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TITLE: Understanding Stromal Fibroblast Heterogeneity in the Pancreatic Tumor Microenvironment

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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) contrary to dogma in the field, stellate cells give rise to a numerically minor subpopulation of PDAC CAFs, 2) we have generated the first transcriptional profile of PSC-derived CAFs and find that these cells express a unique gene expression program enriched in axon guidance cues, extracellular matrix components (aside from collagens), and leukocyte trafficking molecules. 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) contrary to dogma in the field, stellate cells give rise to a numerically minor subpopulation of PDAC CAFs, 2) we have generated the first transcriptional profile of PSC-derived CAFs and find that these cells express a unique gene expression program enriched in axon guidance cues, extracellular matrix components (aside from collagens), and leukocyte trafficking molecules. 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 the Cold Spring Harbor Workshop on Leadership in Bioscience, attend and present at the Cold Spring Harbor Workshop on Pancreatic Cancer, attend and present at the Gordon Conference on Pancreatic Diseases, 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 obtain IACUC approval for our proposed mouse studies, complete regulatory review and approval for our study from ACURO and HRPO, co-stain PDAC sections for our CAF markers of interest together with GFP to mark PSC-derived CAFs, measure GFP+ (PSC-derived) CAF frequencies in p53-mutant versus p53-null PDAC, optimize FACS for GFP+ and Tomato+ CAFs out of PDAC tissues for RNA-seq, confirm RNA integrity

of sorted CAFs, perform RNA-seq on these sorted CAF populations, and begin to identify PSC-specific CAF markers from these RNA-seq datasets.

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

  - I am pleased to report that we accomplished nearly all major goals for year 1 of our proposed project. Accomplishment of training-specific tasks included semi-annual meeting with my mentor committee, during which I got invaluable advice about grant-writing, hiring, budget management, publishing papers, and presenting at conferences. I also attended the Cold Spring Harbor Workshop on Pancreatic Cancer as an instructor, where I provided didactic training and also presented our ongoing work, and learned a great deal from the other instructors at the workshop. I attended the Gordon Conference on Pancreatic Diseases as a Discussion Leader, and also attended and presented our work at numerous monthly or weekly series at OHSU as proposed including the Pancreas Data Discussion Meetings, Knight Cancer Institute Research Meetings, Brenden-Colson Center Monthly Seminar Series, CDCB Department Faculty Forum Lunch series, Rosalie Sears lab meetings, and Sara Courtneidge lab meetings. I also attended the monthly Pancreas Tumor Board on campus, which was very helpful in enhancing my appreciation for the clinical side of pancreatic cancer biology and patient care. I also organized, attended, and presented at the monthly Mouse Models of Human Cancer workshop on campus. Though I was ultimately unable to attend the Cold Spring Harbor Workshop on Leadership in Bioscience, I will plan to attend this or a similar workshop on leadership in the coming year. Accomplishment of research-specific tasks included obtaining approval by IACUC, ACURO, and HRPO for the proposed work. We co-stained PDAC sections for CAF markers of interest as proposed (Figure 1), though we went with a stain for CAF marker Podoplanin (PDPN) instead of Collagen I, as PDPN is localized to the cell body while collagens are secreted, so these results were easier and more straightforward to interpret as a second marker for CAFs. We also quantified the GFP+ CAF compartment of p53-mutant versus p53-null CAFs as proposed, and found a far greater propotion of PSC-derived (GFP+) CAFs in p53-mutant tumors (Figure 2). We optimized FACS and RNA isolation, and performed RNA-seq on our CAF populations of interest. Though analysis is still ongoing, consistent with our timeline in the approved Statement of Work, we have found

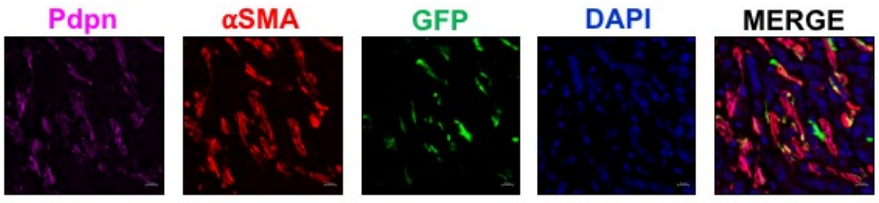

Figure 1. Representative confocal images of PDAC in a *Fabp4-Cre;Rosa26^{mTmG}* host.

substantial transcriptional differences between GFP+ and Tomato+ CAFs (Figure 3), consistent with distinct functions of the PSC-derived CAF population.

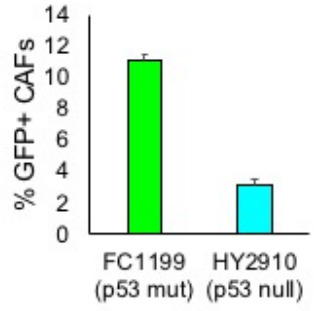

Figure 2. Flow cytometry results assaying GFP+ CAFs (PDPN+ CD31-) in PDAC of the indicated p53 status.

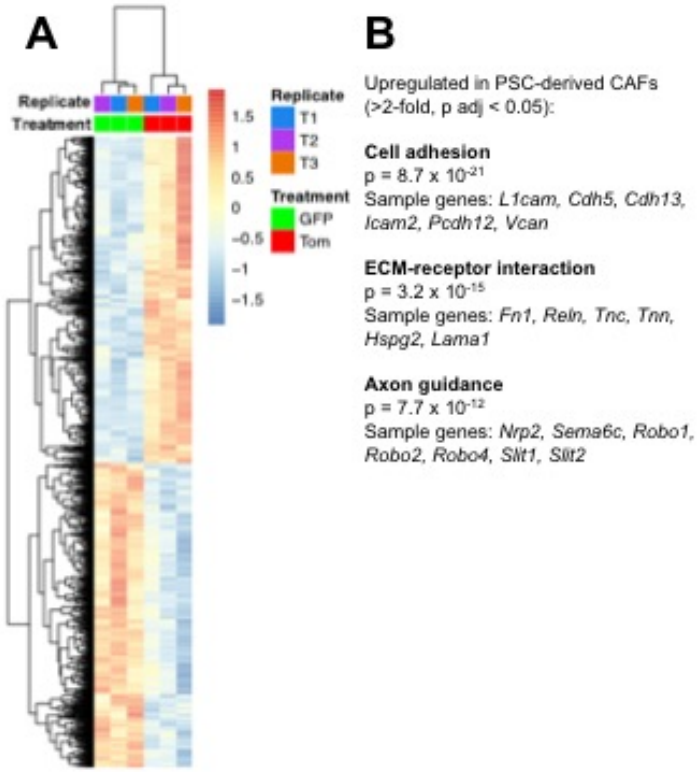

Figure 3. (A) Heatmap of RNA-seq results comparing GFP+ and tdTomato+ PDAC CAFs. **(B)** Gene ontology analysis for genes enriched in GFP+ CAFs.

- **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?**
  - Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

    - Building on our accomplishments from year 1 of the grant, during the next reporting period we plan to continue our analysis of the RNA-seq data and define a PSC-derived CAF gene expression signature. We will also continue our analysis to move toward an improved understanding of differences between the CAF populations of p53-mutant and p53-null tumors by performing these experiments in more mice. We will also move our findings into human samples by staining a PDAC tumor microarray for aSMA and a PSC-derived CAF marker identified in our RNA-seq dataset, and assess a correlation with overall survival. We will also begin to assess the distinct roles of PSC-derived CAFs versus those not of PSC origin in paracrine signaling to tumor cells by performing in vitro experiments and using conditioned media from these distinct CAF populations, and assaying signaling and proliferation of PDAC cells in the presence of these different conditioned media samples. Finally, we will initiate studies in mice to assess the effect of PSC dysregulation on PDAC progression in vivo. 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, attendance of the Cold Spring Harbor Meeting on Biology of Cancer: Microenvironment and Metastasis, 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 staining CAFs for Collagen I as previously described, we stained for PDPN as this signal is restricted to the cell body and far easier to interpret than the Collagen I signal as collagens are secreted.

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

     - Nothing to Report.

   - **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.

     - **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: ??
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?**
    - During the funding period, the PI has obtained support from the American Cancer Society and the National Cancer Institute. Neither has any conceptual overlap with the current project.
- **What other organizations were involved as partners?**
    - Nothing to Report.

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