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14. ABSTRACT

The overarching goal of this Program is to develop the scientific knowledge needed to predict and prevent the progression of IPF. We postulate that IPF is caused by recurrent injury/repair/regeneration at the bronchoalveolar junction secondary to overexpression of MUC5B, mucociliary dysfunction, retention of particles, ER stress, and disruption of normal reparative and regenerative mechanisms in the distal lung. During the first year of funding, we have (1) obtained local and DoD approvals for human and animal research; (2) enrolled 26 first degree relatives of individuals with IPF and completed all study procedures for Project 1; (3) performed ChIP, MNase, and TF binding assays to show that MUC5B promoter region is hyperchippable and that HIF1 and GCF bind in this region (Project 2); (4) imported and bred new strains of mice (St3gal3, Fut2, Ern2, Ift88, and Arl13b) in Projects 3 and 4; (5) developed and assessed the amounts and glycosylation of Muc5b in mouse models at baseline, and identified changes in polymer size and migration after inflammatory challenge (Project 3); (6) identified 10 weeks post-injury as a key timepoint for increased ciliogenesis in Muc5b Tg mice and began characterization of ciliogenesis in human lung, and (7) presented findings at two international conferences and published two manuscripts.

15. SUBJECT TERMS

preclinical pulmonary fibrosis, biomarkers, airway mucin, mucin 5b polymer, mucociliary dysfunction, transcriptional regulation, lung repair, lung regeneration, ER stress, ciliogenesis

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Introduction

The overarching challenge of this Program is to develop the scientific knowledge needed to predict and prevent the progression of Idiopathic Pulmonary Fibrosis (IPF). IPF affects 5 million worldwide, disproportionately affects men, is associated with cigarette smoking and combat-related particulate exposures, increases with age, is inexplicably increasing in prevalence, is a source of morbidity and mortality among military personnel, and is likely underdiagnosed. Patients with IPF are usually diagnosed when the fibroproliferative process has caused permanent and extensive lung parenchymal damage. Given the irreversible nature of this disease, even approved treatments for IPF only modestly slow progression and have not been shown to alter the 3-5 year survival following diagnosis. We have found that: 1) a gain-of-function *MUC5B* promoter variant rs35705950 is the strongest risk factor (genetic and otherwise) for the development of IPF, accounting for at least 30% of the risk of disease; 2) rs35705950 can be used to identify individuals in the preclinical phase of this life-threatening disease; 3) MUC5B represents a key molecule to understand the mechanisms that initiate the fibroproliferative process in the bronchoalveolar epithelium; and 4) focusing on MUC5B may provide a unique opportunity to define the early molecular events that lead to the development of IPF. We propose that a comprehensive, multi-dimensional approach that focuses on MUC5B transcription in airway epithelia, biological consequences of MUC5B overproduction that are mediated by airway epithelia and cilia, and biomarkers to predict preclinical pulmonary fibrosis (PrePF) and identify those at risk of disease progression could conceivably change the approach in IPF from palliative to preemptive.

Keywords

Preclinical pulmonary fibrosis, biomarkers, airway mucin, mucin 5b polymer, mucociliary dysfunction, transcriptional regulation, lung repair, lung regeneration, ER stress, ciliogenesis

Accomplishments

a. What were the major goals of the project?

- *List the major goals of the project as stated in the approved SOW. If the application listed milestones/ target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.*

Project 1

Specific Aim 1: Screen 500 asymptomatic siblings of sporadic IPF cases and perform pulmonary function testing on cases of preclinical pulmonary fibrosis (PrePF).

- Subtask 1: Coordinate with the site PIs to obtain IRB approval at each site for this study.
- Milestone #1: Secure IRB approval at all sites for subject recruitment.
- Subtask 2: Coordinate with the Investigators to consent IPF subjects to contact their siblings for study recruitment.
- Milestone #2: Prepare and submit manuscript on the prevalence and radiographic features of PrePF in the siblings of patients with sporadic IPF.

Specific Aim 2: Develop and validate a biomarker profile that improves the detection of preclinical pulmonary fibrosis (PrePF).

- Milestone #3: Prepare manuscript on development of peripheral blood biomarker profile of PrePF.

Specific Aim 3: Elucidate the determinants of progression in preclinial pulmonary fibrosis (PrePF).

- Major Task 1: Recontact all subjects with Year 1-3 scans positive for PrePF and perform repeat HRCT 2-3 years after study enrollment.
- Major Task 2: Re-contact subjects with Year 1-3 scans positive for PrePF and perform repeat PFTs 2-3 years after initial study enrollment.
- Major Task 3: Recontact all subjects with initial Year 1-3 scans positive for PrePF and perform repeat peripheral blood draw.
- Milestone #4: Prepare manuscript on development of peripheral blood biomarker profile of progressive PrePF.

Project 2

- Goal 1: Elucidate Molecular regulation of MUC5B in relationship to the SNP and publish paper reporting on mechanism of regulation (months 1-12 research, 75% completed; months 12-18 for publication, 100% completed)
- Goal 2: Determine integrated transcriptional control of MUC5B expression in response to pro-fibrotic signals and publish manuscript reporting on integrated regulation of MUC5B expression (months 12-30 research, 85% completed; months 30-42 publication, 50% completed)
- Goal 3: Elucidate the impact of MUC5B variant on airway wound healing and proteostasis and publish manuscript on functional impact of MUC5B variant in airway epithelia (Months 30-48; 75% completed)

Project 3 **Specific Aim 1: Demonstrate that MUC5B/Muc5b overproduction by club cells and T2 cells in distal airways promotes dysfunctional MCC.**	**Timeline Years 1-2**	**Completion**
Major Task 1: Regulatory approval, establishment of mouse colonies.	Months	
Subtask 1: Regulatory approval of animal research.		100% (1/2018)
Milestone #1: Secure IACUC approval at University of Colorado AMC.	0	
Milestone #2: Secure ACURO approval.	0-3	
Subtask 2: Animal breeding for experiments.		#1: 100% (1/2018) #2 established by 1/2018, and will continue through full grant period
Milestone #1: Import C57BL/6J mice and ROSA$^{mT/mG}$ strains.	0-3	
Milestone #2: Breed C57BL/6J, Scgb1a1-Muc5b Tg, SFTPC-Muc5b Tg, Scgb1a1Cre$^{ER/+}$;Muc5b$^{lox/lox}$; SftpcCre$^{ERT2/+}$;Muc5b$^{lox/lox}$; ROSA$^{mT/mG}$ mice.	3-18	
Major Task 2: Demonstrate that Muc5b overproduction in murine PF impairs MCC and mucus transport *in vivo* and *in vitro*.	Months	
Milestone #1: Acute and Chronic MCC in Scgb1a1-Muc5b Tg, SFTPC-Muc5b Tg, Scgb1a1-Muc5b$^{\Delta/\Delta}$; SftpcCre$^{ERT2/+}$;Muc5b$^{\Delta/\Delta}$	0-12	#1: 75% #2: 75%
Milestone #2: Mucus transport in primary cultures of lung epithelia from Scgb1a1-Muc5b Tg, SFTPC-Muc5b Tg, Scgb1a1-Muc5b$^{\Delta/\Delta}$; SftpcCre$^{ERT2/+}$;Muc5b$^{\Delta/\Delta}$ and human cells ±IPF and ±rs35705950 'T' allele	0-12	
Milestone #3: Statistical analysis of Data	12-18	#3: 33%
Major Task 3: Demonstrate that aberrantly glycosylated MUC5B/Muc5b accumulates in the airways in PF.	Months	
Milestone #1: Quantify MUC5B/Muc5b, SCGB1A1/Scgb1a1, SPC, MAL II, and UEA I labels human and mouse lung tissues by histology.	6-18	#1-2: 100% complete (10/2019)
Milestone #2: Demonstrate colocalization of secreted MUC5B/Muc5b with glycan markers	6-18	
Milestone #3: Statistical analysis of Data	12-18	#3: 100% (8/2020) #4-5: 100% (10/2019)
Milestone #4: Manuscript preparation and submission: Data from Aim 1	9-12	
Milestone #5: Manuscript acceptance and publication: Data from Aim 1	12-18	
Specific Aim 2: Determine whether Muc5b-dependent pro-fibrotic effects in mice are induced by aberrant mucin biosynthesis and proteostasis programs in club cells and T2 cells	**Timeline Years 2-3**	
Major Task 1: Characterize the dependence of glycosylation of Muc5b on gene expression levels and cellular source.	Months	
Milestone #1: Demonstrate colocalization of intracellular MUC5B/Muc5b with glycan markers and cell specific markers.	13-24	#1-2: 100% (9/2020)
Milestone #2: Identify changes in polymer size and migration by Western.	13-24	
Milestone #3: Statistical analysis of Data	16-27	90% (1/2021)
Major Task 2: Determine the effects of Muc5b levels and localization on mucin biosynthetic enzyme expression in bleomycin-induced fibrosis.	Months	
Milestone #1: Isolated and purify cells from fluorescent-tagged mice	20-26	#1-2: 80%

Milestone #2: Analyze St3Gal1- St3Gal6, St6Gal1- St6Gal2, Fut1-Fut11, and Agr2 transcript and protein levels.	24-27	(9/2021)
Milestone #3: Statistical analysis of Data	27-30	
Major Task 3: Determine the effects of Muc5b levels and localization on proteostasis dysfunction in bleomycin-induced fibrosis.	Months	
Milestone #1: Identify significant UPR/ER stress markers Atf6, Ern1(IRE-1α), Ern2 (IRE-1β), Ddit3 (CHOP), Hspa5 (Grp78/BiP), Eif2ak3 (PERK), and Xbp1/spliced Xbp1 in Muc5b-overexpressing mice.	24-27	#1-2: 80% (9/2021)
Milestone #2: Confirm protein levels & localization of markers above.	27-30	
Milestone #3: Statistical analysis of Data	30-32	
Major Task 4: Effects of mucin biosynthesis and proteostasis regulators on Muc5b protein synthesis and pro-fibrotic mediator production.	Months	
Milestone #1: Test ER Stress activation in MUC5B-expressing lung epithelial cell lines (A549, NCI-H292, and LC-2/ad) and NHBE's.	24-36	#1: 50% (7/2021)
Milestone #2: Test significance of ER Stress activation using lentiviral overexpression and shRNA-mediated knockdown.	30-36	
Milestone #3: Statistical analysis of Data	24-36	
Milestone #4: Manuscript preparation and submission: Data from Aim 2	27-32	
Milestone #5: Manuscript acceptance and publication: Data from Aim 2	33-36	
Specific Aim 3: Determine the critical mechanisms required for MUC5B/Muc5b to promote pulmonary fibrosis.	**Timeline Years 2-4**	
Major Task 1: In vivo studies.	Months	
Milestone #1: Breed St3gal3, Fut2, Agr2, and Ern2 (IRE-1β) knockout mice for experiments. Obtain other candidates as needed.	13-44	#1: 90% (7/2021)
Milestone #2: Test effects genetic deficiency in in vivo *models above on Muc5b levels, localization, and glycosylation and on epithelial proteostasis, ER stress, and fibrosis.*	24-36	#2: 80% (1/2021)
Milestone #3: Test effects of pharmacologic and enzyme interventions in in vivo *models above on Muc5b levels, localization, and glycosylation and on epithelial proteostasis, ER stress, and fibrosis.*	33-44	#3: 50% (9/2021)
Major Task 2: In vitro studies.	Months	
Milestone #1: Test effects genetic deficiency in models above on mucus transport, and epithelial expression of pro-fibrotic mediators in vitro.	38-41	#1 66% (9/2021)
Milestone #2: Test effects of pharmacologic and enzyme interventions on mucus transport, and expression of pro-fibrotic mediators in vitro.	40-46	#1 33% (9/2021)
Major Task 2: Analysis and dissemination of Research.	Months	
Milestone #1: Statistical analysis of Data	13-48	
Milestone #2: Manuscript preparation and submission: Data from Aim 3 (two papers).	28-42	20% (9/2021)
Milestone #3: Manuscript acceptance and publication: Data from Aim 3 (two papers).	36-48	

Project 4
Aim 1: Determine the effect of Muc5b concentration on expression of cilium-associated genes in distal airway stem cell populations following injury in mice.
Major Task 1: Regulatory approval and animal breeding (scheduled for months 0-9; 100% complete).
Subtask 1: Regulatory approval of animal research.
Milestone #1: Secure IACUC approval at University of Colorado. Milestone set for 09-30-2017, completed 06-09-2017. IACUC renewals was completed 04/22/2020.
Milestone #2: Secure ACURO approval. Milestone set for 12-31-2017, completed 09-05-2017. ACURO renewal was completed 08/03/2020.

Subtask 2: Animal breeding for experiments.
Milestone #2: Breed enough Muc5b-/-, Scgb1a1-Muc5bTg and SPC-Muc5bTg for experiments to commence. Milestone set for 06-30-2018, completed 06-30-2018.

Major Task 2: Markers of ciliogenesis (Arl13b and Foxj1), Muc5b and Mmp7 will be co-localized with basal cell markers (Krt5, Krt14, and p63) and β-catenin following injury (scheduled for months 3-24; 100% complete).
Milestone #1: Treat Muc5b-/-, Scgb1a1-Muc5bTg and SPC-Muc5bTg mice with bleomycin and H1N1 virus. Collect tissue for IF staining. Milestone set for 09-30-2018, completed 09-30-2018.
Milestone #2: Perform IF staining, take images, and perform qualitative analysis of the image data. Milestone set for 03-31-19, completed 03-31-19.
Milestone #3: Perform quantitative analysis of the image data and statistical analysis. Milestone set for 09-30-2019, completed 09-30-2019.

Major Task 3: Identify changes in cilium gene expression in isolated DASC populations at multiple timepoints following injury (scheduled for months 3-18; 90% complete).
Milestone #1: Treat Muc5b-/-, Scgb1a1-Muc5bTg and SPC-Muc5bTg mice with bleomycin and H1N1 virus. Milestone set for 06-30-2018, completed 06-30-2019.
Milestone #2: Perform fresh lung tissue digests, DASC isolation, and RNA extractions. Milestone set for 09-30-2018, completed 09-30-2019.
Milestone #3: Run RT-qPCR on the Fluidigm platform. Milestone set for 12-31-2018, 75% completed.
Milestone #4: Statistical analysis of RT-qPCR data and prioritization of genes for Aim 2. Milestone set for 03-31-2019, 75% complete.

Major Task 4: Publication of findings from Aim 1 (scheduled for months 18-24; 90% complete)
Milestone #1: Prepare and submit manuscript. Milestone set for 09-30-2019, 90% completed.

Aim 2: Demonstrate that changes in cilium gene expression in airway progenitor cells affect injury/repair and fibrosis.
Major Task 1: Establish NHBE cell cultures, optimize lenti-shRNA and lenti-ORF protocols, and treatment concentrations (scheduled for months 0-18; 100% complete).
Milestone #1: Establish NHBE cultures, successfully inhibit and overexpress positive control genes. Milestone set for 06-30-2018, 100% completed.
Milestone #2. Optimize bleomycin and H1N1 virus concentrations. Milestone set for 03-31-2019, 100 % completed 03-31-2021.

Major Task 2: Inhibit and overexpress cilium genes, measure injury/repair, regeneration, and Wnt signaling (scheduled for months 18-36; 50% complete).
Milestone #1: Inhibit and overexpress cilium genes of interest. Milestone set for 12-31-2019, 75% completed.
Milestone #2: Treat cells in which cilium genes are inhibited/overexpressed with bleomycin and H1N1. Milestone set for 03-31-2020, 50% completed.
Milestone #3. Measure wound healing, TEER, Wnt signaling. Milestone set for 06-30-2020, 50% completed.
Milestone #4: Statistical analysis of the data and prioritization of genes for Aim 3. Milestone set for 09-30-2020, 25% completed.

Major Task 3: Determine the influence of cilium gene deletion on injury/repair, lung regeneration, and fibrosis in mice (scheduled for months 3-39; 100% complete).
Milestone #1: Breed Arl13 flox/flox and Ift8 flox/flox to Krt5-CreER mice. Breed CKO mice to Muc5b Tg or deficient lines. Treat with tamoxifen. Milestone set for 03-30-2019, 75% completed.
Milestone #2: Treat mice with bleomycin and H1N1. Collect tissue for analysis. Milestone set for 03-31-2020, 90% completed.
Milestone #3. IF staining for Arl13b, Foxj1, Muc5b, Mmp7 Krt5, Krt14, p63, and β-catenin. Milestone set for 09-30-2020, 100% completed.
Milestone #4. Measure collagen content of the lung by hydroxyproline and SHG assays. Milestone set for 09-30-2020, 100% completed.
Milestone #5: Statistical analysis of the data and prioritization of genes for Aim 3b. Milestone set for 12-31-2020, 100% completed.

Major Task 4: Publication of findings from Aim 2 (scheduled for months 36-42; 50% complete).
Milestone #1: Prepare and submit manuscript. Milestone set for 03-31-2021, 50% completed.

Aim 3: Determine the contribution of the MUC5B promoter variant on expression of cilium-associated genes in distal airway stem cell populations in IPF lung.

Major Task 1: Markers of ciliogenesis (ARL13B and FOXJ1), MUC5B and MMP7 will be co-localized with basal cell markers (KRT5, KRT14, and p63) and Wnt signaling marker β-catenin following injury (scheduled for months 0-36; 100% complete).
Milestone #1: Perform IF staining, take images, and perform qualitative analysis of the image data in IPF and control lungs. Milestone set for 03-31-2019, 100% completed.
Milestone #2: Perform quantitative analysis of the image data and statistical analysis. Milestone set for 09-30-2020, 100% completed.

Major Task 2: Measure expression of cilium genes identified in Aims 1-2 in DASCs from IPF and control lungs with and without Muc5b promoter variant (scheduled for months 0-42; 50% complete).
Milestone #1: Perform fresh lung tissue digests, DASC isolation, and RNA extractions from IPF and control lungs. Milestone set for 09-30-2020, 100% completed.
Milestone #2: Run RT-qPCR Taqman assays for genes from Aims 1-2. Milestone set for 12-31-2020, 50% completed.
Milestone #3: Statistical analysis of RT-qPCR data. Milestone set for 03-31-2021, 0% completed.

Major Task 3: Publication of findings from Aim 3 (scheduled for months 42-48; 0% complete).
Milestone #1: Prepare and submit manuscript. Milestone set for 09-30-2021, 0% completed.

b. What was accomplished under these goals?

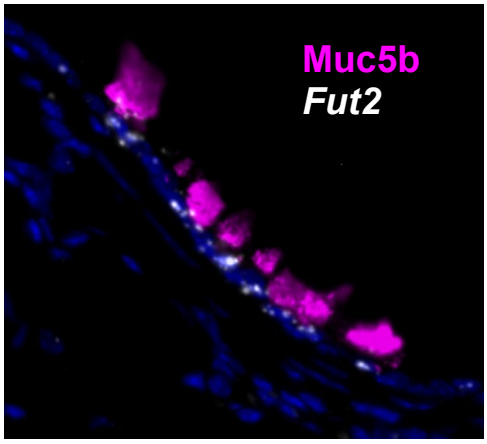
- *For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

Project 1
Specific Aim 1:
• Recruitment of human participants is ongoing at open sites
• 200 first degree relatives of people with IPF referred for study participation
• 131 first degree relatives of people with IPF consented to study participation
• 56 first degree relatives of people with IPF have completed some, but not all study procedures
• 76 first degree relatives of people with IPF have completed all study procedures (informed consent, health questionnaire, blood draw, HRCT scan)
• Radiologic and clinical evaluation by thoracic radiologists and interstitial lung disease specialist clinicians of completed subjects is in process and ongoing
• No adverse events in the human subjects study
Specific Aim 3:
Follow up of preclinical cohort continues
• 320 subjects consented to follow up
• 55 subjects have completed some, but not all study procedures
• 257 subjects completed follow up (informed consent, health questionnaire, blood draw, HRCT scan)
• Radiologic and clinical evaluation by thoracic radiologists and interstitial lung disease specialist clinicians of completed subjects is in process and ongoing
• Manuscript is in process of being written and submitted for publishing
• No adverse events in the human subjects study

Project 2:

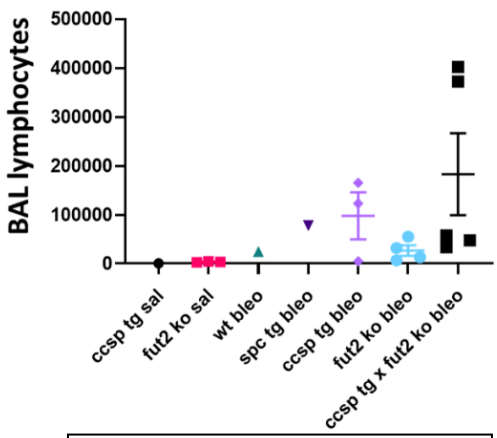
- Performed Micro-C assays in multiple relevant cell types to define 3D chromatin structure of the MUC5B locus
- Analyzed chromatin structure in relationship to MUC5B regulation using ATAC-seq over a differentiation time course
- Elucidated impact of hypoxia on MUC5B expression
- Developed epithelial-fibroblast co-culture model to define the contribution of MUC5B to fibrotic response.
- Utilized synergistic activation mediator (SAM) approach to over-express MUC5B in A549 epithelial cells

Project 3:
Muc5b and Fut2 are co-expressed in airways from bleomycin challenged C57BL/6J mice.

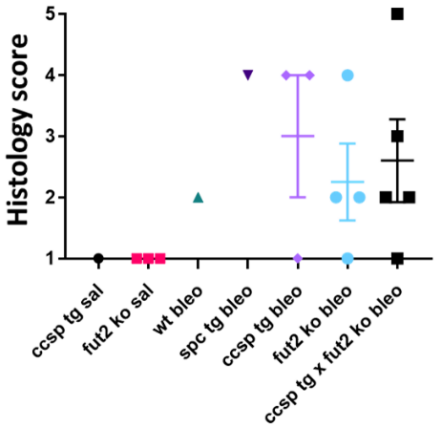


Lungs from bleomycin challenged mice were fixed in methacarn and labeled for Muc5b protein (magenta) and for *Fut2* mRNA (white, RNAscope). We are conducting stereological assessments to determine the quantities and locations of each of these signals.

Fut2 expression may be protective against fibrosis in bleomycin challenged mice (prelim. data below).

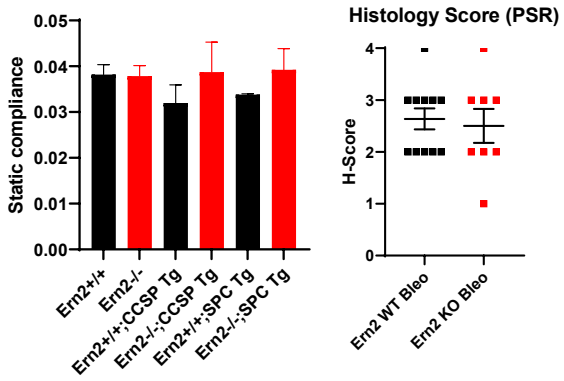


Lungs from bleomycin challenged mice were lavaged for enumeration of leukocytes. Lymphocytic inflammation is prominent in mice 21 d after bleomycin challenge. Fut2 knockout animals have increased lymphocytes, suggesting that Fut2 protects from lung injury.

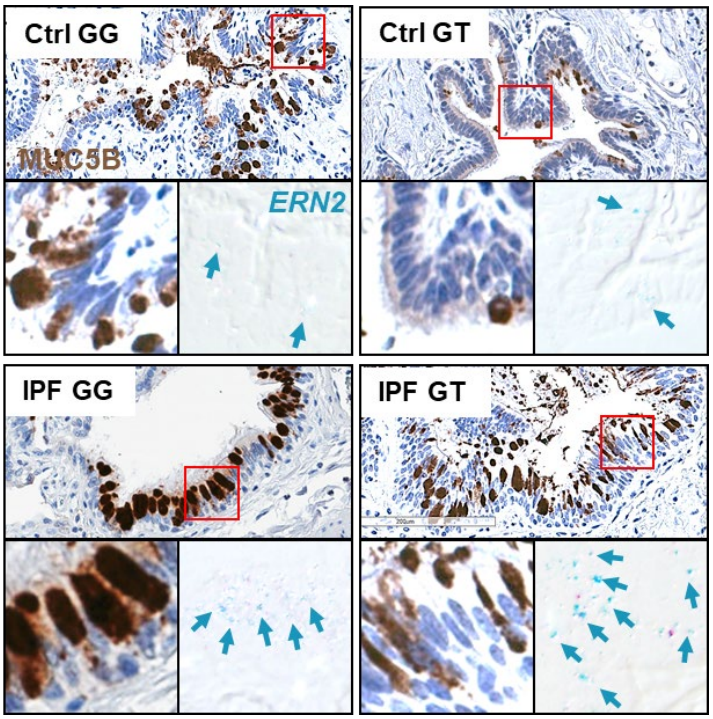


Lungs from bleomycin challenged mice were fixed in methacarn and labeled for collagen using picrosirius red staining. Initial semi-quantitative assessments above suggest that absence of Fut2 worsens fibrosis. Additional samples have been accrued and are being used for stereological quantification.

***Ern2* deficiency does not affect the development of PF in mice with normal Muc5b expression levels, but there is a trend towards protection in Muc5b-overexpressing mice.**



Left: Bleomycin challenged mice (day 21) were euthanized, tracheostomized, and placed on a flexiVent and used to calculate quasi-static compliance. CCSP- and SPC-promoter driven Muc5b transgenic mice have worsened compliance, which is consistent with exaggerated fibrosis in Muc5b overexpressing animals. Ern2 deficiency appears to provide protection (n = 3-7 per group). **Right**: We completed histologic analysis of Ern2 WT and KO mice on wt Muc5b backgrounds. Picrosirius red staining (PSR) is consistent with compliance data.



Top: Healthy control lungs from subjects with GG and GT alleles for SNP rs35705950. MUC5B protein (brown) is found in small airways, and its expression is accompanied by small amounts of ERN2 assessed at the transcript level using RNAscope (teal).

Bottom: IPF lungs from subjects with GG and GT alleles for SNP rs35705950. MUC5B staining is more abundant, and high levels are accompanied by increased ERN2.

Project 4:

Aim 1: Determine the effect of Muc5b concentration on expression of cilium-associated genes in distal airway stem cell populations following injury in mice.

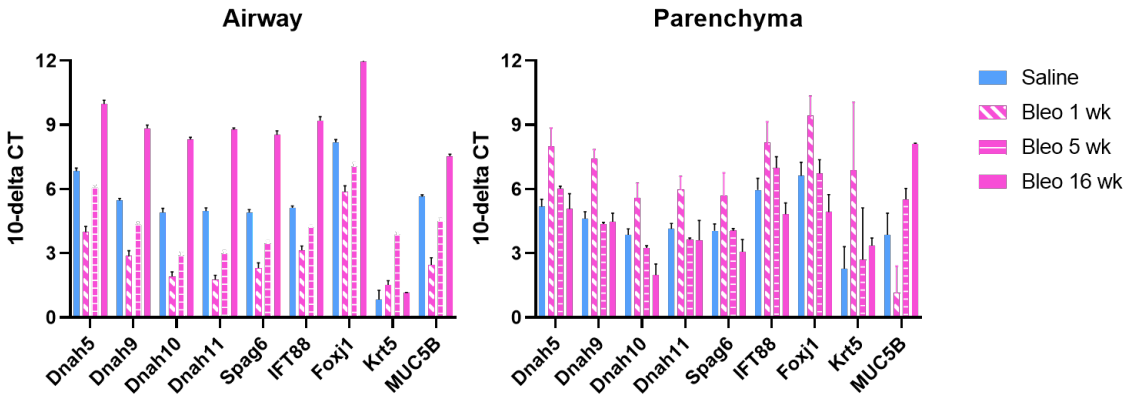
Major Task 1: Regulatory approval and animal breeding (scheduled for months 0-9; 100% complete). This task was completed in previous years.

Major Task 2: Markers of ciliogenesis (Arl13b and Foxj1), Muc5b and Mmp7 will be co-localized with basal cell markers (Krt5, Krt14, and p63) and β-catenin following injury (scheduled for months 3-24; 100% complete). The task, as originally proposed, was completed at the end of Year 2 and additional data were included in Year 3. No additional work was performed in Year 4.

Major Task 3: Identify changes in cilium gene expression in isolated DASC populations at multiple timepoints following injury (scheduled for months 3-18; 90% complete).

We have isolated epithelial cells from the airway tree and parenchyma of C57BL/6J mice at 0 (saline), 1 week, 5 weeks, and 16 weeks post-bleomycin to measure expression of key cilia structural genes. Three different categories of the ciliated cell-related genes were examined, including (1) cilia-related transcription factors Foxj1 and cMyb, (2) cilia intraflagellar transport gene Ift88 (intraflagellar transport 88), and (3) cilia motility and structural integrity genes DNAH5 (dynein axonemal heavy chain 5), Dnah9 (dynein axonemal heavy chain 9), Dnah10 (dynein axonemal heavy chain 10), Dnah11 (dynein axonemal heavy chain 11), and Spag6 (sperm associated antigen 6). The expression of the cilia-related genes was significantly down-regulated in the airway at 1 wk

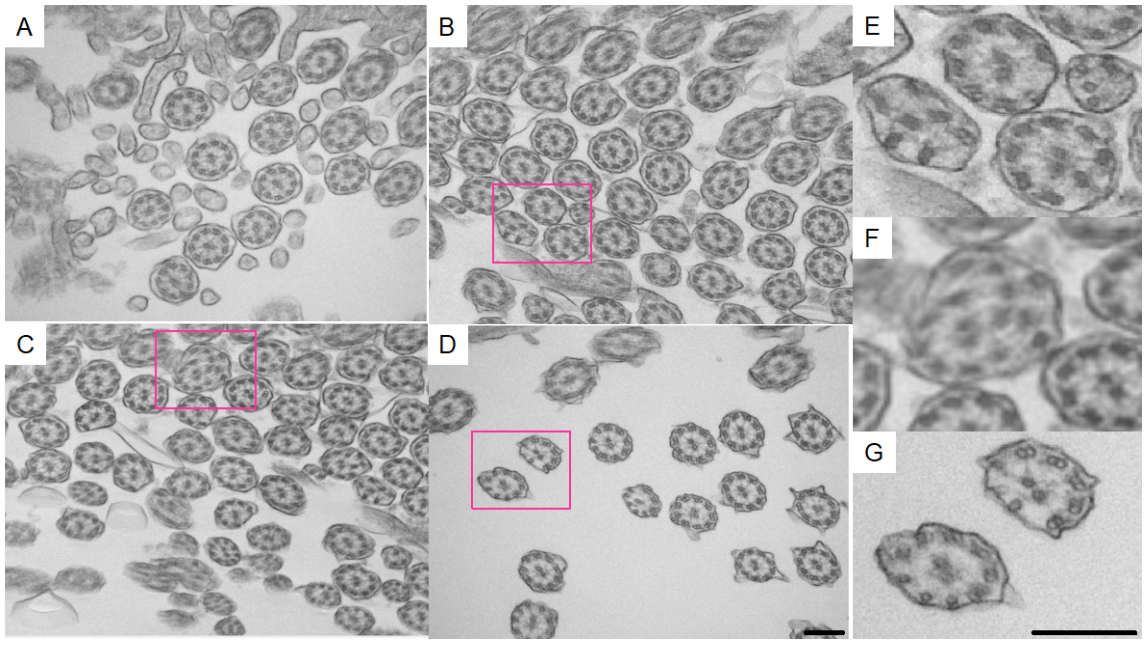
bleomycin treatment which is consistent with acute damage response by cigarette smoke in human airway epithelium [1] and our previous data. Expression of cilium genes returns to saline levels at 5 weeks and increased at 16 wks which is consistent with cMyb and Foxj1 staining data. Consistent with IF data, mRNA levels of cilia structure genes are also abnormally increased. Interestingly, we had opposite results in parenchyma region which also is consistent with our prior observations by IF. These results imply that airway and parenchymal cells respond differently and likely invoke different mechanisms.



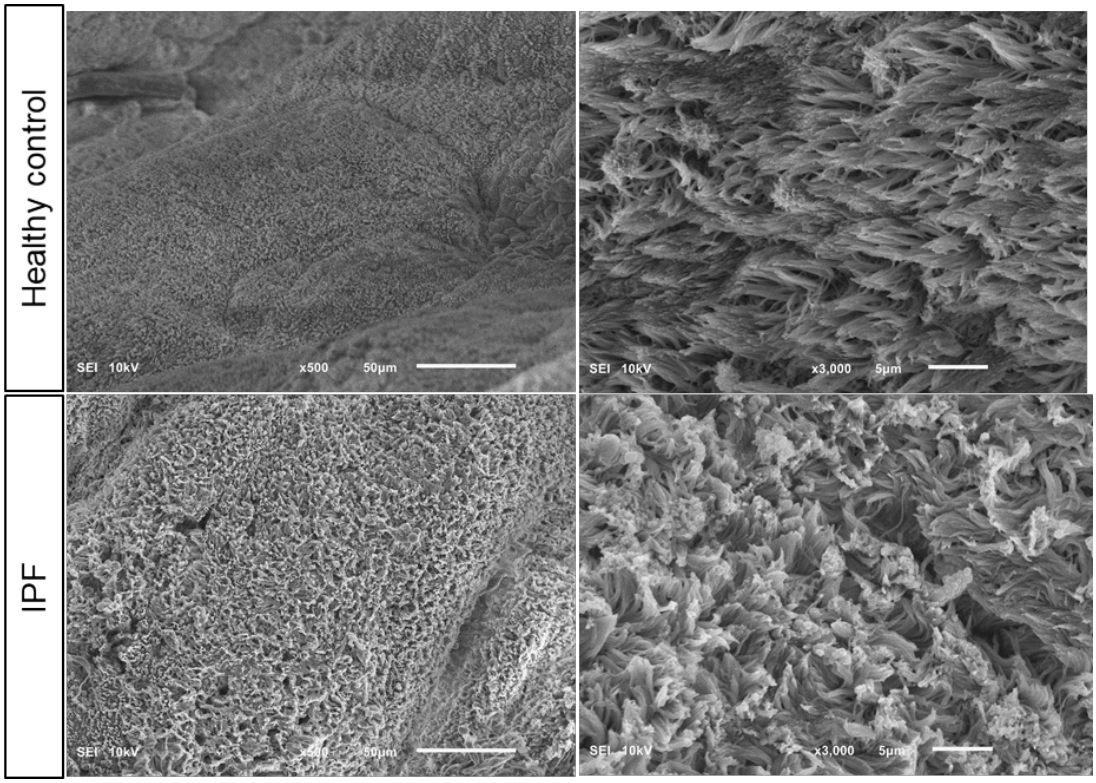
We are in the process of analyzing RNA-seq data from airway and parenchyma cells separately to identify mechanism by which airway and parenchymal cells differently respond to bleomycin and affect mucociliary clearance. All data have been collected and quality controlled and this analysis is currently in progress, with expectation of finishing it in Q1 of Year 5 (NCE year).

Given the small numbers and difficulty of isolating pure Krt5 cell populations, we are in the process of breeding enough mice to isolate Krt5 positive cells using the mTmG tag in Krt5-CreERT2-RosamT/mG; upon treatment with tamoxifen, Krt5 cells will be labeled with GFP and we can use flow cytometry to sort them using GFP. These experiments will be completed by the end of Q2 of Year 5 (NCE year).

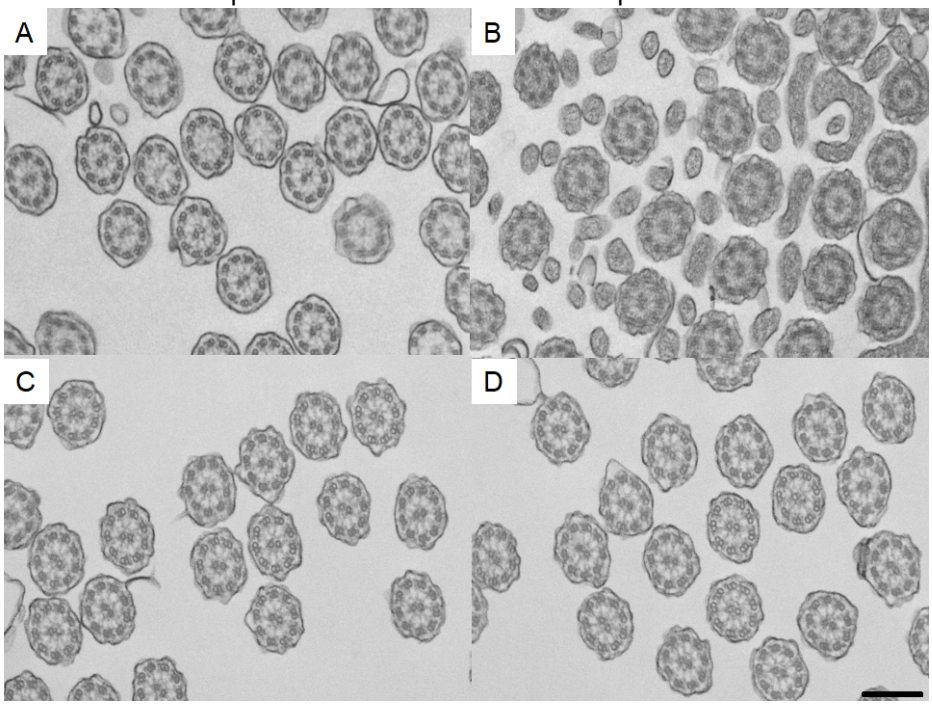
Major Task 4: Publication of findings from Aim 1 (scheduled for months 18-24; 90% complete)
Milestone #1: Prepare and submit manuscript. Milestone set for 09-30-2019, 90% complete.
The manuscript draft is complete and is currently under review by co-authors. Manuscript will be submitted by the end of October 2021. During Yea4, we decided to add an experiment to check whether we observe structural defects in cilia in IPF lungs, similar to what we observed in bleomycin-treated mice in Major Task 2 of this aim in previous years. In transmission electron microscopy (TEM) imaging, we observed that, in contrast to motile cilia structure of healthy tissue containing the 9+2 axoneme structure (A), IPF tissue had incomplete 9+2 structure or ultrastructure (B~G) especially in small airways.



Scanning electron microscopy (SEM) imaging also showed that uncoordinated motile cilia as compared to control. Defect in cilia structure and orientation is crucial for mucociliary clearance which is consistent with our previous ciliary beat frequency data. Our results raise the possibility that defective repair/regeneration process may play a role in pathogenesis in IPF airway epithelia.



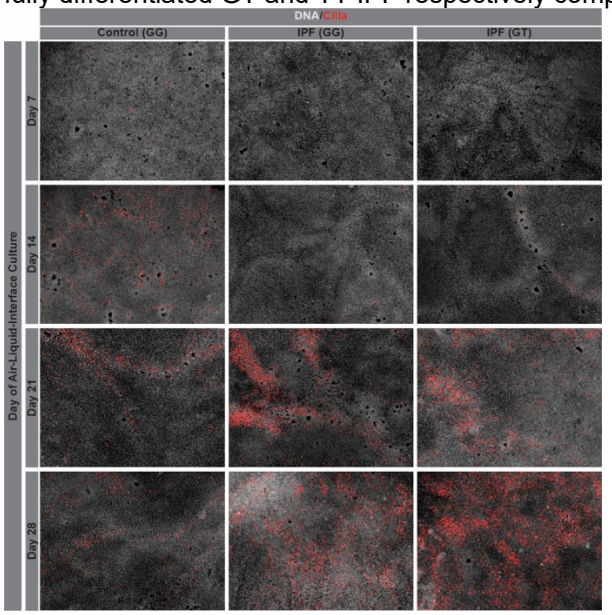
However, we were not able to detect defect in structure in IPF large airways (B~D) compared to healthy individual (A) in TEM imaging which is also consistent with our previous ciliary beat frequency data in organoids. We believe these results are important additions to the manuscript.

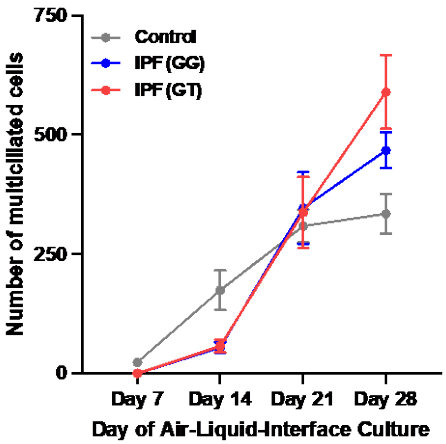


Aim 2: Demonstrate that changes in cilium gene expression in airway progenitor cells affect injury/repair and fibrosis.

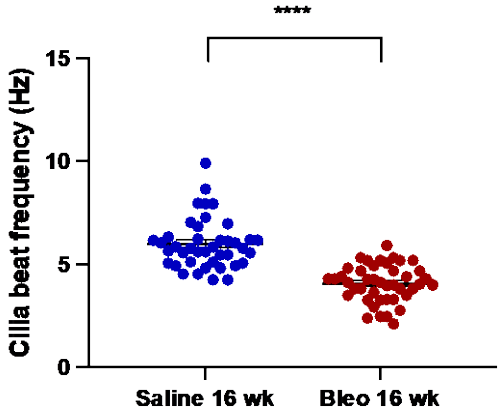
Major Task 1: Establish NHBE cell cultures, optimize lenti-shRNA and lenti-ORF protocols, and treatment concentrations (scheduled for months 0-18; 100% complete).

We have been routinely culturing NHBE cells and have optimized bleomycin concentration (to observe an increase in MUC5B protein secretion with no cell death). Before we begin experiments that change expression of cilia genes, we characterized the extent of ciliogenesis in IPF compared to control donor cells. We observed delayed ciliogenesis in IPF compared to controls at early tiempoints dring differentiation of the airway epithelia but increased ciliogenesis in IPF compared to control after cells are fully differentiated. This matches the timecourse of ciliogenesis we observed in the bleomycin animal model. We also observed 37% and 76% increased ciliogenesis in fully differentiated GT and TT IPF respectively compared to GG IPF at 28 days post ALI culture.



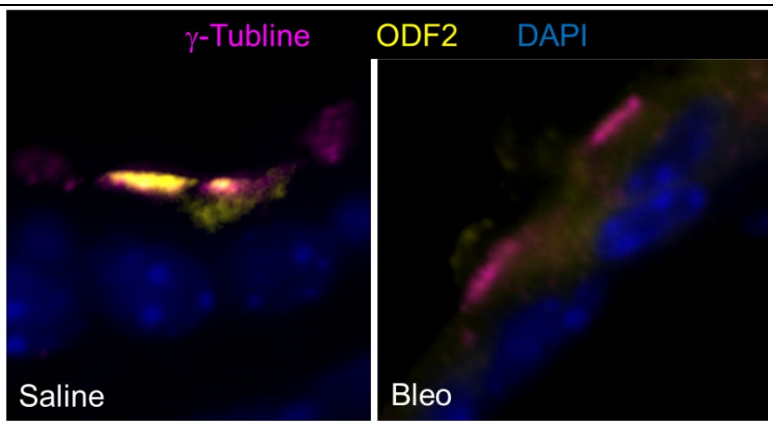


In addition to NHBE cultures, we have implemented the published protocol for airway-derived organoid cultures (Sachs. EMBO J. 2019; 38: e100300) and a method for measuring ciliary beat frequency (CBF) using kymographs. In previous years, we observed a decrease in CBF in small (but not large) airways of IPF comapred to control subjects. We are in the process of replicating these findings in organoids derived from additional individuals. During the time that we did not have access to human tissue, we worked on a protocol to develop organoid cultures from mouse proximal and distal airways. In mouse organoids, we were able to isolate airway tree from mouse lung so that we can grow proximal airway-derived organoids. We were abale to grow airway-derived organoid using proximal airway but not with distal airways because of the lack of stem cells in the distal airway. Using this model we were able to measure CBF and compared CBF in bleomycin treated mice with saline control group. We observed (i) 33% reduced CBF in organoids treated with bleomycin as opposed to saline control and (ii) uncoordinated cilia moving in the same multiciliated cell even though it was not quantifiable.

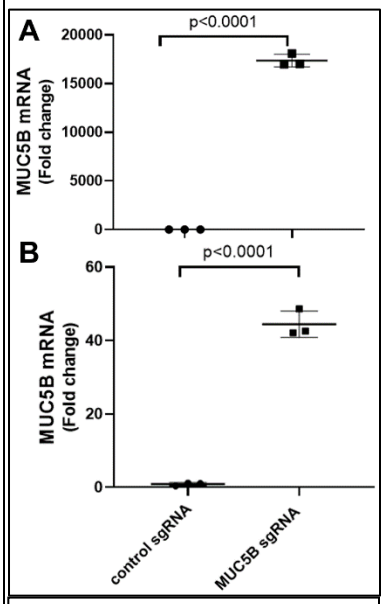


p-value < 0.0001

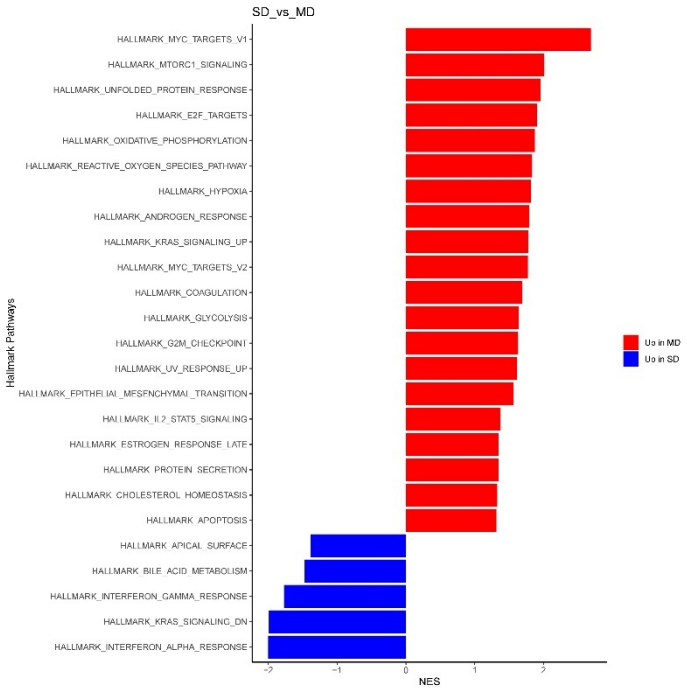
The uncoordinated cilia movement is mostly caused by defect in association of basal body and basal feet. So, we stained basal body (gamma-tubulin) and basal feet (Odf2) and found that basal feet was not associated with basal body in bleo treated mice airway epithelial cells whereas basal body is aligned on apical membrane in saline control.



Major Task 2: Inhibit and overexpress cilium genes, measure injury/repair, regeneration, and Wnt signaling (scheduled for months 18-36; 50% complete).



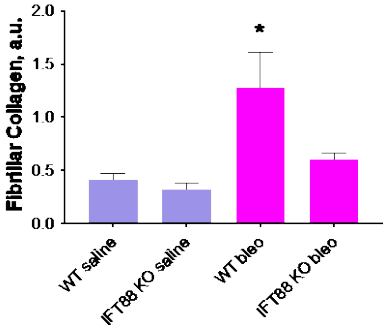
CRISPRa overexpression of MUC5B in airway epithelial cells grown in submerged culture **(A)** and at air-liquid interface **(B).**

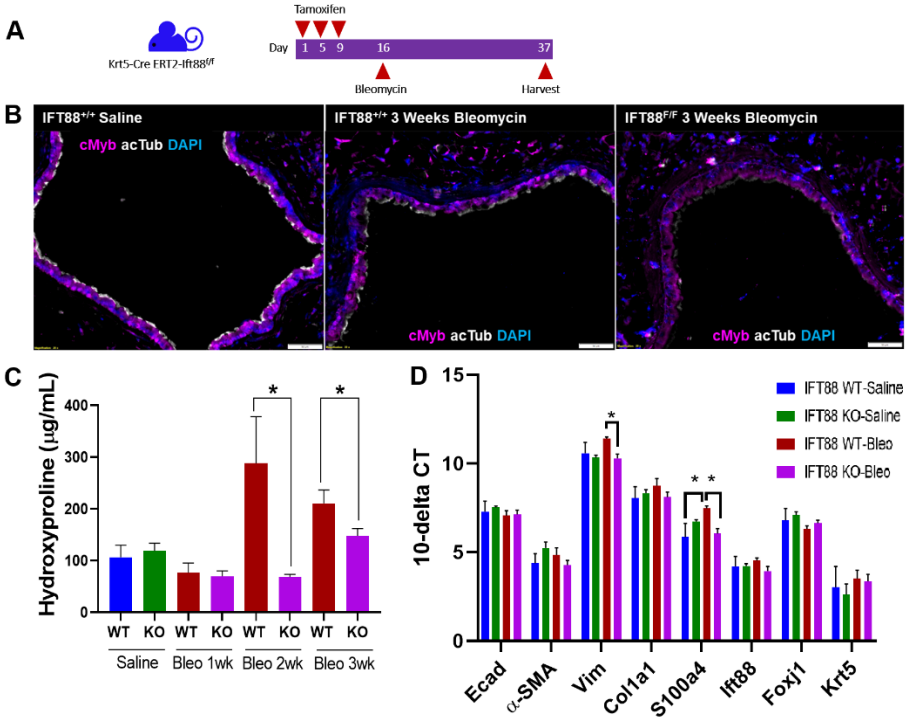


We have successfully inhibited miR-34 (25-100 fold downregulation, depending on the concentration of the anatgomiR), a key micro RNA involved in multiciliogenesis and are in the process of analyzing cells from these cultures. We are also in the process of using CRISPR-dCas9 technology (CRISPRa and CRISPRi systems as well as lentiviral constructs) to overexpress and inhibit MUC5B and ciliogenesis related genes in NHBE and airway-derived organoid cultures. At this time, we have successfully overexpressed MUC5B in airway epithelial cells using CRISPRa technology and have perfromed RNA-seq analysis of transcriptional profiles of these cells. Gene set enrichemtn analysis (GSEA) demosntrates that overexpression of MCU5B in airway epithelial cultures reuslts in multiple pathways. We are in the process of a focused analysis of expression of ciliogenesis genes in this system. Once key targets are identified, we will use the same technology to overxpress and inhibit these ciliogenesis genes. This work with be completed by the end of Q3 of Year 5 (NCE year)

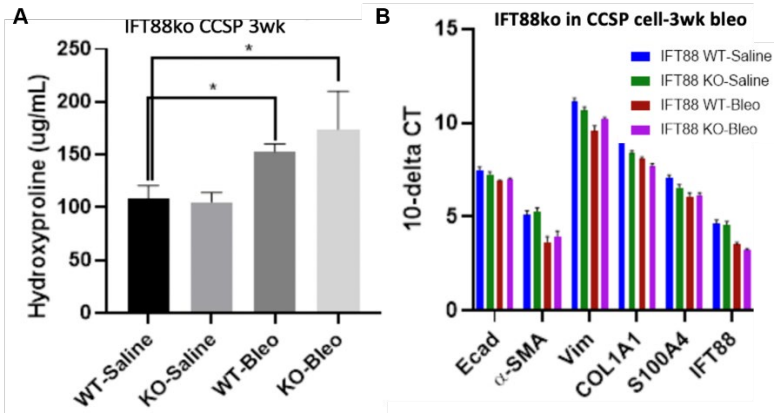
Major Task 3: Determine the influence of cilium gene deletion on injury/repair, lung regeneration, and fibrosis in mice (scheduled for months 3-39; 100% complete).

Our work in previous years demonstrated that mice with a conditional deletion of Ift88 in Krt5 basal cells (Krt5-CreERT2-IFT88$^{flox/flox}$ mice treated with tamoxifen) have reduced fibrosis by hydroxyproline measurements at 3 week post-bleomycin, have reduced or delayed multiciliogenesis and cystic structure formation in the parenchyma. This past year, we confirmed the hydroxyproline results at 3 weeks by confocal/multiphoton-excitation fluorescence microscopy with second harmonic generation (SHG) imaging of fibrillar collagen. We also examined fibrosis at 1 and 2 weeks and demonstrated reduced fibrosis at 2 weeks. We hypothesized that Ift88 knock-out in Krt5 cells reduces bleomycin-induced fibrosis in part via inhibiting epithelial to mesenchymal transition (EMT). We measured EMT markers (alpha-SMA, Vim, Col1a1, S100a4) using RT-qPCR in in whole lung tissue. Bleomycin slightly increased mesenchymal markers at 3 weeks posts-treatment and interestingly, two of the marker genes were reduced in the Ift88 conditional deletion in Krt5 mice. These data imply that reduction in lung fibrosis in Ift88 conditional deletion in Krt5 cells is mediated in part by reduced EMT.





To determine whether club cell progenitors play a role in this process, we examined mice with a conditional deletion of Ift88 in club cells (Scgb1a1-CreERT2-IFT88$^{flox/flox}$ mice treated with tamoxifen). We did not observe differences in lung fibrosis at 3 weeks nor differences in expression of EMT markers, suggesting that only primary cilia on Krt5 basal cell pay a role in delaying multiciliogenesis and reducing lung fibrosis.

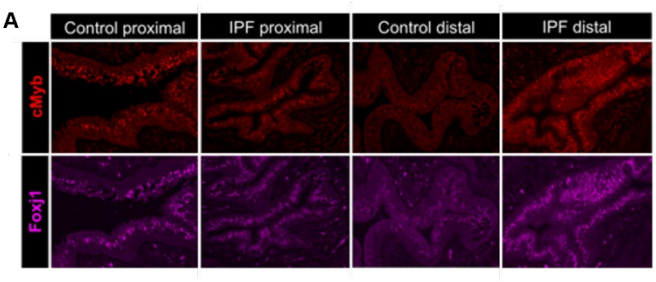


Major Task 4: Publication of findings from Aim 2 (scheduled for months 36-42; 50% complete).
Dr. Kim is in the process of writing organoid manuscript that reports the results of reduced ciliary beat frequency both in IPF small airways and in bleomycin-treated mice. This manuscript will be submitted by the end of Q1 of Year 5 (NCE year).

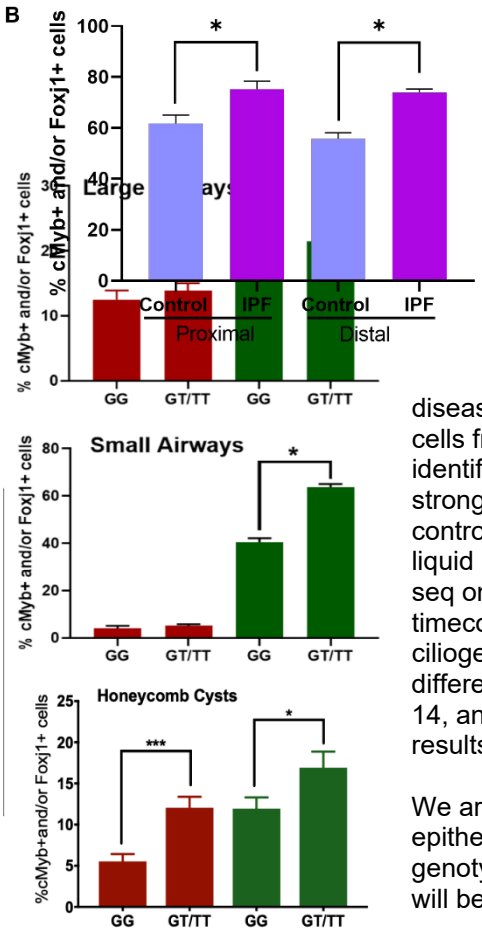
Aim 3: Determine the contribution of the MUC5B promoter variant on expression of cilium-associated genes in distal airway stem cell populations in IPF lung.

Major Task 1: Markers of ciliogenesis (ARL13B and FOXJ1), MUC5B and MMP7 will be co-localized with basal cell markers (KRT5, KRT14, and p63) and Wnt signaling marker β-catenin following injury (scheduled for months 0-36; 100% complete).

Using the same panel of markers for ciliogenesis as in Aim 1, we have continued to quantify the extent of ciliogenesis in airway epithelia of IPF and control subjects. We observe an increased in multiciliogenesis markers MYB and FOXJ in both proximal and distal airways in IPF compared to controls.



We also completed the analysis by MUC5B genotype in IPF tissue. There are no differences in MYB or FOXJ1 positive cells in large airways by disease or genotype. Analysis of small airways revealed significantly increased numbers of FOXJ1 positive cells in GT/TT compared to GG IPF subjects ($p<0.0001$). Importantly, there are statistically significant differences by *MUC5B* genotype in both MYB and FOXJ1 in honeycomb-cyst regions, supporting the idea that these lesions may be a failed lung regeneration attempt in diseased lung. We have previously shown that there are no differences by genotype in large airways.



Major Task 2: Measure expression of cilium genes identified in Aims 1-2 in DASCs from IPF and control lungs with and without Muc5b promoter variant (scheduled for months 0-42; 50% complete).

We have completed isolation and cryopreservation of airway cells from IPF explanted lungs and non-diseased donor lungs. We currently have 20 IPF and 20 non-diseased samples banked. We previously performed bulk RNA-seq on fresh cells from 10 IPF and 10 control lungs. We analyzed the transcriptome data to identify genes that are highly correlated with MUC5B expression and identified strong enrichment for ciliogenesis genes in IPF airway epithelial but not in control airway epithelia. We also previously cultured and differentiated on air-liquid interface cells from 3 IPF and 3 control lungs, and performed bulk RNA-seq on cells that have bene on ALI for 4, 8, 12, and 14 days to characterize the timecourse of expression of ciliogenesis genes. This analysis reveals decreased ciliogeneiss in IPF at early timepoints (days 4 and 8) when cells are not differentiated, similar ciliogenesis between cases and controls at days 12 and 14, and increased ciliogenesis in IPF in fully differentiated cells at day 28. These results are in agreement with the results in animal models in Aim 1.

We are in the process of performing single cell RNA-sequencing on distal airway epithelia from 10 IPF and 10 control subjects, half with GG and half with GT/TT genotype. Sequencing data delivery is expected by the end of Q1 and analysis will be performed in Q2 and Q3 of Year 5 (NCE year).

Major Task 3: Publication of findings from Aim 3 (scheduled for months 42-48; 0% complete).

This manuscript will be prepared in Q3 and submitted by the end of Q4 of Year 5 (NCE year).

Biostats Core:

The core, in collaboration with other projects, has completed a number of genetic analyses that have refined our understanding of the location of both common and rare variants that contribute to risk of pulmonary fibrosis, and that are used in the derivation and validation of the predictive models for Aims 2 and 3 of Project 1 that inform all of the projects. We have also completed most re-analysis of our systems biology data that integrates genotype, methylation at CpG sites, and RNA-seq based gene expression data. Over the last year, we have completed the following analyses:

We completed a set of analyses detailing the inter-relationships between genotype, gene expression and IPF to contribute to a manuscript in preparation. Specifically, we conducted all of the analyses required to assess the

potential for mediation by gene expression of the relationship between an IPF risk variant and IPF; we conducted these for the primary risk variant in the DSP gene and the primary risk variant in the promoter of the MUC5B gene. Since the number of data points and resulting files are very large, we produced the tables included in the submitted manuscript.

We completed updated PEER factor analyses of the RNA-seq samples that are included in the analyses that are restricted to those with all three data types being used in the systems biology analyses: SNP data, RNA-seq data, methylation data. These analyses revealed that adjustments for age, sex and genetic principal components are sufficient to account for any cryptic sources of variation in the RNA-seq data not attributable to genetics or methylation. Similar PERR factor analyses on the methylation data suggested that in addition to age, sex and genetic principal components, methylation chip is also necessary to adjust for potential confounding and make best use of precision variables. These are the variables that were taken into the next set of analyses described below.

Table 1: Summary of eQTLs and mQTLs for SNPs with significant relationships for both QTL types

	eQTL - Cases		***eQTL - Controls***		***mQTL - Cases***	***mQTL - Controls***
SNP	# Significant eQTL	Gene Names	# Significant eQTL	Gene Names	# Significant mQTL	# Significant mQTL
rs4449583	4	SNCAIP/CDHR2/LPCAT1	39	ADRA1B/FGFR4/PLCXD3 +36 more	2927	0
rs6963345	1	TRIM4	0		18	0

We conducted analyses to identify pairs of expression and methylation in regions of close physical genetic distance proximity to each other that were a) correlated with each other and b) associated with the same IPF genetic risk variant. We identified several such pairs, and further investigated the extent to which an association between the genetic risk variant and expression could be explained by the variant association with methylation. Table 1 shows the summary information for SNPs that have a) and b) satisfied. As an illustration of the relationships discovered, Figures 1-3 show the relationship between SNP rs6963345 and expression (Figure 1), methylation (Figure 2) and how the correlation between methylation and expression differ by rs6963345 genotype (Figure 3). These results are being prepared in a manuscript to be submitted for publication.

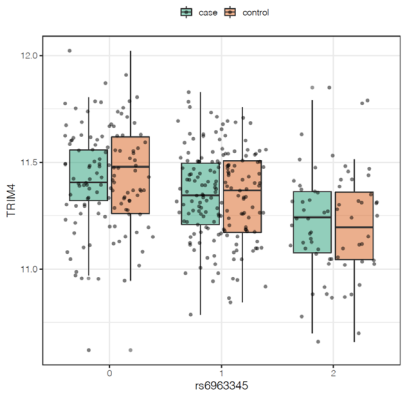


Figure 1: Association between rs6963345 and expression of *TRIM4*.

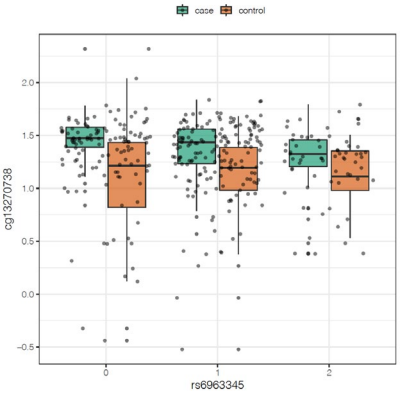


Figure 2: Association between rs6963345 and methylation (cg13270738)

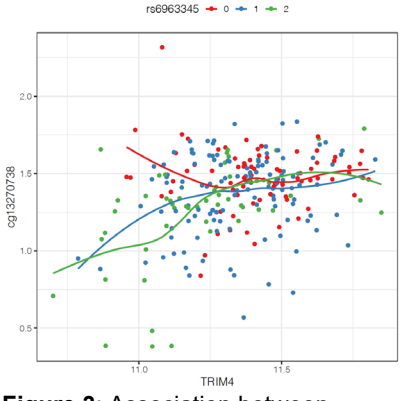


Figure 3: Association between expression of TRIM4 and cg13270738 by genotype at rs6963345.

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

- *If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."*
- *Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

Project 1: Nothing to report
Project 2: Nothing to report
Project 3: Nothing to report
Project 4: Dr. Kim was scheduled to participate in the Cilia, Mucus and Mucociliary Gordon research seminar for trainees in April 2020 but this was postponed to 2021 due to COVID-19.
Biostatistics Core: Nothing to report

d. How were the results disseminated to communities of interest?

- *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
- *Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

Project 1: CT scan results are shared with participants following central review of their imaging at the University of Colorado.
Project 2: Nothing to report
Project 3: Publications, primarily. Due to the pandemic, there was no travel in the past year.
Project 4: Dr. Kim presented her airway-derived organoid work at the EMBL Symposium "Organoids: Modelling Organ Development and Disease in 3D Culture" held virtually Oct 21-25 2020 as well as as an oral presentation by Dr. Eunjoo Kim at the International Society for Stem Cell Research (ISSCR) 2021 virtual meeting on 6/22/2021. Dr. Kim presented her work locally at the Pulmonary Research in Progress and Dr. Yang presented the work at the Department of Medicine Research and Innovation Conference; both presentations were done in the fall of 2020.
Biostatistics Core: Nothing to report

e. What do you plan to do during the next reporting period to accomplish the goals?

- *If this is the final report, state "Nothing to Report."*
- *Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

Project 1:

- Referral, recruitment, and enrollment of research participants will continue during the no cost extension period
- Continue to administer informed consent to interested people and enroll participants in the study. For enrolled participants, continue to complete study procedures (blood draw, CT scan)
- Phenotyping of participants and sharing CT scan results will continue in next reporting period

Project 2:

- We will continue analysis of fibrotic gene expression in MUC5B over-expressing A549 cells (Aim 3).

Project 3: We are completing quantification of fibrosis in ENR2 KO mice with and without Muc5b. We are also quantifying ERN2 and MUC5B signals in human specimens.
Project 4:
Aim 1: Determine the effect of Muc5b concentration on expression of cilium-associated genes in distal airway stem cell populations following injury in mice.
Major Task 3: Identify changes in cilium gene expression in isolated DASC populations at multiple timepoints following injury (90% complete).
We are in the process of starting bleomycin and H1N1 treatments to isolate Krt5+ cells after injury and perform gene expression analysis.

Major Task 4: Publication of findings from Aim 1 (90% complete)
We plan on submitting the publication that describes the results from Aim 1 by the end of October 2021.

Aim 2: Demonstrate that changes in cilium gene expression in airway progenitor cells affect injury/repair and fibrosis.
Major Task 2: Inhibit and overexpress cilium genes, measure injury/repair, regeneration, and Wnt signaling (50% complete).
We will use CRSIPRa, CRISPRi, lenti-shRNA and lenti-ORF protocols to alter expression of ciliogenesis genes both in ALI cultures and in airway-derived organoids.

Major Task 4: Publication of findings from Aim 2 (50% complete)
Organoid manuscript will be submitted by the end of 2021.

Aim 3: Determine the contribution of the MUC5B promoter variant on expression of cilium-associated genes in distal airway stem cell populations in IPF lung.
Major Task 2: Measure expression of cilium genes identified in Aims 1-2 in DASCs from IPF and control lungs with and without Muc5b promoter variant (scheduled for months 0-42; 50% complete).
We will finish data collection by the end of 2021 and analysis of single cell RNA-sequencing data of airway cells isolated from IPF and control lungs, stratified by the *MUC5B* genotype, by middle of 2022.

Major Task 3: Publication of findings from Aim 3 (scheduled for months 42-48; 0% complete).
We will submit result for publication by the end of September 2022.

Biostats Core: we will complete the manuscript reporting the multiomic results.

Impact

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

Project 1:
This study's enrollment of healthy relatives of people with sporadic IPF is spreading the idea that IPF can run in families even when there is no known family history, which is important for explaining risk of IPF. As we continue to reach out to physicians about patient recruitment, we share the knowledge that genetics are a key risk factor for IPF.
Project 2: We have extended our key finding from prior reporting periods regarding the close proximity of RNA polymerase II loading and the MUC5B -3 kb genetic variant to other contexts. Specifically, based on this finding we have defined other novel genetic associations that link SNPs, targets genes, and lung diseases. Our data have helped inform a general new strategy for linking enhancer regions with genetic risk of lung disease.
Project 3:
We have championed a concept that is driving the pulmonary fibrosis field in a new direction. Along with other Program Project Grant teams, we are demonstrating that mucociliary dysfunction is an important and treatable phenomenon in lung fibrosis. Building on our findings related to mucin assembly and ER proteostasis, we are now also analyzing how assembly of large mucin molecules is regulated by specialized secretory machinery.
Project 4:
Our work has identified critical timepoints at which ciliogenesis is overactive following lung injury, in the context of overproduction of the airway mucin MUC5B. We have also demonstrated changes in cilium gene expression in isolated airway epithelia and at the single cell level in lung tissue from IPF and control subjects.
Biostatistics Core: nothing to report

b. What was the impact on other disciplines?

Project 1: Nothing to report
Project 2: Nothing to report
Project 3: We have also been able to extend this work into other pulmonary diseases (specifically asthma and lung adenocarcinoma) as well as gastrointestinal infections and injury. We are also contributing to the basic biology of non-conventional protein assembly and secretion mechanisms.
Project 4: Nothing to report
Biostatistics Core: Nothing to report

c. What was the impact on technology transfer?

Project 1 – nothing to report
Project 2 – nothing to report
Project 3 – nothing to report
Project 4 – nothing to report
Biostatistics Core – nothing to report

d. What was the impact on society beyond science and technology?

Project 1 – nothing to report
Project 2 – nothing to report
Project 3 – nothing to report
Project 4 – nothing to report
Biostatistics Core – nothing to report

Changes/Problems

a. Changes in approach and reasons for change

- *Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*

Project 1: No significant changes are anticipated. We will continue to emphasize physician and patient outreach to improve recruitment into the study.
Project 2: Nothing to report.
Project 3: Early in Year 4, we were recovering from COVID-19 pandemic related slow-downs in product availability. In total, this had an estimated 20% negative impact on productivity during Year 4.
Project 4:
Changes in Aim 1: We will use single cell RNA sequencing instead of qPCR for examination of gene expression in mouse airway epithelial cells (and bulk RNA-seq for Krt5 basal cells).
Changes in Aim 2: (a) We have added organoid cultures to complement ALI cultures. (b) We have added CRISPRa and CRISPRi in addition to lentiviral approaches for manipulation of gene expression in cell culture.
Changes in Aim 3: We will use single cell RNA sequencing instead of qPCR for examination of gene expression in human airway epithelial cells.
Biostatistics Core: nothing to report

b. Actual or anticipated problems or delays and actions or plans to resolve them

Project 1: Recruitment has fluctuated over the past reporting period due to the COVID-19 pandemic and the emergence of more transmissible variants such as the delta variant. Participants were reluctant to undergo elective study procedures during a pandemic. We are reaching out more consistently and more frequently to referral sites to increase the pace of referrals. A study recruitment website is now publicly available and allows potential participants to self-refer to the study
Project 2: We had some ongoing delays due to COVID impacts and are thus wrapping up publications, etc. during the NCE period.
Project 3: Our benchwork was slowed down by approximately 50% over a 3 month period due to COVID-19 shutdown and re-opening. Since June, we have operated with 100% personnel on-site under conditions of physical distancing and shift work. During shutdown we were able to perform on-line histopathology studies, maintain our mouse colony, and conduct data analyses. We anticipate remaining open during the coming months, but we are preparing for similar virtual research activities as needed.
Project 4: COVID19 continues to cause delays to our work with limited availability of reagents for ALI cultures but we are working as quickly as we can to complete experiments in Aim 2. All other tasks are in the process and we have no concerns that they will be completed.
Biostatistics Core: nothing to report

c. Changes that had a significant impact on expenditures

- *Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

Project 1: no changes
Project 2: no changes
Project 3: no changes
Project 4: no changes
Biostatistics Core: no changes

d. Significant changes in the use or care of human subjects, vertebrate animals, biohazards, and/or select agents

- *Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

Project 1: no changes
Project 2: no changes
Project 3: no changes
Project 4: no changes
Biostatistics Core: no changes

e. Significant changes in use or care of human subjects

Project 1: no changes
Project 2: no changes
Project 3: no changes
Project 4: no changes
Biostatistics Core: no changes

f. Significant changes in use or care of vertebrate animals

Project 1: no changes
Project 2: no changes
Project 3: no changes
Project 4: no changes
Biostatistics Core: no changes

g. Significant changes in use of biohazards and/or select agents

Project 1: no changes
Project 2: no changes

Project 3: no changes
Project 4: no changes
Biostatistics Core: no changes

Products

a. Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

- ***Journal publications****. List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*
- ***Books or other non-periodical, one-time publications.*** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*
- ***Other publications, conference papers, and presentations.*** *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Project 1: Nothing to report
Project 2:
Abstract, 2021 Aspen Lung Conference
PRECISION RUN-ON SEQUENCING (PRO-SEQ) ASSIGNS REGULATORY FUNCTION TO ACUTE LUNG INJURY-ASSOCIATED GENETIC VARIANTS
Project 3:

1. Fakih D, Rodriguez-Piñeiro AM, Trillo-Muyo S, **Evans CM**, Ermund A, Hansson GC. Normal murine respiratory tract has its mucus concentrated in clouds based on the Muc5b mucin. *Am J Physiol Lung Cell Mol Physiol*. 2020 318:L1270-L1279. PMID:32348677. PMCID: PMC7347266.
2. Laube BL, Carson KA, **Evans CM**, Richardson VL, Sharpless G, Zeitlin PL, Mogayzel PJ Jr. Changes in Mucociliary Clearance Over Time in Children With Cystic Fibrosis. *Pediatr Pulmonol*. 2020. 55:2307-14. PMID: 32427408. PMC PMC7674244.
3. Ganguly K, Krishn SR, Rachagani S, Jahan R, Shah A, Nallasamy P, Rauth S, Atri P, Cox JL, Pothuraju R, Smith LM, Ayala S, **Evans CM**, Ponnusamy MP, Kumar S, Kaur S, Batra SK. Secretory Mucin 5AC Promotes Neoplastic Progression by Augmenting KLF4-Mediated Pancreatic Cancer Cell Stemness. *Cancer Res*. 2021. 81:91-102. PMID: 33127746; PMCID: PMC7990052.
4. Morgan LE, Jaramillo AM, Shenoy SK, Raclawska D, Emezienna NA, Richardson VL, Hara N, Harder AQ, NeeDell JC, Hennessy CE, El-Batal HM, Magin CM, Grove Villalon DE, Duncan G, Hanes JS, Suk JS, Thornton DJ, Holguin F, Janssen WJ, Thelin WR, **Evans CM**. Disulfide disruption reverses mucus dysfunction in allergic airway disease. *Nat Commun*. 2021. 12:249. PMID: 33431872; PMCID: PMC7801631.
5. McShane A, Bath J, Jaramillo AM, Ridley C, Walsh AA, Evans CM, Thornton DJ, Ribbeck K. Mucus. *Curr Biol*. 2021. 31:R938-45. PubMed PMID: 34375594. *PMC In progress.*

Project 4: Nothing to report
Biostatistics Core: Nothing to report

b. Website(s) or other Internet site(s)

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Project 1 – nothing to report
Project 2 – nothing to report
Project 3 – nothing to report

Project 4 – nothing to report
Biostatistics Core – nothing to report

c. Technologies or techniques

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Project 1 – nothing to report
Project 2 – nothing to report
Project 3 – nothing to report
Project 4 – nothing to report
Biostatistics Core – nothing to report

d. Inventions, patent applications, and/or licenses

Identify inventions, patent applications with date, and /or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Project 1 – nothing to report
Project 2 – nothing to report
Project 3 – nothing to report
Project 4 – nothing to report
Biostatistics Core – nothing to report

e. Other products

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Project 1 – nothing to report
Project 2 – New cell line generated using CRISPR-SAM (endogenous MUC5B over-expression)
Project 3 – nothing to report
Project 4 – nothing to report
Biostatistics Core – nothing to report

Personnel Effort

Project 1

Name:	**David Schwartz, MD**
Project Role:	PI/Project Lead
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2

Contribution to project:	Responsible for the design and execution of the study, and the day-to-day functioning, trouble-shooting, integration, training, and long-term planning of the study.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Joyce Lee, MD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	Longitudinally assess genetically at-risk cohorts for the appearance of autoantibodies and for the subsequent progression to clinical disease.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Jill Norris, PhD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	
Contribution to project:	1
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Tasha Fingerlin**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Oversees all of the statistical analyses related to the biomarker discovery and validation work in relationship to Project 1.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Corinne Hennessy**
Project Role:	Professional Research Assistant
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	6
Contribution to project:	Responsible for organizing, tracking, and curating the DNA and biological samples for this project, and the follow up genotyping efforts and biomarker assays (mRNA and protein).
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Rachel Warren**
Project Role:	Study Coordinator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	12
Contribution to project:	Serves as Study Coordinator for this project. Ms. Bochantin is responsible for coordinating the efforts of the co-investigators, acquiring all of the clinical data and making arrangements to obtain high-resolution CT (HRCT) scans, peripheral blood (DNA, RNA from

	PBMCs, and plasma), and pulmonary function tests (PFTs) on asymptomatic siblings of established IPF patients in Years 1-2 and the follow-up HRCT scans and PFTs in Years 3-4 on subjects with PrePF.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Mark Steele, MD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	Contribute to recruitment and accrual of patients and their families at University of Colorado and phenotype enrolled participants.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Kevin Brown**
Project Role:	Co-Investigator, MD
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Coordinate the accrual of patients and their families at National Jewish Health
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Daniel Kass, MD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Coordinate the accrual of patients and their families at University of Pittsburgh
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Paul Wolters, MD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Coordinate the accrual of patients and their families at UCSF
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Wendi Mason, MD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Coordinate the accrual of patients and their families at Vanderbilt University
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Steven Rowe**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Direct mucociliary clearance enrollment, study conduct, and analysis
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Tejaswini Kulkarni**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	Assists with patient identification and pre-screening. Will be involved in analysis when data are complete.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Justin Wade**
Project Role:	Clinical Trials Coordinator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	Recruits subjects and helps perform MCC imaging studies
Funding support:	*Complete only if the funding support is provided from other than this award*

Project 2

Name:	**Anthony Gerber**
Project Role:	Co-Project Lead
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	Directing research team
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Sarah Sasse**
Project Role:	Co-investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	Performed and analyzed experiments
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Fabienne Gally**
Project Role:	Co-investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	5
Contribution to project:	Performed and analyzed experiments
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Sean Colgan**
Project Role:	Co-Project Lead
Research Identifier (e.g. ORCID ID)	0000-0003-0431-888
Nearest person month worked:	2
Contribution to project:	Directing research team
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Rachael Kostelecky**
Project Role:	Professional Research Assistant
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	12
Contribution to project:	Perform experiments
Funding support:	*Complete only if the funding support is provided from other than this award*

Project 3

Name:	**Christopher Evans**
Project Role:	Project Lead
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	oversight of experimental design, performance, and analysis in Project 3
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Naoko Liu**
Project Role:	Professional Research Assistant
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	6
Contribution to project:	Animal husbandry and analyses of mucins
Funding support:	*Complete only if the funding support is provided from other than this award*

Project 4

Name:	**Ivana Yang, PhD**
Project Role:	Project Lead
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	Design and execution of the study, the day-to-day functioning, trouble-shooting, integration, training, and long-term planning of the study; oversight for Dr. Eunjoo Kim and Ms. Elizabeth Davidson; actively participates in data analysis, data interpretation, and manuscript preparation; conducts weekly meetings with the project personnel
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Eunjoo Kim, PhD**
Project Role:	Postdoctoral Fellow
Research Identifier (e.g. ORCID ID)	

Nearest person month worked:	12
Contribution to project:	Breeding of Ift88-Krt5 and Arl13b-Krt5 CKO animals; i.t bleomycin, and i.n. H1N1 treatments; immunofluorescence analysis of animal tissue; hydroxyproline assays
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Saif Al-Juboori, PhD**
Project Role:	Postdoctoral Fellow
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	6
Contribution to project:	Isolation of distal airway stem cell populations from human and mouse tissue, Wnt signaling assays
Funding support:	*Complete only if the funding support is provided from other than this award*

Biostatistics Core

Name:	**Tasha Fingerlin, PhD**
Project Role:	Biostatistics Core Director
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Oversees all of the statistical analyses related to the biomarker discovery and validation work in relationship to Project 1.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Kelsey Anderson**
Project Role:	Computer Programmer
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	3
Contribution to project:	Responsible for designing and implementing database structures that allow the individual projects to efficiently deposit and retrieve study data, as well as coordinating the integration of systems in such a way as to preserve individual study features while allowing efficient integration of data across projects.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Sean Jacobson**
Project Role:	Junior Biostatistician
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	4
Contribution to project:	Responsible for day-to-day analytic activities for all projects, with duties determined by Dr. Fingerlin in response to investigator needs and priorities. Works directly with Dr. Fingerlin to implement summary reporting, project analyses and data reports for Project Directors and works with the computer programmer to develop the data sets and implement data cleaning and reporting algorithms.
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Camille Moore, PhD**
Project Role:	Senior Biostatician
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Bio-analysis lead work for the biostatistic core
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Brian Vestal, PhD**
Project Role:	Senior Statistician
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
Contribution to project:	Bio-analysis lead work for the biostatistic core
Funding support:	*Complete only if the funding support is provided from other than this award*

Administrative Core

Name:	**David Schwartz, MD**
Project Role:	Administrative Core Lead
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Responsible for the scientific coordination, direction of research emphasis, and administrative activities of the Program
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Bethany Ginzburg**
Project Role:	Administrator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Manage the fiscal and administrative aspects of the Program and coordinate matters with participating departments, the University of Colorado, the subcontract sites, and the Department of Defense
Funding support:	*Complete only if the funding support is provided from other than this award*

a. Has there been a change in the active other support of the Site PI or senior/key personnel since the last reporting period?

- *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
- *If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*

Nothing to report

b. What other organizations were involved as partners?

- *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
- *Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were*

involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed. Provide the following information for each partnership:

- *Organization Name:*
- *Location of Organization: (if foreign location list country)*
- *Partner's contribution to the project (identify one or more)*
  - *Financial support;*
  - *In-kind support (e.g., partner makes software, computers, equipment, etc., available to*
  - *project staff);*
  - *Facilities (e.g., project staff use the partner's facilities for project activities);*
  - *Collaboration (e.g., partner's staff work with project staff on the project);*
  - *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
  - *Other.*

UH2/3-HL123442 (Schwartz) ended on 6/30/2021.

K23HL138131 (Lee) ended on 7/31/2021.

Appendices