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TITLE: Growth and/or Recruitment of a Novel Cell Population with Neural Crest Origin in Lung Fibrosis

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14. ABSTRACT

Pulmonary fibrosis is a debilitating disease characterized by progressive scarring of the lung, which destroys normal lung structure and leads to respiratory failure and death Abnormally increased fibroblasts/myofibroblasts is one of the key pathological changes in lung fibrosis. The origins of these abnormal lung fibroblasts/myofibroblasts are highly heterogeneous. In this project, we plan to determine whether abnormal growth and/or recruitment of a neural crest derived mesenchymal cell population contribute to lung fibrosis. In the past year, we have generated a transgenic reporter mouse line in which neural crest-derived cells were genetically labeled. In normal situation, neural crest-derived cells were detected as nerve fibers adjacent to airway smooth muscles. In contrast, in some bleomycin-induced fibrosis lungs, clusters of neural crest derived cells were detected. This abnormal cellular phenotype varied, and the potential factors affecting this change are currently under investigation. In addition, circulating neural crest-derived mesenchymal progenitor cells were detected in a few fibrosis mice by peripheral blood mononuclear cell isolation and selective culture. These cultured cells, which were negative for epithelium-marker, were MSC-like progenitors and able to differentiate to other type cells.

15. SUBJECT TERMS

Lung fibrosis; Neural crest cells; Lung myofibroblasts; Lung mesenchymal cells; Bleomycin; Peripheral blood mononuclear cells

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1. INTRODUCTION:

Pulmonary fibrosis is a debilitating disease characterized by progressive scarring of the lung, which destroys normal lung structure and leads to respiratory failure and death. In particular, idiopathic pulmonary fibrosis (IPF) is a severe form with median survival ranging from 2.5 to 3.5 years from diagnosis. Thus, there is a critical need to fully understand the cellular and molecular mechanisms underlying lung fibrosis in order to develop new and effective therapies and reduce mortality. Abnormally increased fibroblasts/myofibroblasts and excessive production of extracellular matrix by these cells are key pathological changes in lung fibrosis. The origins of these abnormal lung fibroblasts/myofibroblasts are highly heterogeneous, possibly utilizing different mechanisms for these cell growth and accumulation, which may lead to different responses to therapeutic interventions. Our preliminary study suggests that there may be a new population of mesenchymal cells with neural crest origin specifically detected in fibrosis lung. Therefore, we plan to determine and characterize a new mesenchymal cell population of neural crest origin specifically in fibrosis lungs of bleomycin-treatment mice. In addition, we will also determine changes in circulating neural crest descendants in response to pulmonary fibrogenic injury.

2. KEYWORDS

Lung fibrosis
Neural crest cells
Lung myofibroblasts
Lung mesenchymal cells
Bleomycin
Peripheral blood mononuclear cells

3. ACCOMPLISHMENTS

What were the major goals of this project?

(1) To determine and characterize a new mesenchymal cell population of neural crest origin specifically in fibrosis lung.

(2) To determine changes in circulating neural crest descendants in response to pulmonary fibrogenic injury.

What was accomplished under these goals?

Major Activity 1 (Major Task 1 in SOW):
To generate lung fibrosis models in mice, in which neural crest cells and their descendants are permanently marked.

1) Specific objective: (a) To expand mouse colonies and generate neural crest-specific reporter mice. (b) To generate lung fibrosis models by intratracheal bleomycin administration to the mice.

2) Key outcome: We crossed neural crest driver line Wnt1-Cre (Jackson Laboratory Stock #022137) with fluorescence reporter mice mT-mG to generate Wnt1-Cre/mT-mG reporter mice, in which neural crest-derived cells are supposed to be marked by mGFP expression. By examining the lungs of these reporter mice, only neural crest derived peripheral nerves were detected as mGFP-positive cells around airway smooth muscle cells, validating this neural crest reporter line (Fig.1). We have used these mice to generate single-dose bleomycin-induced lung fibrosis model.

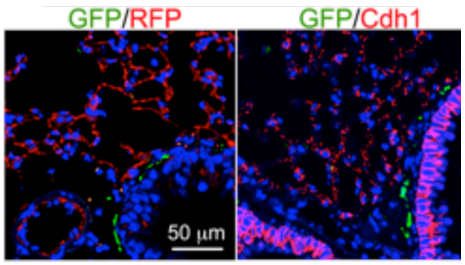

Fig.1. Neural crest-derived peripheral nerves in airways were detected by their mGFP expression in the normal Wnt1-Cre/mT-mG mouse lung.

However, in a later experiment of preparing irreversible lung fibrosis by introducing multiple doses of bleomycin to the mice, we found that the bred reporter mice expressed mGFP in all cells of normal and bleomycin-damaged lungs (Fig.2). We were struggling to find out what caused this change. By discussing with Jackson Laboratory, we learned that this Wnt1-Cre might exhibit Cre recombinase activity in the germ line, which was recently confirmed by the donor (Dinsmore CJ, Ke CY, and Soriano P. The Wnt1-Cre2 transgene is active in the male germline. *Genesis* e23468, 2022). Since the same Wnt1-Cre line was only available as a cryopreservation at Jackson Laboratory, we then obtained an alternative Wnt1-Cre line from Jackson Laboratory (Stock#022501). Unfortunately, after one cycle of cross-breeding, the new Wnt1-Cre/mT-mG reporter had a strange reporter expression pattern in the lung (Fig.3), with mGFP signal in many airway epithelial cells. We are working on another Wnt1-rtTA (Jackson Laboratory Stock# 026194)/TetO-Cre driver line to see whether we can obtained a specific neural crest reporter, but it takes tremedous time to get triple transgenic mouse line.

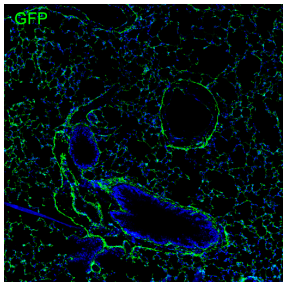

Fig.2. Non-specific expression of mGFP in the lung of the leaking Wnt1-Cre/mT-mG mouse.

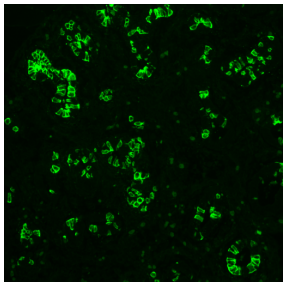
Fig.2. expression of mGFP in the lung of newly obtained Wnt1-Cre/mT-mG mice.

Major Activity 2 (Major Task 2 in SOW):
To determine neural crest derived cells in fibrosis lungs and characterize these cells for their mesenchymal properties

1) Specific objective: (a) To determine whether there are neural crest-derived mesenchymal cells in fibrosis lungs. (b) To characterize abnormal neural crest-derived mesenchymal cells in fibrosis lung.

2) Key outcome: For single dose of bleomycin-induced lung fibrosis model, 2 out of 10 Wnt1-Cre/mT-mG mice had clusters of GFP-positive cells (neural crest derived) in the fibrosis lungs (Fig.4), while the other eight mice did not. Sex and age (2-6 Months) did not affect the cellular phenotype in fibrosis lungs. The data suggest that neural crest-derived cells may contribute to lung fibrosis, but with very low incidence. The potential factors remain unknown.

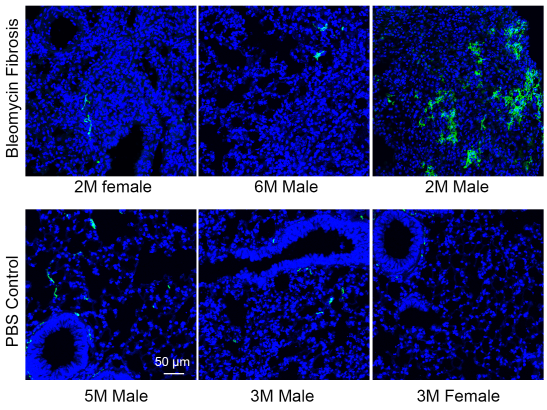

Fig.4. Neural crest-derived cells (green: mGFP) in single dose of bleomycin-induced fibrosis lung of Wnt1-Cre/mT-mG mice. Lung tissues were harvested 3 weeks after bleomycin injury.

We have further examined the fibrosis lung sections in which Wnt1-Cre-driven mGFP-positive cells were detected. By co-immunofluorescence staining (Fig.5), we confirmed that all mGFP$^+$ cells were negative for epithelial marker Cdh1 and found that some of the GFP$^+$-cells (neural crest origin) express Acta2 (myofibroblast marker), Cspg4 (pericyte marker), or Vim (vimentin, a general mesenchymal marker). We were not able to detect neural markers (Tubb3 and Calca) and myofibroblast markers (Pdgfra and Pdgfrb) for the mGFP$^+$ cells.

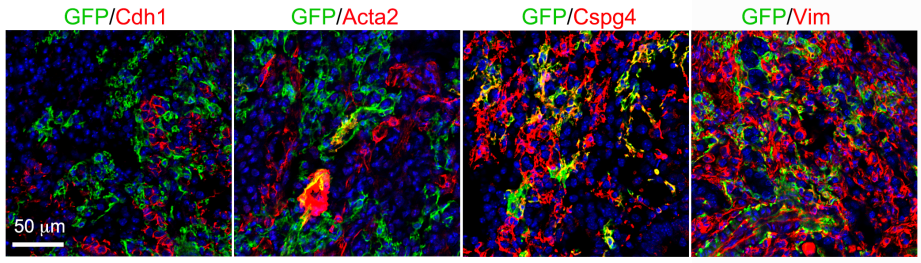

Fig.5. Co-immunostaining of GFP, Cdh1, Acta2, Cspg4, and Vim of the fibrosis mouse lung. Yellow signals result from overlapped red and green signals.

Major Activity 3 (Major Task 3 in SOW):
To measure circulating neural crest-derived monocytoid progenitor cells in lung fibrosis mice.

1) Specific objective: To quantify changes of circulating mononuclear progenitor cells with neural crest origin at different stages of lung fibrosis.

2) Key outcome: We have established the method to isolate and culture circulating monocytoid progenitor cells. Briefly, mouse peripheral blood was collected in a BD Microtainer tube with lithium heparin, and diluted with PBS (1:1), which was then layered onto Histopaque-1077. Following centrifugation at 400 g for 30 min, the peripheral blood mononuclear cell (PBMC) layer was transferred to a fresh tube and washed in PBS. The cell pellet was then resuspended in mesenchymal stem cell (MSC) culture medium and seeded onto a 100 mm culture dish. We have isolated PBMCs from (a) 7 mice that received PBS challenge (control), (b) 5 mice that were 21 days after receiving single dose of bleomycin challenge, (c) 3 mice that were 14 days after the 8th dose of bleomycin challenge (multiple bleomycin-induced chronic lung fibrosis). The PBMCs were then cultured for up to 45 days for colony formation. A single MSC-like cell colony was grown only in 1 control sample and 1 single dose of bleomycin sample. The cultured cells were able to grow more than 20 passages and became stable in culture, which had multipotent differentiation capacity. By immunostaining, these cells are GFP-positive and Tomato-negative (Fig.6), suggesting that they are neural crest-derived circulating mesenchymal progenitor cells. However, it seems that the number of these cells in circulation is extremely low, and bleomycin-

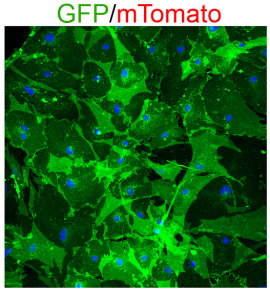

Fig.6. Established cell culture from PBMCs. The cells were stained by the indicated antibodies.

induced lung fibrosis does not significantly increase the numbers of these cells. Thus, direct quantitative analysis of circulating monocytoid cells appears not to be feasible.

Major Activity 4 (Major Task 4 in SOW):
To culture isolated mouse monocytoid cells with neural crest origin and characterize these cells for their mesenchymal properties

1) Specific objective: To characterize neural crest-derived circulating mononuclear cells in culture.

2) Key outcome: For those cells that were established from the PBMC culture as described above in Fig.6, we have further analyzed them by detecting the related cell lineage markers using immunofluorescence staining. As shown in Fig.7, these GFP-positive cells expressed several neural markers including neurofilament medium chain (Nefm) and synaptophysin (Syp). Furthermore, these cells also expressed vimentin (Fig.8), a mesenchymal cell marker. The data suggest that these cultured cells grown from PBMCs may be neural crest-derived ectomesenchymal cells.

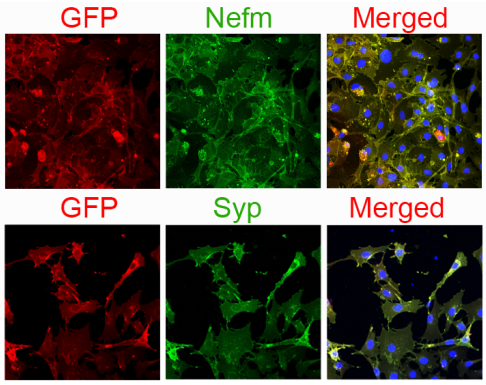

Fig.7. Immunofluorescence co-staining of the cultured cells with GFP and neural markers.

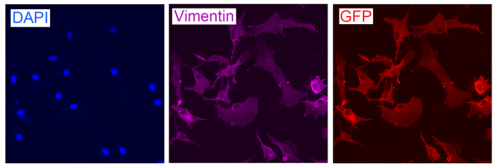

Fig.8. Immunofluorescence co-staining of the cultured cells with GFP and mesenchymal markers.

What opportunities for training and professional development has the project provided?

Nothing to report

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?

We are breeding the Wnt1-rtTA/TetO-Cre/mT-mG neural crest reporter line to repeat multiple doses of bleomycin-induced chronic lung fibrosis experiment, which will be covered by our own animal protocol and budget. This will provide complementary data to validate the results from our previous studies.

4. IMPACT

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

(1) This project tested a new mechanism by which neural crest derived cells might be one of the important sources for abnormal lung fibroblasts and myofibroblasts during fibrosis progression.
(2) The study suggests that the neural crest origin mesenchymal cells in lung fibrosis models are not the major cell type that contributes to the heterogeneity of fibrotic fibroblasts and myofibroblasts.
(3) Neural crest origin monocytoid progenitor cells in circulation and potential recruitment of these cells to fibrotic lung are very rare although these cannot be fully excluded. Therefore, targeting these cells may not be an effective approach for lung fibrosis treatment and prevention.
(4) More sensitive methods will be needed to analyze the neural crest derived progenitor cells in both circulation and lung to evaluate their impacts on lung fibrosis.

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

We had difficult time to deal with non-specific expression of Cre and the subsequent floxed-DNA recombination in all cells in the Wnt1-Cre/mT-mG neural crest reporter line in the 2nd half of the project. This was also recently reported by the original donor (Dinsmore CJ, Ke CY, and Soriano P. The Wnt1-Cre2 transgene is active in the male germline. *Genesis* e23468, 2022). Since the same Wnt1-Cre line is only available as a cryopreservation at Jackson Laboratory, we obtained an alternative Wnt1-Cre line from Jackson Laboratory (Stock#022501). Unfortunately, after one cycle of cross-breeding, the new Wnt1-Cre/mT-mG reporter did not exhibit a specific pattern of neural crest-derived cells. We are still working on another Wnt1-rtTA (Jackson Laboratory Stock# 026194)/TetO-Cre driver line to see whether we can obtained a specific neural crest reporter line that will be suitable for our experiments.

6. PRODUCTS

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Wei Shi
Project Role:	Project Director/Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-6499-2473
Nearest person month worked:	*1.20 Calendar*
Contribution to Project:	Dr. Shi is the PI on this project, and oversees the project, including data generation, analysis, and presentation. He will ensure that the project goals are accomplished in a scientifically rigorous and timely manner.
Funding Support:	DoD, NIH, California Tobacco Related Disease Research Program

Name:	Yongfeng Luo
Project Role:	Postdoctoral Research Associate
Researcher Identifier (e.g. ORCID ID):	0000-0001-8765-0273
Nearest person month worked:	*1.30 Calendar*
Contribution to Project:	Dr. Luo is responsible for bleomycin-induced lung fibrosis mouse models, cell isolation and characterization, immunofluorescence staining, confocal imaging.
Funding Support:	None

Name:	Sue Buckley
Project Role:	Research specialist
Researcher Identifier (e.g. ORCID ID):	0000-0002-9521-5519
Nearest person month worked:	*4.00 Calendar*
Contribution to Project:	Ms. Buckley is responsible for mouse peripheral blood mononuclear cell isolation and culture, and characterization of established culture cells.
Funding Support:	None

Name:	Hong-Jun Wang
Project Role:	Research specialist
Researcher Identifier (e.g. ORCID ID):	0000-0002-1785-3013
Nearest person month worked:	*1.25 Calendar*
Contribution to Project:	Mr. Wang is responsible for mouse breeding and genotyping, lung tissue isolation and histology.
Funding Support:	None

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

None

What other organizations were involved as partners?

None

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** *Not applicable*
- **QUAD CHARTS:** .*Not Applicable*

9. APPENDICES

Nothing to report.