AWARD NUMBER: W81XWH-18-1-0437

TITLE: Understanding Stromal Fibroblast Heterogeneity in the Pancreatic Tumor Microenvironment

PRINCIPAL INVESTIGATOR: Mara Sherman

CONTRACTING ORGANIZATION: Oregon Health & Science University

REPORT DATE: NOVEMBER 2021

TYPE OF REPORT: Final technical report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
NOVEMBER 2021	Final	1 Aug 2018 – 31 Jul 2021

4. TITLE AND SUBTITLE

Understanding Stromal Fibroblast Heterogeneity in the Pancreatic Tumor Microenvironment

5a. CONTRACT NUMBER
W81XWH-18-1-0437

5b. GRANT NUMBER
W81XWH-18-1-0437

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Mara Sherman

E-Mail: shermama@ohsu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oregon Health & Science University

3181 SW Sam Jackson Park Rd

Portland, OR 97239-3011

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Cancer-associated fibroblasts (CAFs) are the key cell type which drives the stromal reaction in pancreatic ductal adenocarcinoma (PDAC), and recent reports suggest that stromal CAFs represent a heterogeneous population of cells from diverse origins, potentially including cell types which support and others which suppress tumor growth. Pancreatic stellate cells (PSCs) are lipid-storing cells in healthy pancreas which can transdifferentiate to an activated CAF phenotype. PSCs have been suggested as the predominant source of fibroblasts in the PDAC tumor microenvironment. However, proper lineage tracing studies have never been performed, and other fibroblast sources are likely. During the funding period, we have analyzed our novel mouse model which allows us to study PSC differentiation and function during pancreatic tumor progression *in vivo* for the first time. Our two most significant findings from the funding period are 1) using a marker combination identified from RNA-seq data generated during year 1 of funding, we identified PSC-derived CAFs in human PDAC at a frequency similar to that seen in mice, 2) we have generated several p53 mutant versus loss-of-function systems and identified a critical role for tumor cell-intrinsic p53 status in regulation of stromal evolution. Together, these findings pave the way for future work on our proposal to better understanding the fibroblastic compartment of the pancreatic tumor microenvironment.

15. SUBJECT TERMS
Pancreatic cancer, tumor microenvironment, stromal heterogeneity, cancer-associated fibroblast, stellate cell

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	18	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. **INTRODUCTION:**

Cancer-associated fibroblasts (CAFs) are the key cell type which drives the stromal reaction in pancreatic ductal adenocarcinoma (PDAC), and recent reports suggest that stromal CAFs represent a heterogeneous population of cells from diverse origins, potentially including cell types which support and others which suppress tumor growth. Pancreatic stellate cells (PSCs) are lipid-storing cells in healthy pancreas which can transdifferentiate to an activated CAF phenotype. PSCs have been suggested as the predominant source of fibroblasts in the PDAC tumor microenvironment. However, proper lineage tracing studies have never been performed, and other fibroblast sources are likely. During the funding period, we have analyzed our novel mouse model which allows us to study PSC differentiation and function during pancreatic tumor progression *in vivo* for the first time. Our two most significant accomplishments from the funding period are 1) we published the findings from this model in *Cancer Discovery*, 2) we have performed long-term PSC ablation studies which show so far that PSC-derived CAFs are important for metastatic progression. Together, these findings pave the way for future work on our proposal to better understanding the fibroblastic compartment of the pancreatic tumor microenvironment.

2. **KEYWORDS:**

Pancreatic cancer, tumor microenvironment, cancer-associated fibroblast, pancreatic stellate cell, stromal heterogeneity

3. **ACCOMPLISHMENTS:**

Training-Specific Tasks:

Major Task 1: Training and educational development in clinical and basic pancreatic tumor microenvironment research

Throughout the funding period, my training and educational development in clinical and basic pancreatic tumor microenvironment research was facilitated by my semi-annual mentor committee meetings. My faculty mentors are experts in tumor microenvironment and/or pancreatic cancer (Lisa Coussens, Rosalie Sears, Sara Courtneidge, and Laura Attardi) and provided scientific guidance themselves in addition to making helpful connections to colleagues who could further guide and train me and my lab members. In addition, my membership in the OHSU Brenden-Colson Center for Pancreatic Care has enabled me to attend monthly meetings with fellow center members including many clinicians (pathologists, oncologists, radiologists, surgeons) who have been extremely helpful in training me and shaping my understanding of the clinically relevant features of pancreatic cancer biology and therapeutic resistance.

Subtask 1: Attend Cold Spring Harbor Laboratory Workshop on Leadership in Bioscience

In lieu of traveling to Cold Spring Harbor for this leadership training program, I participated in a similar program developed at OHSU since the time of my Career Development Award application, the Mentorship Academy. This seven-part training series was a highly meaningful leadership program focused on diverse aspects of mentorship, scientific leadership, and lab management, and benefited me not only in providing guidelines for running a lab but also in helping establish personal connections with peers throughout the School of Medicine who I had not otherwise had an opportunity to meet due to disparate research interests and distant campus homes, but who share common challenges and experiences. I look forward to participating as a trainer in future iterations of the Academy in an effort to pay forward this important component of my career development. I do note that this was free of charge and no travel was required so I did not charge my DOD CDA for this, but thought to explain the training experience as this was an alternative and important means to accomplish this subtask.

Subtask 2: Attend Cold Spring Harbor Laboratory Workshop on Pancreatic Cancer

I was most fortunate to attend the Cold Spring Harbor Laboratory Workshop on Pancreatic Cancer, which greatly improved my understanding of diverse aspects of disease biology from the basic science and clinical perspectives and also enhanced my peer and mentor network. I have since been invited to participate in this workshop as an instructor, which I am most honored to do—I will no doubt continue to learn from these workshops.

Subtask 3: Attend and present work at Gordon Conference on Pancreatic Diseases

I was delighted to attend and present our work at the 2019 Gordon Conference on Pancreatic Diseases, where I presented a poster and got very helpful feedback, and also participated in the meeting as a discussion leader and had meaningful interactions with the conference attendees focused on diverse aspects of the pancreatic tumor microenvironment.

Subtask 4: Presentation of research at OHSU (yearly)

I have had opportunities to present our DOD-funded research at several annual OHSU seminar slots, including the Brenden-Colson Center discussion group, the Mouse Models of Human Cancer meeting, and the Basic and Translational Sciences Seminar Series. These meetings attract different audiences and therefore provided different perspectives and feedback which helped shape our work and facilitated troubleshooting and overcoming technical challenges.

Subtask 5: Attendance of Pancreas Tumor Board (monthly)

I have attended the monthly pancreas tumor board whenever scheduled, and though I find these discussions of patient presentation and care somewhat disheartening at

times, they also provide very meaningful training and strong motivation to continue the work we do with the ultimate goal of helping pancreatic cancer patients.

Subtask 6: Organize and present at Mouse Models of Human Cancer monthly workshop (OHSU)

Throughout the funding period, I was the organizer of the Mouse Models of Human Cancer monthly meeting—this was recently renamed Models of Disease to include those who don't use mouse models (for example, those who use organoid platforms or other model systems) and not to limit the audience to cancer researchers. My trainees also attend these meetings, and we have gotten insightful feedback on our DOD-funded work over the years.

Subtask 7: Attend scientific conferences (AACR Special Conference on Tumor Microenvironment, Keystone Symposium on Tumor Microenvironment)

I presented my DOD-funded work at the AACR Special Conference on Tumor Microenvironment (2019), Keystone Symposium on Tumor Microenvironment (2021), and AACR Special Conference on Pancreatic Cancer (2019), all of which yielded fruitful discussions and helpful feedback.

Subtask 8: Attend and present work at Cold Spring Harbor Laboratory Conference on Biology of Cancer: Microenvironment and Metastasis

I gave a short talk on our DOD-funded work at this conference, where I learned a great deal from other researchers studying cancer-associated fibroblast heterogeneity in different tissue settings.

Subtask 9: Attend and present work at AACR Special Conference on Pancreatic Cancer

I was deeply honored to present our DOD-funded work at the 2019 AACR Special Conference on Pancreatic Cancer (the 2021 meeting was shortly after the end of the funding period) as a Rising Star Keynote Speaker.

Subtask 10: Attend Cold Spring Harbor Laboratory Workshop on Pancreatic Cancer

As mentioned above, I attended this workshop as a trainee and have since been invited back to participate as an instructor (will do this in 2022, and did it during the virtual workshop in 2020).

Research-Specific Tasks:

Major Task 1: Obtain IACUC approval for animal studies

I obtained IACUC approval for animal studies within the first month of the funding period.

Subtask 1: Approval of IACUC protocol for animal studies

I obtained IACUC approval for animal studies within the first month of the funding period.

Subtask 2: Regulatory review and approval by the USAMRMC Animal Care and Use Review Office (ACURO)

I obtained ACURO approval within the first three months of the funding period.

Subtask 3: Regulatory review and approval by the USAMRMC Human Research Protection Office (HRPO)

I obtained HRPO approval within the first three months of the funding period.

Major Task 2: Quantify contribution of PSCs to PDAC CAF population

Subtask 1: Co-stain PDAC sections for aSMA and GFP. Compare GFP-positive cells to aSMA-positive cells to assess origin of stained fibroblasts.

Using our Fabp4-Cre, Rosa26-mTmG reporter mice, we established PDAC using three independent tumor models and stained for aSMA to label myofibroblastic CAFs and for GFP to label CAFs of a stellate cell origin. Across all three models, we found that GFP+ CAFs represented about 10-15% of the total aSMA+ CAF population. This was exciting and surprising to us, as stellate cells were previously thought to give rise to all of the CAFs in the tumor microenvironment, with diversification resulting from cytokine and growth factor gradients within the tumor (Figure 1).

Subtask 2: Co-stain PDAC sections for Collagen I and GFP. Compare GFP-positive cells to Collagen I-positive cells to assess origin of stained fibroblasts.

Similar to the aSMA results described above, we found that Collagen I was produced by both GFP+ and GFP- CAFs, with the vast majority of Collagen I-producing cells of non-stellate cell origin per our GFP lineage reporter. This gains significance in light of recent reports that Collagen I production in the primary PDAC microenvironment and in liver metastases actually restrains tumor progression, such that stroma-targeted therapies would ideally not perturb type I collagen production. As PSC-derived CAFs are only minor contributors to collagen synthesis, these results suggest therapeutic potential of targeting PSC-derived CAFs without impairing one of the established tumor-suppressive or homeostatic functions of the stroma.

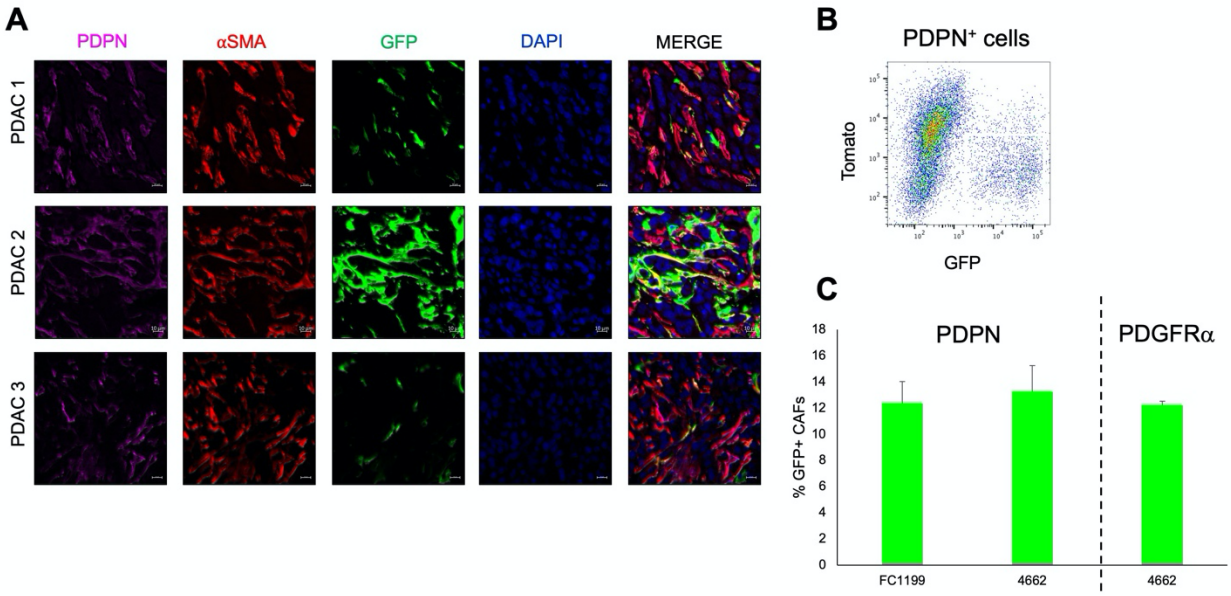

Figure 1, related to Major Task 2. A, PDAC tumors from Rosa26-mTmG;Fabp4-Cre mice were harvested and analyzed by immunohistochemistry for GFP and CAF markers PDPN and aSMA. **B,** PDAC tumors from Rosa26-mTmG;Fabp4-Cre mice were harvested and analyzed by flow cytometry for the lineage reporters Tomato and GFP, as well as the CAF marker PDPN. **C,** The percent of total CAFs derived from PSCs per GFP positivity was quantified from flow cytometry data, using two independent CAF cell surface markers, PDPN and PDGFRa.

Major Task 3: Determine effect of PDAC genetics on origin of CAFs in the tumor microenvironment

Subtask 1: Measure GFP+ fibroblasts (GFP/aSMA co-labeling) in Kras-mutant, p53-mutant (epithelial) versus Kras-mutant, p53-null (mesenchymal) tumors. Compare levels of stellate cell-derived fibroblasts in tumors with different p53 status.

First, we optimized orthotopic transplant models of Kras-mutant, p53-mutant (R172H) and Kras-mutant, p53-null PDAC from the same genetic background to yield size-matched tumors at experimental endpoint that harbored similar levels of total CAFs, both by immunohistochemistry and flow cytometry (looking at PDPN+ cells by flow cytometry or at PDPN+ aSMA+ cells by IHC). These tumors were generated in our Fabp4-Cre, Rosa26-mTmG reporter mice. Then, we prepared tissue sections and stained for GFP and aSMA. While total aSMA+ CAF abundance was not significantly different between the two groups, we found that stromal evolution from a PSC origin is significantly increased in the context of p53-mutant PDAC compared to p53-null PDAC. To assess a causal relationship for p53 status in stromal evolution, we knocked out p53 from p53-mutant parental PDAC cells using CRISPR/Cas9, then implanted the cells and found that loss of p53 significantly reduced PSC-derived CAF abundance as

a proportion of total CAFs, implicating p53 status as a cancer cell-intrinsic regulator of stromal evolution (Figure 2). These findings gain significance in light of recently published work from the Cox and Timpson groups at the Garvan Institute, who compared CAFs from genetically engineered mouse models of pancreatic cancer harboring p53 R172H or p53 loss. They found that CAFs from p53-mutant tumors are uniquely pro-metastatic and promote chemoresistance, phenotypes driven in part by stromal production of Hspg2 (perlecan). We hypothesize that the basis for these findings is increased mobilization of PSC-derived CAFs into the CAF pool in the p53-mutant setting, and consistent with this notion, we find that PSC-derived CAFs express significantly higher levels of perlecan than do CAFs of non-PSC origin.

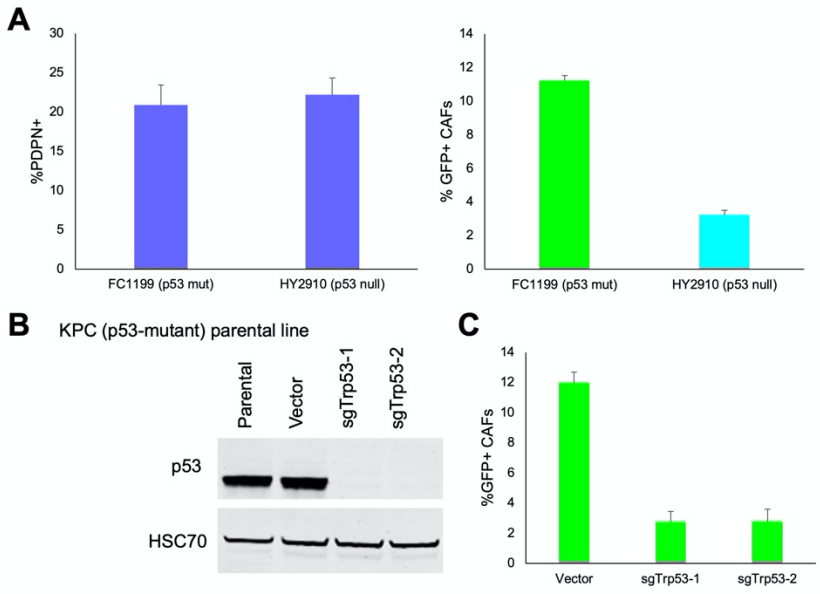

Figure 2, related to Major Task 3. A, PDAC was generated from C57BL/6J pure models harboring mutant KRAS G12D as well as either mutant p53 R172H or p53 loss. Size-matched tumors were harvested and analyzed by flow cytometry for lineage markers GFP and Tomato as well as CAF marker PDPN. **B,** Western blot showing p53 protein expression in a p53-mutant (R172H) parental PDAC lines as well as 2 independent knockout lines generated with CRISPR/Cas9 using 2 different sgRNA sequences. HSC70 is a loading control. **C,** The lines depicted in **B** were implanted into Rosa-mTmG;Fabp4-Cre hosts, and size-matched tumors were harvested and analyzed by flow cytometry for lineage markers GFP and Tomato as well as CAF marker PDPN.

Major Task 4: Perform gene expression profiling on GFP+ (PSC-derived) and GFP- (non-PSC-derived) PDAC CAFs

Subtask 1: Optimize FACS for Pdgfra and GFP out of PDAC tumors.

Optimization of FACS for the purpose of RNA-seq analysis of CAF subsets was a greater technical challenge than originally anticipated. While the tdTomato+ CAFs

(those of a non-PSC origin) were plentiful and relatively easy to isolate with high viability and RNA integrity, isolating viable GFP+ CAFs with high-quality RNA at the end was very difficult. We optimized these experiments by sorting on PDPN instead of PDGFRA (PDPN has emerged as a pan-CAF marker in PDAC since my original DOD grant submission, and we found that we captured significantly more CAFs gating on PDPN than on PDGFRA, which has since been shown to be preferentially expressed by the relatively minor iCAF subset). In addition, we used a large nozzle for the sort, and spiked RNase inhibitor into our single-cell suspension to prevent RNA degradation during the lengthy sorting process. We were delighted to obtain healthy and viable cells from both populations (PDPN+GFP+ and PDPN+GFP-/tdTomato+) and RNA of high quality and quantity after these optimization steps.

Subtask 2: Confirm RNA integrity of sorted CAFs by performing qPCR for fibroblast genes (i.e., Acta2, Col1a1, Il6). Determine whether isolated RNA is of sufficient quality for RNA-seq.

By performing the optimizing steps outlined above, we were able to obtain RNA from sorted CAFs of sufficient integrity for RNA-seq. Fibroblast genes including Acta2, Col1a1, and Il6 were highly expressed in both fractions as expected, and did not differ significantly between the two CAF populations, suggesting that any transcriptional differences between these populations would lie outside these conventional transcripts.

Subtask 3: Perform RNA-seq to obtain gene expression profiles of Pdgfra+GFP+ and Pdgfra+GFP- PDAC CAFs. Determine and compare transcriptional programs in stellate cell-derived versus non-stellate cell-derived CAF populations.

As discussed above, we performed this RNA-seq experiment gating on PDPN instead of PDGFRA, and got very exciting results. While CAFs of PSC versus non-PSC origin had similar levels of expression of common CAF markers such as PDPN itself, aSMA, FAP, and most collagen genes, we found extensive transcriptional differences between these populations, indicating that transcriptional heterogeneity among PDAC CAFs reflects at least in part distinct cells of origin. We found PSC-derived CAFs to express higher levels of gene expression programs associated with cell-cell adhesion, extracellular matrix components (not collagens), and axon guidance (Figure 3).

Subtask 4: Identify PSC-specific fibroblast marker from RNA-seq datasets

We identified several candidates from our RNA-seq datasets that appeared to be unique to PSC-derived CAFs among total CAFs and would thus serve as a basis for co-staining (together with PDPN or aSMA) of human PDAC specimens to identify PSC-derived CAFs. We decided on the combination of aSMA and TIE1, a receptor tyrosine kinase that is highly expressed on endothelial cells but, among CAFs, is unique to those of a PSC origin. We also identified NAV1 as an additional marker, though the NAV1 antibodies for IHC were not as good as those available for TIE1 on human tissues, so we used TIE1 for our subsequent analysis of patient specimens (Figure 3).

Subtask 5: Co-stain for aSMA and PSC-specific fibroblast marker in human PDAC tissue microarray and assess relationship with patient outcome

We co-stained two independent human PDAC tissue sets for aSMA and TIE1, and quantified the aSMA+TIE1+ cells out of total aSMA+ cells to determine the frequency of putative PSC-derived CAFs in these human tumors. These independent patient tissue collections included a tumor microarray containing 4 spatially distinct punches from each of 153 PDAC patient samples (provided by our collaborator Dr. Dawson at UCLA), and a separate set of whole tissue sections (as opposed to small punches on an array) from 43 patients at OHSU. We were very pleased to get consistent results from both patient populations, and to find that the majority of patient samples showed PSC-derived CAF frequencies nicely in line with results from our mouse models (less than 20% of total CAFs). In fact, almost every patient sample showed PSC-derived CAFs as the minority of CAFs in their tumors. We next took advantage of the clinical data available for the patients represented on the tumor microarray to assess whether PSC-derived CAF frequency associated with overall survival, but did not find a significant difference in outcome among patients with relatively high versus relatively low levels of PSC-derived CAFs. We will further perform correlative analyses in patient samples as we come to better understand the functions of these cells.

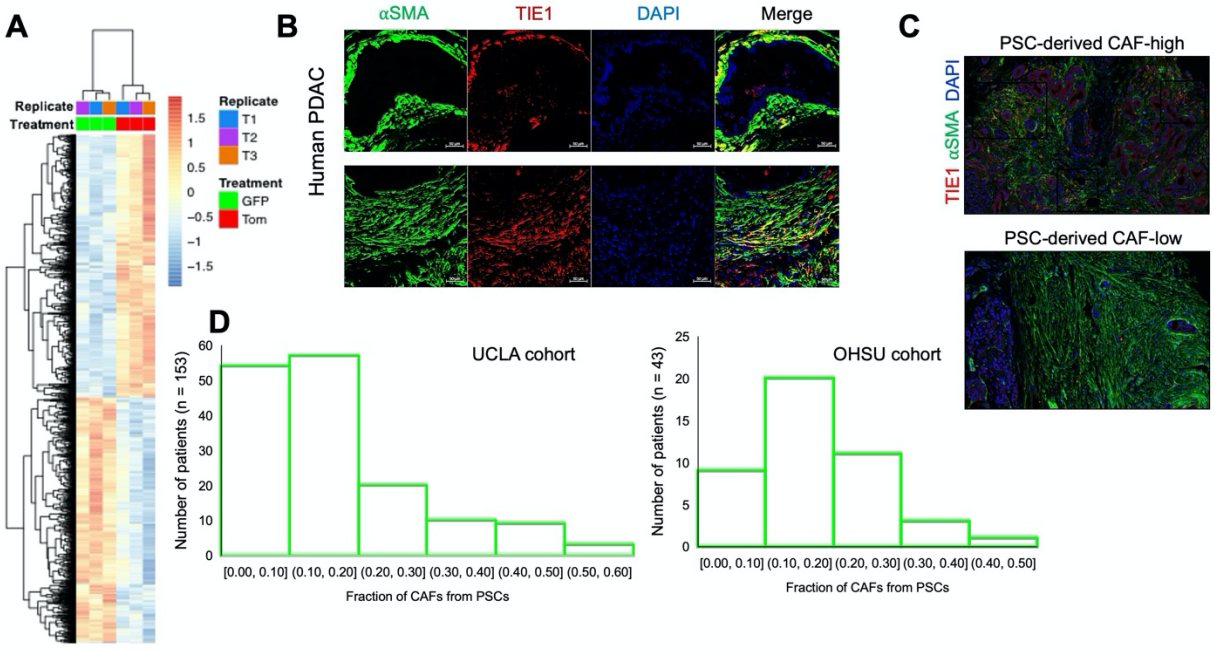

Figure 3, related to Major Task 4. A, Heatmap depicting RNA-seq results from n = 3 PDAC-bearing Rosa-mTmG;Fabp4-Cre mice, comparing gene expression in GFP+ (PSC-derived) versus Tomato+ (non-PSC-derived) CAFs which were obtained from FACS for PDPN+ CAFs for each fluorescent reporter. **B,** Immunohistochemical staining of human PDAC tissues for aSMA and putative PSC-derived CAF marker TIE1, showing colocalization on a subset of CAFs. **C,** Representative images of human PDAC tissues from a tumor microarray, depicting tumors with relatively high versus relative low abundance of CAFs (total aSMA-positive area) from a putative PSC origin (TIE1/aSMA

double positive area). **D,** All patient tumor specimens on each of two independent tumor microarrays were co-stained for aSMA and TIE1, and the frequency of putative PSC-derived CAFs out of total CAFs was quantified for each array, showing these as a minority of CAFs as observed in mouse models.

Major Task 5: Characterize paracrine crosstalk to PDAC cells from PSC-derived and non-PSC-derived CAFs

Subtask 1: Test effect of CM from primary Pdgfra+GFP+ and Pdgfra+GFP- CAFs on growth-permissive signaling (pAKT, pSTAT3) by Western blot. Determine the differential roles of different CAF populations on paracrine induction of mitogenic signal transduction.

We have found that the profound differences between PSC-derived (GFP+) and non-PSC-derived CAFs in vivo are not very effectively retained under standard culture conditions, which drive CAFs to a contractile and myofibroblastic phenotype, making these conditioned media experiments somewhat challenging. However, we were able to overcome this challenge in part by developing a method for ablation of PSC-derived CAFs in vivo (Figure 4), and we are continuing to work on developing culture methods to better retain CAF heterogeneity in vitro, for example by using different hydrogels as growth substrates and by modulating nutrient levels in culture medium.

Subtask 2: Assay proliferation of PDAC cells upon exposure to CM from GFP+ or GFP- PDAC CAFs. Compare the paracrine regulation of cancer cell proliferation by secreted factors from different CAF populations.

Due to the reasons specified above with respect to plasticity of CAF populations in vitro, we chose to use our targeted PSC ablation model and instead measure the impact of PSC-derived CAFs on tumor cell proliferation in vivo. We did not find a difference, consistent with the notion that PSC-derived CAFs are dispensable for primary tumor growth yet critical for efficient metastatic spread from the primary tumor microenvironment.

Subtask 3: Measure paracrine induction of anabolic gene expression in PDAC cells by distinct CAF populations. Compare the paracrine regulation of growth-permissive gene expression by stellate cell-derived versus non-stellate cell-derived CAFs.

We have elected to carry out these experiments in vivo in light of the plasticity of CAF populations in culture (Figure 4), such that we will sort tumor cells out of established tumors that have or lack PSC-derived CAFs, using EpCAM as a cell surface marker, then perform transcriptional profiling. We are still optimizing these experiments but are confident that they will work based on the state of optimization.

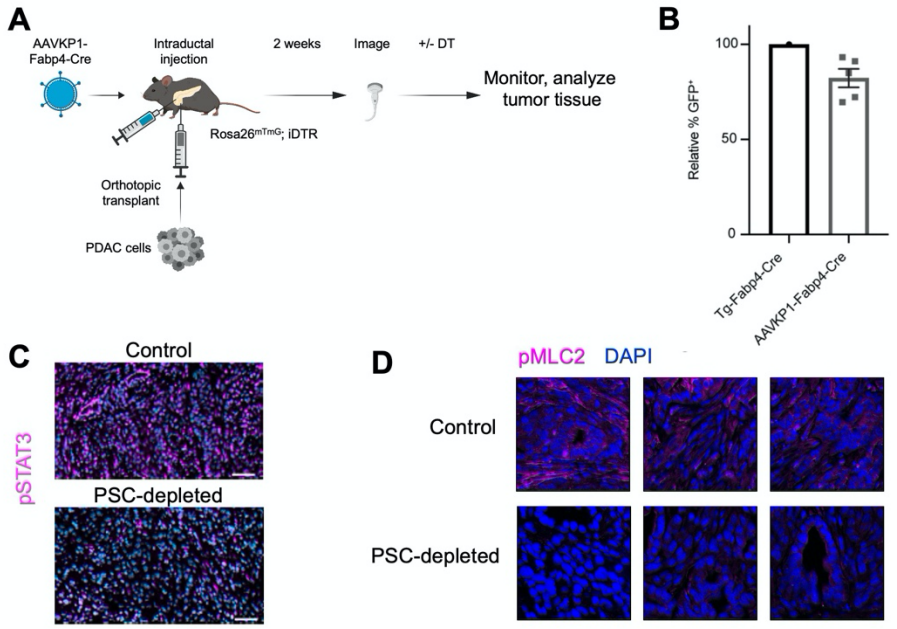

Figure 4, related to Major Task 5. A, Schematic of our model to enable specific targeting of PSCs and derivative CAFs in vivo, wherein we perform orthotopic implantation of PDAC cells as well as retrograde ductal injection of viral Fabp4-Cre directly into the pancreas. As we use hosts harboring a Cre-inducible allele of the diphtheria toxin receptor, this model allows for targeted ablation of PSC-derived CAFs using diphtheria toxin once tumors reach a defined size. **B,** GFP-expressing stellate cells, as a readout of Fabp4-Cre activity in the pancreata of Rosa-mTmG mice, were quantified in mice harboring an Fabp4-Cre allele versus ductal injection of viral Fabp4-Cre. **C,** Immunohistochemical staining for p-STAT3 in control versus PSC-depleted PDAC. **D,** Immunohistochemical staining for p-MLC2 in control versus PSC-depleted PDAC.

Major Task 6: Determine the effect of RARbeta-mediated PSC homeostasis on PDAC growth and survival

Subtask 1: Measure PDAC growth and overall survival in mice lacking Rarbeta in PSCs and derivative CAFs. Determine whether PSC dysregulation via Rarbeta disruption regulates PDAC lethality.

We were very fortunate during the funding period to develop a more robust alternative, and instead of manipulating Rarbeta in PSCs, we developed a means to ablate this cell population as a whole by delivering a viral Fabp4-Cre intraductally into the pancreas of mice harboring a Cre-inducible diptheria toxin receptor allele, then treating with diphtheria toxin when tumors reached a defined size to ablate PSC-derived CAFs specifically without impacting adipose tissue, the hematopoietic system, or other potentially Fabp4-expressing cell populations. These experiments revealed that PSC-derived CAFs are dispensable for primary tumor growth but, excitingly, play critical roles in regulation of extracellular matrix composition and metastatic spread from the

primary tumor microenvironment. Consistent with these findings, ablating PSC-derived CAFs significantly prolongs survival (Figure 5).

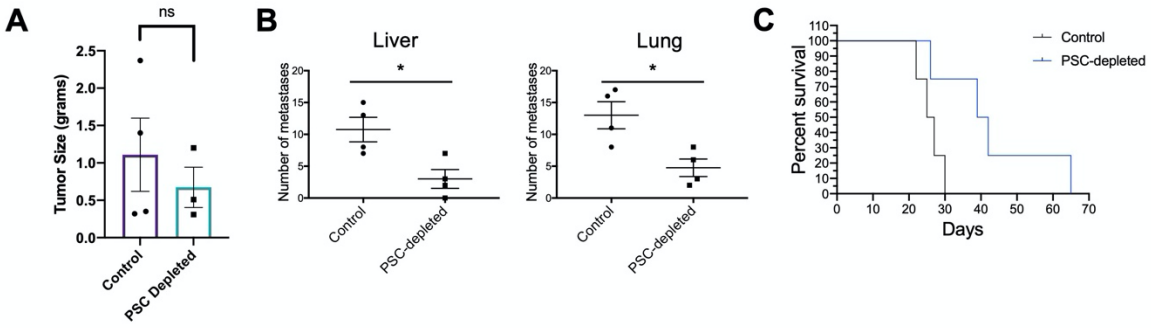

Figure 5, related to Major Task 6. A, Primary tumor size at humane endpoint in iDTR mice which received intraductal Fabp4-Cre and either saline (control) or diphtheria toxin injection starting when tumors reached 3mm in diameter. **B,** Quantification of metastases in the liver and lungs of mice as in **A** from H&E step sections, with each data point depicting an average of 5 step sections through the tissue per mouse. **C,** Overall survival from the time of enrollment (3mm tumor diameter) of control versus PSC-depleted mice.

Major Task 7: Characterize stromal reaction in PDAC in which PSCs are dysregulated

Subtask 1: IHC for stromal markers (Collagen I, aSMA, CD45) in PDAC from mice in Major Task 6. Compare stromal reaction and leukocyte infiltration in mice with dysregulated stellate cells versus controls.

We performed these analyses in mice lacking PSC-derived CAFs compared to controls and found profound changes in extracellular matrix components including perlecan and tenascin C without significant impacts on type I collagen or total aSMA-positive CAF abundance, consistent with ablation of a numerically minor subpopulation of CAFs (Figure 6).

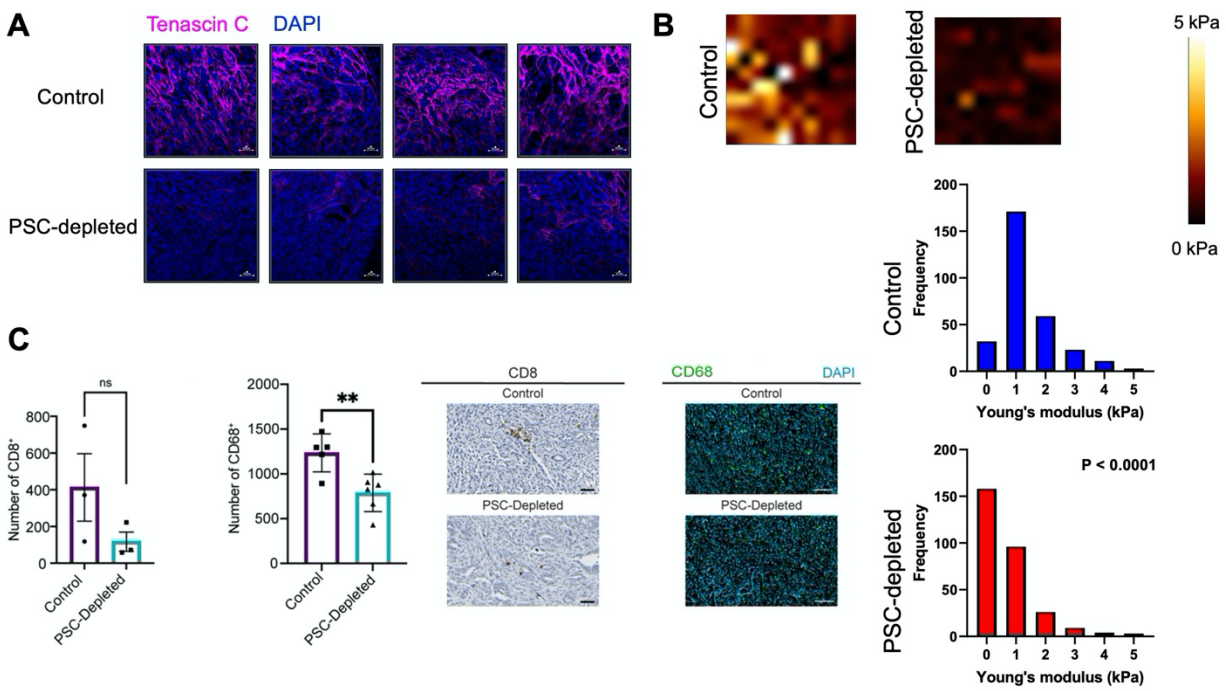

Figure 6, related to Major Task 7. A, Tenascin C immunohistochemical staining on PDAC tumor tissues from iDTR mice injected with AAV-Fabp4-Cre, enrolled when tumors reached 6mm in diameter, treated with saline (control) or diphtheria toxin, and tumors harvested 5 days after the first injection. **B,** Tumors from mice as in **A** were harvested, embedded in OCT, fresh-frozen, and sectioned for analysis by atomic force microscopy. Representative force maps are shown in the top portion of the panel, and quantification of force (Young's modulus) as an average over the tumor area is plotted below. **C,** Tumors from mice as in **A** were analyzed by immunohistochemical staining to quantify intratumoral CD8 T cells and CD68+ myeloid cells.

4. **IMPACT:**

   - **What was the impact on the development of the principal discipline(s) of the project?**

     - The findings of the project so far are significant to the field as they suggest that PSCs actually give rise to only a subset of PDAC CAFs and not most or all of these CAFs as previously thought. In addition, the mouse model that we have developed will likely be of use to the field in studying CAF evolution in PDAC at distinct stages or of distinct genotypes.

   - **What was the impact on other disciplines?**

     - Nothing to Report.

   - **What was the impact on technology transfer?**

        - Nothing to Report.

    - **What was the impact on society beyond science and technology?**

        - Nothing to Report.

5. **CHANGES/PROBLEMS:**

    - **Changes in approach and reasons for change**

        - Instead of modulating PSC function in vivo through manipulation of RAR, we have developed a means to ablate these cells based on recently published methods for ductal injection of viral Cre particles into the pancreas. This is a far more robust means by which to manipulate these cells as RAR ablation could potentially be compensated by activation of tangential transcription factor networks, whereas ablation of these cells will give us a strong readout for the functions of these cells in the tumor microenvironment for the first time.

    - **Actual or anticipated problems or delays and actions or plans to resolve them**

        - Experimental progress has been delayed due to the COVID-19 pandemic. We are presently back in the lab as of June 9, 2020 and hope to continue our work at a solid pace.

    - **Changes that had a significant impact on expenditures**

        - Nothing to Report.

    - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

        - Nothing to Report.

    - **Significant changes in use or care of human subjects**

        - Nothing to Report.

    - **Significant changes in use or care of vertebrate animals.**

        - Nothing to Report.

    - **Significant changes in use of biohazards and/or select agents**

        - Nothing to Report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
  - **Journal publications.**
    - Helms et al., Mesenchymal lineage heterogeneity underlies non-redundant functions of pancreatic cancer-associated fibroblasts, *Cancer Discovery*, in press, PMID: 34548310.
  - **Books or other non-periodical, one-time publications.**
    - Nothing to Report.
  - **Other publications, conference papers, and presentations.**
    - Nothing to Report.
- **Website(s) or other Internet site(s)**
  - Nothing to Report.
- **Technologies or techniques**
  - Nothing to Report.
- **Inventions, patent applications, and/or licenses**
  - Nothing to Report.
- **Other Products**
  - This DOD funding enabled us to generate data and hypotheses that served as the basis for an R01 application that was funded by the NIH/NCI, grant R01 CA250917, $233,131 direct costs per year and $355,498 total per year for 5 years, totaling $1,165,655 in direct costs and $1,777,490 overall. Specifically, support from this DOD grant for Major Task 3 and Major Task 4 enabled us to generate data in support of a causal role for cancer cell p53 status in regulation of CAF phenotypes and to demonstrate transcriptional differences between CAFs of a PSC versus non-PSC origin, and we proposed to further investigate the significance of those findings in our now-funded R01 application.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Mara Sherman
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	eRA Commons ID: MHSHERMAN
Nearest person month worked:	3.6
Contribution to Project:	Mentoring, data analysis, mouse work, RNA-seq.
Funding Support:	N/A

Name:	Sohinee Bhattacharyya
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	eRA Commons ID: BHATTACHARYYA.SOHINEE
Nearest person month worked:	10.8
Contribution to Project:	Benchwork, data collection and analysis, mouse work
Funding Support:	N/A

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report.
- **What other organizations were involved as partners?**
  - Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**
   - Not applicable.