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14. ABSTRACT

Idiopathic PF (IPF) is a progressive disorder characterized by peripheral lung injury and replacement of alveoli with cystic structures surrounded by excessive collagen rich matrix. Recently, an unexpected link was made between the IPF and *MUC5B.* The risk variant increases gene expression, but mechanisms through which overproduced MUC5B potentiates fibroproliferative remodeling in the distal lung are unknown. Determining how MUC5B affects the PF development is crucial. MUC5B is a massive glycoprotein that assembles via disulfide bond formation. In mouse models of acute and chronic lung injury—including PF—disrupting disulfides with inhaled reducing agents acutely restores mucociliary function and prevents fibrosis by disrupting Muc5b ('MUC' in humans; 'Muc' in mice). The chief goal of the research proposed here is to elucidate molecular mechanisms of MUC5B assembly that could lead to selective intervention targets. MUC5B polymerizes at its N- and C-termini, via D3 and CK domains, but precisely how N-terminal D3 and C-terminal CK domains affect MUC5B *in vivo* is not known. These will be interrogated using gene-editing in mice at baseline, in lung injury, and in models of IPF.

15. SUBJECT TERMS
lungs, mucins, fibrosis, gene editing

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TABLE OF CONTENTS

	Page
1. Introduction	3
2. Keywords	4
3. Accomplishments	4
4. Impact	5
5. Changes/Problems	6
6. Products	6
7. Participants & Other Collaborating Organizations	7
8. Special Reporting Requirements	8
9. Appendices	8

REPORT OUTLINE

1. **INTRODUCTION:** This project seeks to develop an innovative genetic screening platform to study mucin molecules, with the ultimate goal of identifying conceptually novel targets for mucus-directed therapies. Although mucus is required for innate defense in the lungs, mucus dysfunction is a significant problem in lung diseases. Idiopathic pulmonary fibrosis (IPF) is a progressive disorder characterized by peripheral lung injury and aberrant repair with excessive collagen-rich matrix. Recently, a link was discovered between the risk of developing IPF and a polymorphism in MUC5B, a gene encoding a mucin glycoprotein localized to central conducting airways. The polymorphism resides in the MUC5B promoter, enhances its expression, and results in increased MUC5B protein in small bronchiolar airways. MUC5B aggregation in small airways and honeycomb cysts is a well-known pathology in late stages of IPF, and emerging data implicate mucus dysfunction as a defining characteristic present throughout the course of PF, even in early pre-clinical stages. Dysregulated mucus causes mucociliary clearance (MCC) defects, leading to prolonged inflammation and the promotion of distal lung remodeling. This proposal seeks to determine how MUC5B aggregates and causes fibrosis in distal airspaces. MUC5B polymers are formed by disulfide bond-mediated assembly (Fig. 1), and disulfide reduction improves mucus function and prevents PF. Although mucolytics may be potent for rescue, there are few efficacious prevention options available. Determining sites on MUC5B required for its polymerization during synthesis could lead to strategies that are preventative and more selective. The overarching concept guiding this proposal is that controlling MUC5B polymerization could enhance mucociliary defense in injured or diseased lungs. Immediate goals are to determine how Muc5b polymerization occurs and how manipulating Muc5b assembly could improve host defense. These objectives address a significant problem by directly confronting challenges related to the size and nature of mature polymeric mucins. Unlike cytokines or growth factors (e.g.) where ligands or antagonists can easily be introduced directly into the lungs or systemic routes, MUC5B/Muc5b polymers are massive (10s to 100s of mega-Daltons) and complex, making exogenous administration untenable. To overcome this obstacle, CRIPSR/Cas9 targeting will be used to directly mutagenize endogenously produced Muc5b in a highly specific manner in vivo. Accordingly, the studies proposed here test the hypothesis that CRISPR/Cas9 gene-editing will elucidate specific cysteine residues required for MUC5B polymer assembly thus revealing targets for reducing lung injury, inflammation, and mucus dysfunction. Two specific aims are proposed to 1) test the hypothesis that conserved D3 and CK cysteines are required for Muc5b polymerization (Fig. 2), and 2) test the hypothesis that selective inhibition of Muc5b disulfide polymerization sites restores mucus gel function, thereby reducing lung injury and PF in vivo.

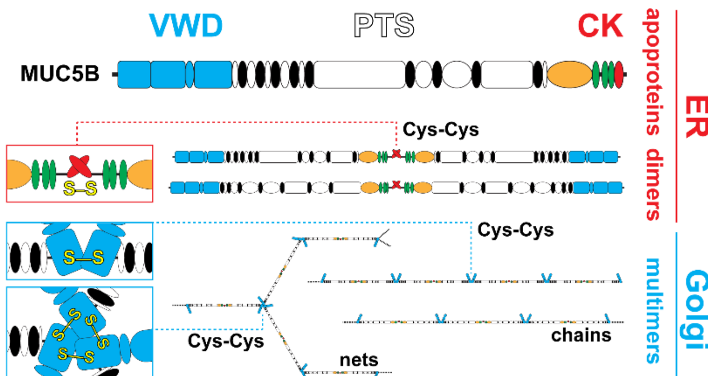

Fig. 1. MUC5B structure and assembly. MUC5B is translated in the ER where apoproteins dimerize via protein disulfide isomerase (PDI) enzyme-mediated disulfide bonding (S-S) at C-terminal cysteine knot (CK) domains . In the Golgi, during glycosylation (not shown), the mucins polymerize via N-terminal disulfides in VWF-D like (VWD) domains. Glycans omitted for clarity.

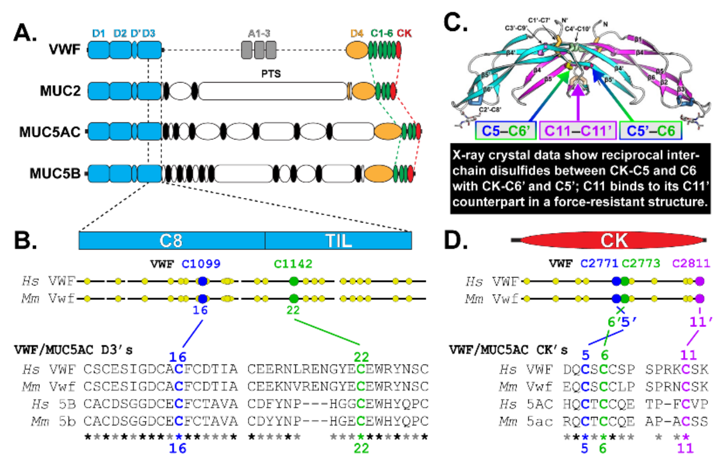

Fig. 2. Conserved mucin and VWF disulfide assembly sites. (**A**) N-terminal D1/D2/D'/D3 and C-terminal D4, VWC, and CK domain architectures are conserved in mucins and VWF. D3 and CK domains are known polymerization regions in VWF. (**B**) Two D3 domain modules (C8 and TIL, trypsin inhibitor-like) contain cysteines **C1099** and **C1422** required for inter-chain assembly in VWF. These are strongly conserved in human and mouse MUC5B/Muc5b. They are numbered 1-30 here based on their orders in their D3 domains. D3-**C16** and **C22** align with VWF and are thus hypothesized to be required for MUC5B/Muc5b polymerization. (**C**) VWF C-termini dimerize as interleaved β sheets secured by inter-chain disulfides. The so-called cysteine knot (CK) domain they form has been crystallized for human VWF. CK-C's are numbered as in D3's above. In VWF, CK-**C5** and **C6** form reciprocal crossed inter-chain disulfides (**C5-C6'** & **C5'-C6**). CK-**C11** forms a disulfide with its complement (**C11'**) that further stabilizes **5-6'** and **5'-6** pairs. (**D**) Conserved CK-**C5**, **C6**, and **C11** sites in mouse Muc5b will be interrogated along with D3 pairs in **B**.

2. **KEYWORDS:** lung, mucin, mucus, fibrosis, MUC5B, disulfide, polymerization, gene editing

3. **ACCOMPLISHMENTS:**

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

Major Task 1: Regulatory approval, generate targeting reagents.	Months	% completion
Subtask 1: Regulatory approval of animal research.		100%
Milestone #1: Secure IACUC approval at University of Colorado AMC.	0	
Milestone #2: Secure ACURO approval.	0-3	
Subtask 2: Generate mice by CRISPR/Cas9 & HDR.		
Milestone #1: Submit sequences for generating mouse strains.	0-1	
Milestone #2: Produce clones for 3 guide RNA's; 5 oligos for HDR.	1-2	
Major Task 2: Produce and screen mice by *in vivo* site-directed mutagenesis.	Months	
Milestone #1: Produce 5 lines of Tg mice.	3-4	80%
Milestone #2: Screen F0 mice (5 lines; at least 15 homozygotes per line).	4-5	80%
Milestone #3: Transfer 5 lines from genetics core to PI's vivarium space.	5	*80%*
Milestone #4: Breed F0 mice to C57BL/6J (1st cross to result in N1)	5-6	60%
Milestone #5: Analyze Muc5b polymers in F0 line	5-6	*60%*
Milestone #6: Statistical analysis of Data	6	*0%*
Major Task 3: Produce established transgenic lines; perform inflammatory challenges	Months	
Milestone #1: Breed N1 mice (5 lines) with C57BL/6J (2nd cross to result in N2).	8-9	40%
Milestone #2: Inbreed N2 mice (5 lines); produce F1 mice for experiments	9-11	20%
Milestone #3: Expose F1 experimental mice (5 lines) to bleomycin, Aspergillus extract, and NTHi lysate.	11-15	0%
Milestone #5: Analyze mucus microrheology, physiology, inflammation, mucins, histology.	12-16	0%
Milestone #6: Statistical analysis of Data	13-16	*0%*
Milestone #7: Write-up and publish results of data. Share technologies.	14-18	10%
Milestone #8: Develop future study designs, seek funding to perform long-term PF studies in mice, and humans;	14-18	0%

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

Major Task #1 was completed entirely.

For Major Task #2, major activities include the production of 4 of the 5 point mutant lines proposed for our research. These include the D3-C22S, CK-C5, CK-C6, and CK-C11 lines. (see Fig. 2)

The D3-C22S line yielded 10 founders out of 38 F0 pups. The lines were bred to C57BL/6 mice to generate N1 animals, and three litters were observed on 11/9/20, 11/11/20, and 11/12/20. The F0 founders have been bred and will now be transferred to the PI's mouse colony and to test for Muc5b polymerization changes by immunoblotting. They were also used to produce and D3-C15S lines due to a recent report that this cysteine residue can serve as a site of polymerization in the intestinal mucin Muc2.

On 4/27, we did IVF with Muc5b C22S/C22S males and wildtype females. The resulting embryos were injected with either C15S or C16S plus guide RNA and Cas9. We had 5 pregnant females. In one cage, 18 pups were born and in the other, 24. The pups were born on 5/17. We are genotyping these animals presently.

For the CK-C5 and -C6 lines, disruption of each resulted in early post-natal mortality (>20 mice cannibalized by dams). Because these cysteines are used for heterotypic bonds (C5 on one molecule forms a disulfide with C6 on another molecule), we suspected that misfolding induced ER stress resulting in cellular toxicity. We were able to salvage mice at early postnatal timepoint (days 5-7), and we are examining them histologically. CK-C11S mice are being produced currently.

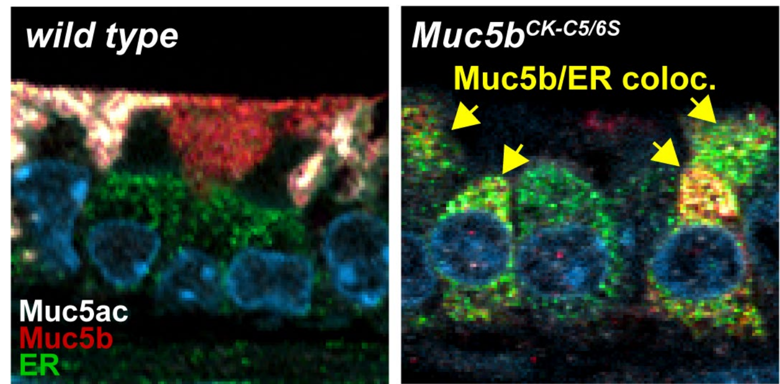

Figure 3. Localization of Muc5b in the ER of uninflamed *Muc5b*$^{C5/6S}$ mice. Wild type mice (left) express and secrete Muc5ac (white) and Muc5b (red), but Muc5b is diminished and is restricted to the ER (green) in *Muc5b*$^{C5/6S}$ mice (right).

Because of the nature of these results with CK cysteine mutations, we repeated our study using a different homology directed repair oligonucleotide that mutates C5 and C6 alone. This line of mice produced a striking phenotype in which the mutated Muc5b molecule was sequestered in the endoplasmic reticulum and degraded (Fig. 3). We are investigating whether this phenotype drives ER stress in a pro-fibrotic manner, or if it shunts Muc5b into a protective ER associated degradation response.

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

Nothing to Report.

- **How were the results disseminated to communities of interest?**

Nothing to Report.

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

We have targeted D3-C15 and -C16 in using sperm from a founder C22S male mouse and ova from wild type C57BL/6 females. The progeny are expected to yield C15S and C16S mutants singly and in combination with C22S, thereby increasing the likelihood that we are able to fully disrupt Muc5b D3 assembly.

The other CK line, CK-C11 has been designed is scheduled to be injected in July 2021.

Dr. Jaramillo has tested for biochemical characterization of mucin polymers in D3-C22S mice by western blotting. Since there were no changes in polymerization, she paused on starting lung fibrosis and injury models until more mice are on hand.

During this time, however, the CK-C5/C6 line has produced very intriguing results, wherein the production go a misassembled Muc5b polymer results in suppression of the protein. We are investigating mechanisms of ER associated degradation through proteasomal and autophagic mechanisms.

4. **IMPACT:**

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

Nothing to Report.

- **What was the impact on other disciplines?**

Nothing to Report.

- **What was the impact on technology transfer?**

We are preparing a technology disclosure related to the potential for ER associated degradation to serve as a means for suppressing Muc5b expression in vivo.

- **What was the impact on society beyond science and technology?**

  Nothing to Report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

  Our overall approach remains unchanged.

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

  A recently paper investigating structure of a related mucin called Muc2 was published (PMID: 31310764) after submission and award of this grant. The data are controversial, but they suggest that the 15th cysteine in the D3 domain of Muc5b may be the one that is involved in N-terminal polymerization as opposed to C16. We have injected mice to produce the C15S mutation alone and in combination with C22S.

- **Changes that had a significant impact on expenditures**

  The COVID-19 pandemic and closure in response to it delayed our mouse generation by approximately 2 months. Fortunately, both the Evans lab and the Mouse Genetics Core were considered critical operations, so by June 2020 thing were back on track for mouse production. In addition, there were additional losses in time that caused an additional 3 months delay due to respect to staffing and histology cores and the availability of vivarium resources for lung fibrosis and injury models. As of September 2020, these are all operational but running at lower capacity due to COVID-related staffing restrictions. Because we are behind schedule, we have not produced enough mice to perform bleomycin challenge studies. For this reason, we requested and were granted an extension of this award to 2022.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  Nothing to Report.

- **Significant changes in use or care of human subjects**

  Nothing to Report.

- **Significant changes in use or care of vertebrate animals.**

  Nothing to Report.

- **Significant changes in use of biohazards and/or select agents**

  Nothing to Report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  Nothing to Report.

- **Website(s) or other Internet site(s)**

  Nothing to Report.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*Christopher Evans*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-5600-7314*
Nearest person month worked:	*2.4 calendar months*
Contribution to Project:	*Dr. Evans has overseen the project and assisted with planning and study design.*
Funding Support:	*Additional funding for the PI comes from NIH and Cystic Fibrosis Foundation resources.*

Name:	*James Needell*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*4 calendar months*
Contribution to Project:	*Mr. Needell has overseen the mouse breeding and husbandry.*
Funding Support:	*Additional funding for Mr. Needell comes from NIH resources.*

Name:	*Ana Maria Jaramillo*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-8089-4275*
Nearest person month worked:	*6 calendar months*
Contribution to Project:	*Dr. Jaramillo managed the generation of mouse strains, screens for presence of transgenes, and analysis of effects on Muc5b assembly.*
Funding Support:	*Additional funding for Dr. Jaramillo comes from Cystic Fibrosis Foundation resources.*

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

  Nothing to Report.

- **What other organizations were involved as partners?**

  Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**

  Nothing to Report.

- **QUAD CHARTS:**

  Nothing to Report.

9. **APPENDICES:** Nothing to Report.