Mechanisms of inflammation in cystic fibrosis and potential for reversibility

RESUBMISSION FOR AN F-31 GRANT FROM THE NICHD

LEIGH C HENDERSON

1. Introduction

Due to the extensive changes from the original application, the resubmission's research training plan does *not* identify changes using bracketing, indenting, or changed typography.

The largest critique of my previous submission was lack of hypothesis-driven research. This resubmission is driven by the following hypotheses:

1. Oxidative stress induces HDAC2 protein modification and subsequent degradation resulting in increased inflammatory signaling, which contributes to cystic fibrosis (CF) lung disease.
2. Pro-inflammatory signaling will normalize to non-cystic fibrosis levels after CFTR potentiation.

These hypotheses serve also to narrow the focus of the proposal to specific pathways that may contribute to cystic fibrosis lung disease. I can accomplish the proposed work as a predoctoral student.

Previously, I proposed to create a cell model differentiated from induced pluripotent stem cells (iPSCs). Reviewers pointed out that the differentiation protocol was not well established and optimization would take unjustifiably long for a predoctoral project. To address these critiques, I developed a method to knockout genomic CFTR in primary airway epithelial cells (AECs) – resulting in a new CF AEC model. I also created a CFTR-null line of Calu-3 lung carcinoma cells. I switched from an iPSC-derived model to primary and immortalized AEC models for several reasons. First, improved culture techniques recently made gene-editing feasible primary AEC. My co-sponsor, Dr. Y, has developed a method by which primary AEC thrive in culture for at least one year and a dozen passages. Second, primary and immortalized AECs are currently a closer model to *in vivo* AECs than those differentiated from stem cells. Available differentiation protocols result in generation of airway progenitor cells, a subpopulation of which are epithelial-like. However, it is not well known how well epithelial-like airway progenitor cells resemble AECs *in vivo* because the differentiation protocols are so new (with the most recent published in fall 2013). Third, use of primary and immortalized AECs increases the feasibility of the project for me as a graduate student.

Other critiques addressed include the need for establishment of gene-editing technology in the laboratory and an expert mentor in gene-editing. In the months stretching from June-December 2013, my laboratory designed and created CRISPR plasmids for gene-editing, transfected the plasmids into the non-CF Caco-2 cell line, isolated clones with edited CFTR, and showed the clones to be CFTR-null (manuscript in preparation). I have been a critical part of the development of CRISPR technology in my laboratory and involved at each step of the process. Edited clones include those with single base pair insertions/deletions, large-scale deletions, and specific base pair changes such as the G551D mutation. Gene-editing technology is firmly established. Dr. X (sponsor) has been the primary gene-editing mentor. His expertise in molecular genetics, beginning with the identification of CFTR as the causative gene in cystic fibrosis, is well fitted for application to the new field of gene editing. Indeed, the CRISPR technique for gene-editing was published less than a year before my laboratory began to use it (Mali et. al. 2013 and Le Cong et. al. 2013). Several other laboratories that I collaborate with are also beginning to use CRISPR technology and have provided helpful advice on the topic, namely those of Dr. Z (across the hall) and Dr. Q (at Northwestern University, Illinois).

My sponsor, Dr. X, also fine-tuned the training plan section in response to reviewer critiques. The plan now specifies the skills (ex. epithelial electrophysiology and state of the art primary cell culture techniques) and opportunities (ex. contributing to an R01 grant being submitted by Dr. X) made available to me through the proposed work.

2. Specific Aims

Monogenic disorders arise from mutations in a single gene. New technologies make it possible to correct or create such mutations at the genome level. Specifically, gene-editing enables the creation of matched disease/control cell models that differ genomically only at the disease-causing gene. Research into the mechanisms of monogenic disease pathology will benefit immensely from such cell models.

Cystic fibrosis (CF) is a monogenic disease caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Mutant CFTR in airway epithelial cells causes dehydration of airway mucus leading to bacterial infection. Chronic cycles of bacterial infection and inflammation are responsible for the progressive nature of CF lung disease. Lung disease is the leading cause of morbidity and mortality among the 70,000 people with CF worldwide, the majority of whom are children.

Airway epithelial cell (AEC) models derived from transformed cells have contributed greatly to the current understanding of CF lung disease pathology. Despite their usefulness, non-endogenous regulation of CFTR expression and lack of isogenicity between disease and control lines impose significant limitations on the information that can be gained from existing AEC models. I have developed a gene-editing procedure to create more physiologically relevant AEC models. Specifically, I can create CFTR-null primary human tracheal epithelial cells by introduction of frame-shifting insertions or deletions to genomic CFTR. My edited primary cells present a novel opportunity to study effects of CFTR's absence in chromosomally stable cells that maintain the properties of native AEC.

I propose to use this new model to examine inflammatory signaling in CF AEC. Results from the studies will inform therapeutic development with the goal of slowing progression of chronic CF lung disease.

Aim 1: Elucidate mechanisms of HDAC2 degradation which contribute to inflammatory signaling in CF

Excessive inflammation mediated by airway epithelial cells damages lung tissue and contributes to CF lung disease. My mentor's laboratory found that increased release of pro-inflammatory cytokine IL-8 correlates with hyperacetylation of the IL-8 promoter. Furthermore, hyperacetylation is linked to reduced histone deacetylase 2 (HDAC2) protein activity and abundance without a corresponding reduction in mRNA. This suggests differential HDAC2 regulation in CF cells occurs at the protein level. Several protein modifications are reported to decrease HDAC2 activity and promote degradation. In chronic obstructive pulmonary disorder (COPD), oxidative stress-induced HDAC2 modifications result in decreased activity/abundance and contribute to increased inflammatory signaling. Drugs targeting this pathway are effective in fighting COPD. HDAC2 modification pathways remain unexplored in CF. CF airway epithelial cells are exposed to high levels of oxidative stress *in vivo* and are more vulnerable to oxidative damage because of reduced expression of antioxidant genes. I hypothesize oxidative stress induces HDAC2 protein modification and subsequent degradation resulting in increased inflammatory signaling in CF airway epithelial cells. Elucidation of this pathway may result in new drug targets for the prevention and treatment of CF lung disease.

Aim 2: Determine the time course of inflammatory stress response after CFTR potentiation

CF cells undergo chromatin modifications at inflammatory genes and thus it is unknown whether correction of the basic defect, CFTR dysfunction, will normalize inflammatory signaling by CF AECs. This question is particularly pressing due to the advent of Ivacaftor, a CFTR potentiator drug. Ivacaftor treatment improves lung health in CF patients by unknown mechanisms. I hypothesize pro-inflammatory signaling normalizes to non-CF levels upon CFTR potentiation. Ivacaftor is only effective at potentiating CFTR with specific mutations, the most common of which is G551D. I propose to address my hypothesis in human nasal epithelial cells isolated from CF patients with the G551D mutation. If pro-inflammatory signaling does not respond to Ivacaftor treatment, then CF patients on Ivacaftor may still benefit from anti-inflammatory therapies.

3. Research Strategy

(a) Significance

Cystic fibrosis (CF) is a monogenic disorder caused by mutations to the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. In airway epithelial cells (AECs), *CFTR* maintains airway surface liquid viscosity by modulating the osmotic gradient. In CF, highly viscous airway surface liquid predisposes patients to pulmonary bacterial infections. Ensuing inflammation leads to lung tissue damage and, eventually, to lung disease. Inflammation begins when airway epithelial cells release pro-inflammatory chemoattractants. Neutrophils recruited to the airway release bactericidal elastase and oxidants, which can also damage lung connective structure and cells. Bronchiectasis and lung disease develop over time as a result of these recurrent processes (15). To address pulmonary inflammation, CF patients are prescribed high-dose ibuprofen. Additional treatments include airway clearance techniques to clear thick mucus from the lungs and inhaled antibiotics. Despite treatment, 47% of CF patients in the United States 18-29 years-old have moderate to severe lung disease (19). New therapeutic targets are needed.

Elucidate pathways amenable to therapeutic intervention

Chromatin remodeling contributes to exuberant inflammatory signaling in CF AEC even in the absence of bacterial infection (5, 28). Specifically, a dramatic reduction in histone deacetylase-2 (HDAC2) contributes to hyperacetylation at pro-inflammatory genes (6). The studies proposed here will investigate the mechanisms underlying reduced HDAC2 levels, providing insight into means to manipulate acetylation state for therapy.

Determine which attributes of CF inflammatory signaling are reversible and the time course of doing so

Comparing profiles of CF cells to the same cells with *CFTR* potentiated will allow us to determine which pathways altered in CF cells can be restored to non-CF levels as a consequence of *CFTR* potentiation. These studies would then also identify pathways for which alternative methods for intervention are necessary.

Use of a more relevant cell model to study disease process

Most of the current understanding about CFTR function and its effects is derived from studies in cells transformed virally or by cancer-causing mutations that led to adenocarcinomas. Transformed cells are chromosomally unstable and selected for their ability to replicate. Viral transformation can also increase inflammatory response (23), which would interfere with the proposed experiments. Transformed AEC often lose expression of cilia and formation of tight junctions; it is hoped that they maintain other native properties relevant to biological questions under study. Furthermore, introduction of exogenous *CFTR* expression and/or regulation to create matched CF/non-CF lines may disrupt native functions of CFTR. Improved primary AEC culture techniques combined with new gene-editing technologies have made it possible to create matched CF/non-CF primary AEC (see preliminary data). This non-transformed model uniquely provides an isogenic [except at *CFTR*], chromosomally stable environment with endogenous regulation of *CFTR* (Table 1). Aim 1 makes use of gene-edited CFTR-null primary human tracheal epithelial cells. Aim 2 makes use of drug-potentiated human nasal epithelial cells derived from CF patients.

Table 1: Advantages of gene-corrected cell models (last two rows) over existing airway epithelial cell models of cystic fibrosis.

Cell Model (control / CF)	Description	Ideal characteristics		
		Non-transformed	Isogenic CF and control lines	Endogenous regulation of *CFTR*
9/HTEo$^-$ pCEP / pCEP-R	CFTR function is inhibited by the overexpression of the protein's R domain		✓*	
S9 / IB3	CFTR function is rescued by expression from a virus in S9 but not the CF-patient derived IB3 cells		✓*	
Primary cell inhibited with $CFTR_{inh}$-172	CFTR is inhibited with a drug	✓	✓	
16HBE14o$^-$ sense / antisense	CFTR function is inhibited by plasmid-based expression of part of human *CFTR*		✓*	
Calu-3 $shCFTR_{ALTER}$ / *shCFTR*	CFTR is inhibited by transposon-integrated short hairpin RNAs against CFTR		✓*	
CF_{15} / CF_{15} advCFTR	CFTR function is transiently rescued by expression from an adenovirus (advCFTR)		✓*	
Primary cell CFTR(+/+) / CFTR(mut/mut)	Genomic *CFTR* is mutated or corrected by CRISPR-mediated editing in primary AECs	✓	✓	✓
Calu-3 CFTR(+/+) / CFTR(mut/mut)	Genomic *CFTR* is mutated by CRISPR-mediated editing in Calu-3 AECs		✓*	✓

*Due to frequent genomic rearrangements in immortalized cell lines, isogenicity between CF and control lines is not certain. References for cell models: 9/HTEo$^-$ pCEP / pCEP-R (42); S9/IB3 (20); Primary cell inhibited with *CFTR*$_{inh}$-172 (41); 16HBE14o$^-$ sense/antisense (30), Calu-3 *shCFTR*$_{ALTER}$ */shCFTR* (38), CF_{15} (26) and CF_{15} advCFTR (43).

In addition to primary airway epithelial cells, I will also study a novel CF lung adenocarcinoma cell line. I created this model by knocking out genomic CFTR in Calu-3 cells with CRISPR gene-editing technology. A large proportion of the literature on CFTR's role in AEC is derived from the non-CF Calu-3 lung adenocarcinoma cell line. Mine is the first stable CF Calu-3 model and has the unique advantage of endogenous regulation of *CFTR* (Table 1). If the findings in the Calu-3 model are similar to those in the primary AEC models, then it can be surmised that cell transformation does not affect the studied inflammatory processes. Then the Calu-3 model will be useful for future studies and provided as a resource to other investigators.

b) Approach

Aim 1: Elucidate mechanisms of HDAC2 degradation that contribute to inflammatory signaling in CF

Dr. X's laboratory has previously shown that reduced histone deacetylase-2 protein in CF airway epithelial cells correlates with promoter hyperacetylation and increased expression of the pro-inflammatory gene IL-8 (Fig. 1). IL-8 recruits neutrophils to the airways; excessive neutrophil recruitment characteristic of CF results in airway damage (15). Increased inflammatory signaling was not an artifact of CF-specific exposure to chronic airway infections because the CF cell models used were matched to controls derived from the same individual. Furthermore, infants with CF exhibit slightly increased pro-inflammatory IL-8 signaling in broncheoalveolar lavage fluid in the absence of bacterial infections (28). CFTR dysfunction contributes directly to increased inflammatory signaling by AEC via yet unknown mechanisms.

HDAC2 protein level and activity are reduced in CF AECs without a corresponding reduction in mRNA (6). This suggests that HDAC2 protein synthesis or degradation is affected. S-nitrosylation, nitration, phosphorylation, and ubiquitination of the HDAC2 protein are reported to decrease activity and promote degradation (1, 31, 37) and are therefore logical candidate processes to explore. In fact, HDAC2 modifications play a role in the pathogenesis of other pulmonary disorders. In chronic obstructive pulmonary disorder (COPD), HDAC2 modifications contribute to increased expression of pro-inflammatory genes in airway epithelium (3). Several drugs targeting this pathway are proving effective in fighting COPD (4). HDAC2 degradation pathways remain unexplored in CF. CF airway epithelial cells are exposed to high levels of oxidative stress and are more vulnerable to oxidative damage because of reduced expression of antioxidant genes, a reproducible but unexplained phenomena of CF cells (14). *I hypothesize oxidative stress induces HDAC2 protein modification and subsequent degradation resulting in increased inflammatory signaling in CF airway epithelial cells*. Elucidation of this pathway may result in new drug targets for the prevention and treatment of CF lung disease.

Measure IL-8 and HDAC2 mRNA and protein expression; Measure IL-8 promoter acetylation

IL-8 and *HDAC2* gene expression assays will be run as previously reported (5, 6). Based on prior studies in other other cell models, it is expected that CF cells will express ~2-15x more IL-8 mRNA (Fig 1A) and the same amount of HDAC2 mRNA (5, 6). IL-8 protein will be quantified by ELISA as previously reported (39, 41) or by Luminex assay in collaboration with the laboratory of Dr. R. HDAC2 protein will be quantified by western blotting. It is expected that CF cells will express ~2x more IL-8 (39, 41) and ~3-6x more HDAC2 protein than non-CF cells (Fig 1D-E). Histone 4 acetylation patterns at the *IL-8* promoter are well characterized and will be assayed as previously reported (5). It is expected that histone 4 acetylation will be ~5-100x higher in CF cells (Fig 1B). Histone 3 acetylation patterns at the *IL-8* promoter (2) will also be assayed.

Induce oxidative and bacterial stress

I will expose AEC to physiologically relevant oxidative and bacterial stress. AEC *in vivo* are exposed to hydrogen peroxide (H_20_2) released by neutrophils in the airway lumen (45). Excessive neutrophil load is characteristic of CF airways (15). Therefore, oxidative stress will be induced by exposure to 200 uM H_20_2 in serum-free medium for 3 hours (5). Over 75% of CF patients are infected with *Pseudomonas aeruginosa* by age three (11). By adulthood, *P. aeruginosa* and several other species of bacteria are usually the most numerous species in the lungs of healthy CF patients (7, 10). Among *P. aeruginosa* proteins, flagellin stimulates the greatest response in airway epithelial cells (see review (44)). Therefore, bacterial stress will be induced by exposure to *P. aeruginosa* flagelin (50ng/ml) for 6 hours (46). Flagellin will be purified from a clinical isolate of *P. aeruginosa,* available from

Dr. R, as previously described (49). A mock purification of flagellin from a strain of *P. aeruginosa* lacking flagella will serve as a negative control.

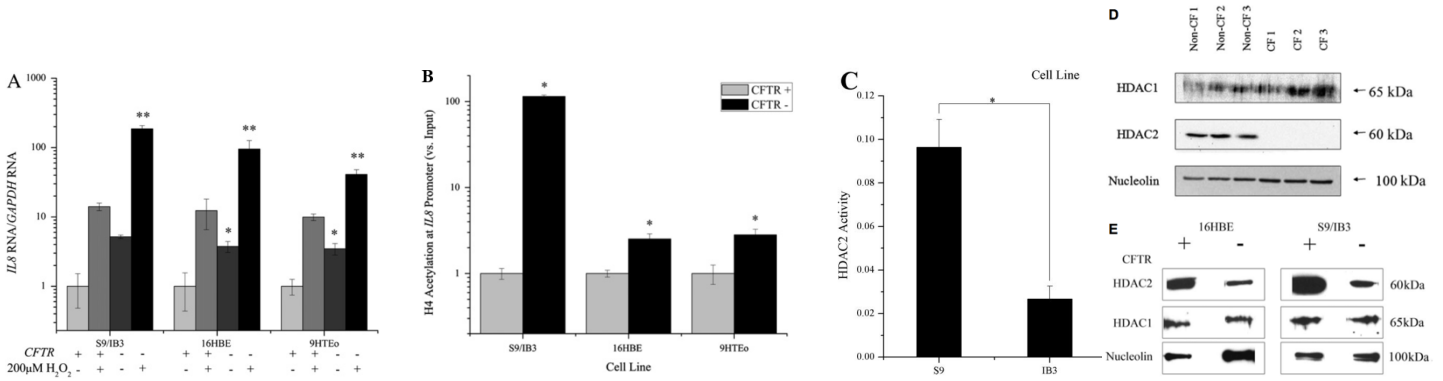

Figure 1: (A) IL-8 expression is increased in three CF AEC lines under basal conditions and stress induced by hydrogen peroxide. **(B)** Increased IL-8 expression correlates with hyperacetylation at histone 4 of the IL-8 promoter, **(C)** decreased HDAC2 activity in CF AEC, and decreased HDAC2 protein in **(D)** primary and **(E)** immortalized AEC models. Figures reproduced from references (5, 6), authored by Dr. X's (sponsor's) laboratory.

Measure the rate of HDAC2 synthesis and degradation; Measure HDAC2 activity

Reduced levels of HDAC2 protein without a change in mRNA levels could be explained by reduced HDAC2 synthesis or increased degradation. I will measure HDAC2 synthesis and degradation rates by stable isotope labeling by amino acids in cell culture (SILAC) (29, 36). In SILAC, cells are cultured in media in which the isotopic form of an amino acid replaces the natural form. Newly synthesized proteins incorporate the labeled amino acid. Synthesis rates are measured after a sufficient time of incubation with the isotope; degradation rates are measured after a 'chase' period. Measurement is performed by mass spectrometry after purification of the protein of interest. Dr. S will provide mentoring in isotopic labeling. Leigh Henderson (PI) has experience in the technique and is a co-author on several of Dr. S's isotopic labeling papers (8, 9, 12). In addition to HDAC2 synthesis and degradation rates, HDAC2 activity will be assessed by colorimetric enzymatic assay kit (6). Prior data indicates decreased HDAC2 activity in CF AEC (Fig. 1C).

Examine HDAC2 protein modifications in CF airway epithelial cells

Post-translational modifications to HDAC2 influence the protein's stability and activity. **S-nitrosylation** at cysteine residues 262 and 274 decreases deacetylation at chromatin targets of HDAC2 (31, 35). **Ubiquitination** results in proteasomal degradation (29). **Nitration** at tyrosine residue 253 increases ubiquitin-mediated proteasomal degradation (37). **Phosphorylation** at unidentified serine and threonine residues decreases enzymatic activity and increases ubiquitin-mediated proteasomal degradation (1).

I hypothesize that decreased HDAC2 levels in CF cells exposed to stress can be explained by increased levels of post-translational modifications on HDAC2. **S-nitrosylation** will be measured by biotin-switch assay in which free thiol groups are blocked with S-methyl methanethiosulfonate, s-nitrosyl groups are converted to free thiols with ascorbate and then labeled with biotin-HPDP, and then purified biotinylated proteins are analyzed by western blotting for HDAC2 (34). **Nitration, phosphorylation, and ubiquitination** will be measured by immunoprecipitation of HDAC2 followed by western blotting for nitrotyrosine, phosphoserine, phosphothreonine, or ubiquitin (1, 29, 37). Primary CF AEC have undetectably low amounts of HDAC2 (Fig 1D), therefore HDAC2 accumulation will be encouraged by incubation of the cells with proteasome inhibitors ALLM and/or MG-132 for 24 hours preceding protein modification assays.

Determine the effect of CF-relevant HDAC2 modifications on IL-8 expression and promoter acetylation

HDAC2 deacetylates the *IL-8* promoter and reduces gene expression. By decreasing HDAC2 amount/activity, the modifications discussed above should increase promoter acetylation and gene expression. To test this model, I will first target HDAC2 modifications found to be altered in CF. A variety of drugs target HDAC2 modifications: S-nitrosoglutathione (GSNO) promotes s-nitrosylation of HDAC2; cell-permeable glutathione (GSH-e) removes s-nitrosylthiol groups from HDAC2 (31); and valproic acid specifically ubiquitinates HDAC2 and other class I HDACs (29). In addition to drugs, I will also use mutant HDAC2 proteins incapable of S-nitrosylation (35) or nitration (37) at specific residues. The Flag- or His-tagged mutant HDAC2 plasmids will be introduced into airway epithelial cells using Lipofectamine 2000; Lipofection has already optimized for use in

both primary HTE and Calu-3 cells by Leigh Henderson (PI). Tagged wild type HDAC2, empty vector, and untagged HDAC2 plasmids will provide controls. Upon altering HDAC2 modification status, I will measure HDAC2 binding to the *IL-8* promoter, *IL-8* promoter acetylation (by immunoprecipitation as previously reported (6)), and IL-8 expression as well verify alteration of the targeted HDAC2 modification.

Identify pathways responsible for modifying HDAC2 in CF

If HDAC2 modifications are found to contribute to increased IL-8 expression, then pathways responsible for modifying HDAC2 will be studied. For example, if S-nitrosylation of HDAC2 contributes to increased IL-8 expression, then I hypothesize that decreased Nrf-2 activity contributes to an increase in S-nitrosylation of HDAC2 via the pathway outlined in Figure 2. Each step in the pathway is supported by previous literature, as discussed below, but the pathway itself has not been studied in CF.

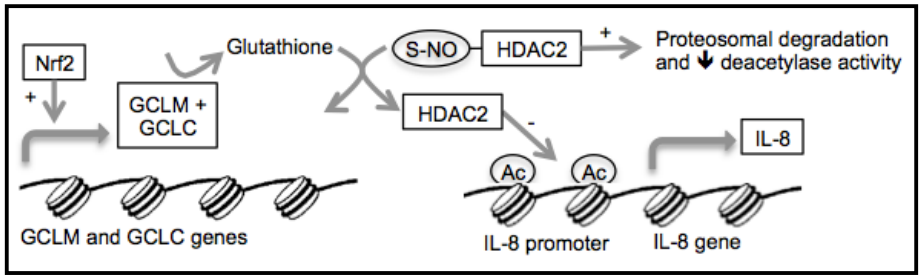

Figure 2: I hypothesize that decreased levels of Nrf2 protein lead to increased IL-8 production.

Dysfunction of CFTR is shown to reduce the activity and level of Nrf2, a transcription factor that binds to antioxidant responsive elements in the promoters of genes to promote production of antioxidant proteins (14). *GCLM* and *GCLC* are under transcriptional control of Nrf-2. Together, GCLM and GCLC catalyze the production of the antioxidant metabolite glutathione (GSH). The reduced form of glutathione (GSH) reacts with reactive oxygen species to form glutathione-disulfide (GSSG). Notably, GSH removes s-nitrosothiol groups from proteins including HDAC2 (31). S-nitrosylation of HDAC2 results in decreased deacetylase activity and increased degradation (31, 35).

To test the hypothesized pathway, each step will be targeted and the downstream effects studied. GSH levels may be raised with GSH-e; GSH synthesis antagonized with BSO; Nrf2 activity antagonized with PI3K inhibitors such as NVP-BKM120; Nrf2 localization into the nucleus activated by ML334; and Nrf2 degradation inhibited by tBHQ and sulforaphane. GSH will be measured by gas chromatography mass spectrometry (13, 33), which also allows for the measurement of GSH synthesis rate using a stable isotope of cysteine. Nrf2, GCLM, and GCLC levels will be assayed by qRT-PCR and western blot.

Primary human tracheal epithelial (HTE) cell models

The Epithelial Cell Culture Core, directed by Dr. Y (co-sponsor), isolates airway epithelial cells from autopsied or lung transplant-recipient tracheas under BSL2 safety conditions. Leigh Henderson (PI) receives these isolated HTE and proceeds with CRISPR-mediated gene-editing (16, 21) in core facilities. Briefly, cells are lipofected with plasmids encoding Cas9 and guide RNA (gRNA). Cas9 is a bacterial endonuclease; the version I use (Addgene 44719) is mammalian-codon optimized and co-transcribed with a 2A peptide sequence followed by green fluorescent protein (GFP). Upon expression, 1) Cas9 and gRNA form a complex, 2) the complex binds with DNA complementary to a sequence in the gRNA, and 3) Cas9 cleaves a double strand break at the site of binding (32). To knockout CFTR, I use two gRNAs: one targets *CFTR* exon 12 near F508 and the other, exon 11 near G551. These gRNAs were chosen for high efficiency at creating CFTR mutations in the Caco-2 cell line, in which combinations of 1-2 gRNAs against *CFTR* exons 1, 11, 12, and 23 were trialed (manuscript in preparation). A large deletion results from non-homologous end joining between the two break sites. Smaller insertions or deletions result from non-homologous end joining during repair of a single break site. Gene-editing often results in different mutations on the two alleles of *CFTR*; if both the alleles have frame-shift mutations, as in the clone in Fig 3C-D, then the result is CFTR knockout. Gene-edited clones are isolated from the population by 1) fluorescently activated cell sorting for GFP+ cells (Fig. 3A), 2) plating at low density (Fig. 3B), and 3) removal to a new plate with the aid of cloning cylinders.

HTE can be stored at -80°C in suspension with 10% DMSO for at least 1 year. For experiments requiring formation of an epithelium, cells are cultured on Transwell polyester filter inserts at air-liquid interface (40, 41). Under these conditions, HTE form tight junctions and express cilia at the apical membrane (Fig 4A-B), characteristics of AEC *in vivo*. Furthermore, HTE maintain conductance through CFTR for at least 6 passages (Fig. 4E). To confirm CFTR knockout, conductance through CFTR will be assayed in gene-edited CFTR-null

HTE. Assays will be performed on an Ussing chamber under the guidance of Dr. Y (co-sponsor). Dr. Y has longstanding expertise in trans-epithelial ion conductance (17, 18, 41, 47, 48).

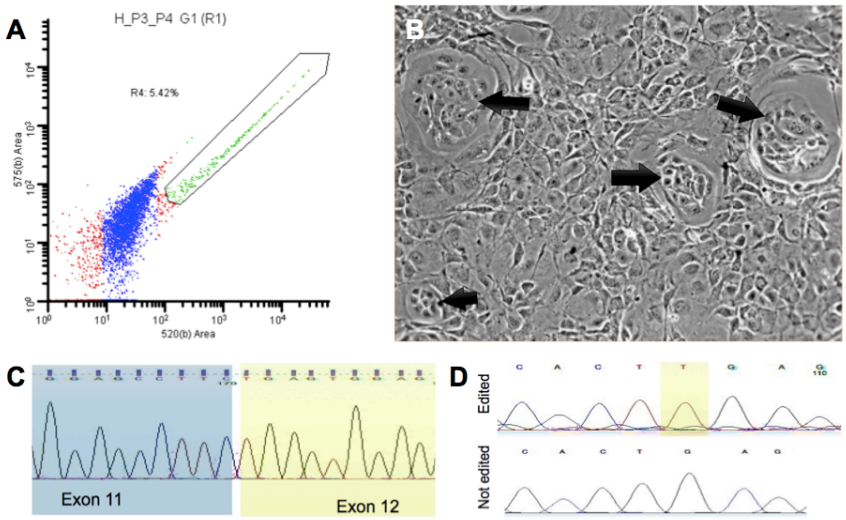

Figure 3: Gene-editing in primary human tracheal epithelial (HTE) cells. **(A)** GFP+ cells isolated by FACS. **(B)** Black arrows point to HTE clones, surrounding cells are irradiated fibroblasts. **(C)** PCR amplification using primers spanning CFTR exons 11 and 12 resulted in this amplicon, which shows joining of the exons. **(D)** The same HTE clone as in panel C displays a T-insertion on the second allele of CFTR.

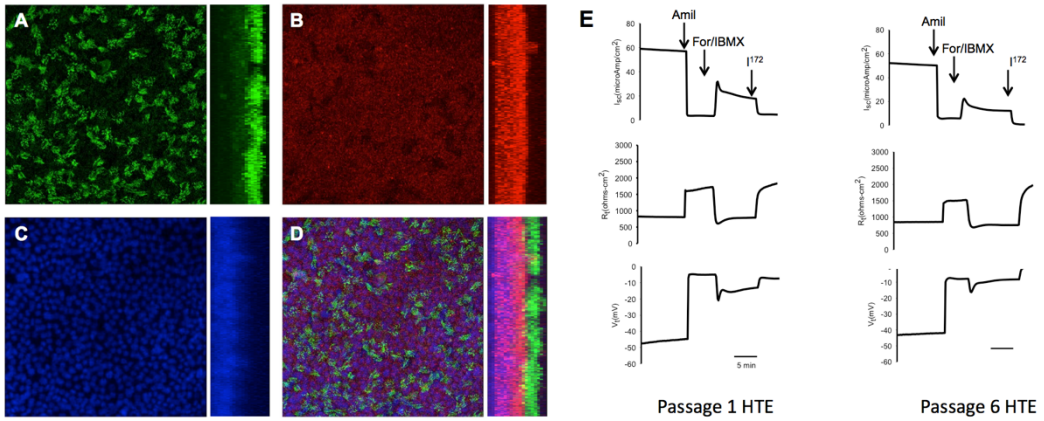

Figure 4: *In Vitro* cultures of primary HTE express cilia, functional tight junctions, and functional CFTR. Panels **A-D** show the same 60x field of non-CF primary airway epithelial cells grown on a filter at air-liquid interface, immunostained, and imaged on a confocal microscope. Right panels are reconstructed orthogonal images presented as viewed in the y-z plane. Cilia marked by anti-acetylated alpha tubulin (green, **A**) appear at the apical surface, tight junctions marked by anti-ZO-1 (red, **B**) at the cell body, and nuclei marked by DAPI (blue **C**) at the cell center. **(D)** Overlay**. (E)** After 6 passages in culture, HTE cells maintain the ability to generate a current across a monolayer of cells. Amiloride added to the apical surface blocks sodium ion conductance and decreases the current and voltage and increases resistance (↓I_{sc} ↑R_T ↓V_T) as expected. Forskolin added apically activates adenylate cyclase and IBMX inhibits phosphodiesterase, leading to the accumulation of cAMP, and anion flow through opened CFTR (↑I_{sc} ↓R_T ↑V_T). CFTR inhibitor-172 (I^{172}) closes CFTR and results in a decrease of CFTR-specific current (↓I_{sc} ↑R_T ↓V_T). Panel E data courtesy Dr. Calvin Cotton (co-sponsor).

Potential problems & alternative strategies

Primary HTE expansion after gene-editing is limited. Dr. Y (co-sponsor) and I are exploring ways to extend lifespan, including altered culture techniques and addition of telomerase. With the current methods, however, it is expected that enough cells from each clone will be available for 1-3 assays (dependent on assay requirements). Multiple clones are generated from each tracheal donor, but it is not expected that there will be enough clones from one individual to perform all proposed assays. Primary HTE from different tracheal donors will therefore be utilized. Variable genetic profile and airway health history between donors may affect results. Still, CFTR function will account for differences between non-edited and edited clones from the same donor.

Off-target DNA cleavage by Cas9 can result in mutations elsewhere in the genome. Off-target mutations occur most often at sequences that closely match the target sequences of the guide RNA (24). Collaborators in the laboratory of Dr. U have identified only one likely off-target site for the *CFTR* exon 12 gRNA (none are predicted for the exon 12 gRNA). I will amplify and sequence this site in all clones; mutant clones will be discarded. To further guard against experimental artifacts from off-target effects, 1) non-edited control clones will be generated

by lipofection without gRNA plasmids but with the Cas9 plasmid and 2) assays will be performed in replicate with multiple gene-edited clones from the same tracheal donor. Dr. Y and Dr. T are in the process of performing trans-epithelial conductance measurements and whole transcriptome sequencing (RNA-seq) on HTE clones to determine clonal variation. Results will inform the number of replicate clones needed for statistical significance.

Future directions

Although this proposal focuses on the role of HDAC2 in raising IL-8 expression, CF AEC also express increased levels of other inflammatory cytokines (5). It would be interesting to explore whether the pathways identified by this work also affect these other cytokines.

Aim 2: Determine the time course of inflammatory stress response after CFTR potentiation

Ivacaftor is the only clinically approved drug that targets the basic defect in CF. Ivacaftor potentiates CFTR by increasing channel open probability (22, 27). The drug is only effective against certain CFTR mutations, the most common of which is G551D (found in 4.3% of CF patients). Although clinically approved in 2012, mechanistic links between Ivacaftor and improved lung health remain unstudied at the basic science level. As previously discussed, high levels of pro-inflammatory signaling by CF AEC contribute to lung disease. Dr. X's laboratory has shown that CF cells undergo chromatin modifications that lead to increased IL-8 signaling. It is unknown whether established chromatin modifications and raised pro-inflammatory signaling will persist during CFTR potentiation with Ivacaftor. Perhaps chronic stress induced by CFTR dysfunction permanently alters the chromatin state regulating inflammatory genes.

Transient (48hr) expression of normal CFTR in the F508del homozygous CF_{15} nasal epithelial cell line does not affect stress-induced IL-8 response (25, 43), but stable expression of normal CFTR in the F508del/W1282X bronchial epithelial cell line reduces IL-8 response (5). Therefore, *I hypothesize inflammatory signaling and corresponding epigenetic alterations will take >48 hours to normalize in Ivacaftor-treated cells.*

Characterization of the time course of inflammatory response during Ivacaftor treatment and identification of (non)responsive pathways involved will inform expectations for Ivacaftor therapy. Patients may benefit from continued anti-inflammatory therapies if Ivacaftor does not normalize inflammatory response pathways studied.

Primary human nasal epithelial (HNE) cell model and Ivacaftor treatment

The Epithelial Cell Culture Core isolates nasal epithelial cells from de-identified nasal scrape samples. Samples come from the Cystic Fibrosis Center at University Hospitals, where the patients' physicians oversee the informed consent and nasal scrape process. I propose to use HNE cells from patients with at least one G551D allele. Eight qualifying patients are seen at the CF Center. So far, we have collected, expanded, and frozen a stock of HNE from one patient. HNE cells from age and gender matched subjects without respiratory illness will serve as controls. HNE expansion potential is less than that of HTE cells, making HNE better suited for direct assays rather than gene-editing. Ivacaftor dissolved in DMSO, or vehicle control, will be added to cell media. CFTR potentiation will be measured by trans-epitheilial conductance through CFTR as previously described. Dr. Y is currently determining efficacy, potency, and duration of the effective concentration.

Cytokine assays

Ivacaftor's effects on inflammatory signaling in AEC are unknown. Therefore, I will begin by assaying a spectrum of inflammatory markers with the Luminex 10-Plex Panel, which simultaneously measures GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α. RNA expression levels will be checked by qRT-PCR for cytokines found to be altered. If basal inflammatory signaling unchanged, then inflammatory signaling in response oxidative and bacterial-induced stress (discussed above) will also be assayed.

Mechanisms of inflammation

I hypothesize that Ivacaftor treatment will reduce protein modifications to HDAC2 observed in aim 1, normalize HDAC2 protein levels, and normalize acetylation at the IL-8 promoter. Methods discussed in aim 1 will be utilized to test this hypothesis.

Future directions

I would like to test whether a gene-edited Calu-3 G551D model can be potentiated with Ivacaftor. However, trans-epithelial electrical conductance testing for CFTR function in Calu-3 cells is hampered by their inability to

form electrically tight monolayers. Patch-clamp techniques may provide an alternative means to test CFTR function. If Ivacaftor potentiates G551D CFTR in my Calu-3 cell model, then I will determine the time course of inflammatory stress response after CFTR potentiation.

Literature Cited

1. **Adenuga D, Yao H, March TH, Seagrave J, Rahman I**. Histone deacetylase 2 is phosphorylated, ubiquitinated, and degraded by cigarette smoke. *Am. J. Respir. Cell Mol. Biol.* 40: 464–73, 2009.

2. **Angrisano T, Pero R, Peluso S, Keller S, Sacchetti S, Bruni CB, Chiariotti L, Lembo F**. LPS-induced IL-8 activation in human intestinal epithelial cells is accompanied by specific histone H3 acetylation and methylation changes. *BMC Microbiol.* 10: 172, 2010.

3. **Barnes PJ**. Role of HDAC2 in the pathophysiology of COPD. *Annu. Rev. Physiol.* 71: 451–64, 2009.

4. **Barnes PJ**. New anti-inflammatory targets for chronic obstructive pulmonary disease. *Nat. Rev. Drug Discov.* 12: 543–559, 2013.

5. **Bartling TR, Drumm ML**. Oxidative stress causes IL8 promoter hyperacetylation in cystic fibrosis airway cell models. *Am. J. Respir. Cell Mol. Biol.* 40: 58–65, 2009. PMID: 18635816

6. **Bartling TR, Drumm ML**. Loss of CFTR results in reduction of histone deacetylase 2 in airway epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 297: L35–43, 2009. PMID: 19411311

7. **Bauernfeind A, Bertele RM, Harms K, Hörl G, Jungwirth R, Petermüller C, Przyklenk B, Weisslein-Pfister C**. Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. [Online]. *Infection* 15: 270–277, 1987. http://www.ncbi.nlm.nih.gov/pubmed/3117700.

8. **Bederman I, Chandramouli V, Sandlers Y, Henderson L, Cabrera ME**. Time-course of hepatic gluconeogenesis during hindlimb suspension unloading. (Online version of record published before inclusion in an issue). *Exp. Physiol.* (June 15, 2012). doi: 10.1113/expphysiol.2012.067074. PMID: 22707505

9. **Bederman I, Perez A, Henderson L, Freedman J a, Poleman J, Guentert D, Ruhrkraut N, Drumm ML**. Altered de novo lipogenesis contributes to low adipose stores in cystic fibrosis mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* (June 7, 2012). doi: 10.1152/ajpgi.00451.2011. PMID: 22679004

10. **Bittar F, Richet H, Dubus J-C, Reynaud-Gaubert M, Stremler N, Sarles J, Raoult D, Rolain J-M**. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *PLoS One* 3: 1–7, 2008.

11. **Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, Rosenfeld M, Hiatt P, McCoy K, Castile R, Smith a L, Ramsey BW**. Longitudinal assessment of Pseudomonas aeruginosa in young children with cystic fibrosis. *J. Infect. Dis.* 183: 444–452, 2001.

12. **Cakmak A, Qi X, Cicek a. E, Bederman I, Henderson L, Drumm M, Ozsoyoglu G**. A New Metabolomics Analysis Technique: Steady-State Metabolic Network Dynamics Analysis. *J. Bioinform. Comput. Biol.* 10: 1240003–1, 2012. PMID: 22809304

13. **Capitan P, Malmezat T, Breuillé D, Obled C**. Gas chromatographic-mass spectrometric analysis of stable isotopes of cysteine and glutathione in biological samples. [Online]. *J. Chromatogr. B, Biomed. Sci. Appl.* 732: 127–35, 1999. http://www.ncbi.nlm.nih.gov/pubmed/10517229.

14. **Chen J, Kinter M, Shank S, Cotton C, Kelley TJ, Ziady AG**. Dysfunction of Nrf-2 in CF epithelia leads to excess intracellular H2O2 and inflammatory cytokine production. *PLoS One* 3: e3367, 2008.

15. **Chmiel JF, Berger M, Konstan MW**. The role of inflammation in the pathophysiology of CF lung disease. *Clin. Rev. Allergy Immunol.* 23: 5–27, 2002.

16. **Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini L a, Zhang F**. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–23, 2013.

17. **Cotton CU, Boucher RC, Gatzy JT**. Bioelectric properties and ion transport across excised canine fetal and neonatal airways. *J. Appl. Physiol.* 65: 2367–2375, 1988. PMID: 3215836

18. **Cotton CU, Boucher RC, Gatzy JT**. Paths of ion transport across canine fetal tracheal epithelium. *J. Appl. Physiol.* 65: 2376–2382, 1988. PMID: 3215837

19. **Cystic Fibrosis Foundation**. *Cystic Fibrosis Foundation Patient Registry 2012 Annual Data Report*. Bethesda, MD: 2012.

20. **Flotte TR, Solow R, Owens RA, Afione S, Zeitlin PL, Carter BJ**. Gene expression from adeno-associated virus vectors in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 7: 349–356, 1992.

21. **Gaj T, Gersbach C a., Barbas CF**. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* (May 2013). doi: 10.1016/j.tibtech.2013.04.004.

22. **Van Goor F, Hadida S, Grootenhuis PDJ, Burton B, Cao D, Neuberger T, Turnbull A, Singh A, Joubran J, Hazlewood A, Zhou J, McCartney J, Arumugam V, Decker C, Yang J, Young C, Olson ER, Wine JJ, Frizzell R a, Ashlock M, Negulescu P**. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc. Natl. Acad. Sci. U. S. A.* 106: 18825–30, 2009.

23. **Higashimoto Y, Elliott WM, Behzad AR, Sedgwick EG, Takei T, Hogg JC, Hayashi S**. Inflammatory mediator mRNA expression by adenovirus E1A-transfected bronchial epithelial cells. *Am. J. Respir. Crit. Care Med.* 166: 200–7, 2002.

24. **Hsu PD, Scott D a, Weinstein J a, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini L a, Bao G, Zhang F**. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* , 2013.

25. **Hybiske K, Fu Z, Schwarzer C, Tseng J, Do J, Huang N, Machen TE**. Effects of cystic fibrosis transmembrane conductance regulator and DeltaF508CFTR on inflammatory response, ER stress, and Ca2+ of airway epithelia. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 293: L1250–60, 2007.

26. **Jefferson DM, Valentich JD, Marini FC, Grubman SA, Iannuzzi MC, Dorkin HL, Li M, Klinger KW, Welsh MJ**. Expression of normal and cystic fibrosis phenotypes by continuous airway epithelial cell lines. *Am. J. Physiol. Lung Cell. Mol. Physiol.* .

27. **Jih K-Y, Hwang T-C**. Vx-770 potentiates CFTR function by promoting decoupling between the gating cycle and ATP hydrolysis cycle. *Proc. Natl. Acad. Sci. U. S. A.* 110: 4404–9, 2013.

28. **Khan T, Wagener J, Bost T, Martinez J, Accurso F, Riches D**. Early pulmonary inflammation in infants with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 151: 1075–82, 1995.

29. **Krämer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters M a, Brill B, Groner B, Bach I, Heinzel T, Göttlicher M**. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *EMBO J.* 22: 3411–20, 2003.

30. **Kube D, Sontich U, Fletcher D, Davis PB**. Proinflammatory cytokine responses to P . aeruginosa infection in human airway epithelial cell lines. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 280: L493–L502, 2001.

31. **Malhotra D, Thimmulappa RK, Mercado N, Ito K, Kombairaju P, Kumar S, Ma J, Feller-kopman D, Wise R, Barnes P, Biswal S**. Denitrosylation of HDAC2 by targeting Nrf2 restores glucocorticosteroid sensitivity in macrophages from COPD patients. *J. Clin. Invest.* 121: 4289–4302, 2011.

32. **Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM**. RNA-guided human genome engineering via Cas9. *Science* 339: 823–6, 2013.

33. **Monostori P, Wittmann G, Karg E, Túri S**. Determination of glutathione and glutathione disulfide in biological samples: an in-depth review. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 877: 3331–46, 2009.

34. **Nakamura T, Lipton S a**. Emerging role of protein-protein transnitrosylation in cell signaling pathways. *Antioxid. Redox Signal.* 18: 239–49, 2013.

35. **Nott A, Watson PM, Robinson JD, Crepaldi L, Riccio A**. S -nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. *Nature* 455: 411–415, 2008.

36. **Ong S, Mann M**. Stable Isotope Labeling by Amino Acids in Cell Culture for Quantitative Proteomics. In: *Quantitative Proteomics by Mass Spectrometry*, edited by Sechi S. Bethesda, MD: Humana Press, 2007, p. 37–52.

37. **Osoata GO, Yamamura S, Ito M, Vuppusetty C, Adcock IM, Barnes PJ, Ito K**. Nitration of distinct tyrosine residues causes inactivation of histone deacetylase 2. *Biochem. Biophys. Res. Commun.* 384: 366–71, 2009.

38. **Palmer ML, Lee SY, Maniak PJ, Carlson D, Fahrenkrug SC, Grady SMO**. Protease-activated receptor regulation of Cl- secretion in Calu-3 cells requires prostaglandin release and CFTR activation. *Am. J. Physiol. Cell Physiol.* 290: 1189–1198, 2006.

39. **Perez A, Davis PB**. Gene profile changes after Pseudomonas aeruginosa exposure in immortalized airway epithelial cells. *J. Struct. Funct. Genomics* 5: 179–94, 2004. PMID: 15263834

40. **Perez A, Drumm ML, Cotton CU**. Method to isolate and expand murine primary airway epithelial cells. *Pediatr. Pulmonol.* : A179, 2013.

41. **Perez A, Issler AC, Cotton CU, Kelley TJ, Verkman AS, Davis PB**. CFTR inhibition mimics the cystic fibrosis inflammatory profile. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 292: L383–L395, 2007. PMID: 16920886

42. **Perez A, Risma KA, Eckman EA, Davis PB**. Overexpression of R domain eliminates cAMP-stimulated Cl- secretion in 9 / HTEo- cells in culture. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 271: L–85–L92, 1996. PMID: 8760136

43. **Pizurki L, Morris M a, Chanson M, Solomon M, Pavirani a, Bouchardy I, Suter S**. Cystic fibrosis transmembrane conductance regulator does not affect neutrophil migration across cystic fibrosis airway epithelial monolayers. *Am. J. Pathol.* 156: 1407–16, 2000.

44. **Prince A**. Flagellar activation of epithelial signaling. *Am. J. Respir. Cell Mol. Biol.* 34: 548–51, 2006.

45. **Riches DW, Sawyer RT, Fenton MJ, Martin TR**. Innate Immunity in the Lungs. In: *Murray and Nadel's Textbook of Respiratory Medicine*, edited by Mason RJ, Broaddus VC, Martin T, King TEJ, Schraufnagel DE, Murray JF, Nadel JA. Philadelphia, PA: Saunders, 2010, p. 255–284.

46. **Shanks KK, Guang W, Kim KC, Lillehoj EP**. Interleukin-8 production by human airway epithelial cells in response to Pseudomonas aeruginosa clinical isolates expressing type a or type b flagellins. *Clin. vaccine Immunol.* 17: 1196–1202, 2010.

47. **Stutts MJ, Cotton CU, Yankaskas JR, Cheng E, Knowles MR, Gatzy JT, Boucher RC**. Chloride uptake into cultured airway epithelial cells from cystic fibrosis patients and normal individuals. [Online]. *Proc. Natl. Acad. Sci. U. S. A.* 82: 6677–6681, 1985. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=391273&tool=pmcentrez&rendertype=abstract.

48. **Veizis EI, Carlin CR, Cotton CU**. Decreased amiloride-sensitive Na+ absorption in collecting duct principal cells isolated from BPK ARPKD mice. *Am. J. Physiol. Renal Physiol.* 286: F244–54, 2004. PMID: 14559716

49. **Zhang J, Xu K, Ambati B, Yu F-SX**. Toll-like Receptor 5-Mediated Corneal Epithelial Inflammatory Responses to Pseudomonas aeruginosa Flagellin. *Invest. Ophthalmol. Vis. Sci.* 44: 4247–4254, 2003.

7. Project Summary/Abstract

Lung disease is the major cause of morbidity and mortality in cystic fibrosis (CF), a devastating disease that afflicts ~30,000 individuals in the US and ~70,000 worldwide. Life expectancy for individuals with CF is currently 38 years. Lung function typically begins to decline during the teenage years and lung disease sets in between 20-30 years of age. Cycles of bacterial infection, inflammation, and airway obstruction precipitate lung disease. CF is caused by mutation of a single gene: cystic fibrosis transmembrane conductance regulator (*CFTR*). CFTR dysfunction contributes to airway inflammation, which is present even in uninfected CF infants and is excessive for bacterial loads in infected patients. CF airway epithelial cells contribute to excessive inflammation via increased pro-inflammatory signaling.

Prior airway epithelial cell models for CF are not ideal for one of two reasons. First, some disease models do not have genetically matched controls. Genetic matching is important because CF lung disease severity is altered by dozens of single nucleotide polymorphisms located throughout the genome. Second, some disease models have been manipulated by heterologous expression of functional CFTR or interference with CFTR. While these models have proved useful, they do not address questions that involve endogenous CFTR regulation. This project will utilize a novel cell model: gene-edited primary human airway epithelial cells with genetically matched disease/control cells and endogenous CFTR regulation.

Increased inflammatory signaling in CF airway epithelial cells correlates with epigenetic alterations and decreased levels of the epigenome-altering protein histone deacetylase 2 (HDAC2). This proposal explores pathways leading to decreased HDAC2 levels. One such pathway is targeted to treat non-CF lung diseases such as chronic obstructive pulmonary disorder (COPD). If commonalities are uncovered, then treatments available for COPD may be useful for CF. Reducing airway inflammation in pediatric patients would forestall development of lung disease and improve patient quality of life.

Ivacaftor, a drug that directly addresses CFTR dysfunction, was recently approved for treatment of CF in a minority of patients. The mechanistic links between Ivacaftor and improved lung health remain unstudied at the basic science level. This proposal explores airway epithelial cell inflammatory response to Ivacaftor treatment. Results will inform clinical expectations for inflammatory resolution and the potential utility of co-treatment with anti-inflammatory drugs.

8. Project Narrative

The airway epithelial cells of patients with cystic fibrosis release excessive inflammatory signals that help precipitate lung disease. This proposal will elucidate mechanisms behind excessive inflammatory signaling as well as the potential for clinical reversibility.