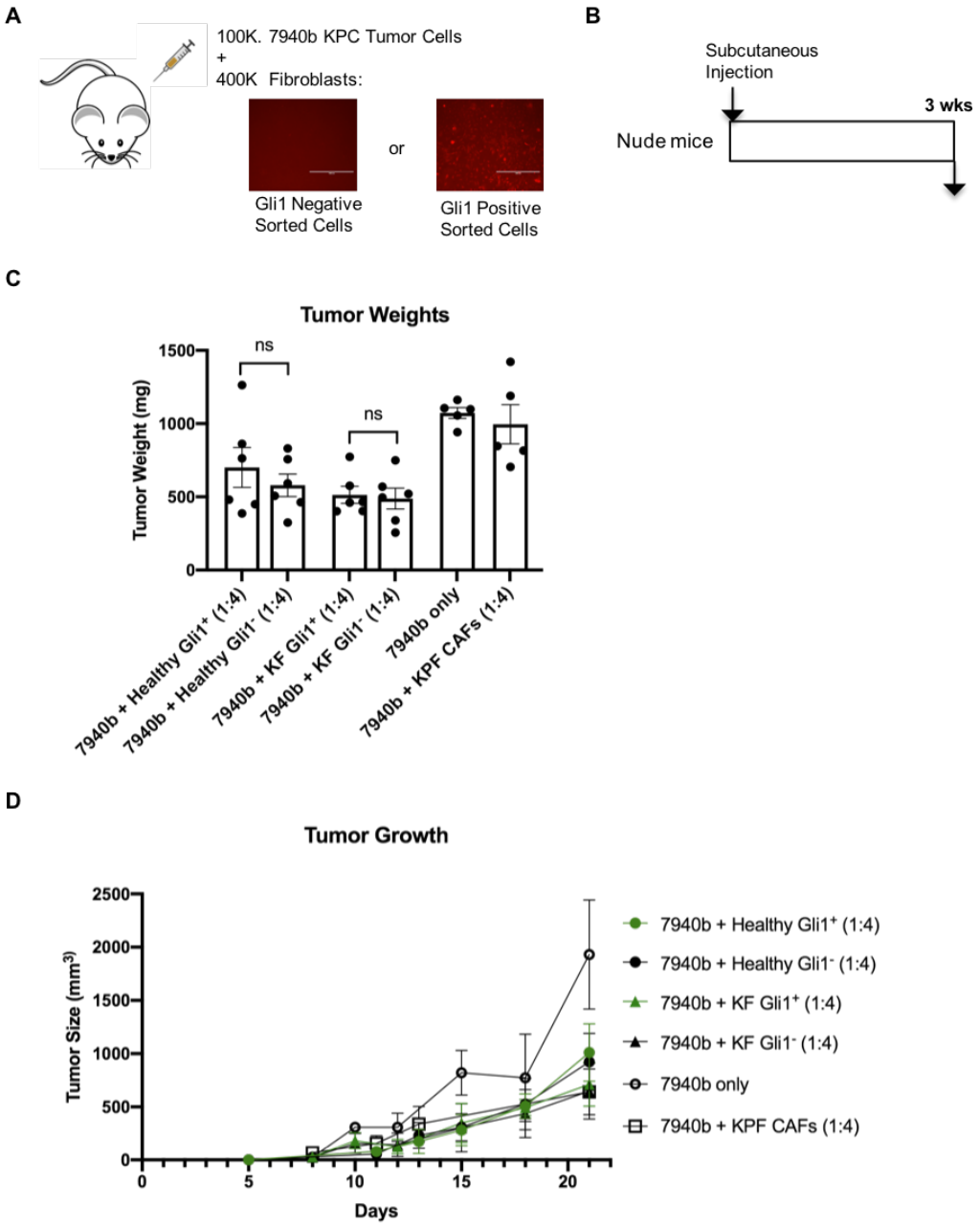

Figure 3-2 Subcutaneous tumor growth and weight was not affected by fibroblasts originating from Gli1^{+} cells. (A) Adult nude mice were given subcutaneous Matrigel injections consisting of 100K 7940bl KPC tumor cells and 400K fibroblasts on both sides of their flank. Tomato positive or negative fibroblasts were sorted from either healthy Gli1$^{CreER/+}$;RTom or lineage-traced KF;Gli1$^{CreER/+}$;RTom mice. Control injections of 100K 7940bl alone or 100K 7940bl with 400K KPF CAFs were used. (B) Cells were injected in adult nude mice. Tumor size was monitored for three weeks before harvesting the tumors for size and weight. (C) Tumor weight upon harvest for the experimental groups. There was no significant difference in tumor weight between Gli1 positive and negative tumors (n≥5). (D) Tumor size over the period of tumor injection showed no significant difference in tumor growth between experimental groups.

Gli1 and Hoxb6 fibroblasts can be interrogated *in vitro* as well. I have sorted out positive and negative healthy labelled cell cultures based on YFP expression (**Figure 3-3**A,C). Since Gli1 is a Hedgehog effector gene, we initially sought to determine if both the $Gli1^+$ and $Gli1^-$ populations respond to Hedgehog signaling via a small molecule agonist called SAG (**Figure 3-3**B,D). Both $Gli1^+$ and $Gli1^-$ cultures responded to the extrinsic SAG signal and upregulated Gli1, suggesting that the Gli1 may initially label a plastic, context-dependent population. Similarly, $Hoxb6^+$ and $Hoxb6^-$ cultures trend towards responding to SAG. Further characterization of the fibroblast populations can proceed with these cultures, such as utilizing their conditioned media to detect polarization differences in macrophages or placing them in iCAF and myCAF culture conditions to further test their plasticity.

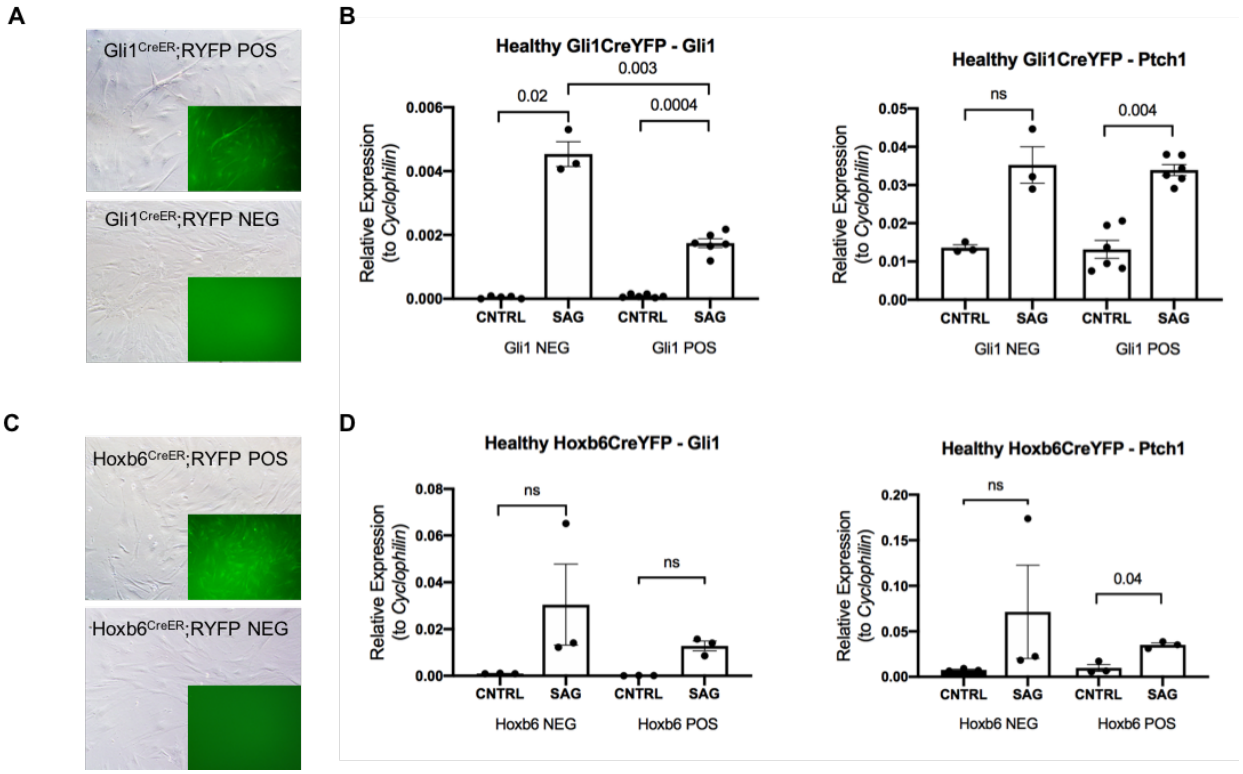

Figure 3-3 Response to Hedgehog agonist, SAG, is not limited to Gli1 or Hoxb6- sorted populations (A) Healthy Gli1$^{CreER/+}$;RYFP cell cultures were generated by sorting YFP positive or negative CD45^{-}PDGFR^{+} cells. Fluorescent imaging confirmed successful fibroblast sort. (B) Confluent, serum-starved Gli1^{CreER};RYFP cells were exposed to 600nM SAG for two days. Both Gli1 positive and negative cultures increased Hedgehog target, Gli1, and Hedgehog receptor, Ptch1, expression in response to SAG exposure. (C) Healthy Hoxb6^{CreER};RYFP cell cultures were generated by sorting YFP positive or negative CD45^{-} PDGFR^{+} cells. Fluorescent imaging confirmed successful fibroblast sort. (D) Confluent, serum-starved Hoxb6^{CreER};RYFP cells were exposed to 600nM SAG for two days. Both Hoxb6 positive and negative cultures increased Hedgehog target, Gli1, and Hedgehog receptor, Ptch1, expression in response to SAG exposure.

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Finally, another avenue to functionally characterize these fibroblasts is with RNA analysis. Sorting via FACS allows us to isolate CD45 negative populations that do not express the fibroblast marker PDGFRα (PDGFRα^{-}YFP^{-}), fibroblast populations that do not express our lineage label (PDGFRα^{+} YFP^{-}), and fibroblast populations which do (PDGFRα^{+} YFP^{+}). Overall, the quantity of experimental mice has been low, and the resulting quantity and quality of fibroblast RNA even lower when sorted, but with more time to develop large KPF lineage-traced tumors, additional RNA interrogation of gene expression can proceed. Initial data has been collected on the sorted populations, looking at Gli1; HH receptor, PTCH1; myofibroblast marker, ACTA2; PSC marker, FABP4; and adipocyte marker, DLK1 (**Figure 3-4**).

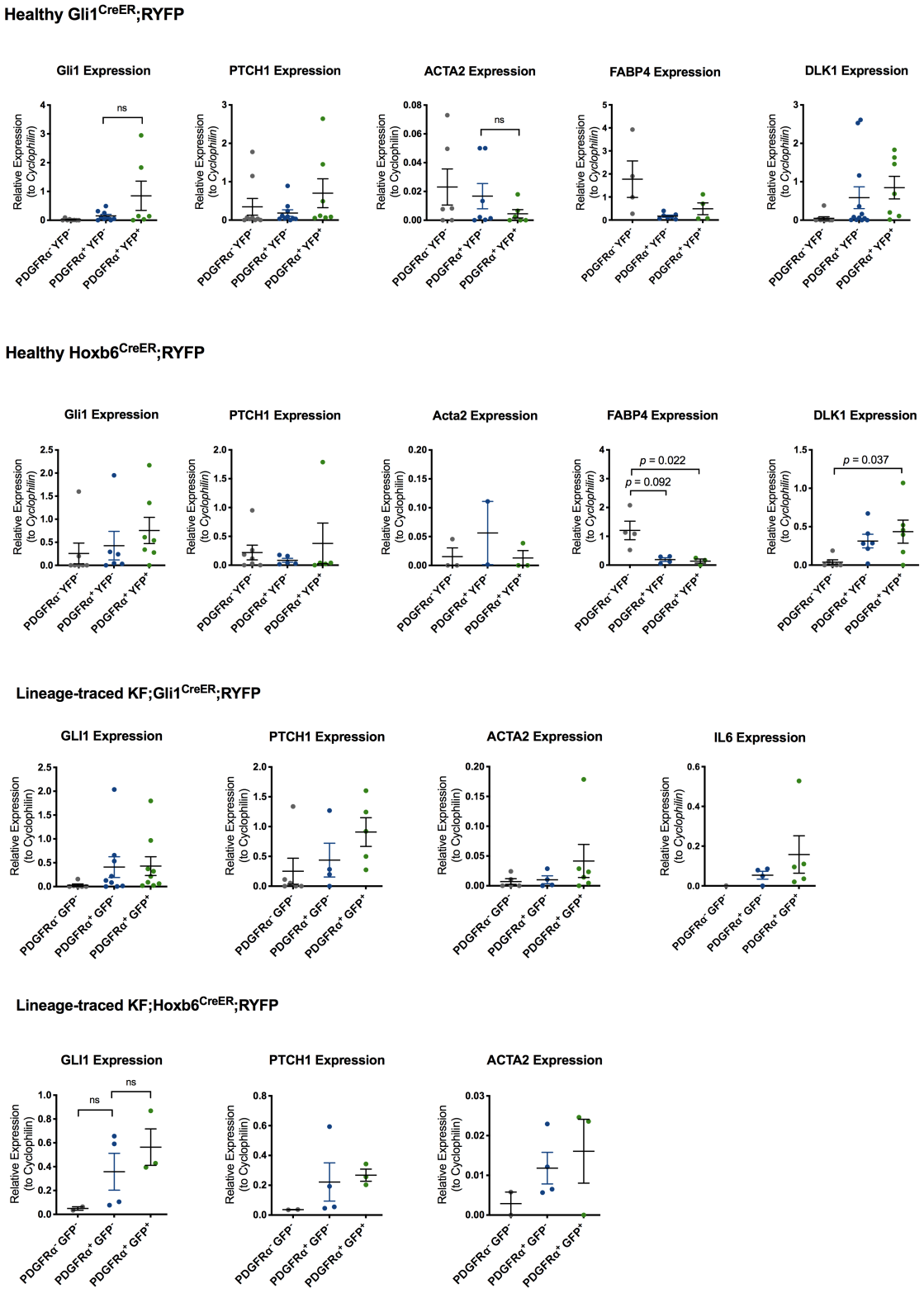

Figure 3-4 RNA analysis of sorted fibroblast populations. Data presented as mean +/- SEM.

Utilizing the genetic models to answer other scientific inquires

The dual recombinase models used to lineage-trace Gli1 and Hoxb6 cells are genetically complex and took several years to generate. Additional outstanding questions can be addressed using these tools, such as the developmental contributions to the adult mesenchyme and the contributions of healthy fibroblasts to pancreatitis.

Hoxb6 was initially of interest to us since it is prominently and exclusively expressed in the pancreas mesoderm until E16.5[48]. We had initially hypothesized that Hoxb6 could function as a pan-mesenchymal marker in the adult pancreas. While that proved to be false, only a fifth of the adult fibroblasts express Hoxb6, our lineage tracing model could be utilized to study developmental inquires. Another developmental mesenchymal marker, Nkx3.2, was lineage-traced throughout development and adulthood and found to differentiate into pericytes[49]. By exposing a pregnant female to tamoxifen chow, we can label Hoxb6 cells in utero and trace their contribution to the healthy mesenchyme, and even to the cancer-associated stroma. It is still relatively unexplored how developmental mesenchymal populations persist into adulthood and how their functional role may change.

My research has focused on how Gli1 and Hoxb6 contribute to the fibrosis of carcinogenesis, though cancer is not the only fibrotic disease of the pancreas. Chronic pancreatitis is a painful disease with limited therapy options and a characteristic fibroinflammatory environment not dissimilar to pancreatic cancer[72,82]. We can lineage-trace our resident fibroblasts throughout a model of chronic pancreatitis to determine if and how they contribute to that model of fibrosis. It would illuminate how unique a response the Gli1 expansion to carcinogenesis was and further interrogate the fibroblast heterogeneity and origins of chronic pancreatitis.

Investigating other sources of cancer-associated fibroblasts

Healthy Gli1-expressing fibroblasts contributed to about a fifth of the cancer-associated fibroblast population. We assumed that resident $Gli1^+$ fibroblasts are the cells responsible for such a contribution, but it is possible that bone-marrow derived $Gli1^+$ cells may be translocating to the pancreas. In a quick investigation into that possibility, we performed a bone marrow transplant experiment (**Figure 3-5**). Bone marrow from bl6 $Gli1^{lacz/+}$ mice was injected into wildtype bl6 mice. After a month of recovery, the mice received orthotopic injections of bl6 KPC tumor cells. Then, the tumors were analyzed for positive β-galactosidase staining which would indicate cells expressing Gli1 that derived from the bone marrow. We did not observe any positive staining in our mice, suggesting that $Gli1^+$ cells likely do not originate from the bone marrow and we can assume they arose from resident pancreatic cells.

While a certain amount of recombination inefficiency may result in our underestimation of the Gli1 contribution, there likely are alternative origins of the CAFs. A previous limitation to lineage-tracing fibroblasts was a lack of specific mesenchymal markers and no dual recombinase labelling system. We now can utilize the Flp-based KF model[29] to label other cell populations, and with the advent of widespread single cell sequencing, we can mine the data of healthy fibroblast populations to find additional markers to lineage-trace. FABP4 is an adipogenic gene recently used to label pancreatic stellate cells. Crossing in a FABP4-CreERT allele into the KF model would allow us to trace PSCs throughout carcinogenesis. Similarly, obtaining NG2-CreERT,

WT1-CreERT, or PDPN-Cre mice can provide information about the fate of pericytes, mesothelium, and other fibroblasts during carcinogenesis.

As we begin to deconvolute the fate, function, and drivers of fibroblast populations, we are improving our knowledge on the main regulators of tissue repair and carcinogenesis. With the data accumulated so far, it remains unclear if these fibroblast subsets are responsible for pro-tumor or anti-tumor effects. We ultimately seek to exploit any functional knowledge to target CAF populations that promote tumor growth and survival, so that we may improve the outcome for this deadly disease.

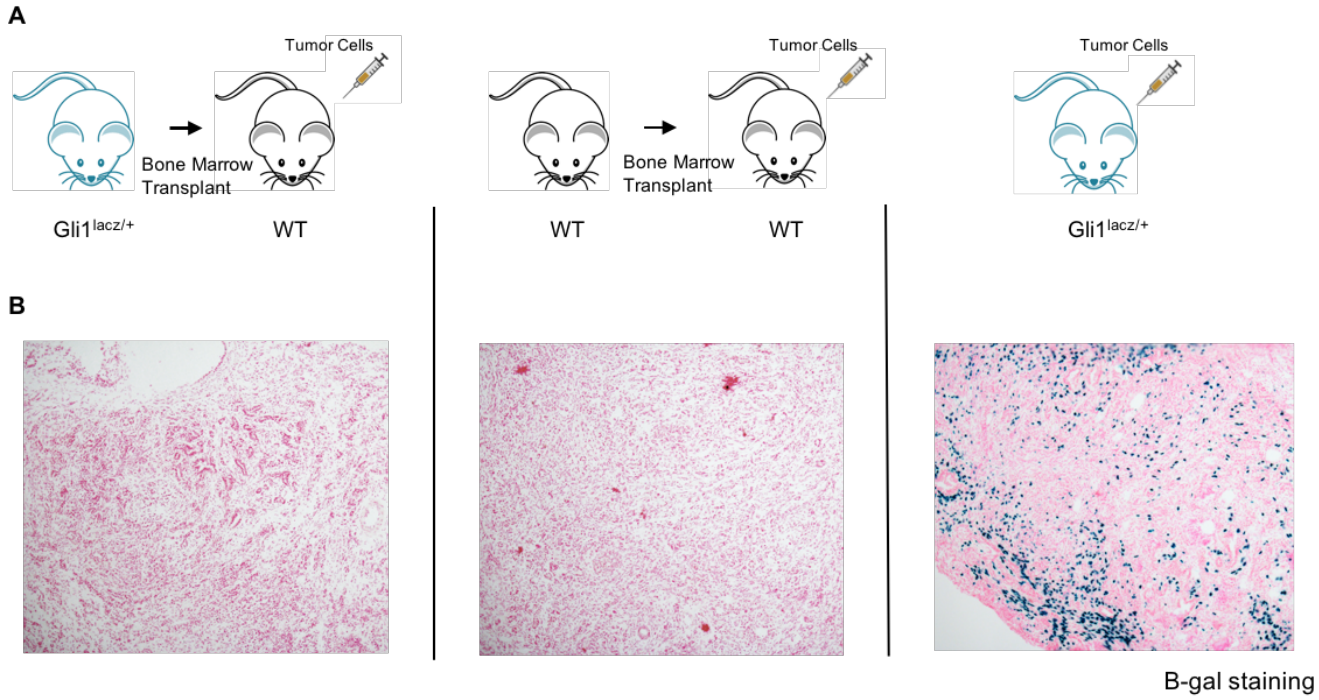

Figure 3-5 Bone marrow transplant experiment to determine origin of Gli1^{+} cells (A) Experimental set-up for the three conditions. The main experimental condition had bl6 Gli1$^{lacz/+}$ bone marrow transplanted into irradiated wildtype bl6 mice before bl6 KPC tumor cells were injected. The negative control condition had bl6 wildtype bone marrow transplanted into irradiated wildtype bl6 mice before bl6 KPC tumor cells were injected. The positive control condition only had bl6 Gli1$^{lacz/+}$ mice receiving pancreatic orthotopic injections of bl6 KPC tumor cells. (B) Representative 10x images of the β-galactosidase staining performed on the three conditions. No positive staining was observed in the experimental group (n≥3).

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