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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

1. 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

- Elucidate the molecular regulation of the *MUC5B* promoter relative to the *MUC5B* promoter variant rs35705950.
- Determine integrated transcriptional control of MUC5B expression in response to pro-fibrotic signals.
- Define the impact of the *MUC5B* promoter variant rs35705950 on epithelial-mediated pro-fibrotic innate immune responses.

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: 50%
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	75% (9/2020)
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: 75% (9/2020)
Milestone #2: *Analyze St3Gal1- St3Gal6, St6Gal1- St6Gal2, Fut1-Fut11, and Agr2 transcript and protein levels.*	24-27	

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: 25% (9/2020)
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: 25% (9/2020)
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: 50% (9/2020)
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% (9/2020)
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: 25% (9/2020)
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	
Milestone #2: Test effects of pharmacologic and enzyme interventions on mucus transport, and expression of pro-fibrotic mediators in vitro.	40-46	
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	
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; 75% 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, 50% completed.
Milestone #4: Statistical analysis of RT-qPCR data and prioritization of genes for Aim 2. Milestone set for 03-31-2019, 50% 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; 90% 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, 75% completed.
Major Task 2: Inhibit and overexpress cilium genes, measure injury/repair, regeneration, and Wnt signaling (scheduled for months 18-36; 25% complete).
Milestone #1: Inhibit and overexpress cilium genes of interest. Milestone set for 12-31-2019, 50% completed.
Milestone #2: Treat cells in which cilium genes are inhibited/overexpressed with bleomycin and H1N1. Milestone set for 03-31-2020, 20% completed.
Milestone #3. Measure wound healing, TEER, Wnt signaling. Milestone set for 06-30-2020, 20% completed.
Milestone #4: Statistical analysis of the data and prioritization of genes for Aim 3. Milestone set for 09-30-2020, 0% 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; 75% 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, 75% completed.
Milestone #3. IF staining for Arl13b, Foxj1, Muc5b, Mmp7 Krt5, Krt14, p63, and β-catenin. Milestone set for 09-30-2020, 75% completed.
Milestone #4. Measure collagen content of the lung by hydroxyproline and SHG assays. Milestone set for 09-30-2020, 75% completed.
Milestone #5: Statistical analysis of the data and prioritization of genes for Aim 3b. Milestone set for 12-31-2020, 75% completed.

Major Task 4: Publication of findings from Aim 2 (scheduled for months 36-42; 25% complete).
Milestone #1: Prepare and submit manuscript. Milestone set for 03-31-2021, 25% 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; 95% 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, 90% 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, 25% 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?

Project 1
Specific Aim 1:
- Recruitment of human participants is ongoing at open sites
- 165 first degree relatives of people with IPF referred for study participation
- 119 first degree relatives of people with IPF consented to study participation
- 61 first degree relatives of people with IPF have completed some, but not all study procedures
- 58 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
- 319 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
- No adverse events in the human subjects study

Project 2:
ATAC-seq performed on freshly brushed human airway cells and now analyzed via a tool called motif displacement. This method, which has emerged from Aims 1 and 2 in this project will be an important methodological resource for future investigations. To that end, we have integrated data from our chromatin profiling studies with single cell ATAC-seq data from normal and IPF patient lungs. As shown in Figure 1, ATAC-seq profiling of the MUC5B locus in patient samples indicates open chromatin at the region, aligned with the observations we have described in previous progress reports. Taken together, integrating these single cell data with our data establishing at least two chromatin conformations and RNA Pol II loading at the -3kB enhancer, we propose a chromatin priming model for MUC5B mis-expression in IPF (Figure 2).

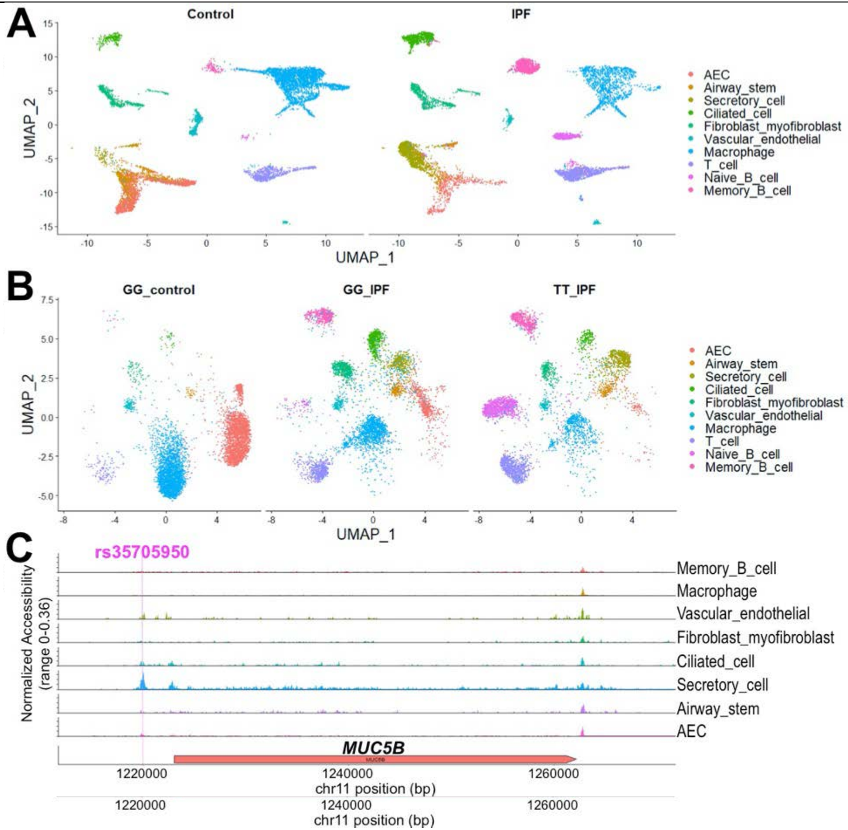


Figure 1. **Integrated single-nucleus sequencing reveals promiscuous chromatin accessibility at the -3 kb transversion site in lung tissue from IPF patients. (A) Cell types identified in snRNA-seq in lung tissue from IPF patients and unaffected controls using UMAP-based cell clustering. (B) snATAC-seq cell clustering in IPF and control lungs by rs35705950 genotype using gene expression information from paired snRNA-seq to define cell types. (C) snATAC-seq data aggregated by cell type and visualized as chromatograms at the MUC5B locus. (AEC; alveolar epithelial cell)**

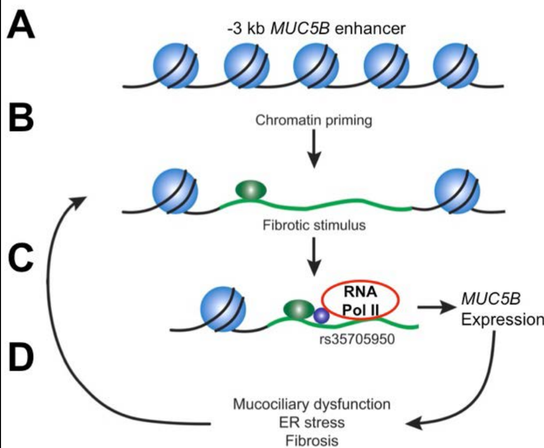


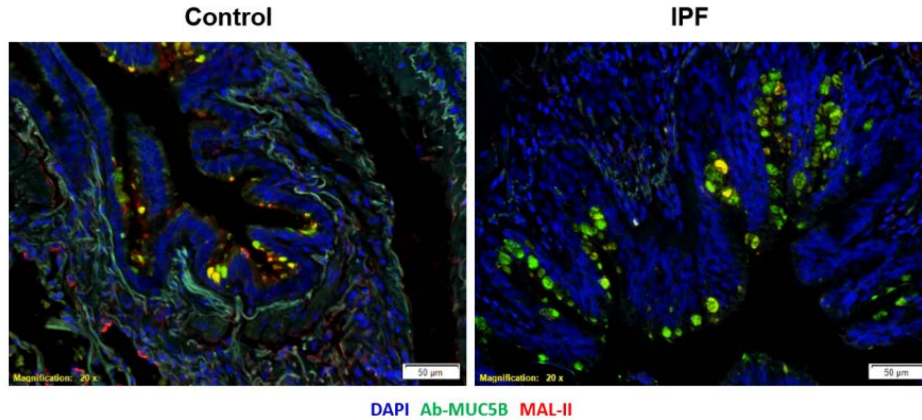
Figure 2. **Model of epigenetic priming and positive feedback. (A) Basal positioned nucleosomal packaging of the MUC5B -3 kb enhancer. (B) In response to pleiotropic stimuli, chromatin remodeling occurs, priming enhancer DNA for interactions with transcription factors. (C) In the setting of fibrotic lung disease, the enhancer is activated by a range of transcription factors acting through semi-degenerate binding sites for STAT, ETS and Forkhead box family members, among others, leading to recruitment of RNAPII and induction of MUC5B expression. (D) MUC5B expression in turn promotes endoplasmic reticulum (ER) stress and mucociliary dysfunction, leading to additional activation of MUC5B in adjacent cells, thus comprising a positive feedback circuit.**

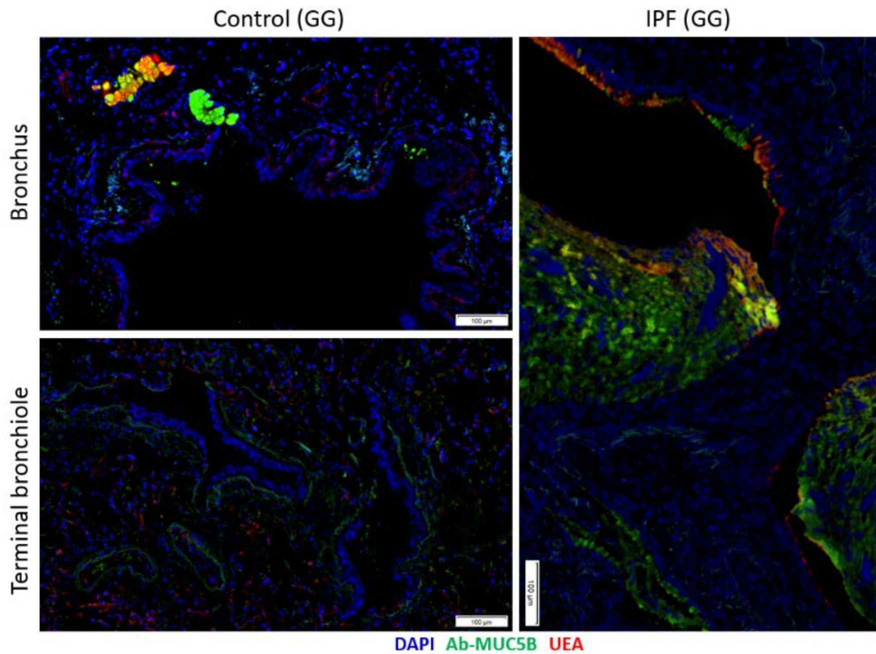
Through the use of CRISPR-based Synergistic Activated Mediator approaches, we have been able to mimic the MUC5B SNP and over-express MUC5B in pulmonary cell lines. With these cells, we have pursued work proposed in Aim 3 to address the influence of MUC5B gain of function on ER stress responses. A primary recent focus has been to develop a stable cell line that mimics the rs35705950 MUC5B gain of function SNP.

Using CRISPR-based synergistic activated mediator (SAM) approach, we have generated an A549 cell line that over-expresses endogenous MUC5B transcript and protein. Using these cells, we have

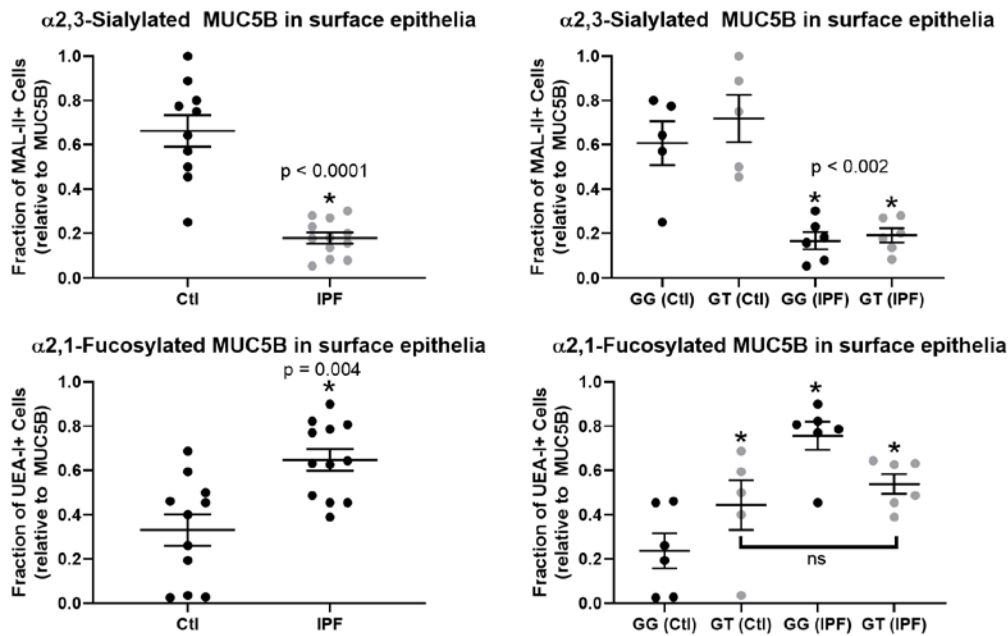
performed ER stress responses through the utilization of tunicamycin and revealed some degree of specificity, where the ER stress targets XBP1 and IRE-1 show enhanced induction in MUC5B OE cells relative to controls (Figure 3). No differences in CHOP or GRP78 were noted. These results implicate the over-expression of MUC5B in selective increases in ER stress responses.

Project 3
See Section A above for overview. Below are additional details of findings:

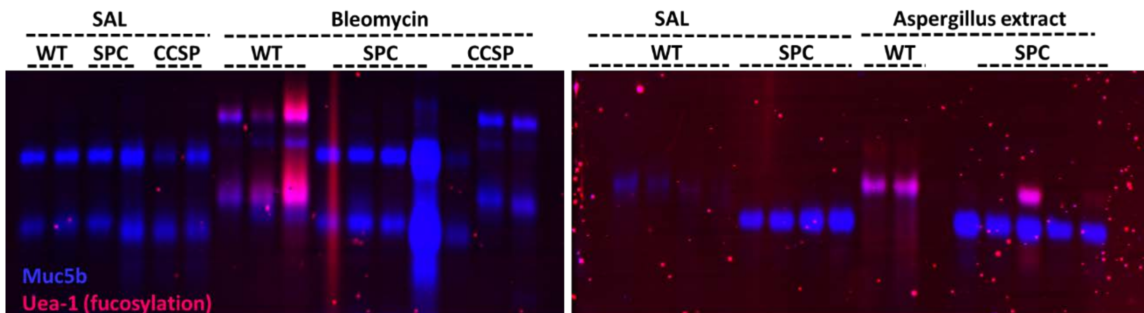




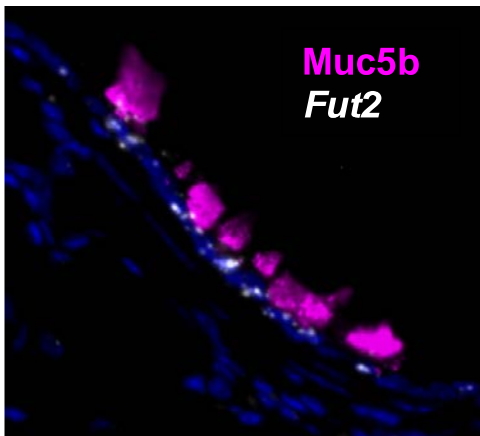
IPF is associated with decreased α2,3-sialylation and increased α1,2-fucosylation of MUC5B.



Muc5b overexpressing mice are resistant to transitioning to fucosylated Muc5b glycoforms

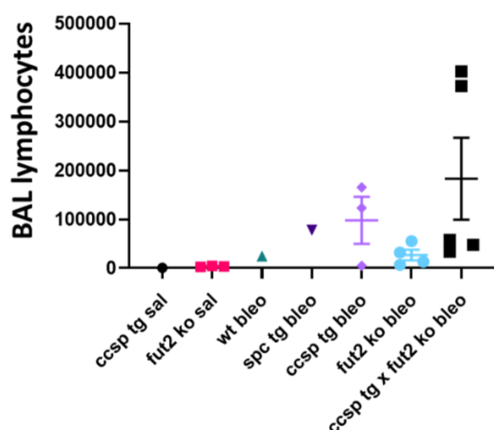


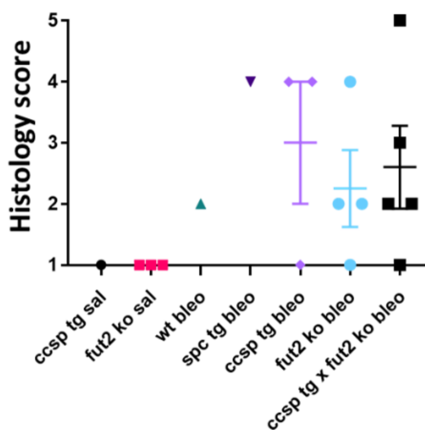
Muc5b and Fut2 are co-expressed in airways from bleomycin challenged C57BL/6J mice.



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.

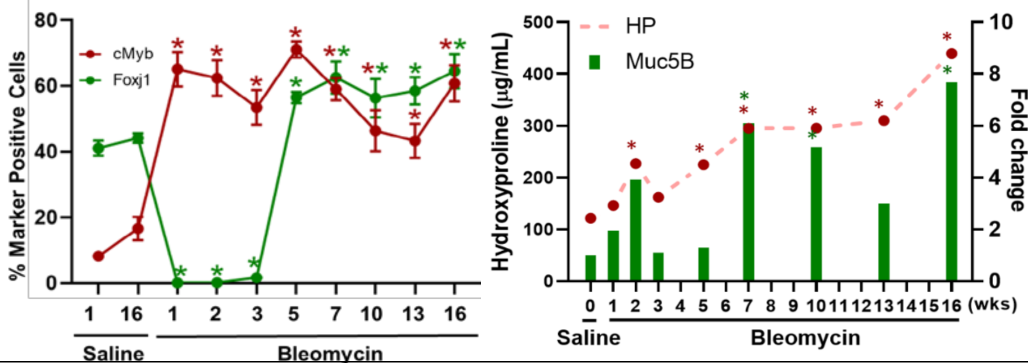
Project 4:

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

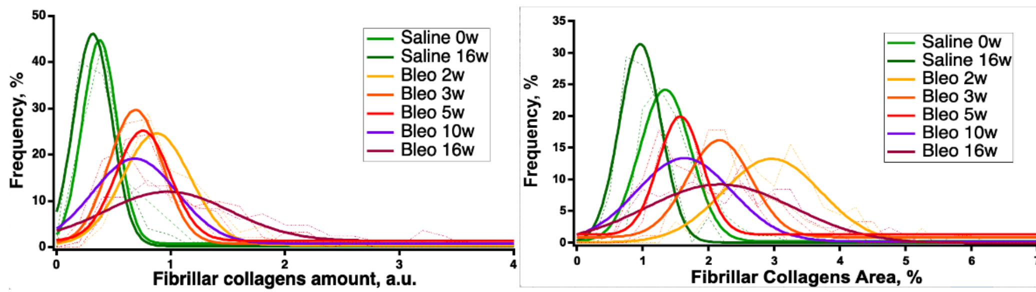
Major Task 1: Regulatory approval and animal breeding (scheduled for months 0-9; 100% complete). We obtained IACUC approval at the University of Colorado 06-09-2017 and ACURO approval 09-05-2017 prior to start of funding and therefore ahead of the proposed milestone. Muc5b strain breeding commenced immediately after funding started and we have been able to breed sufficient numbers of animals to stay on track with experiments proposed in Aim 1. We completed UCD IACUC and ACURO renewals on 04/22/2020 and 08/03/2020, respectively.
This task is completed.

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. However, we have added three new experiments to finalize this task for publication:

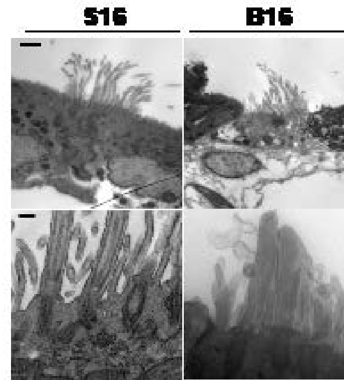
1. Measurement of Muc5b in lavage fluid to demonstrate correlations of Muc5b, markers of multiciliogenesis, and fibrosis, as measured by hydroxyproline assays.



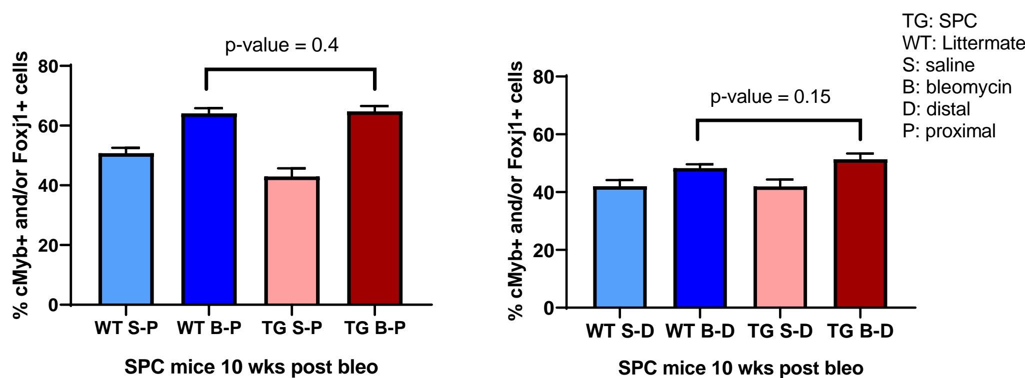
2. Second harmonic generation (SHG) quantification of fibrillar collagen content in the lung at multiple timepoints following bleomycin.

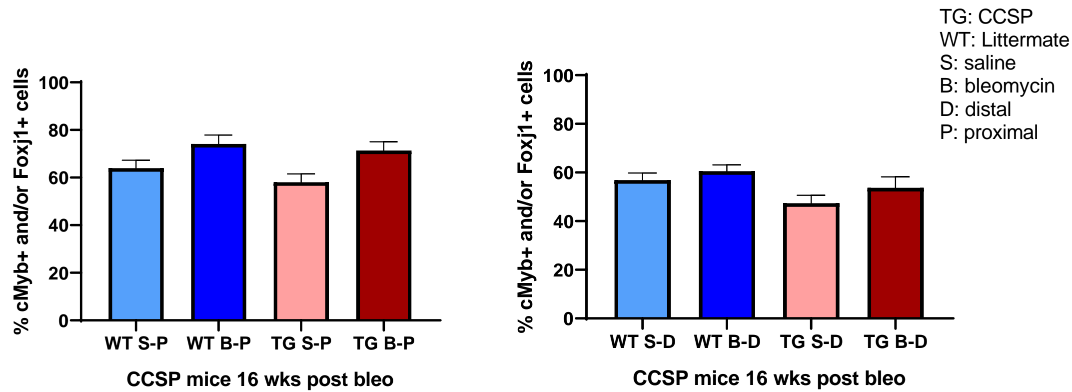


3. Transmitting electron microscopy (TEM) imaging of cilia that regenerated following deciliation with bleomycin. Cross sectional images at 16 weeks post treatment demonstrated four major categories of ciliary abnormalities in the bleomycin group (B16) compared to saline group (S16): (1) abnormal cilia with single axonome, (2) abnormal giant compound cilia with 9+2 complete axonomes, (3) abnormal giant compound cilia showing completed and incomplete axonomes, and (4) intracytoplasmic cilia, microtubular doublets or complexes. This suggests that aberrant repair of damaged cilia of multiciliated cell results in ciliary abnormalities indicating that altered cilia structure could be one of the early markers of lung fibrosis and it may affect ciliary function in the airways.

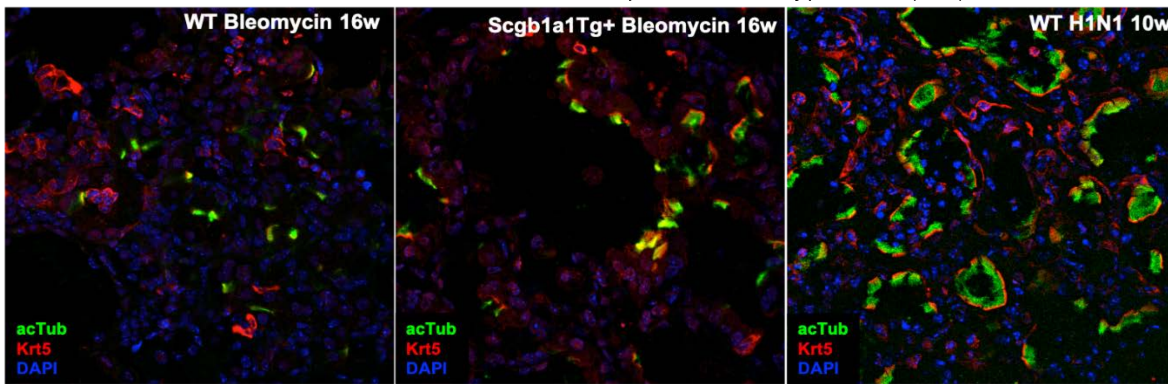


To determine the effect of excess Muc5Bb on multiciliogenesis, we challenged Scgb1a1-Muc5bTg SPC-Muc5b Tg and Tg-negative littermate control mice with bleomycin. We have observed a trend that the ciliogenesis marker positive cells are slightly increased in SPC-Muc5b Tg mice (n=4) as compared to Tg negative mice (n=5) upon bleomycin even though it is not statistically significant. Now we are adding more mice to increase number of mice to analyze. We did not observe an increase in Scgb1a1-Muc5b Tg mice. This difference in response is likely because SPC-Muc5b Tg mice have higher overexpression of Muc5b compared to Tg negative mice and because that overexpression is in the distal lung whereas Scgb1a1-Muc5b Tg mice only have modest overexpression of Muc5b mostly in proximal airways.



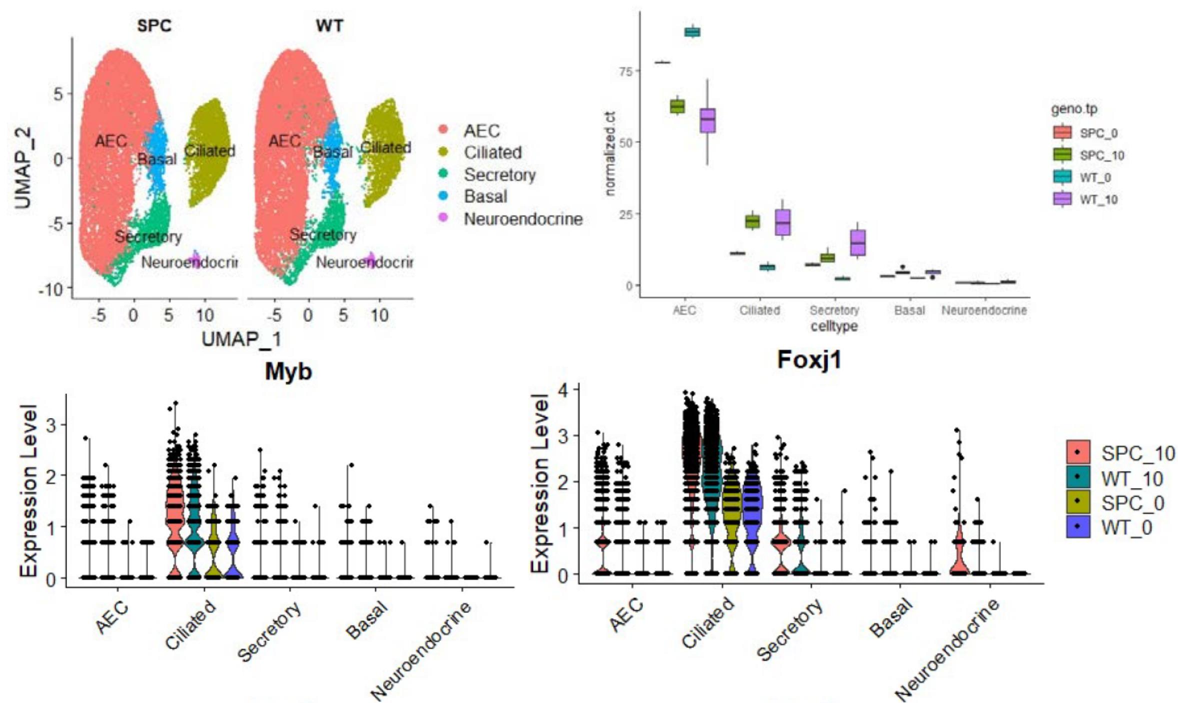


Importantly, we have observed honeycomb-like cyst structures lined with Krt5+ basal and multiciliated cells (acTub) in bleomycin and H1N1 models. Scgb1a1-Muc5b Tg+ mice have bigger cyst-like structures with more multiciliated cells detected, compared to wild type mice (WT).



Major Task 3: Identify changes in cilium gene expression in isolated DASC populations at multiple timepoints following injury (scheduled for months 3-18; 75% complete).

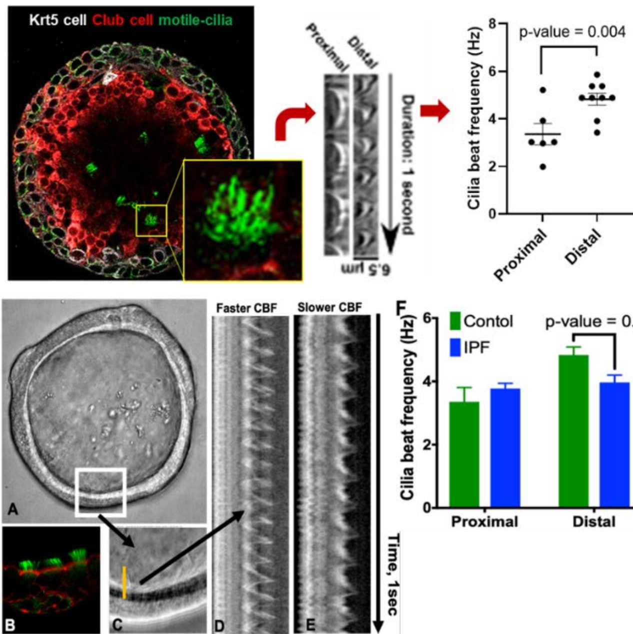
Given the small numbers and difficulty of isolating pure Krt5 cell populations, we are in the process of breeding enough mice to isolate Krt5 positive cells using the mTmG tag in Krt5-CreERT2-RosamT/mG; upon treatment with tamoxifen, Krt5 cells will be labeled with GFP and we can use flow cytometry to sort them using GFP. While waiting for these mice, we performed bulk RNA-seq on the sorted EpCam+ cells from SPC-Muc5bTg and Tg- littermates treated with a single dose of i.t. bleomycin at 1 week, 3 weeks, and 10 weeks post-bleomycin. Our analysis shows that differences in expression of cilium genes in Tg+ compared to Tg- mice are small most likely because the Epcam+ cell population is comprised of mainly alveolar type II cells. However, we observe a trend of decreased cilium gene expression at 1 week and then increased at 3 weeks and 10 weeks. Gene Set Enrichment Analysis (GSEA) confirmed the trends in cilium gene expression over time and identified Notch signaling as a pathway of interest; this result is not surprising given the importance of Notch signaling in multiciliogenesis. Overall, these bulk data demonstrate that there is probably more pronounced deciliation followed by more overactive mulcticiliogenesis in the distal airways of SPC-Muc5bTg+ mice compared to Tg- littermates. Based on these bulk data, we have collected single cell profiles (scRNA-seq) of SPC-Muc5bTg and Tg- littermates at baseline and at 10 weeks post-bleomycin. At baseline, we observe an increase in ciliated cell number in SPC-Muc5bTg+ mice compared to Tg- littermates. As expected from our other data, we observe a large increase in ciliated cell numbers 10 weeks post-bleomycin in both Tg+ and Tg- animals, with no significant difference by



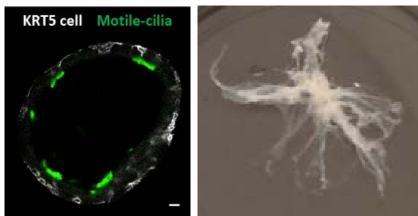
genotype. However, we do observe an
increase in expression of cMyb and Foxj1 in the ciliated cell cluster of Tg+ compared to Tg- animals. We continue to refine the analysis of this dataset.

<u>Major Task 4: Publication of findings from Aim 1 (scheduled for months 18-24; 90% complete)</u>
Milestone #1: Prepare and submit manuscript. Milestone set for 09-30-2019, 90% complete.
We have a draft of the manuscript completed and are waiting for results of one more experiment before we can submit. Submission has been delayed to Q4 of 2020 while we wait for the results from this last experiment.

Aim 2: Demonstrate that changes in cilium gene expression in airway progenitor cells affect injury/repair and fibrosis.
<u>Major Task 1: Establish NHBE cell cultures, optimize lenti-shRNA and lenti-ORF protocols, and treatment concentrations (scheduled for months 0-18; 90% complete).</u>



We have been culturing NHBE cells, have optimized bleomycin concentration (to observe an increase in MUC5B protein secretion with no cell death). H1N1 experiments are on hold due to COVID19-related PPE (N95 mask) shortages and will be completed in 2021. In addition to NHBE cultures, we have decided to use airway-derived organoids to have complementary 2D and 3D culture systems. We have implemented the published protocol for organoid cultures (Sachs. EMBO J. 2019; 38: e100300) and a method for measuring ciliary beat frequency (CBF) using kymographs. We first determined that CBF in distal airway is faster than proximal airways, consistent with previously published results. We were able to compare one IPF to one control sample prior to COVID19 lab shutdown and 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 now that we once again have access to human tissue.



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. We optimized the digestion protocols to isolate intact airway tree from mouse lung so that we can grow proximal and distal derived organoids separately. Using this model we will be able to measure CBF upon bleomycin treatment, Muc5B overexpression or cilium gene knock out.

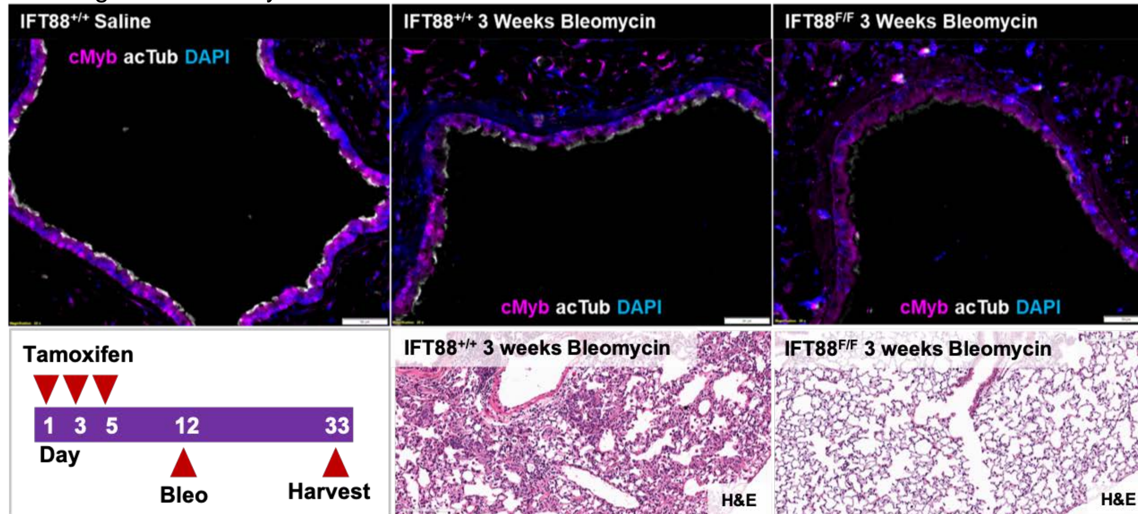
<u>Major Task 2: Inhibit and overexpress cilium genes, measure injury/repair, regeneration, and Wnt signaling (scheduled for months 18-36; 25% complete).</u>

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. These cell culture experiments were delayed due to COVID19-related lab shutdown and limited availability of plastics (plates with inserts for ALI cultures).

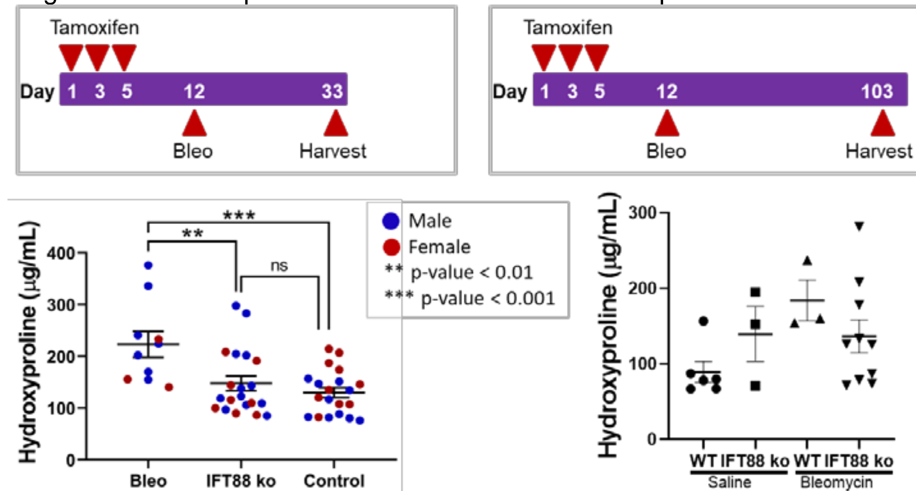
<u>Major Task 3: Determine the influence of cilium gene deletion on injury/repair, lung regeneration, and fibrosis in mice (scheduled for months 3-39; 75% complete).</u>

To assess the effect of cilium gene deletion in primary cilia on lung fibrosis in the bleomycin model, we have continued to work with the mice with the conditional deletion of Ift88 in Krt5 cell using Krt5-CreERT2-IFT88$^{flox/flox}$ transgenic mouse. i.p. injection of 70 mg/kg tamoxifen for 4 days results in sufficient recombination, as monitored by allele-specific primers. Immunofluorescence demonstrates presence of Club cell-derived ciliated cells, suggesting that we will need to also breed Ift88 flox/flox and Scgb1a1-CreER mice to address the contribution of these club cell progenitors; this breeding is in progress. To determine whether Ift88 deletion in Krt5 cells reduces lung fibrosis, we previously assessed lung fibrosis biochemically by measuring HP content and the results demonstrated that Krt5-CreERT2-Ift88$^{flox/flox}$ mice had significantly less fibrosis than Krt5-CreERT2-Ift88$^{+/+}$ mice following challenge with bleo. During the past year, we further characterized this model. We showed that multiciliogenesis in Krt5-CreERT2-Ift88$^{flox/flox}$ mice is down to almost undetectable with bleomycin treatment while Krt5-CreERT2-Ift88$^{+/+}$mice showed induced cMyb in the airways as expected. We also observed paucity of cyst-like structures with conditional deletion of Ift88 at this same timepoint (3 weeks post-bleomycin). Taken together, our results indicate that deletion of a key cilium gene in primary cilia in Krt5 progenitor cells affects multicifibrosis and multiciliogenesis, fibrosis, and honeycomb cyst formation. Currently, we are quantifying cMyb and Foxj1 positive cells at 10 wks post-bleomycin to see whether multiciliogenesis is reduced in Krt5-CreERT2-Ift88$^{flox/flox}$ mice as

compared to Krt5-CreERT2-Ift88$^{+/+}$mice at this later timepoint. Also, we will measure CBF in airways of Krt5-CreERT2-Ift88$^{flox/flox}$ using organoid to demonstrate that IFT88 ko reduces fibrosis without affecting to mucociliary clearance.



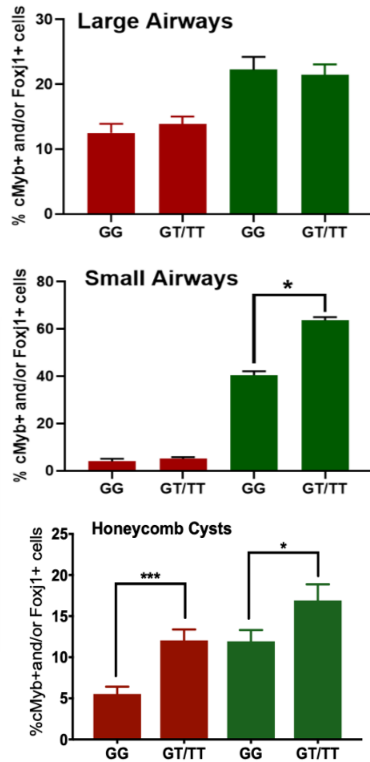
Because of difficulties we have had with breeding of the Arl13flox/flox mice and promising results in the Krt5-CreERT2-IFT88$^{flox/flox}$ cross, we have extended experiments with this line beyond what was originally proposed to encompass later timepoints (to assess whether lack of fibrosis and honeycomb cysts is persistent) and to club cell progenitors (breeding to Scgb1a1-CreER mice). We have preliminary data to show that difference in fibrosis persist to 10 weeks post-bleomycin, although the response is more variable. We also observed that deleted IFT88 allele was not detectable at 10 weeks post-bleomycin (it is detectable at 3 weeks post-bleomycin) implying that cells with Ift88$^{flox/flox}$ were replaced by cells with wild type Ift88 during regeneration of airway upon bleomycin injury. Because of this, we will determine when mice lose Ift88$^{flox/flox}$ by using qPCR so that we know how long Ift88$^{flox/flox}$ is required to reduce fibrosis formation upon bleo treatment.



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; 50% 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. 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 and are in the process of finishing the counting of small airways in control samples



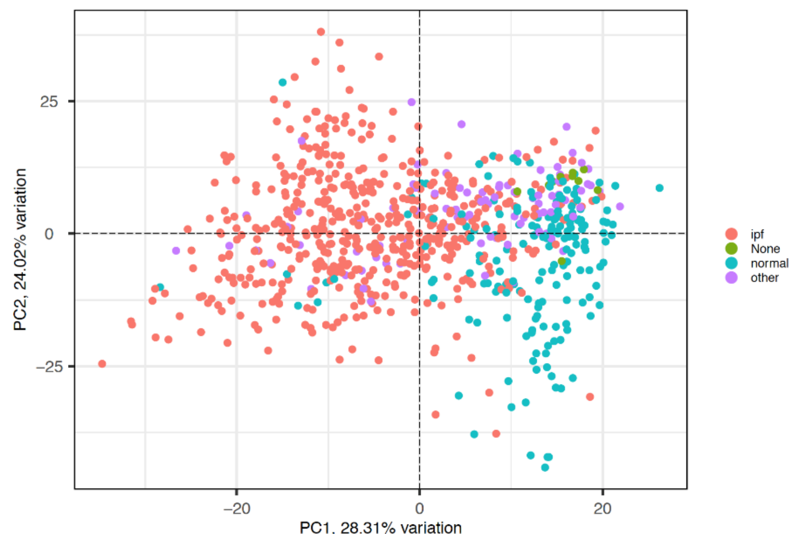
Major Task 2: Measure expression of cilium genes identified in Aims 1-2 in DASCs from IPF and control lungs with and without Muc5b promoter variant (scheduled for months 0-42; 50% complete).

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 proximal and distal airway epithelia from 10 IPF and 10 control subjects, half with GG and half with GT/TT genotype. Analysis of this large single cell sequencing dataset will be the final experiment in this task.

Biostatistics Core:

As from the beginning of the project, Dr. Fingerlin is available to all project investigators as it relates to study design or other questions.

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 initial analyses of our systems biology data that integrates genotype, methylation at CpG sites, and RNA-seq based gene expression data. We present below some of our preliminary findings and continue to work with Drs. Yang and Schwartz to refine the interpretation and additional analyses.



Data preparation

To integrate the three types of data (genotype, methylation, expression), we first identified the study participants who had data for each of the three types. We then reassessed all of the quality control metrics such as rate of missingness, differential missingness between cases/controls, and indicators of batch or positional effects, that could influence the internal validity of our statistical inferences. This reduced sample showed no evidence for systematic differences between the full sample for any of the types of data, nor did we identify any potential systematic biases or confounders.

Among the sample with all three types of data, we recapitulated most of the associations found among the full cohort. For instance, we observed strong association signals for differences between cases and controls in methylation, as expected based on other studies and our full cohort. **Figure 1** shows that a principal component of methylation values, which is a summary measure of methylation that captures >28% of the variation in the methylation, is associated with IPF.

We conducted further 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 many 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.

For example, on Chromosome 3, IPF risk variant rs2293607 is associated with expression of *ADIPOQ* (**Figure 2**) and a methylation site near *LRRC34* (**Figure 3**). Methylation near *LRRC34* is strongly associated with expression of *ADIPOQ*, and this association persists after adjustment for rs2293607 and case/control status, but the relationship between methylation and expression is strongest among those with two copies of the rare allele (**Figure 4**). While we are prioritizing the pairs and relationships based on several factors, here we give an example of one variant and pair of expression/methylation is indicative of the richness of our data and the ability of this approach to provide an integrated view of the individual and joint relationships among genotype, expression, and methylation.

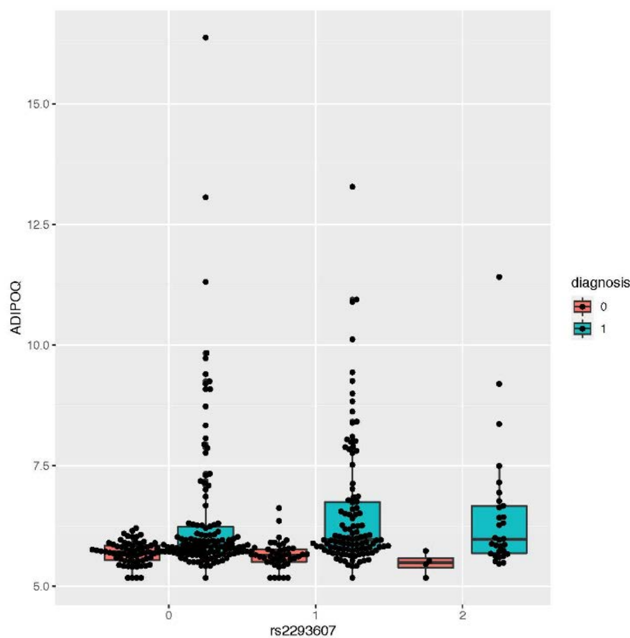
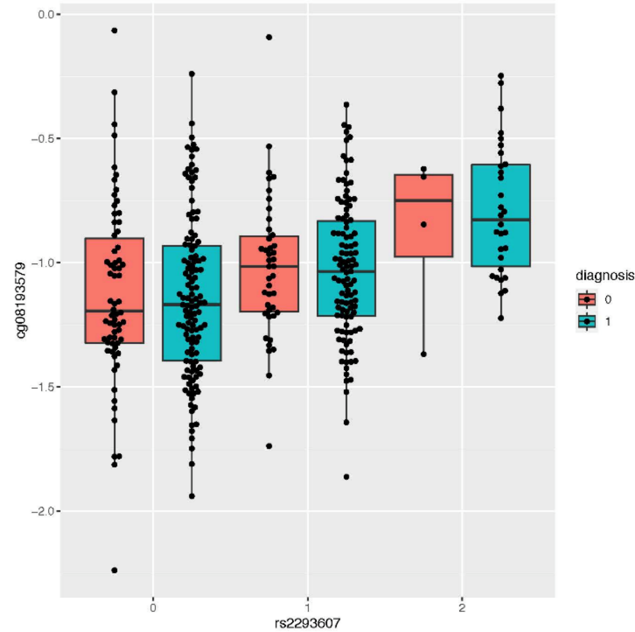
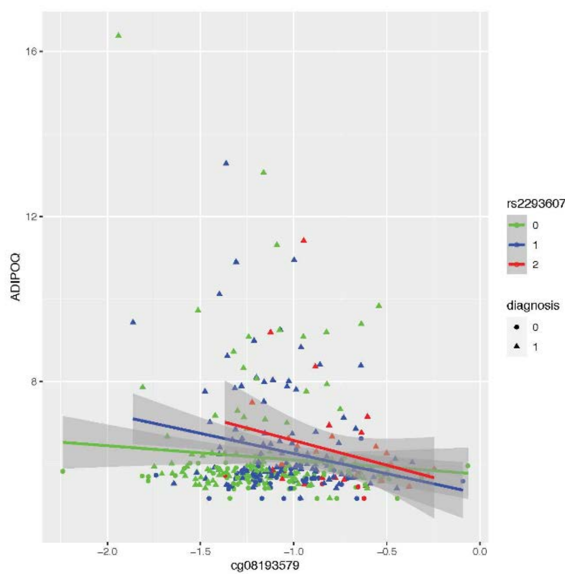


Figure 2: ADIPOQ expression by genotype

Figure 3: Methylation by genotype

Figure 4: Correlation between methylation and expression by genotype and IPF vs. Normal





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

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?

Project 1: CT scan results are shared with participants following central review of their imaging at the University of Colorado.
Project 2: A manuscript on MUC5B chromatin was submitted for publication. COVID-related cancellation of scientific conferences have limited communication of results.
Project 3: Data were published in two manuscripts:

- Fakih et al, Am J Physiol Lung Cell Mol Physiol. 2020 Jun 1;318(6):L1270-L1279. PMID: 32348677
- Campbell et al, J Exp Med. 2019 Dec 2;216(12):2714-2723. PMID: 31582416

Project 4:
Dr. Kim was scheduled to present her ciliogenesis at the FASEB Lung Epithelium meeting in August 2020. The meeting was canceled because of COVID19. Dr. Kim presented her ciliogenesis work at the National Jewish Health/University of Colorado Research in Progress in July 2020. Dr. Yang presented the work at the University of Colorado Department of Medicine Research and Innovation Conference in September 2020. Dr. Kim is presenting her airway-derived organoid work at the EMBL Symposium "Organoids: Modelling Organ Development and Disease in 3D Culture" held virtually Oct 21-25.
Biostatistics Core: Nothing to report

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

Project 1:

- Referral, recruitment, and enrollment of research participants will continue
- 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:

- Continue characterization of the Unfolded Protein Response (UPR) using newly developed cell lines.
- Pilot chromatin conformation capture assays, using Micro-seq (Aim 2)

Project 3:
In the next 6 months, we expect to complete all research related to Specific Aim 2, finishing all Major Tasks up to submission of a manuscript focused on fucosylation, sialylation, and Ern2 dependence of pulmonary fibrosis phenotypes in mice, along with correlative data in human samples. These studies will also extend into Specific Aim 3, which will test the roles of ER stress interventions in relevant models.
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 (75% complete).
We will finish the analysis of single cell RNA-seq data in Muc5b Tg mice treated with bleomycin. We will isolate Krt5+ cells after injury with bleomycin and H1N1 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 2020.

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 (90% complete).
The only part of the task left to finish is optimization of H1N1 concentration and we will complete this by the end of Q2 of 2021, provided that PPE is available.

Major Task 2: Inhibit and overexpress cilium genes, measure injury/repair, regeneration, and Wnt signaling (25% complete).

We will finish optimization of CRSIPRa, CRISPRi, lenti-shRNA and lenti-ORF protocols and use them to alter expression of MUC5B and ciliogenesis genes both in ALI cultures and in airway-derived organoids.

Major Task 3: Determine the influence of cilium gene deletion on injury/repair, lung regeneration, and fibrosis in mice.
We will finish phenotyping of mice with Ift88 conditional deletion in Krt5 cells at 10 weeks and phenotype mice with Ift88 conditional deletion in Scgb1a1 cells at 3 weeks and 10 weeks.

Major Task 4: Publication of findings from Aim 2 (90% complete)
We plan on submitting the publication that describes the results from Aim 2 by the end of Q3 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 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; 95% complete).
We will finish quantification of the markers in small airways from control lungs.

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 and analysis of single cell RNA-sequencing data of airway cells isolated from IPF and control lungs, stratified by the *MUC5B* genotype.
Biostatistics Core: We will continue to refine the multi-omic analyses and results to allow application of the predictive models once the final cohort is assembled and we have the genomic data.

2. 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: Nothing to report
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.
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.
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

3. Changes/Problems

a. Changes in approach and reasons for change

Project 1: No significant changes are anticipated. We will continue to emphasize physician and patient outreach to improve recruitment into the study.
Project 2: The COVID19 pandemic is delaying some aspects of the work, including development of critical reagents. We anticipate that this will continue to be problematic.
Project 3: Nothing to report.
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

- *Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

Project 1: Recruitment picked up in the beginning of the reporting period, but slowed down following the COVID-19 pandemic as people were reluctant to undergo 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: Research labs are currently working at 50% capacity due to COVID-19. We anticipate that productivity will be limited next quarter.
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: While we are slightly delayed in completing gene expression studies on cells isolated from mouse lungs in Aim 1, we are a little bit ahead of the schedule in single cell analysis of human lung tissue in Aim 3.
Biostatistics Core: nothing to report

c. Changes that had a significant impact on expenditures

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

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

4. Products

a. Publications, conference papers, and presentations

- ***Journal publications***.
- ***Books or other non-periodical, one-time publications.***
- ***Other publications, conference papers, and presentations.*** *Identify any other publications, conference*

Project 1: Nothing to report
Project 2: Manuscript submitted
Project 3: Data were published in two manuscripts:

- Fakih et al, *Am J Physiol Lung Cell Mol Physiol*. 2020 Jun 1;318(6):L1270-L1279. PMID: 32348677
- Campbell et al, J Exp Med. 2019 Dec 2;216(12):2714-2723. PMID: 31582416

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

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

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

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

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

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

5. 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:	**Julie Powers**
Project Role:	Clinical Coordinator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	4
Contribution to project:	Overseeing clinical coordination team, completing IRB and regulatory submissions, managing referral sites and their IRB and regulatory submissions, administering informed consent with new participants, and coordinating the phenotyping process of participant CT scans and health records
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:	**Victor Thannickal**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
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:	**Kate Slaten**
Project Role:	Clinical Trials Coordinator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
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:	**Oliver Eickelberg, MD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Provide expertise in procedures and methods for isolation and study of specific cell populations from IPF lung explants and biopsies; supervision to Dr. Yan Hui on isolation of cell populations
Funding support:	*Complete only if the funding support is provided from other than this award*

Name:	**Melanie Königshoff, MD**

Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	1
Contribution to project:	Provide expertise in Wnt signaling and lung regeneration following injury; supervision to Dr. Yan Hui on Wnt investigation in the proposal
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:	1
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:	3
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:	**Soumontha Chanthaphonh**
Project Role:	Administrator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	2
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?**

Dr. David Schwartz was awarded a R38, and UG3 from NHLBI, as well as a VA Merit.

Drs. David Schwartz and Ivana Yang were awarded a new R01 from NHLBI.

The addition of these new research projects will have no effect on their performance related to this award.

R38HL143511-01A1 (MPI: Schwartz, Abman, Buttrick)
Colorado StARR Program in Medicine and Pediatrics (CSPMP)
NHLBI
03/01/2020-02/29/2024
0.0 calendar

UG3HL151865 (PI: Schwartz)
Preclinical Pulmonary Fibrosis, an opportune rare disease cohort
NHLBI
08/01/2020-07/31/26
1.2 calendar

1I01BX005295 (PI: Schwartz)
lncRNAs, Linking Genetic Susceptibility to Molecular Phenotype in IPF
VA Eastern Colorado Health Care System
10/01/2020-09/30/2024
1.8 calendar

R01HL149836 (MPI: Schwartz/Clouthier/Yang)
Genes and Transcripts that Interact with MUC5B in Pulmonary Fibrosis
NHLBI
06/01/20-05/31/24
1.8 calendar (Schwartz and Yang)

Dr. Tasha Fingerlin is a co-Investigator on a recently awarded R01, RHL149741A. (PI: Henson). In addition, she was awarded a research project from the State of Colorado Office of Economic Development, CTGG1 2020-2688, as Principle Investigator. The addition of these new research projects will have no effect on her performance related to this award.

Dr. Joyce Lee has the following new active clinical trials:

A Phase 3, Randomized, Double-Blind, Placebo-Controlled Efficacy and Safety Study of Pamrevlumab in Subjects with Idiopathic Pulmonary Fibrosis (IPF)
FibroGen, Inc.
04/30/2020-04/29/2025

A Multicenter, Randomized, Double-blind, Placebo-controlled, Phase 2 Study of the Efficacy and the Safety and Tolerability of BMS-982678 in Participants with Pulmonary Fibrosis
Bristol-Myers Squibb
05/15/2020-05/14/2025

New active grant for Dr. Christopher Evans:

W81XWH-19-1-0172 (Evans)

Department of Defense
Gene-Editing to Determine MUC5B Mucin Polymer Targets in Lung Injury, Repair, and Fibrosis
09/30/2019-03/31/2021
1.8 calendar

Dr. Steve Rowe was awarded an administrative supplement from NHLBI.

3R35HL135816-04S1 (Rowe)
Administrative Supplement: A ferret model of SARS-CoV-2 infection
08/15/2020 – 07/31/2021
0 calendar

Dr. Jim Loyd retired from Vanderbilt University in March 2020 and Dr. Wendi Mason became site PI in his stead.

b. What other organizations were involved as partners?

Nothing to report

6. Appendices