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TITLE: Understanding Stromal Fibroblast Heterogeneity in the Pancreatic Tumor Microenvironment

PRINCIPAL INVESTIGATOR: Mara Sherman

CONTRACTING ORGANIZATION: Oregon Health & Science University

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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

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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 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.

2. **KEYWORDS:**

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

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

  - The major goals of the project for this funding period included training-specific tasks and research-specific tasks. The training-specific tasks were to meet semi-annually with my mentor committee, attend and present at the Cold Spring Harbor Workshop on Pancreatic Cancer, attend and present at the AACR Special Conference on Pancreatic Cancer, present our research at regular OHSU seminar series and meetings, attend our monthly Pancreas Tumor Board, and organize and present at the Mouse Models of Human Cancer monthly workshop. The research-specific tasks were to use PDAC cell lines with mutant or null p53 status and analyze contribution of PSCs to the CAF pool in tumors associated with these distinct genotypes, continue to analyze RNA-seq data from PSC-derived versus non-PSC-derived CAFs to understand PSC-derived CAF function and identify potential markers for these cells to analyze in human PDAC, co-stain and quantify putative PSC-derived CAFs in a human tumor microarray, analyze paracrine crosstalk from PSC-derived CAFs to PDAC cells, and begin to analyze tumor growth and overall survival upon PSC dysregulation in vivo.

- **What was accomplished under these goals?**

Training-Specific Tasks:

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

Mentorship Committee: Evaluation of Progress

Status: Complete

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

Status: Not started (currently enrolled in OHSU Mentorship Academy, which has similar objectives)

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

Status: Complete

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

Status: Complete

Subtask 4: Presentation of research at OHSU (yearly)

- Pancreas Data Discussion Meetings
- Knight Cancer Institute Research Meetings
- Brenden-Colson Center Monthly Seminar Series
- Cell, Developmental & Cancer Biology Department Faculty Forum lunch series
- Rosalie Sears' Lab Meetings
- Sara Courtneidge's Lab Meetings

Status: In progress

Subtask 5: Attendance of Pancreas Tumor Board (monthly)

Status: In progress

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

Status: In progress

Mentorship Committee: Evaluation of Progress

Status: In progress

Subtask 7: Attend Scientific Conferences (AACR Special Conference Tumor Microenvironment, Keystone Symposium Tumor Microenvironment

Status: Complete

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

Status: Instead attended EACR-AACR Joint Conference on Tumor Microenvironment, Complete

Mentorship Committee: Evaluation of Progress

Milestones Achieved: Submission of R01 application, Publication of Novel Results

Status: Complete

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

Status: Complete

Mentorship Committee: Evaluation of Progress

Status: Complete

Mentorship Committee: Evaluation of Progress

Milestones Achieved, Submission of second R01 application, Publication of Novel Results

Status: In progress

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

Status: Complete (shifted to even years, attended the 2020 meeting as there will not be a workshop in 2021)

Mentorship Committee: Evaluation of Progress

Status: Not started

Research-Specific Tasks:

Specific Aim 1: Determine the relative contribution of pancreatic stellate cells to the pancreatic tumor microenvironment.

Major Task 1: Obtain IACUC approval for animal studies

Subtask 1: Approval of IACUC protocol for animal studies

Status: Complete

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

Status: Complete

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

Status: Complete

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

Subtask 1: Co-stain PDAC sections for □SMA and GFP. Compare GFP-positive cells to aSMA-positive cells to assess origin of stained fibroblasts.
Mouse model used: Rosa26lox-mTred-Stop-lox-mGFP; Fabp4-Cre (n=20)
Cell line used: KPC FC1199 (murine, gift from Dr. David Tuveson)

Status: Complete. Results reveal that GFP-positive stellate cells give rise to a numerically minor population of all aSMA-positive CAFs.

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.
Mouse model used: Rosa26lox-mTred-Stop-lox-mGFP; Fabp4-Cre (n=20; same cohort as Subtask 1)
Cell line used: KPC FC1199 (murine)

Status: Complete. Results reveal that GFP-positive stellate cells give rise to a numerically minor population of all Collagen I-positive CAFs.

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

Subtask 1: Measure GFP+ fibroblasts (GFP/□SMA 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.

Mouse model used: Rosa26lox-mTred-Stop-lox-mGFP; Fabp4-Cre (n=20)
Cell lines used: KPC FC1199 (murine, p53-mutant), LSL-Kras-1 (murine, p53-null; gift from Dr. Haoqiang Ying)

Status: Complete. Results reveal significantly less contribution of stellate cells to the CAF pool in p53-null tumors than in p53-mutant tumors (Figure 1).

Milestone(s) Achieved: Evaluate PSCs as a cell of origin of PDAC CAFs; determine extent of PSC contribution to the PDAC tumor microenvironment in the context of distinct tumor genotypes

Specific Aim 2: Analyze the distinct gene expression program of stellate cell-derived fibroblasts in PDAC.

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. Confirm fibroblast gene expression, lack of epithelial gene expression in Pdgfra-expressing cells, and find a fluorophore that does not bleed into GFP to enable effective sorting of GFP-positive and –negative populations.
Mouse model used: Rosa26lox-mTred-Stop-lox-mGFP; Fabp4-Cre (n=10)
Cell line used: KPC FC1199 (murine)

Status: Complete. FACS now works reproducibly and our protocol yields high numbers of viable cells.

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.
Mouse model used: Rosa26lox-mTred-Stop-lox-mGFP; Fabp4-Cre (n=15; same cohort as Subtask 1)
Cell line used: KPC FC1199 (murine)

Status: Complete. We have optimized our protocol to get RNA integrity numbers over 9 (out of 10) across samples and preps, which is suitable for RNA-seq.

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.
Mouse model used: Rosa26lox-mTred-Stop-lox-mGFP; Fabp4-Cre (n=8)
Cell line used: KPC FC1199 (murine)

Status: Complete. Our RNA-seq was successful and showed broad transcriptional differences in PSC-derived and non-PSC-derived CAFs, suggesting functional non-redundancy between these CAF populations of distinct cellular origins.

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

Status: Complete. We have identified the marker combination of aSMA and TIE1 for stellate cell-derived CAFs based on RNA-seq data and validation in mouse samples.

Subtask 5: Co-stain for aSMA and PSC-specific fibroblast marker in human PDAC tissue microarray and assess relationship with patient outcome. Divide patient samples into PSC-high and PSC-low populations and assess outcome to assess a relationship between CAF origin and disease progression or overall survival. (staining and analysis performed Dr. David Dawson, UCLA; PI will only receive data from the TMA) (n=150 patient samples on established TMA)

Status: Complete. Results showed heterogeneity in CAF origin across tumor samples, but stellate cell-derived CAF frequencies were consistent with frequencies measured in our mouse models, supporting the relevance of these models for studying the pancreatic tumor microenvironment (Figure 2).

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.
Mouse model used: Rosa26lox-mTred-Stop-lox-mGFP; Fabp4-Cre (n=15)
Cell lines used: KPC FC1199 (murine), LSL-Kras-1 (murine), KPC FC1245 (murine, gift from Dr. David Tuveson)

Status: In progress

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.
(same mouse cohort and cell lines used as in Subtask 1)

Status: In progress

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.
(same mouse cohort and cell lines used as in Subtask 1)

Status: In progress

Milestone(s) Achieved: Determine differential transcriptional programs in PSC-derived and non-PSC-derived PDAC CAFs; characterize functional response of PDAC cells to distinct CAF populations via paracrine signaling; identify a marker of PSC-derived CAFs and validate findings in human PDAC

Specific Aim 3: Assess the consequence of PSC dysregulation for pancreatic tumorigenesis.

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

Subtask 1: Measure PDAC growth and overall survival in mice lacking Rar□ in PSCs and derivative CAFs. Determine whether PSC dysregulation via Rar□ disruption regulates PDAC lethality.
Mouse model used: Rarblox/lox; Fabp4-Cre (and lox/+ controls) (n=30)
Cell line used: KPC FC1199 (murine)

Status: In progress. For these experiments, we are using our viral transduction system for ablation of PSC-derived CAFs, as this is more robust than the Rarb approach (Figure 3).

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

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

Status: In progress.

Milestone(s) Achieved: First functional assessment of dysregulation of endogenous PSCs for PDAC growth and overall survival; evaluation of the role of RAR□ in endogenous PSCs in vivo

Conference presentations during the funding period:

Cold Spring Harbor Workshop on Pancreatic Cancer, “Origins and functions of pancreatic cancer-associated fibroblasts,” June, 2020.

AACR-EACR Conference on Tumor Microenvironment, “Mechanisms and consequences of pancreatic cancer stromal evolution,” March, 2020.

AACR Special Conference on Pancreatic Cancer, “Mechanisms and consequences of pancreatic cancer stromal evolution,” September, 2019.

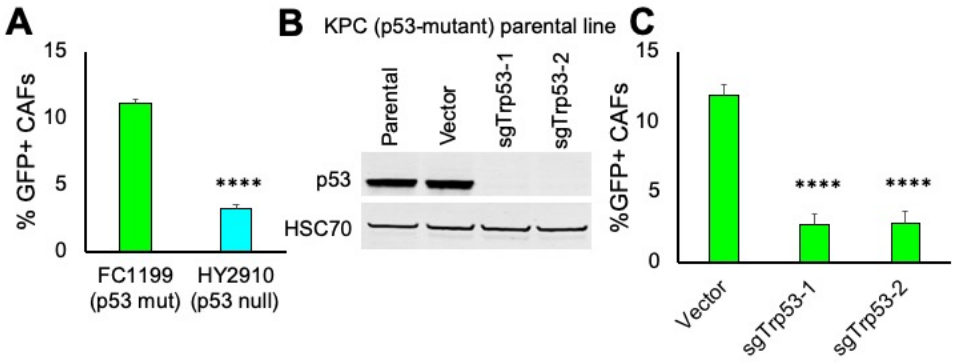

Figure 1. (A) Results from flow cytometry to determine %GFP+ (PSC-derived) of total PDPN+ CAFs in the indicated PDAC models (n = 4 per line, ***P < 0.001 by unpaired t-test). (B) Western blots for p53 in the indicated control and sgRNA-transduced PDAC lines. (C) Flow cytometry results showing %GFP+ CAFs in tumors from the indicated lines (n = 4 per line, ****P < 0.0001 by one-way ANOVA).

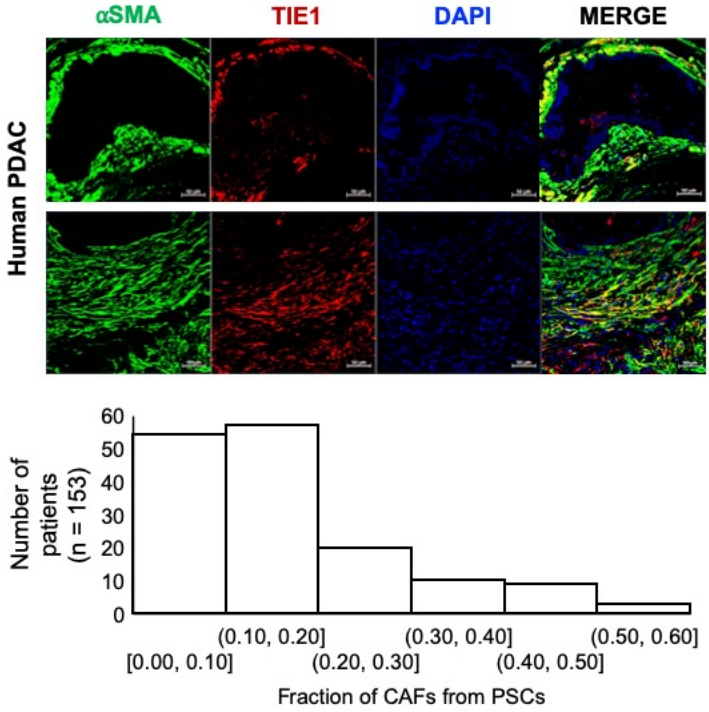

Figure 2. (Top) Representative confocal images of human PDAC samples stained for the indicated markers of PSC-derived CAFs. (Bottom) Quantification of TIE1/SMA+ out of total SMA+ CAFs in human PDAC samples (n = 153).

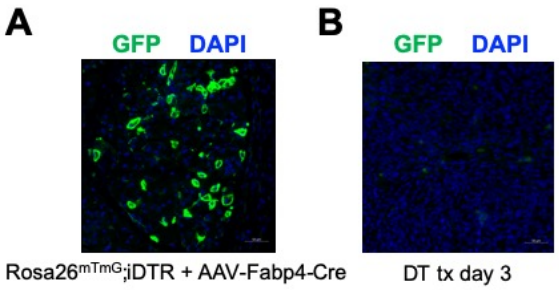

Figure 3. Representative confocal images of pancreas tissue from Rosa26mTmG; iDTR hosts injected with AAVKP1-Fabp4-Cre and harvested at day 7 (A) or treated with DT starting day 7 and harvested on day 10 (B) (n = 5 per time point).

- **What opportunities for training and professional development has the project provided?**
  - As discussed above, the project has provided substantial opportunity for professional development for the PI, including regular meetings with the mentor committee. Training for the PI has included regular seminar series at OHSU, complete with helpful feedback from the local research community, as well as presentations as the international meetings listed above, with feedback from the broader pancreatic cancer research community. Training for the postdoctoral fellow on the project has included exposure to animal models of pancreatic cancer, flow cytometry, immunohistochemistry, and molecular biology.
- **How were the results disseminated to communities of interest?**
  - Results were presented at the AACR Special Conference on Pancreatic Cancer on September 29, 2019.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Building on our accomplishments from year 2 of the grant, during the next reporting period we plan to continue our analysis of the functions of PSC-derived CAFs by dysregulating these cells during pancreatic tumorigenesis in vivo. To this end, we will use our AAV system optimized during this funding period to ablate PSCs and derivative CAFs, monitor tumor growth and survival, and analyze tumor tissues to analyze paracrine signaling as well as features of the tumor microenvironment which we predict to be regulated by PSC-derived CAFs based on the RNA-seq datasets obtained from year 1 of funding. In addition to these research-specific tasks, I will also perform training-specific tasks during the next reporting period. These will include continued participation in the regular seminar series and meetings at OHSU listed above, continued semi-annual meetings with my mentor committee, attendance of the AACR Special Conference on

Pancreatic Cancer (to be virtual this year), attendance of the Cold Spring Harbor Meeting on Mechanisms and Models of Cancer (also virtual), and submission of an R01 application.

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.**

    - Nothing to Report (aiming to submit a manuscript within the next funding period).

  - **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**

  - Nothing to Report.

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.