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The Role Of Fibroblast Growth Factor Receptors In Inflammatory Airway Diseases

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THE ROLE OF FIBROBLAST GROWTH FACTOR RECEPTORS IN INFLAMMATORY AIRWAY DISEASES

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ABSTRACT

Chronic inflammation plays a role in the development and progression of airway diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). COPD is caused primarily by chronic cigarette smoke exposure. Conversely, CF is a single gene disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*).

Although the causes of COPD and CF are different, there are common pathological features. Studies show that chronic inflammation plays a significant role in the pathogenesis of both COPD and CF. Moreover, chronic inflammation induces ageing-associated pathomechanisms like cellular senescence, which exacerbates chronic inflammation. Previous work demonstrates that both chronic inflammation and cellular senescence contribute to COPD development. Furthermore, chronic inflammation is also a hallmark of CF. However, the molecular mechanisms by which chronic inflammation contributes to the complex pathology of COPD and CF have not been fully elucidated. Furthermore, while cellular senescence has been implicated in the pathomechanism of COPD, the role of cellular senescence in CF has not been characterized.

The present work demonstrates that fibroblast growth factor receptor (FGFR)-mediated signaling contributes to chronic inflammation in both COPD and CF. Studies on

FGFR4 knock out mice demonstrated that FGFR4 plays an essential role in lung inflammation. Furthermore, FGFRs contribute to chronic inflammation in CF as well. Additionally, we show that cellular senescence, a major contributor to chronic inflammation, is activated in CF lungs and the activation of cellular senescence is regulated by FGFRs. To confirm the role of FGFRs in the induction of cellular senescence we demonstrated that FGFR inhibition in CF airways significantly decreased inflammation, cellular senescence and improved mucociliary clearance. Given that chronic inflammation and cellular senescence in COPD and CF are activated through FGFR-mediated signaling, FGFR inhibition could be a potential therapeutic intervention for these disorders.

Taken together, this work demonstrates the role of FGFRs in regulating inflammation and cellular senescence in airway inflammatory diseases (COPD and CF). This work also highlights the potential of FGFR inhibitors as therapeutic agents for chronic inflammation and cellular senescence. Overall, this work provides impetus for future investigations of FGFRs as regulators of inflammation and cellular senescence in airway inflammatory diseases and potentially other chronic inflammatory diseases.

Keywords: cystic fibrosis, chronic obstructive pulmonary disease, chronic inflammation, cellular senescence, fibroblast growth factor signaling

DEDICATIONS

This work is dedicated to my parents Jack and Debra Easter, to my bonus parents Dr. Marilyn Winkleby and Dr. Michael Fischetti and to my brother Jack Easter Jr. for their continued support throughout my academic career.

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INTRODUCTION

Inflammatory Airway Diseases

Persistent and chronic inflammation within the respiratory tract is a hallmark of several airway diseases including chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis (CF) (1, 2). Chronic inflammation in the lung stems from a combination of genetic predispositions and environmental factors including pathogens, noxious gas, irritants, allergens, and pollutants. Although these lung diseases have distinct origins, they share common underlying disease phenotypes not limited to chronic inflammation. Other characteristics include airway obstruction, mucus hypersecretion and impaired mucociliary clearance (1-4).

Chronic inflammation is a characteristic of COPD and CF. Chronic inflammation in COPD stems from inhaled noxious gases, mainly cigarette smoke which leads to the activation of epithelial and alveolar macrophages (5). These alveolar macrophages and epithelial cells secrete cytokines and chemokines such as tumor necrosis factor (TNF-) α, interleukin (IL-) 1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), and CXCL8 (IL-8) that recruit additional inflammatory cells such as CD8+ T cells, neutrophils, monocytes, and lymphocytes (6). These inflammatory responses cause structural damage in the airway, airway obstruction and respiratory symptoms.

Chronic inflammation is a hallmark of CF. Thick mucus in CF airways impairs mucociliary clearance and immune cell function leading to recurrent lung infections that increase inflammation in the CF lung (7). Although recurrent infections cause inflammation in the CF lung, there is evidence that inflammation is endogenous to the CF lung and precedes infection. Studies have shown that CF neonates and young children showed increased IL-8 in their bronchoalveolar lavage fluid in the absence of bacterial infection (7, 8). Furthermore, defects in CFTR cause dysregulation of several intracellular signaling pathways including nuclear factor-kappa B (NF-κB) leading to excess secretion of proinflammatory mediators and a decrease in anti-inflammatory mediators (9). Together, these changes result in excessive production of cytokines such as IL-1β, IL-6, and IL-8 which drive inflammation regardless of infection status (10). Together the dysregulation of signaling pathways and chronic infection contribute to chronic inflammation in CF.

Besides chronic inflammation, COPD, and CF share airway obstruction, mucus hypersecretion and impaired mucociliary clearance (11). Furthermore, airway obstruction is narrowing and swelling of the airway due to thick mucus clogging the airway or tightening of the airway which leads to respiratory symptoms such as coughing, wheezing, and difficulty breathing (12). Mucus hypersecretion by goblet cells and enlarged submucosal glands is a hallmark of these diseases and contributes to airway obstruction (13, 14). Additionally, mucociliary clearance is impaired in these diseases (11). mucociliary clearance is the airways primary defense from foreign particles and microorganisms (15). The airway epithelium is covered with ciliated epithelial cells that beat in unison to move a mucus layer of trapped foreign particles up and out of the lungs

(16). Despite having different underlying causes of airway disease, COPD, and CF share common phenotypes of disease.

Chronic Obstructive Pulmonary Disease

COPD is a progressive chronic inflammatory disease and is considered one of the leading causes of death worldwide (17, 18). Risk factors for the development of the disease include inhaled tobacco smoke, noxious particles, and atmospheric pollutants (18). Clinical features of COPD include (1) emphysema with parenchymal destruction and loss of alveolar septa; (2) chronic bronchitis that consists of chronic bronchial inflammation, which is clinically defined as a chronic cough and sputum production for 3 months over two continuous years (17, 19, 20). The diagnosis is based on symptoms and spirometry values with varying degrees of severity of chronic bronchitis, emphysema, or both (21). Inflammation plays a significant role in disease development of COPD. The inflammatory response outweighs the protective restorative mechanisms, which leads to lung damage and subsequent loss of lung function. The contributing immune cells to this inflammatory process in COPD are neutrophils, macrophages, and T cells (22, 23). The accumulation of inflammatory cells leads to unbalanced proteolysis because of the large number of neutrophils and macrophages in the lung that are secreting proteases (24). The endogenous antiproteases are outnumbered which leads to the destruction of the lung tissue and elastin degradation by these excess proteases. This process contributes to the emphysematous phenotype in COPD lungs (17, 24). In summary, COPD is a chronic inflammatory lung disease characterized by chronic bronchitis and emphysema.

Cystic Fibrosis

CF is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) which leads to insufficient protein levels and/or function of CFTR (25, 26). CFTR is an ATP-binding cassette transporter that functions as an ion channel that transports chloride ions and bicarbonate across the membrane of cells which helps maintain hydration and ion balance in several organs in the body including the lungs, digestive tract, pancreas, liver, and gallbladder (26). Although CF is a multi-organ disease, respiratory failure is the most common cause of morbidity and mortality (27, 28). Lung pathologies of CF include chronic airway inflammation, mucus obstruction, and recurrent respiratory infections (27, 29). The CFTR ion channel in healthy lungs serves to maintain a hydrated air surface liquid volume of the airway epithelial layer for mucus to be transported and protect the lungs (30, 31). In CF lungs, CFTR dysfunction leads to dehydrated airways which contributes to mucus dehydration and ciliary beat dysfunction (26). The consequences of dehydrated airways in CF lungs are thick sticky mucus that obstructs airways and creates an environment for recurrent airway infections and chronic inflammation.

Comparison of Pathologies in COPD and CF

Despite different disease etiology, CF being a disease caused by a genetic mutation and COPD being caused by environmental factors mainly long-term cigarette smoke exposure, they share many common disease pathologies. Both diseases are associated with progressive airflow obstruction, chronic inflammation, and recurrent infectious exacerbations (11). Airway epithelial cells play an important role in maintaining normal airway function (11). The airway epithelium experiences molecular and structural changes

that play critical roles in disease initiation and progression of both COPD and CF (3). These structural and molecular changes in the airway and alveolar epithelium impact the airway microenvironment which affects host defenses and repair processes leading to airflow limitations, a characteristic of both diseases (32).

Airway hydration is maintained by the CFTR channel and amiloride-sensitive epithelial sodium channel (ENaC) (33). The microanatomy responsible for effective mucociliary clearance consists of the air surface liquid (ASL): a mucus layer that traps particles and pathogens, and a periciliary liquid layer (PCL) underneath, which maintains hydration for effective mucociliary transport by ciliated cells on the airway epithelium (33). To maintain sufficient airway hydration the CFTR and ENaC ion channels balance chloride secretion and sodium absorption (34). Mucus dehydration is a common pathology in COPD and CF which causes impaired mucociliary clearance and inflammation in the lungs (2, 11).

Alterations in ASL composition and disruption of airway microenvironment have been connected to the pathogenesis of CF and COPD (3). Mutations in CFTR protein in CF airways leads to a disruption in chloride and bicarbonate transport into the airway, causing dehydration and acidification of the ASL (35). This results in the production of thick, acidic, and altered mucus secretions which impairs mucociliary clearance, promotes chronic bacterial infection and inflammation. ASL dehydration mediated by CFTR dysfunction also contributes to altered mucus structure, increased viscosity and abnormal mucus secretion which contribute to mucus plugging, impairment of mucociliary clearance and inflammation (31).

Moreover, cigarette smoke is a major risk factor for COPD. Previous studies have shown that cigarette smoke induces acquired CFTR dysfunction in patients with normal CFTR (32). Smoke-induced CFTR dysfunction results in ASL dehydration, delayed mucociliary transport and increased mucus expression (36). Together with mucin hypersecretion, smoke induced CFTR dysfunction provokes mucus hyper concentration and plugging in COPD airways. In ciliated cells from COPD patients ciliary beat frequency was significantly reduced compared to controls (37). Reduced mucociliary clearance contributes to accumulation and adhesion of mucus in the airways which causes irritants and noxious agents to get trapped in the airway which promotes inflammation in the form of neutrophils and macrophages (3). These recruited immune cells release excess proteases and promote emphysema. Additionally, βEnaC transgenic mice, a model that develops CF-like airway surface dehydration also exhibits chronic mucus obstruction, airway inflammation and emphysematous hallmarks in these mice (38). Together these studies highlight the role of airway dehydration in the pathogenesis of both COPD and CF through dysfunction of EnaC and CFTR.

Additionally, efficient tissue repair and regeneration following injury is necessary for tissue homeostasis and prevention of disease. Exposure to repeated noxious/ inflammatory stimuli impairs epithelial regeneration and repair pathways. The impaired ability of epithelial cells to restore barrier functions contributes to aberrant remodeling and structural damage that further impairs epithelial functions and create a cycle of exogenous insults and impaired repair (11, 39). Both CF and COPD are characterized by dysfunctional airway epithelial repair and remodeling that impair lung architecture and contribute to disease pathogenesis and progression.

Chronic Inflammation

Inflammation is a natural defense mechanism that recognizes harmful and foreign stimuli and removes them and begins the healing process (40, 41). Chronic inflammation is a slow, long-term inflammation that lasts for long periods of time, even up to years. Chronic inflammation can result from a variety of causes including failure to clear an infection, inhaled substances of industrial chemicals that cannot be eliminated by enzymatic breakdown or phagocytosis, autoimmune disorders, auto-inflammatory disorders, recurrent episodes of acute inflammation and inflammation induced by oxidative stress, mitochondrial dysfunction, or free radicals (42, 43). A typical inflammatory response is characterized by acute upregulation of inflammatory mediators such as neutrophils that will disperse once the threat is neutralized. However, in chronic inflammation recurrent triggers will prevent resolution of acute inflammation and promote a low grade chronic, systemic inflammation that activates other immune cells such as macrophages and dendritic cells that will activate the adaptive immune system which includes T-cells and B-cells (43). Which continues the cycle of chronic inflammation and contributes to tissue dysfunction and chronic disease.

Chronic Inflammation in COPD and CF

In airway diseases, various stimuli such as pathogens, toxins, pollutants, irritants, and allergens trigger inflammation in the airway epithelial cells. This inflammatory response involves the recognition of pathogen patterns by Toll-like receptors (TLRs), which then activate inflammatory mediators including NF-κB, chemokines like IL-8, and TNF-α which recruit immune cells (44). These activated immune cells undertake processes

such as phagocytosis of pathogens, resolution of inflammation, and initiation of wound healing to repair damaged epithelial cells, ultimately leading to tissue remodeling. In COPD cigarette pollutants trigger TLRs and apoptotic, necrotic, and dead cells release damage associated molecular pattern molecules (DAMPs) (45). Activated inflammatory cells recruit neutrophils and monocytes to the injury site. Neutrophils release elastase and matrix metalloproteinase 9 (MMP9) which results in mucus hypersecretion, elastin degradation and emphysema (46).

In cystic fibrosis (CF), the inflammatory response is triggered by TLRs recognizing pathogen-associated molecular patterns (PAMPs). This recognition leads to activation of inflammatory mediators such as neutrophils, macrophages, and NF-κB (11). NF-κB signaling upregulates IL-8 which recruits monocytes. Together neutrophils, macrophages, and monocytes clear CF-related pathogens aiding in resolution. These studies describe the inflammatory response in COPD and CF.

Once inflammation is established in COPD it is persistent despite smoking cessation (47). Studies have shown that even after smoking cessation, there is a progressive small airflow obstruction which is due to accumulation of inflammatory mucous exudates in the lumen and increase in the tissue volume of the bronchial wall. This increase in tissue volume of the bronchial wall is due to lymphoid follicles infiltrating immune cells (48). Chronic inflammation is a lasting phenotype of COPD lungs. Chronic inflammation in CF stems from chronic infections and underlying molecular changes in immune cells that stem from CFTR dysfunction that leads to immune cell dysfunction (29, 49).

Thick mucus in CF airways leads to airway obstruction and hypoxia that results in sterile inflammation mediated mainly by epithelial cells, monocytes macrophages, and

neutrophils which secrete IL-1α and IL-1β which contribute to mucus hypersecretion and expression of airway mucins (7). Inflammation in CF airways is mainly mediated by neutrophils which results in increased production of reactive oxygen species (ROS) that inhibit antimicrobial activity in CF airways (50, 51). Overall, the dysfunctional immune response in CF causes hyperinflammation and increases susceptibility to infection.

In combination with hyperinflammation, thick mucus creates a favorable environment for infections in CF lungs. Interestingly, over the course of their early and adolescence years, CF patients are colonized with several different types of microbes including *S. aureus*, *Haemophilus Influenzae* and *H. parainfluenzae* are some of the most common colonizers in early childhood (52). As CF patients age to adulthood, P. aeruginosa becomes the dominant colonizer of CF lungs. Chronic bacterial infections in CF lungs promote a destructive cycle of inflammation including neutrophil recruitment, frustrated phagocytosis, and the production of neutrophil-derived antimicrobials (50). In a state of chronic pulmonary inflammation in CF lungs, both bacterial and host factors can damage lung tissue, leading to increased inflammation and irreversible changes to airway and parenchymal anatomy including bronchiectasis, cystic changes, mucus plugging, and emphysema (2, 10, 11). Overall, both immune dysfunction and bacterial infections contribute to chronic inflammation in CF airways. Our results suggest that not only do immune dysfunction and bacterial infections contribute to CF disease progression, but cellular senescence does as well.

Cellular Senescence

Cellular senescence was first discovered by Hayflick in the 1960s in fibroblast that lost their proliferative ability due to permanent cell cycle growth arrest, this would be later known as replicative senescence. Since then, cellular senescence has been characterized as a critical mechanism in wound repair, tumor suppression, regeneration, and aging (53-57). Acute senescence is beneficial during development, wound healing, tissue repair and protective in malignancy. However, senescent cells have been shown to accumulate with age and in disease affected tissues. Additionally, elimination of senescent cells results in increased lifespan and reduced organ failure (58). Furthermore, studies have shown that transplanting senescent cells into young mice led to increased cellular senescence, increased organ failure, and mortality, demonstrating that senescent cells can drive aging and disease (59).

At its core, cellular senescence is defined as an irreversible cell cycle growth arrest that is induced by several cellular stressors including oxidative stress, telomere shortening and chronic inflammation (56). There are two main types of cellular senescence, replicative senescence, and stress-related senescence. Replicative senescence is driven by progressive shortening of telomeres which activates the DNA damage response and tumor suppressor p53(p53) and cyclin dependent kinase inhibitor $p21^{CIP1}$ (p21) thus starting the process of cellular senescence. Stress-related senescence on the other hand stems from cellular stressors such as chronic inflammation or oxidative stress. This may also activate the DNA damage response but can also activate cyclin-dependent kinase inhibitor $p16^{INK4}$ (p16) signaling pathway which starts the senescence process by causing growth arrest in the cell

by inhibition of cyclin-dependent kinase-2/4. Downstream of p16/p21 activation in cellular senescence, proinflammatory mediators NF-κβ, p38 MAPK (mitogen-activated protein kinase) and Jak (Janus-activated kinase) are activated, resulting in secretion of multiple inflammatory proteins known as the senescence associated secretory phenotype (SASP). SASPs include cytokines, chemokines, proteins, and growth factors. Some of the most common SASPs markers include interleukin (IL)1-β, IL-6, and IL-8 however there are several other SASPs markers such as chemokines, growth factors and secreted proteases (60) Secretion of SASPs markers by senescent cells causes paracrine senescence, fibrosis, and tissue destruction (61). Senescent cells are characterized by a myriad of cell markers (Figure 1) such as increased expression of senescence-associated β-gal (SA β-gal) (due to the acidic lysosomes of senescent cells compared to normal cells), are apoptotic resistant marked by expression of B-cell leukemia/lymphoma 2 (BCL2) and B-cell lymphoma-extra-large (BCL-xL), p16, and p21 markers for cell cycle growth arrest, IL1-β, IL-6, and IL-8 for SASP markers (62-64). Cellular senescence contributes to disease pathogenesis and progression in Alzheimer's disease, and chronic lung diseases such as idiopathic pulmonary fibrosis and COPD.

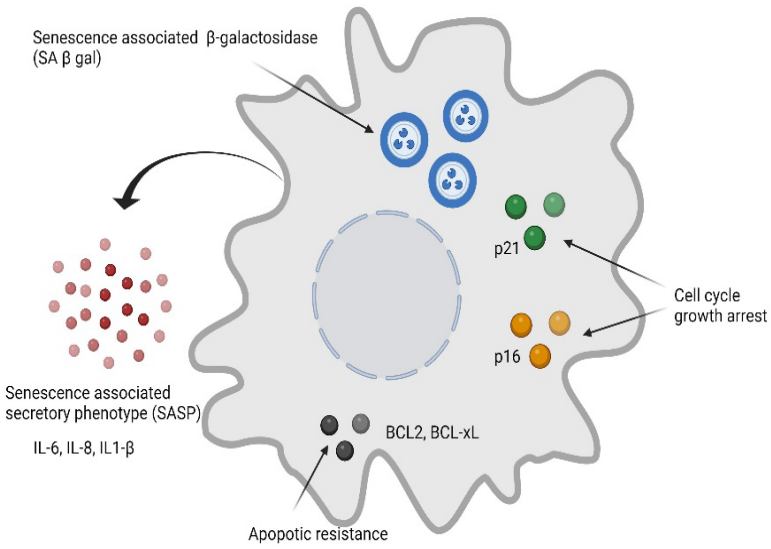

Figure 1. Senescence is defined by multiple markers. Senescent cells exhibit a distinct profile characterized by increased expression of senescence-associated β-galactosidase (SA-β-gal), evident by blue staining. Enhanced expression of p16INK4a and p21CIP1/WAF1 halts the cell cycle progression, promoting cell cycle arrest. Elevated levels of senescence-associated phenotype (SASP) factors such as IL1-β, IL-6, IL-8. Increased apoptotic resistance marked by increasing in BCL2 and BCL-xL. Collectively, these diverse markers provide a comprehensive understanding of the multifaceted nature of cellular senescence. (Created with Biorender.com)

Cellular Senescence in COPD and CF

Several studies have shown that senescent cells are increased in patients with COPD compared to aged-matched controls (65). For instance, a higher number of p16 or p21 positive senescent cells were observed in epithelial airway cells, type II pneumocytes, endothelial cells, pulmonary artery smooth muscle cells, fibroblast and endothelial progenitor cells from COPD patient lungs compared to controls (66). Additionally, other studies have demonstrated significant positive correlations between cellular senescence markers and measures of airflow obstruction, a key functional outcome of COPD (67). Moreover, other studies have shown that cigarette smoke exposure, which is a major risk factor for the development of COPD, induced cellular senescence in airway epithelial cells (19). Taken together these studies support the contribution of cellular senescence to the development of COPD.

CF disease was initially characterized as a pediatric disease. However, increasing numbers of people with CF live into adulthood, where the adult population with CF now outnumbers the pediatric population. At this point, the median predicted survival for people with CF is over 56 years old (68). The shift in the median age of CF patients can be attributed to the advancements in treatments for CF including highly effective modulator therapy that corrects CFTR dysfunction (68). Considering the CF population is aging, and chronic inflammation is a hallmark of aging and CF, it is important to consider the role of aging in CF disease. Recently, the hallmarks of aging have been characterized in CF disease (Figure 2) (69). A hallmark of aging that is associated with chronic inflammation is cellular senescence (65, 70).

A previous study has shown that a few cellular senescence markers are increased in CF lungs such as p16INK4a (p16), a cyclin-dependent kinase inhibitor, phospho-Histone H2A.X (γH2A.X), and phospho-checkpoint 2 kinase (phospho-Chk2) compared to healthy controls (71). However, these markers are also DNA damage markers which only encompass part of the markers that define a cell as senescent. It's known that defining cellular senescence is challenging due to the availability, suitability of biomarkers and its overlap with pro-inflammatory markers and other cellular processes. Thus, several different types of markers are needed to characterize cellular senescence. Therefore, more experimental data is required to characterize cellular senescence in CF, its mechanisms, and to determine if there is a direct connection between cellular senescence and CF disease.

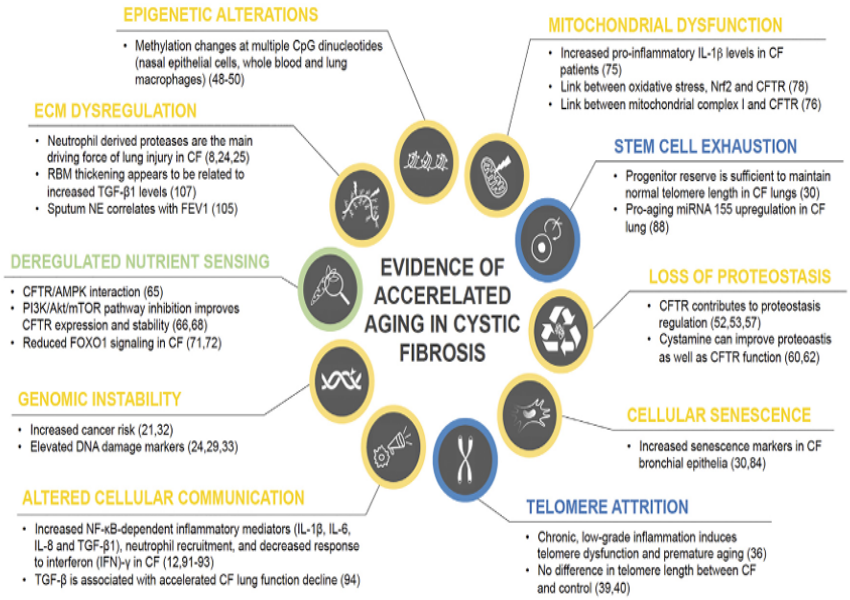

Figure 2. Evidence of the hallmarks of aging found in CF
Current evidence of hallmarks of aging found in CF. Research findings on the role of each of the ten hallmarks of aging in CF disease are summarized and color coded based on their level of evidence with blue = no evidence for involvement in CF pathology, green = weak evidence, yellow = strong evidence.

Note: From from Künzi L, Easter M, Hirsch MJ, Krick S. Cystic Fibrosis Lung Disease in the Aging Population. Front Pharmacol. 2021 Apr 15;12:601438. doi: 10.3389/fphar.2021.601438. PMID: 33935699; PMCID: PMC8082404. Copyright frontiers. Reprinted with permission.

Crosstalk Between Chronic Inflammation and Cellular Senescence

Despite inflammation being critical for defending against pathogens and foreign stimuli, inflammation may turn into a hazardous factor to health for the aging population (72). Chronic and persistent low-grade inflammation during ageing is known as inflammaging which is a hallmark of aging (73). Inflammaging is characterized by maintaining a low-grade, sustained background of inflammation even in the absence of acute infection and clinically diagnosed disease (42, 73). Inflammaging is a risk factor that contributes to reduced tissue repair and regenerative capacity which is a characteristic of age-related diseases. Immune mediators IL-6, IL-8 and CXCR2 play an essential role in the initiation and maintenance of cellular senescence (74, 75). Additionally, senescent cells are proinflammatory and can induce and maintain chronic inflammation (75). Senescent cells mediate their proinflammatory nature through their SASP where they secrete proinflammatory mediators that contribute to chronic inflammation, induce paracrine senescence and tissue dysfunction (60, 62). Several of the SASP markers are proinflammatory cytokines that contribute to inflammation in surrounding tissue, Additionally, SASP markers IL-6 and IL-8 have been shown to induce senescence in surrounding cells (76). Moreover, studies have shown that TGF-β-driven paracrine senescence limits liver regeneration following acute injury with acetaminophen demonstrating the negative effect of paracrine senescence on tissue repair (77). SASP markers include extracellular matrix components as well, which have been shown to contribute to remodeling of the tissue microenvironment and disruption of tissue homeostasis (78). Accumulation of senescent cells has been shown to contribute to disease,

with several studies demonstrating that removal of senescent cells reverses disease and disease symptoms (79).

The Biology of Fibroblast Growth Factors

Fibroblast Growth Factors

Fibroblast growth factors (FGFs) encompass a large family of polypeptide growth factors that are conserved throughout metazoan evolution (80). In vertebrates, there are 22 FGF isoforms which are highly conserved in gene structure and amino acid sequence including a conserved common core domain (Figure 3) (81). FGFs play important roles in development, cell proliferation, differentiation, tissue maintenance, and regulation of homeostasis (82). To perform their biological functions, FGFs bind to fibroblast growth factor receptors (FGFRs). FGFs can be classified as intracrine, paracrine and endocrine FGFs. Intracrine FGFs, FGF11-FGF14 are not secreted extracellularly due to their lack of a secretion signal (80, 82). They act on intracellular molecules in an FGFR-independent manner and interact with voltage gated sodium channels with a neuronal mitogen-activated protein kinase (MAPK) scaffold protein. Paracrine FGFs include FGF/1/2/5, FGF3/4/6, FGF7/10/22, FGF8/17/18, and FGF9/16/20 subfamilies. All paracrine FGFs perform their biological functions by binding and activating FGFRs. Heparin/heparan sulphate (HS) acts as a cofactor that stabilizes the interaction between paracrine FGFs and FGFRs (83). Paracrine FGFs signal to neighboring cells and tissues given their high affinity for extracellular matrix HS. Endocrine FGFs, FGF19, FGF21 and FGF23 have a low affinity for HS which allows them to circulate and affect distant cells and tissues. Additionally, endocrine FGFs require a co-receptor klotho with FGFRs to mediate their biological

functions (84). Endocrine FGFs regulate cell metabolism and homeostasis of lipids, glucose, energy, bile acids, phosphate, and vitamin D (84).

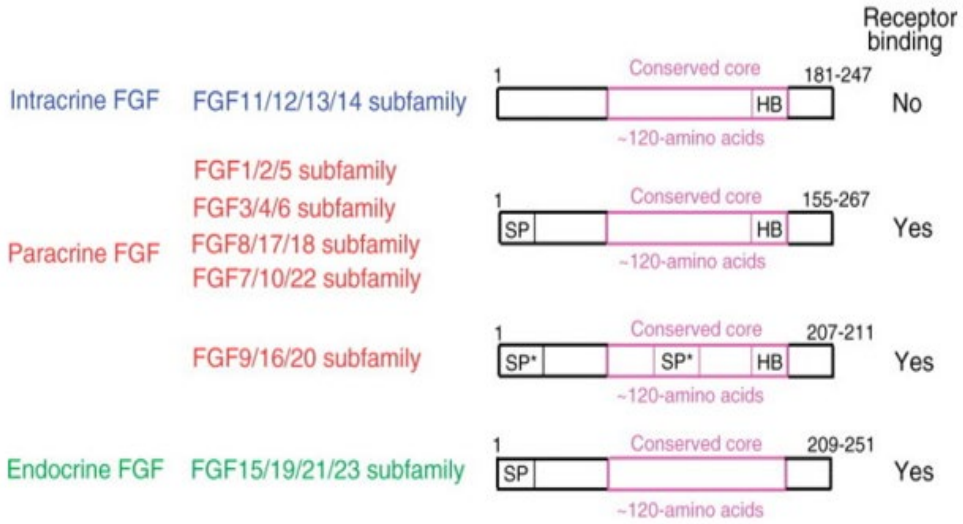

Figure 3. Fibroblast Growth Factor Subfamilies.
Note: From Itoh N, Ornitz DM. Fibroblast growth factors: from molecular evolution to roles in development, metabolism, and disease. J Biochem. 2011 Feb;149(2):121-30. doi: 10.1093/jb/mvq121. Epub 2010 Oct 12. PMID: 20940169; PMCID: PMC3106964. Reprinted with permission.

Fibroblast Growth Factor Receptors

Paracrine and endocrine FGFs bind to FGFRs which belong to the family of receptor tyrosine kinases. In mammals, FGFRs are encoded by four separate genes generating for isoforms, termed FGFR1-4. FGFRs are comprised of three extracellular immunoglobulin-like domains, a single-pass transmembrane domain, and a cytoplasmic tyrosine-kinase domain (81). FGFR1-3 exist in two splice variants, termed b and c, that differ in their immunoglobulin (Ig)-like domain 3. Overall, there are a total of seven FGFR variants: FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c and FGFR4 (Figure 4) (82). When a ligand interacts with an FGFR, it causes a conformational change in the receptor, leading to FGFR dimerization and activation of intercellular tyrosine kinase domains that cross phosphorylate each other on tyrosine residues on their activation loops (80). FGFR signaling is transduced by the cytoplasmic adaptors phosphoinositide phospholipase C (PLC) γ and FGF receptor substrate 2α (FRS2α) (81). After ligand binding and auto-phosphorylation of FGFR, PLCγ binds to one specific phosphorylated tyrosine residue within the FGFR cytoplasmic tail. PLCγ-catalyzes the production of diacylglycerol and inositol 1,4,5-triphosphate that can increase cytoplasmic calcium. Increased cytoplasmic levels of calcium activate several calcium-sensing signal mediators, including the protein phosphatase calcineurin which dephosphorylates nuclear factor of activated T-cells (NFAT) and activates NFAT to translocate into the nucleus and modulate expression of specific target genes (81, 85, 86).

Conversely, FRS2α is constitutively bound to FGFR independent of the receptor activation state (85). FGFR/FRS2α-mediated signaling results in the activation of

Ras/MAPK including extracellular signal-regulated kinase (ERK), p38 MAPK and Jun N-terminal kinase (JNK) (87). ERK is known to preferentially be activated in response to growth factors whereas p38 MAPK and JNK signaling respond to stress stimuli (88). Downstream signaling of FGFRs encompasses Ras/Raf/MAPK, phosphatidylinositol 3-kinase/ protein kinase B(PI3K/Akt), and PLCγ (80, 89). Through activation of these downstream signaling pathways, FGFRs participate in various vital physiological functions such as proliferation, differentiation, cell migration and survival. Mutations and aberrant expression of FGFRs are linked to various cancers and is considered an oncogenic signaling pathway (90). Several small molecule inhibitors have been developed to target FGFRs in cancer and some are being used in clinical trials (91).

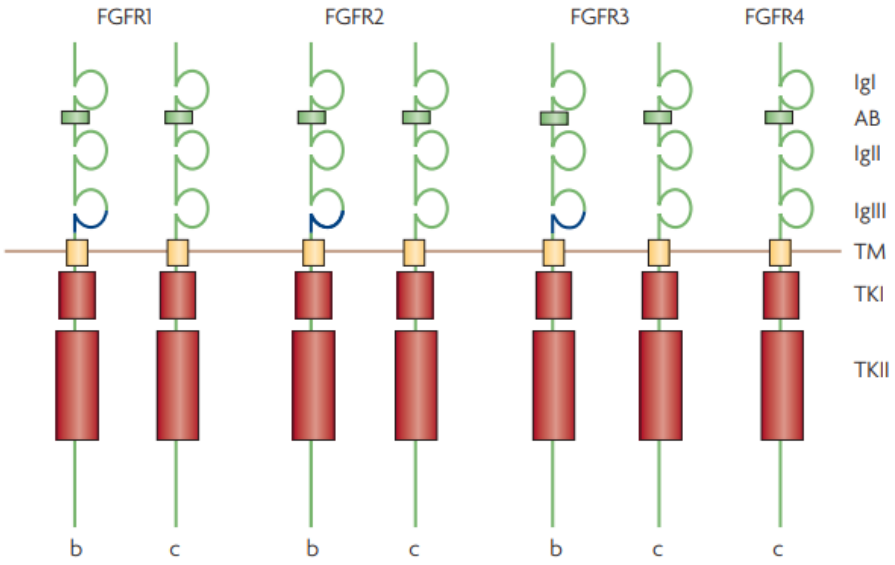

Figure 4. Fibroblast Growth Factor Receptor Isoforms.

The four vertebrate FGFRs structure consist of three Ig loops (IgI, IgII, IgIII) and an acid box (AB) within their extracellular domains. The 'b' isoforms of FGFR1-3 feature blue-colored alternatively spliced sequences in IgIII which distinguish them from the c isoforms. These receptors possess a transmembrane domain (TM) and intracellularly harbor split tyrosine kinase enzyme domains (TKI and TKII). FGF ligands bind to the C-terminal part of IgII and the N-terminal portion of IgIII, while CAM (cell adhesion molecule) ligands bind to the acid box.

Note: From Mason I. Initiation to end point: the multiple roles of fibroblast growth factors in neural development. Nat Rev Neurosci. 2007 Aug;8(8):583-96. doi: 10.1038/nrn2189. PMID: 17637802. Reprinted with permission.

Fibroblast Growth Factor 23

Fibroblast growth factor 23 (FGF23) was discovered in 2000 as the gene being responsible for autosomal dominant hypophosphatemic rickets (92). FGF23 is a hormone that belongs to the endocrine FGF family and is responsible for regulating phosphate and vitamin D metabolism (81). FGF23 is secreted by osteocytes and osteoblast into the circulation and acts in the bone, kidney, parathyroid, lungs, liver, and possibly other organs (93). Canonical FGF23 signaling requires klotho as a co-receptor for the binding to FGFR1c which in the kidneys regulates phosphate by reducing sodium-phosphate co-transporters (94, 95). Conversely, FGF23 acts in the heart, parathyroid, bones, and erythrocytes mainly through FGFR3 and FGFR4 in a klotho independent fashion, known as non-canonical FGF23 signaling (93, 96). Dissecting the functional structure of the protein, the N-terminal region of FGF23 interacts with FGFRs and shares homologies with other FGF family members, whereas its C-terminal region binds its co-receptor, klotho (95, 96). Prior to secretion of FGF23 by osteoblasts, post-translational modifications occur and promote FGF23 to circulate as an active protein known as intact FGF23 (97, 98). Additionally, FGF23 can circulate as proteolytic cleaved fragments known as cleaved FGF23which was thought to be inactive however recent studies have shown that these FGF23 fragments play a role in iron availability during acute inflammation (97, 99). This occurs via subtilisin-like pro-protein convertases, such as furin, which are responsible for FGF23's proteolytic cleavage and recognition of its consensus sequence. Full-length FGF23 is O-glycosylated by polypeptide N-acetylgalactosaminyltransferase 3 (GalNT3) at Thr residues which shields FGF23 from proteolytic cleavage and promotes a gross half-life of 45-60 minutes in humans, 20-30 minutes in mice and 5 mins in rats (97, 98, 100).

Klotho

Klotho was first identified as an anti-aging protein because mice lacking klotho developed multiple ailments associated with premature aging including shortened lifespan (101). Conversely, mice overexpressing klotho have increased lifespan by up to 30% (102). Klotho has two known human isoforms, the full-length protein which is a single-pass transmembrane protein and a secreted soluble protein (102). Klotho is a co-receptor for FGF23/FGFR1c signaling (103). Studies have shown that klotho increases the binding affinity of FGF23 to FGFR1 by 20-fold (104). Membrane bound klotho is known for its role in regulating phosphate homeostasis acting as a co-receptor for FGFR1c as well as other FGFR isoforms (81). Full-length klotho (membrane-bound) exists on the cell membrane and can be post-translationally modified by α-secretases. A disintegrin and metalloproteinase domain-containing proteins 10 and 17 (ADAM10 and ADAM17) and β-secretase, β-APP cleaving enzyme 1 (BACE1) (102). The cleaved product is a soluble klotho protein ∼130 kDa that lacks the transmembrane and intracellular domains. Soluble klotho is known for its regulation of oxidative stress and ion homeostasis (105). Moreover, β-klotho is an isoform in the klotho family which exists as a membrane-bound protein expressed in the liver and adipose tissue and signals with FGFR1 and FGFR2 (106). FGF/FGFR/β-klotho signaling is responsible for regulating glucose and lipid metabolism (105). Overall, klotho is a key part of FGF/FGFR signaling.

FGF23/FGFR Induced Signaling

FGF23 is a pro-inflammatory and phosphaturic hormone, which signals through two main receptors: FGFR1 (klotho dependent signaling) and FGFR4 (klotho independent signaling) (81). FGF23 mediates its physiological functions via

FGF23/FGFR1/klotho/ERK signaling in the kidney by regulating phosphate homeostasis (93). FGF23/FGFR4/PLCγ/NFAT signaling does not require klotho as a coreceptor and mediates FGF23's pathological functions in the liver, heart and lungs contributing to inflammation in these tissues (103, 107, 108). Moreover, Previous studies have shown that FGF23 binds to FGFR4 with high affinity in the absence of klotho or insufficient klotho levels (81, 107). For example, in COPD airways, klotho levels are low and FGF23 levels are high which leads to increased FGF23/FGFR4 signaling and airway inflammation (103). In conclusion, FGF23 has both physiological and pathological functions depending on the tissue and state of klotho expression.

Fibroblast Growth Factor Signaling in Inflammatory Airway Diseases

FGF/FGFR Signaling in Inflammatory Airway Diseases

FGF/FGFR signaling has been shown to play a role in the pathogenesis of inflammatory airway diseases such as COPD and asthma (109). FGF1, FGF2, and FGF7 have been shown to be associated with COPD (110). FGF1 and FGF2 with FGFR1 have been shown to be involved in airway remodeling in COPD patients (111). FGF2 is increased in the bronchial epithelium and localized in the nucleus of airway smooth muscle cells (109, 111). Additionally, FGF1/FGFR1 levels are elevated in airway smooth muscle cells and airway epithelial cells in COPD patients compared to healthy patients (112). In vitro experiments demonstrated that FGF1 and/or FGF2 through FGFR1, induced cell proliferation in human airway smooth muscle cells. Intratracheal injection of FGF2 increases pulmonary blood flow in injured lungs and reduces the volume of emphysematous lungs (113). In elastase-induced pulmonary emphysema, FGF2 can induce

regeneration in animal models (114). FGF7 has been shown to protect against emphysema by reducing inflammation and alveolar cell death in mice (109). Additionally, FGF7 gene delivery into skeletal muscle of mice protects against elastase-induced emphysema (115). COPD can also be caused by heterozygous loss-of-function mutations in FGF10 (116). Together these studies demonstrate the important role FGFs have in COPD lungs.

Endocrine FGFs such as FGF23 have been shown to contribute to airway inflammatory diseases. Moreover, FGF23 levels are increased in plasma from both COPD and CF patients (103, 117). Furthermore, studies show that FGF23 contributes to inflammation in COPD and CF airways. Given the significant role that FGFs play in airway inflammatory diseases, the role of FGF23 in airway inflammatory diseases should be explored. Together, These studies demonstrate the significant role that FGFs play in airway inflammatory diseases, with FGF2, FGF7, FGF10 and FGF23 playing significant roles airway inflammatory diseases.

FGFs in Aging

Several FGFs have been shown to be associated with aging. In aged tissues like the brain, bone, and skin, FGF ligand expression and FGFR activation is decreased (118). Moreover, FGF1, FGF2, FGF4 with FGFR1 or FGFR2 signaling pathways play an important role in stem cell self-renewal and/or inhibiting senescence in mesenchymal, endothelial, hematopoietic, ectodermal, and neural stem cell populations (119). Given FGFs associations with aging more research needs to be done to understand their role in aging and age associated pathways as targeting FGF signaling mechanisms could be a promising therapy to reverse the effects of aging.

FGF23/FGFR Signaling in Aging

Interestingly, both FGF23 null mice and klotho null mice exhibit several premature aging-like features (98, 102). Genetic ablation of FGF23 in mice results in shortened lifespan with kyphosis, hypogonadism, infertility, osteopenia, pulmonary emphysema, and general atrophy of tissues (120). Klotho null mice exhibit short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, and emphysema (121). Recent studies imply that premature aging-like features in klotho null mice are not directly caused by the absence of klotho but by altered high phosphate levels due to the lack of FGF23 being able to perform its physiological function of regulating mineral metabolism because klotho is required for these functions (122). More research is needed to understand the connection between mineral metabolism and its effect on aging. On the other hand, mice that overexpress klotho have a 30% increased lifespan (101). Klotho expression declines with age, kidney failure, diabetes, and neurodegenerative disease (105). Decreased klotho levels are associated with increased all-cause mortality in the United States (123). FGF23/FGFR is well characterized for its role in inflammatory diseases however given that FGF23 and its co receptor klotho have connections with aging, more research is needed to characterize FGF23/FGFRs role in aging and aging pathways.

FGF23 Signaling in COPD

Plasma FGF23 levels are significantly elevated in COPD patients and are associated with an inflammatory phenotype with goblet cell hyperplasia (103). In the bronchial epithelium exposure to cigarette smoke significantly decreases klotho levels and this decrease is further exacerbated in bronchial epithelial cells exposed to cigarette smoke and FGF23 (103). Moreover, cigarette smoke significantly increases FGFR4 expression in a

dose dependent manner. This state of klotho deficiency and elevated FGF23 leads to activation of FGFR4/PLCy/NFAT signaling which activates pro-inflammatory cytokine IL1-β which induces inflammation in the bronchial epithelium (Figure 5) (103).

Furthermore, in human bronchial epithelial cells, FGF23 stimulates O-linked β-N-acetylglucosamine (O-GlcNAc) stress response which regulates NFAT mediated IL-6 activation which could be involved in chronic inflammation in COPD (124). Also, FGF23 levels are higher in COPD patients with frequent exacerbations (defined by having at least two exacerbations per year, experience accelerated loss of lung function, deterioration in quality of life and increase in mortality) (125). In summary these studies show that FGF23/FGFR4 signaling contributes to airway inflammation in COPD (Figure 5). Other studies have shown that FGF23/FGFR signaling also contributes to CF airway inflammation.

FGF23 Signaling in CF

FGF23 levels are significantly increased in plasma from CF patients, however klotho levels remain unchanged between control and CF patients (117). Moreover, CF airways exhibit increased expression of both FGFR1 and FGFR4. Interestingly, transforming growth factor β (TGFβ) is a CF lung disease modifier gene and is associated with more severe lung disease in CF (126). Proinflammatory mediators such as TGFβ have been shown to increase secretion of FGF23 from osteocytes and this could explain the elevated FGF23 levels in CF (127). Moreover, CF bronchial epithelial cells treated with TGFβ and FGF23 demonstrated significant increases in secretion of pro-inflammatory mediator IL-8 (Figure 5) (117). This is mediated through TGFβ upregulation of FGFR1

and together with FGF23, TGFβ induces IL-8 via FGFR1/ERK and TGFβ signaling. Interestingly, soluble klotho can attenuate this increase in IL-8 via inhibition of TGFβ signaling in the CF bronchial epithelium (Figure 5). Together these studies demonstrate that FGF23/FGFR1 signaling contributes to airway inflammation in CF.

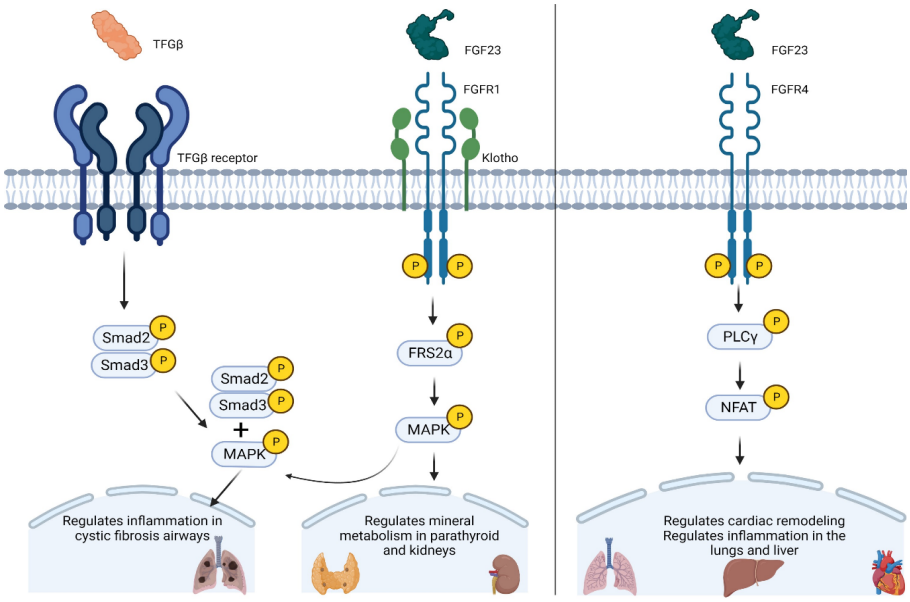

Figure 5. Diagram illustrating the physiological and pathophysiological role of FGF23.

Left panel: Illustrates TFGβ and FGF23/FGFR1/MAPK signaling in inflammation in CF. Middle panel demonstrates the role FGF23/FGFR1 in regulating mineral metabolism in the parathyroid and kidneys. Right panel: Explains the inflammatory role that FGF23/FGFR4 plays in the lung, liver, and heart. (Created with Biorender.com)

RATIONALE AND GOALS

Chronic inflammatory diseases have been recognized as one of the most significant causes of death in the world. Chronic inflammatory diseases include COPD and CF. COPD is one of the leading causes of death in the US and affects 16 million Americans. On the other hand, CF is one of the most common single gene disorders in the world and affects 30,000 Americans. Chronic inflammation in the airways is a common phenotype of both COPD and CF that contributes to disease progression. The mechanisms associated with chronic inflammation in COPD and CF have not been fully elucidated. Previous literature demonstrates that FGF23 and its receptors play a role in chronic inflammation in COPD and CF. However, these studies did not characterize the role of FGFRs in inflammation in healthy lungs or explore the connection between cellular senescence and FGFRs in airway inflammatory diseases. This work aims to uncover the role of FGFRs in inflammation in healthy lungs as well as elucidate the connection between FGFRs and cellular senescence in CF. Given that cellular senescence drives inflammation, understanding the underlying mechanisms may provide a therapeutic target to combat cellular senescence and inflammation. Moreover, several studies show that FGFR inhibitors are effective for reducing disease outcomes in diseases where FGFRs play a role. This work aims to show that FGFR inhibitors could be a feasible treatment for cellular senescence and chronic inflammation in airway inflammatory diseases.

FIBROBLAST GROWTH FACTOR RECEPTOR 4 DEFICIENCY MEDIATES INFLAMMATION IN THE ADULT LUNG

by

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Abstract

Fibroblast growth factor receptor (FGFR) 4 has been shown to mediate pro-inflammatory signaling in the liver and airway epithelium in chronic obstructive pulmonary disease (COPD). In past reports, FGFR4 knockout (*Fgfr4-/-)* mice did not show any lung phenotype developmentally or at birth, unless FGFR3 deficiency was present simultaneously. Therefore, we wanted to know whether the loss of FGFR4 had any effect on the adult murine lung.

Our results indicate that adult *Fgfr4-/-* mice demonstrate a lung phenotype consisting of widened airway spaces, increased airway inflammation, bronchial obstruction, and right ventricular hypertrophy consistent with emphysema. Despite downregulation of FGF23 serum levels, IL-1β and IL-6 in the *Fgfr4-/-* lung and abrogation of p38 signaling, primary murine *Fgfr4-/-* airway cells showed increased expression of IL-1β and augmented secretion of IL-6, which correlated with decreased airway surface liquid depth as assessed by μOCT. These findings were paralleled by increased ERK phosphorylation in *Fgfr4-/-* airway cells when compared to their control wild type cells. Analysis of a murine model with constitutive activation of FGFR4 showed an attenuation of some pro-inflammatory mediators in the lung and airway epithelium.

In conclusion, we are the first to show an inflammatory and obstructive airway phenotype in the adult murine *Fgfr4*-/- lung, which might be due to upregulation of ERK phosphorylation in the *Fgfr4-/-* airway epithelium.

Introduction

FGF receptors (FGFRs), a sub-family of receptor tyrosine kinases, consist of four family members including FGFR1, 2, 3, and 4 (1). Dependent on different FGF ligands, FGFRs exert diverse functions through activation of downstream signaling pathways. In organs that are contributing to phosphate homeostasis such as the kidney and parathyroid gland, signaling occurs through FGFR1 by binding FGF23 and its co-receptor klotho (2, 3). In organs that do not express klotho, or in a state of klotho deficiency, FGF23 can activate FGFR4 and induce the phosphorylation of PLCγ leading to hypertrophic growth in cardiac myocytes and the production of inflammatory cytokines in the liver (4, 5). FGFR3 and FGFR4 are abundantly expressed in both epithelium and mesenchyme in the developing mammalian lung (6-8). Weinstein et al. generated mice homozygous deletion for *Fgfr4*; however, these did not exhibit any overt abnormalities in the lungs or other organs after birth. Mice homozygous for targeted disruption of both *Fgfr3* and *Fgfr4* had lungs that were completely blocked in alveologenesis without formation of secondary septae (9).

Bronchopulmonary dysplasia (BPD), a pulmonary disorder of the newborn and associated with hyperoxia induced lung injury, is also significantly linked with a polymorphism in the FGFR4 gene (9, 10). An established murine BPD model also demonstrated decreased expression of *Fgfr3* and *Fgfr4* (11). In addition, inactivation of both *Fgfr3* and *Fgfr4* in the embryonic mouse lung mesenchyme, but not the epithelium, led to a defect in elastogenesis with upregulation of Mfap5, the gene encoding the extracellular matrix component MAGP-2, a critical elastic component of extracellular

matrix microfibrils (12). In the adult lung, FGFR4 is overexpressed in non-small cell lung cancer and can induce proliferation (13).

We have recently shown that FGFR4 signaling is also activated in chronic obstructive pulmonary disease (COPD) (14). Human bronchial epithelial cells from COPD patients, which were differentiated at the air liquid interface, showed an increased expression of FGFR4, which seemed to mediate secretion of interleukin 1b via activation of PLCγ/NFAT. Inhibition of FGFR4 attenuated this effect implying a pathological role for FGFR4 in the COPD lung. However, there is not much known about the effect of FGFR4 deficiency in healthy adult lungs. The loss of FGFR4 has been shown to affect metabolism; Huang et al., showed that *Fgfr4*-/- mice developed features of the metabolic syndrome including an increase in white adipose tissue mass, insulin resistance, glucose intolerance and hyperlipidemia, when challenged with a high-fat diet (15). The aim of this report is to analyze the physiological role of FGFR4 in the adult lung. Based on our recent findings, we initially hypothesized that FGFR4 deficiency is protective against airway inflammation.

Results

FGFR4 Deficiency in Mice Leads to an Airway Phenotype Resembling COPD

We analyzed the lungs of 6-month-old *Fgfr4-/-* mice and compared them to their age-matched wild type littermates. We did not detect any FGFR4 protein or mRNA expression in *Fgfr4-/-* lungs (Figure 6A). Interestingly, paraffin embedded slides from murine *Fgfr4-/-* lungs revealed airway enlargement (Figure 6B), which was reflected in a significant increase in mean linear intercepts (Figure 6C). Using flexiVent to analyze lung function, *Fgfr4-/-* lungs showed a significant increase in static compliance (Module

Preservation) and a marked decrease in the FEV0.05/FVC ratio (Figure 6E) indicating airway obstruction. In addition, there was a significant increase in total cell count, monocytes/macrophages, and neutrophils in the BAL fluid without any difference in lymphocyte count (Figure 6F and G). Collectively, full *Fgfr4-/-* mice at 6-months of age showed evidence of airway inflammation and airway enlargement consistent with emphysema.

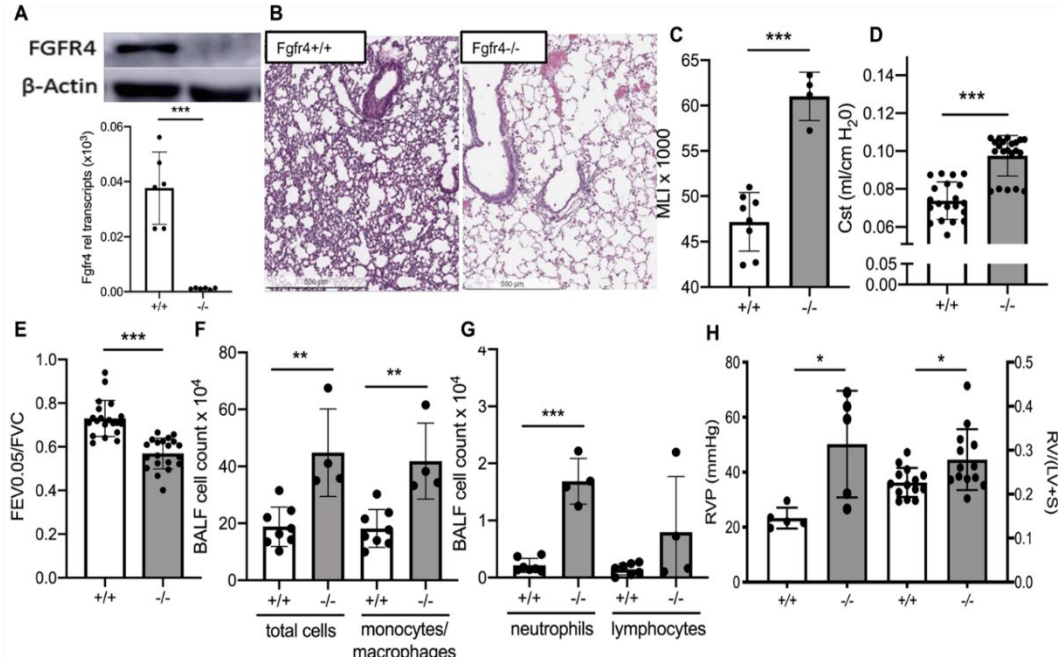

Figure 5. In vivo effect of FGFR4 deficiency.
A) Immunoblot using an anti-FGFR4 antibody (upper image) and bar graphs showing Fgfr4 mRNA levels (lower diagram) in whole lung tissue from *Fgfr4-/-* and *Fgfr4+/+* mice. B) Immunohistochemistry using hematoxylin staining of paraffin embedded lung tissue (20X) demonstrating widened airway spaces in *Fgfr4-/-* mice. C) Bar graphs showing morphometric analysis (mean linear intercepts) of lung tissue from *Fgfr4-/-* and *Fgfr4+/+* mice. D) Quasi-static compliance (Cst), E) ratio of forced expiratory volume (FEV) and forced ventilatory capacity (FVC), assessed by flexiVent. F) Bar graphs showing total cell count, total macrophage and monocyte count and G) total neutrophil and lymphocyte count from bronchoalveolar lavage fluid. H) Bar graphs indicating hemodynamic analysis of right ventricular pressure and Fulton index (RV/LV+S) of *Fgfr4-/-* and *Fgfr4+/+* hearts. Statistical analysis was done using ANOVA or Student's t-test showing means ± SEM with *p<0.05, **p<0.01, and ***p<0.001 with n = 4-15 mice per group.

FGFR4 Deficiency in Mice Affects the Right Heart

In addition to the inflammatory lung phenotype, 6-month-old *Fgfr4-/-* mice demonstrated an increase in right ventricular pressure as assessed by right heart catheterization and evidence of right ventricular hypertrophy as assessed by Fulton index, when compared to their age matched litter mates (Figure 6H).

FGFR4 Deficiency Affects FGF23 Levels, Inflammation and Abrogates Phosphorylation of p38 MAPK in Murine Lungs

Interestingly, Fgfr4-/- mice had significantly decreased circulating FGF23 serum levels (Figure 7A), but increased expression of FGF23 mRNA in *Fgfr4-/-* lungs without any changes in FGFR1 or TGF-β mRNA levels (Figure 7B). Furthermore, IL-1β and IL-6 mRNA levels were downregulated two-fold in total lung lysates from *Fgfr4-/-* mice (Figure 7C). Analysis of potentially affected signaling pathways revealed that *Fgfr4-/-* lungs showed negligible change in phosphorylation of PLCγ, but an almost complete abrogation of p38 phosphorylation and downregulation of both phospho-ERK and total ERK expression (Figure 7D, E).

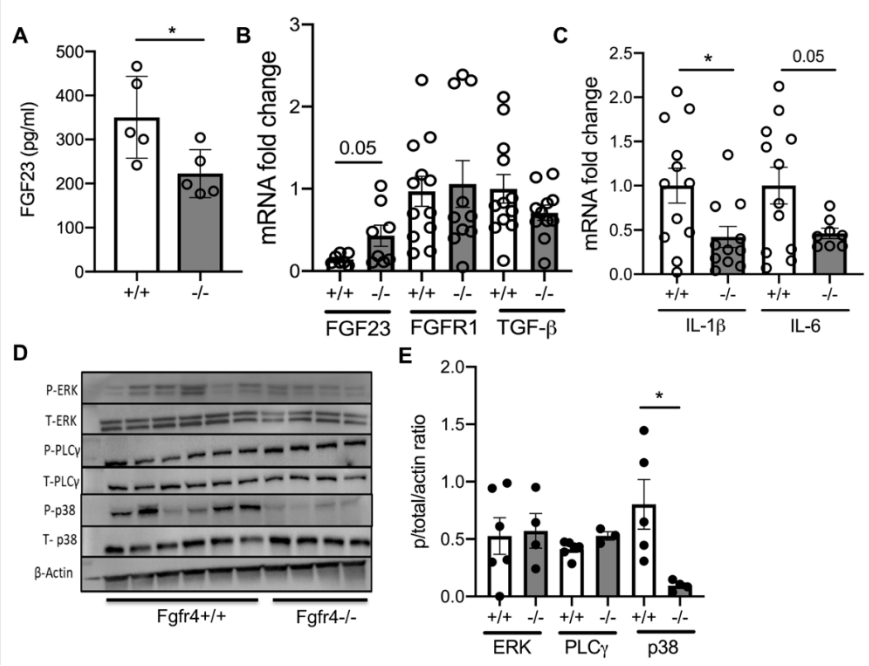

Figure 6. Effect of FGFR4 deficiency in the murine lung
A) Bar graphs indicating FGF23 serum levels from *Fgfr4-/-* and *Fgfr4*+/+ mice. B) mRNA fold change in FGF23, FGFR1 and TGF-β levels and C) pro-inflammatory mediators such as IL-1β and IL-6 in total lung tissue from *Fgfr4-/-* and *Fgfr4*+/+ mice. D) Representatives immunoblot analyses and E) quantification by densitometry of phospho-ERK, total ERK, phospho-PLCγ, total PLCγ, phospho-p38 and total p38 from total lung tissue of *Fgfr4-/-* and *Fgfr4*+/+ mice. (All n = 3-6 mice per group showing mean ± SEM with *P<0.05).

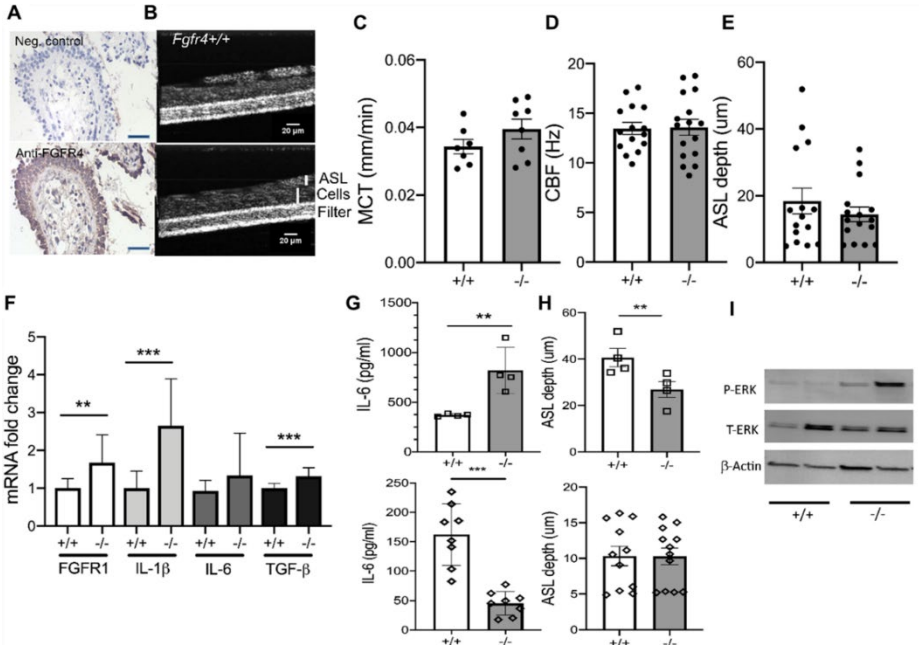

Figure 8. Effect of FGFR4 deficiency in the murine airway epithelium

A) Immunohistochemistry using anti-IgG (negative control) (upper image) and anti-FGFR4 antibody (lower image) and counter hematoxylin staining in a representative human lung section showing staining of the airway epithelium (arrows). B) Representative images of *Fgfr4-/-* and *Fgfr4+/+* MTECs, differentiated on filters using μOCT. C) Quantification of mucociliary transport (MCT), D) ciliary beat frequency (CBF) and E) airway surface liquid (ASL) depth from differentiated *Fgfr4-/-* and *Fgfr4+/+* MTECs. F) mRNA fold changes of FGFR1, IL-1β, IL-6 and TGF-β levels in *Fgfr4-/-* and *Fgfr4+/+* MTECs. G) IL-6-protein levels, assessed in supernatant from *Fgfr4-/-* and *Fgfr4+/+* MTECs, when divided into different isolation groups and H) their respective ASL depth analysis. I) Representatives immunoblot analyses of undifferentiated (UD) and differentiated (D) *Fgfr4-/-* and *Fgfr4+/+* MTECs assessing expression of phospho-ERK, total ERK and β-actin. (3 separate experiments from 3-6 mice per group. All bar graphs are mean ± SEM with *P<0.05, **P<0.01 and ***P<0.005).

FGFR4 Deficiency Affects Mucociliary Clearance and Inflammation in Murine Tracheal Epithelial Cells

To further elucidate the role of FGFR4 in the airway epithelium (Figure 8A) (14), mucociliary clearance was assessed in primary murine tracheal epithelial cells (MTECs) from *Fgfr4-/-* mice and their wild type littermates. MTECs were differentiated at the air liquid interface (ALI) and mucociliary clearance parameters analyzed by μOCT (Figure 8B). There was no difference in mucociliary transport, ciliary beat frequency, and airway surface liquid (16) depth between wild type and *Fgfr4-/-* MTECs (Figure 8C, D and E). We did note, however, an increase in FGFR1, IL-1β and TGF-β mRNA levels in *Fgfr4-/-* MTECs, when compared to MTECSs from wild type littermates (Figure 8F). MTECs did not show expression of FGF23 (data not shown). Analysis of IL-6 protein levels in cell culture media did not show any significant difference between *Fgfr4-/-* and *Fgfr4+/+* MTECs when data were pooled (not shown), but showed significant differences, when sub-stratified according to the different isolations. MTEC isolations with higher IL-6 levels showed a further increase in the Fgfr4-/- cultures (Figure 8G, upper graph), which was reversed in the MTEC isolations with lower IL-6 protein levels (Figure 8G, lower graph). MTECs were isolated from 3-6 *Fgfr4+/+* and *Fgfr4-/-* mice, pooled and half of them were plated submerged (undifferentiated), the other half were differentiated at the air liquid interface for 3-4 weeks – these were used for ASL measurements. We compared the differentiated MTECs of the same isolation to their respective *Fgfr4+/+* control MTECs. Post-stratification analysis of ASL depth revealed that the "high IL-6 secretor group" showed a significantly decreased ASL depth (Figure 8H, upper graph), whereas there was no difference in ASL depth in the "low IL-6 secretor group" (Figure 8H, lower graph).

Analysis of potentially involved signaling pathways in both undifferentiated (UD) and differentiated (D) MTECs revealed an upregulation of phospho ERK (Figure 8I).

In summary, MTECs isolated from *Fgfr4-/-* mice demonstrated heterogeneity in IL-6 protein levels but did not exhibit any significant differences in mucociliary clearance. Interestingly, *Fgfr4-/-* MTEC cultures with high IL-6 protein secretion showed a decrease in ASL depth and increased phosphorylation of ERK.

Constitutive FGFR4 Activation Inhibits Expression of Inflammatory Mediators in Murine Lungs and Tracheal Epithelial Cells

Since *Fgfr4-/-* lungs show airway inflammation and airway enlargement consistent with emphysema, we wanted to assess the effect of constitutive FGFR4 activation. Therefore, we analyzed lung tissue and MTEC cultures from *Fgfr4-Arg/Arg385* mice. This knockin mouse model exhibits a gain-of-function mutation in *Fgfr4* replacing a glycine with an arginine residue in the transmembrane domain, leading to increased FGFR4 stability and prolonged phosphorylation (4, 17). Microscopic assessment of the pulmonary tissue of *Fgfr4-Arg/Arg385* mice did not show any differences compared to wild type littermates (Figure 9A). Circulating FGF23 plasma levels were slightly decreased in *Fgfr4-Arg/Arg385* mice (Figure 9B). mRNA analysis of lung tissue demonstrated significant decreases in inflammatory mediators such as TGF-β, IL-1β and IL-6 (Figure 9C), whereas *Fgfr4-Arg/Arg385* MTECs showed significant increases in FGFR1 and a significant decrease in IL-6 mRNA expression (Figure 9D). In summary, *Fgfr4-Arg/Arg385* lungs did not show any airway or parenchymal changes and inflammatory mediator expression was downregulated in MTECs and total lungs, when compared to age-matched MTECs/lungs from wild type littermates.

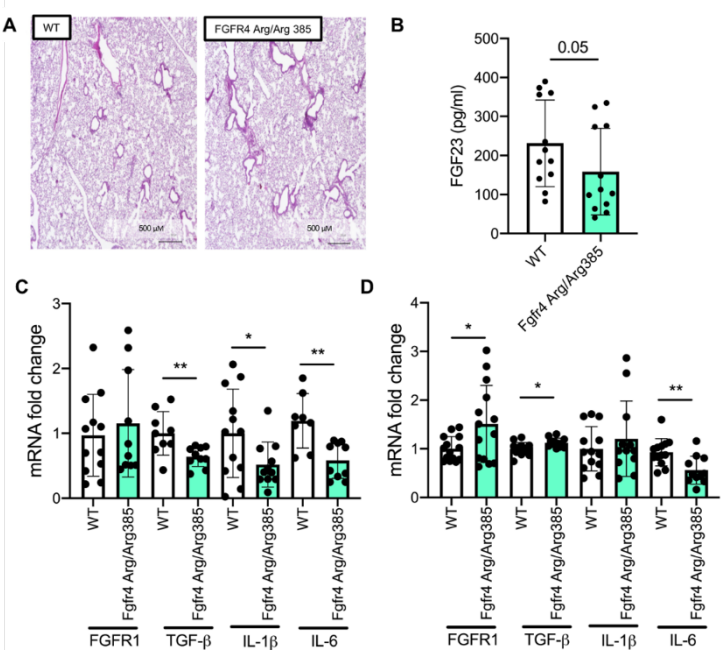

Figure 7. Effect of constitutive FGFR4 activation in the murine lung and airway epithelium.

A) Hematoxylin staining in representative murine lung sections of control and *Fgfr4-Arg/Arg385* mice. B) Bar graphs indicating serum Fgf23 levels and C) fold changes of FGFR1, TGF-β, IL-1β and IL-6 mRNA levels in total lung tissue and D) MTECs, isolated from *Fgfr4-Arg/Arg385* mice and their wild type littermates. (3 separate experiments from 3-6 mice per group. All bar graphs are mean ± SEM with *P<0.05 and **P<0.01).

Methods

Study Approval

All animal protocols were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham. Both *Fgfr4-/-* and *Fgfr4-Arg/Arg385* mouse models were provided by Dr. Christian Faul. Both mouse models were generated on the C57BL/6 background as previously described (4). All animals were housed and bred in UAB facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), where they were under the supervision of a team of veterinarians and staff. They were monitored daily by the investigators. UAB complies with NIH policies on animal welfare, the Animal Welfare Act, and all other applicable federal, state, and local laws.

Primary Murine Tracheal Epithelial Cell Cultures

Primary murine trachea epithelial cells (MTECs) were isolated from *Fgfr4*+/+, *Fgfr4-/-* and *Fgfr4-Gly/Gly385 (control) Fgfr4-Arg/Arg385 mouse tracheas* and plated on collagen IV-coated clear 12mm-transwell filters (Corning, Corning, NY). MTECs were cultured and some of them differentiated for 2-4 weeks at the air liquid interface according to an adapted protocol as described previously (14).

Western Blot

All protein lysates were obtained from MTECs and murine lungs using RIPA buffer with phosphatase and protease inhibitors. Proteins were separated into 4-20% precast Ready Gels (Bio-Rad, Hercules, CA, USA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Pierce, Thermo Fisher Scientific, Waltham, MA, USA). Membranes

were blocked with 5% low-fat milk in Tris-buffered saline (pH 7.4) with 0.05% Tween 20 (TBST) for 30 minutes and incubated overnight with the following primary antibodies: rabbit anti-FGFR4, rabbit total and phospho- anti-ERK1/2, rabbit total and phospho-anti-p38 MAPK, rabbit total and phosphor anti-PLCγ1 (Cell Signaling Technologies, Danver, MA, USA) and mouse anti-β-actin-peroxidase (Sigma, St. Louis, MO, USA). After 5 washes with TBST, membranes were incubated with a goat anti-rabbit peroxidase conjugated (Invitrogen) at 1:5000 in TBST for 45 minutes. Positive signals were visualized by chemiluminescence on a ChemiDoc XRS system (Bio-Rad). Images were acquired using Image Lab software (Bio-Rad). Densitometry was measured using ImageJ software (NIH).

ELISA

An enzyme-linked immunosorbent assay (ELISA) for the quantitative detection of mouse IL-6 (Invitrogen, Vienna, Austria) was used on basolateral medium from MTECs. IL-6 protein levels in the basolateral medium from MTECs ranged from 25-1500 pg/ml. We had one outlier ALI culture, which showed IL-6 protein levels of > 3000 pg/ml, which was excluded after statistical outlier analysis.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from MTECs, and murine lungs as previously described (18). Real-time quantitative PCR reactions were performed using the following TaqMan probes: Fgfr4 Mm00433314, Fgf23 Mm00445621, Il-6 Mm00446190, Il-1beta Mm00434228, Tgf-beta1 Mm01178820 and Gapdh Mm99999915 (Invitrogen, Carlsbad, CA, USA).

Micro-Optical Coherence Tomography (μOCT)

Micro-Optical Coherence Tomography imaging was performed on differentiated MTECs using the same technique as previously described (19, 20).

Analysis of Lung Function in Mice

Six-month old mice were anesthetized and pulmonary function was evaluated using the ratio of forced expiratory volume/forced vital capacity (FEV0.5/FVC) as a measure for airway obstruction and quasi-static compliance (Cst) with a flexiVent (SCIREQ; Montreal, Canada) as described previously (21).

Bronchoalveolar Lavage

Bronchoalveolar lavage fluid (BALF) was collected as previously described (31). Briefly, 1ml of phosphate buffered saline was instilled via tracheal cannula into the lungs and aspirated. The collected BALF was then centrifuged at 500g for 5 min at 4°C to pellet the cells. The cell pellet was then resuspended in 100-150μl of phosphate-buffered saline to determine total cell count. Cytospins were prepared as previously described and Wright-Giemsa staining for differential cell counts was performed using the HEMA 3 Stain Set (Fisher Scientific, Hampton, NH, USA) as directed by the manufacturer (14).

Lung Histology and Morphometric Analysis

Mice were euthanized and perfused via the right ventricle with 3ml of phosphate buffered saline. The lungs were inflated using 1ml of 10% neutral buffered formalin and fixed for 24 hours. After the lungs were dehydrated in 70% ethanol, they were processed and embedded in paraffin. Sections were cut 3-5 mm, mounted on slides, and stained with hematoxylin. Anti-rabbit FGFR4 antibody (sc-124), Santa Cruz Biotechnology, Dallas,

TX, USA, was used for staining total lung tissue, embedded in paraffin. Morphometric analysis was performed assessing mean linear intercepts after fixation of the murine lung tissue as previously described in detail (21, 22).

Statistics

Data were analyzed with Prism5 (GraphPad Software, Inc., La Jolla, CA) and shown as mean ± SEM using Student's *t* test and analysis of variance or Kruskal Wallis test with appropriate posttests for at least three independent experiments. Significance was accepted at $p < 0.05$.

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FIBROBLAST GROWTH FACTOR RECEPTORS MEDIATE CELLULAR SENESCENCE IN CYSTIC FIBROSIS AIRWAY EPITHELIUM

by

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Abstract

By 2025, the number of adults living with cystic fibrosis (CF) will increase by approximately 75% due to drastic improvements in life expectancy attributable to advances in treatment and care including the development of highly effective modulator therapy. Chronic airway inflammation in cystic fibrosis (CF) contributes to morbidity and mortality and aging processes like 'inflammaging' and cell senescence could contribute to CF pathology. Our results show that single cell RNA sequencing data, human primary bronchial epithelial cells from non-CF and CF donors, a CF bronchial epithelial cell line, and *Cftr* knockout (*CFTR*$^{-/-}$) rats all demonstrated increased cell senescence markers in the CF bronchial epithelium. This was associated with upregulation of fibroblast growth factor receptors (FGFRs) and mitogen-activated protein kinase (MAPK) p38. FGFR or MAPK p38 inhibition attenuated cell senescence and improved mucociliary clearance and air surface liquid volume in *Cftr*$^{-/-}$ rats. CFTR modulator therapy itself did not seem to have any effect on cell senescence in CF airways. In summary, FGFR/MAPK p38 signaling contributes to senescence in CF airways which is reduced by FGFR blockade making this a potential novel senolytic treatment in an aging CF population.

Introduction

Cystic Fibrosis (CF) is the most common autosomal recessive disorder, affecting more than 70,000 people worldwide (1). Respiratory failure is the leading cause of morbidity and mortality in people with CF (pwCF) (2). The emergence of highly effective modulator therapies (HEMT) led to a significant decrease in disease burden and increased life expectancy, but chronic airway inflammation continues to persist thereby affecting

many cellular processes leading to accelerated aging and lung function decline (3). Investigations of the aging biology in chronic lung diseases have advanced and several cellular processes, termed “the hallmarks of aging”, have been used to characterize and study accelerated aging processes in lung diseases (4). Although, little is known about the aging processes in the CF lung.

Cellular senescence is an aging hallmark defined by irreversible cell cycle growth arrest due to cellular stressors, like inflammation (5). Senescent cells are apoptotic resistant, have increased expression of senescence associated β-gal (SA β-gal), and develop a senescence associated secretory phenotype (SASP) causing tissue damage, inflammation, and paracrine senescence (6). Molecular markers of senescence include B-cell leukaemia/lymphoma 2 (BCL2), B-cell lymphoma-extra-large (BCL-xL) for apoptotic resistance, cyclin-dependent kinase inhibitor 2A (p16), and cyclin-dependent kinase inhibitor 1 (p21) for cell cycle growth arrest, IL-6, IL-8 and IL1-β for SASP and increased expression of SA β-gal (7-9). Cellular senescence contributes to disease pathogenesis and progression in chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF) and Alzheimer’s Disease. Senolytic drugs targeting senescent cells in these diseases have proven to be beneficial reversing disease course in preclinical models (10).

Fibroblast growth factor receptors (FGFRs) encompass a subfamily of receptor tyrosine kinases that consists of four family members (FGFR1, 2, 3 and 4) with diverse functions (11, 12). FGFR1 and 4 are increased in CF and COPD airways and regulate airway inflammation (13, 14). FGFR1 signaling contributes to airway inflammation in CF by activating the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK-MAPK) signaling cascade (13). FGFR4 induces airway inflammation through

phospholipase C γ (PLCγ)/calcineurin and NFAT signaling (13, 14). FGFR signaling plays a complex role in cellular senescence with both pro and anti-senescence qualities (15-17). However, no studies to date have examined the consequences of cellular senescence and accelerated aging in the CF bronchial epithelium. This will be the first study to characterize cellular senescence in both *in vitro* and *in vivo* models of CF lung disease. We show that MAPK p38 signaling through FGFRs regulates cellular senescence in the CF bronchial epithelium making FGFR blockade a novel and potential future amenable therapeutic target for senolytic therapies targeting the CF lung, which is independent of CFTR function.

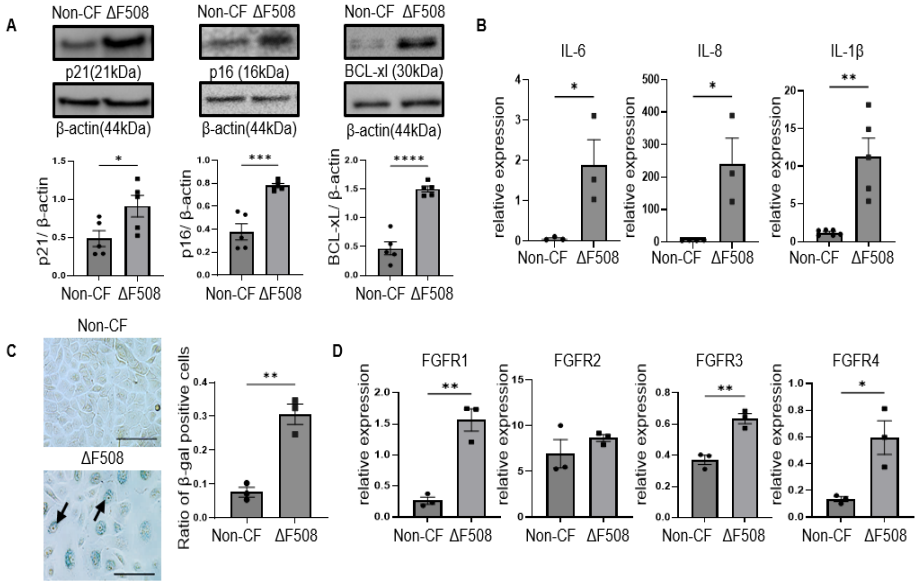

Figure 8. Cellular senescence markers are increased in CF primary human bronchial epithelial cells, cultured at the air liquid interface (ALI).

A) Bar graphs showing representative immunoblot images and densitometric analyses for p16, p21 and BCL-xL from ALI cultures from CF ΔF508 and non-CF donors (N=5). B) Bar graphs demonstrating relative mRNA levels of SASPs markers normalized to GAPDH and C) representative images for senescence associated β- galactosidase staining using brightfield imaging of the same CF ΔF508 and non-CF donor ALI cultures including quantification of β- galactosidase staining using the ratio of SA-β gal positive cells per brightfield by image J (N=3), arrows show β- gal positive cells (Scale bar= 100uM, Magnification 40x) D) Bar graphs indicating relative mRNA levels of FGFR1-4 normalized to GAPDH in the same two groups. Statistical analysis was done using Student's t-test showing means ± SEM with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ from 3-5 different donors per group with experiments repeated three times.

Results

Primary Human Bronchial Epithelial Cells from CF Donors Express Increased Cellular Senescence Markers

Primary human bronchial epithelial cells, cultured at the air liquid interface (ALI) from CF donors, homozygous for the ΔF508 mutation, and non-CF controls were assessed for the expression of an established set of cell senescence markers (1). Using western blot imaging and densitometric analysis, we observed increases in p21, p16 and BCL-xL protein levels in the CF ALI cultures (Figure 10A). mRNA levels of the senescence associated secretory phenotype (SASP) (IL-6, IL-8, IL1-β) were also significantly increased in the CF ALI cultures (Figure 10B). Additionally, SA β-gal staining was significantly increased in CF ΔF508 ALI cultures, when compared to non-CF controls (Figure 10C). Since we have previously shown an association between FGFR signaling and IL-8 secretion in the CF epithelium, including upregulation of FGFR1 (2), we compared the FGF receptor expression between control and CF ALI cultures. Interestingly, most FGFRs were markedly increased in CF ΔF508 primary human bronchial epithelial cells compared to non-CF control ALI cultures (Figure 10D). In summary, markers of cellular senescence and FGFR expression are increased in CF ΔF508 primary human bronchial epithelial cell ALI cultures compared to non-CF controls.

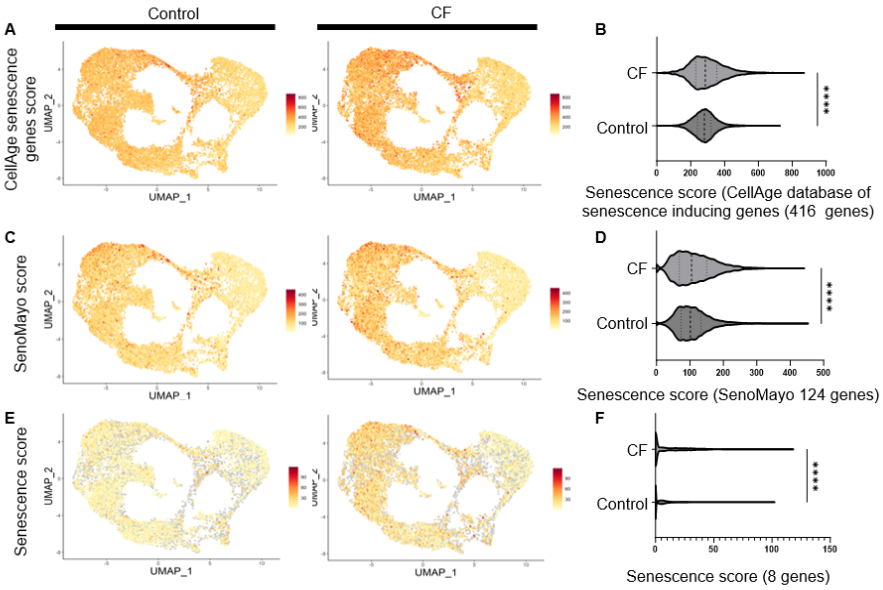

Figure 9. Single-cell RNA sequencing data reveals an increase in cell senescence markers in CF epithelial cells compared to control epithelial cells.

Single cell RNA (scRNA) transcriptome of control and CF epithelial cells (GSE150674 Control (CO) and Cystic Fibrosis (CF) epithelial cell count CO n= 23119 and CF n= 17590) from 19 control and 19 CF donor lungs from donors with end stage CF lung disease and healthy controls, CF ΔF508 homozygous patients (n=10,131 cells and n=8 donors) and healthy donors (n=23119 cells and n=19)were separated from the whole dataset and analysed using BBrowser3 to generate UMAPs and violin plots showing senescence scores from three separate gene databases: A,B):CellAge database of senescence inducing genes (416 genes), C,D) SenMayo (124 genes) and E,F): cellular senescence markers used in this study (cdkn1a, cdkn2a, blc2, bcl2l1, il6, il1b, il6 and glb1). Statistical analysis was done using Student's t-test showing with *p < 0.05, **p < 0.01, ***p < 0.001 and **** p<0.0001.

Single-Cell RNA Sequencing Data from Primary CF Airway Epithelial Cells Demonstrate Evidence of Cellular Senescence

To validate our findings in a separate and larger CF patient cell cohort, single-cell RNA sequencing (scRNA-seq) data of airway epithelial cells from the previously published GSE150674 dataset was used (3). This dataset includes donors with end stage CF lung disease and healthy controls and ages ranging 6-60 years in CF (n=17,590 cells and n=19 donors) and 18-63 years in healthy donors (n=23119 cells and n=19 donors). For the analysis, we filtered the CF cell population to cells homozygous for the DF508 mutation (n= 10,131 cells and n=8 donors with age range of 25-60 years) and created 3 senescence signature scores visualized via uniform manifold approximation and projections (UMAP) and violin plot from three gene sets: CellAge senescence inducing genes (408 genes) database (4), SenoMayo (124 genes) (5) and our previously used cellular senescence marker panel (*CDKN1A, CDKN2A, BCL2, BCL2L1, IL6, IL1β and GLB1*) (Figure 11). UMAPs and violin plots examining the senescence score from the CellAge database showed a significant increase in the CF cohort compared to controls (Figure 11A, 11B). Furthermore, the SenoMayo score mean was significantly increased in the CF group compared to controls, (Figure 11D) with noticeable differences in the UMAP between CF and control groups (Figure 11C). Moreover, we created a cellular senescence score using cellular senescence and SASP markers found in our study: *CDKN1A, CDKN2A, BCL2, BCL2L1, IL6, IL1β and GLB1* which demonstrated significant differences in the cellular senescence score visually and via violin plot in the CF epithelial cell group compared to control (Figure 11 E,F). Overall, these data support the evidence of cellular senescence in the CF airway epithelium using multiple senescence scores with large datasets of genes associated with cellular senescence.

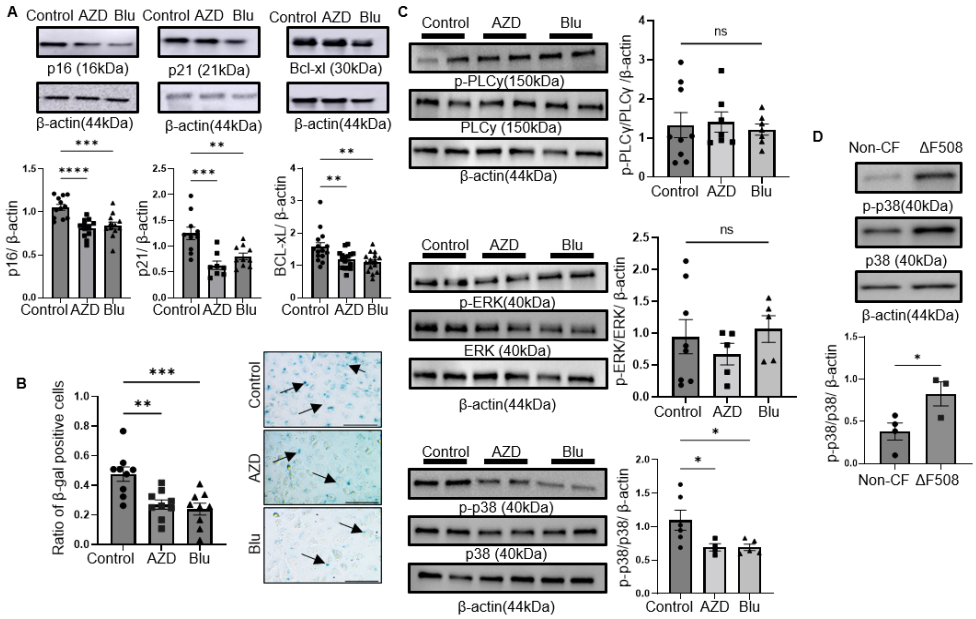

Figure 10. FGFR inhibition decreases cellular senescence markers which leads to a decrease in phosphorylation of p38 MAPK.

A) Representative immunoblot images and densitometric analyses of the cellular senescence markers p16, p21, and BCL-xL from CFBEs, which were treated with AZD4547 0.1uM or BLU9931 0.1uM for 24 hours. B) Representative images of SA-βgal staining's in CFBEs treated with AZD4547 and BLU9931 and quantification by capturing three images from different regions of the cell culture plates and counting all cells and all of β-galactosidase positive cells from the three images and making a ratio of β-galactosidase positive cells to total cells, arrows indicate β- gal positive cells (scale bar= 100uM, Magnification 40x). C) Representative immunoblots and densitometric analysis for p-ERK/ERK, p-PLC/PLCγ and p-p38/p38 MAPK in CFBEs treated with AZD4547 and BLU9931 for 24 hours. D) Representative immunoblot images and densitometric analyses from primary bronchial epithelial ALI cultures of CF (ΔF508) donors and non-CF control donors for p-p38/p38 MAPK expression. Statistical analysis was done using Student's t-test showing means ± SEM with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; 3 independent experiments were done in triplicates.

FGFR Inhibition Decreases Cellular Senescence Markers Which Leads to A Decrease in Phosphorylation Of p38 MAPK

CF bronchial epithelial cells (CFBEs) were treated with different FGFR inhibitors, including the clinically used FGFR inhibitor AZD4547 (2, 6-8) and the FGFR inhibitor Blu3391 (9). Expression of p16, p21 and BCL-xL showed a marked attenuation following treatment with each inhibitor (Figure 12A). Furthermore, the ratio of SA β-gal positive cells in the CFBEs treated with AZD4547 or Blu3391 were significantly lower compared to vehicle treated cells (Figure 12B). Next, we examined the activation of the downstream signaling mediators of FGFRs including ERK, PLCγ and p38 MAPK (2, 9-13). CFBEs, treated for 24 hours with AZD4547 or Blu3391, did not show any significant differences in phosphorylation of PLCy or ERK (Figure 12C). However, there was a significant decrease in p38 MAPK phosphorylation in CFBEs treated with AZD4547 or Blu3391 (Figure 12C). Primary human airway CF ALI cultures exhibited a baseline increase in p38 MAPK phosphorylation compared to non-CF controls (Figure 12D). In summary, FGFR inhibition attenuated cellular senescence in the CFBEs, which is partially mediated by FGFRs and p38 MAPK signaling.

Inhibition of p38 MAPK Decreases Cellular Senescence Markers in Cfbes

To further investigate whether p38/MAPK mediates cellular senescence in the CF bronchial epithelium, CFBEs were treated with a p38/MAPK inhibitor (SB203580) for 24 hours. A significant decrease in protein expression of p16, p21 and BCL-xL was observed following pharmacological blockade of p38/MAPK (Figure 13A). In addition, the SASP cytokines IL-8 and IL-6 were also attenuated by p38/MAPK inhibition in CFBEs (Figure 13B). These findings were also accompanied by a significant decrease in the ratio of SA

β-gal positive cells in CFBEs treated with the p38 MAPK inhibitor (Figure 13C). Together, these data suggest that multiple cellular senescence markers are regulated by p38 MAPK in the CF bronchial epithelium.

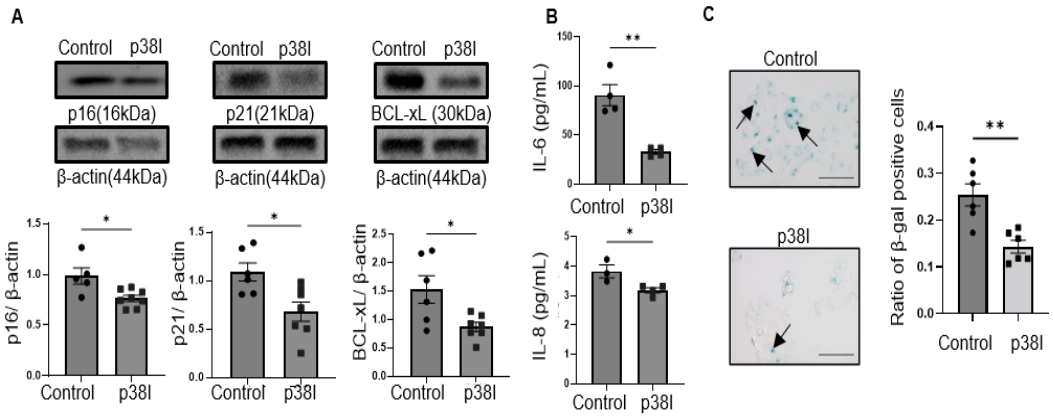

Figure 11. Inhibition of p38 MAPK decreases cellular senescence markers in CFBEs.

A) Representative immunoblot images and densitometric analyses showing p16, p21 and BCL-xL expression of CFBEs treated with SB203580 at 20uM for 24 hours compared to controls B) Bar graphs showing protein levels of IL-6 and IL-8 in CFBE supernatant after treatment with SB203580 for 24 hours. C) Representative images of SA-βgal staining in control and SB203580-treated CFBEs including quantification, arrows indicate β- gal positive cells (scale bar= 100uM, Magnification 40x). Statistical analysis was done using Student's t-test showing means ± SEM with *p < 0.05, **p < 0.01, and ***p < 0.001 with n =3-5 experiments.

Cellular Senescence Markers are Increased in Lung Tissue and the Airway Epithelium of $Cftr^{-/-}$ *Rats Compared to Littermate Controls*

Six-month old $Cftr^{-/-}$ rats, a model that exhibits CF-like airway disease (14), were used to validate our findings of increased cellular senescence in vivo. Lung tissue from 6-month-old $Cftr^{-/-}$ rats and controls were assessed via immunohistochemistry and showed increased staining of p16, p21 and BCL-xL in the bronchial epithelium (Figure 14A). Furthermore, SA- β-gal staining was increased in the $Cftr^{-/-}$ rat lungs compared to controls (Figure 14B). mRNA levels for *Bcl2*, *Cdkn2a*(p16), *Cdkn1a*(p21) and SASP markers (IL-6, IL-1β IL-8) from total lung tissue were also significantly increased in comparison to $Cftr^{+/+}$ lungs (Figure 14 C, D). In summary, there is evidence of cellular senescence in the bronchial epithelium from a well-established *in vivo* model exhibiting CF airway disease.

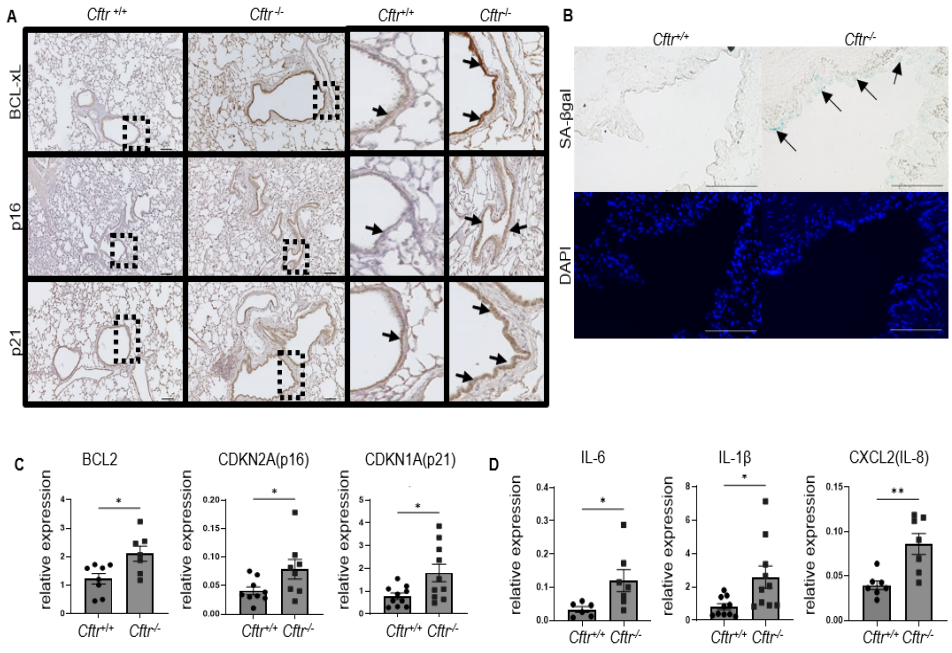

Figure 12. Cellular Senescence markers are increased in 6-month-old *Cftr-/-* rat lungs compared to controls.

A) Immunohistochemical staining for BCL-xL, p16 and p21 in *Cftr*$^{-/-}$ rat lung tissue compared to controls demonstrating an increased signal in the bronchial epithelium, arrows specify airway epithelial regions stained for BCL-xL, p16 and p21 (scale bar= 100uM, magnification 10x). B) Senescence associated β-galactosidase stain and nuclear counterstain (DAPI) in lung tissue from *Cftr*$^{-/-}$ rats and littermate controls, arrows indicate areas of airway epithelial with β-gal staining (scale bar= 100uM, magnification 40x). C, D) Relative mRNA levels of BCL2, p16, p21 and SASPs markers IL-6, IL-8 and IL1-β normalized to GAPDH, from total lung tissue of control and *Cftr*$^{-/-}$ rats. Statistical analysis was done using Student's t-test showing means ± SEM with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ with n = 5-10 rats per group and experiments done in triplicates.

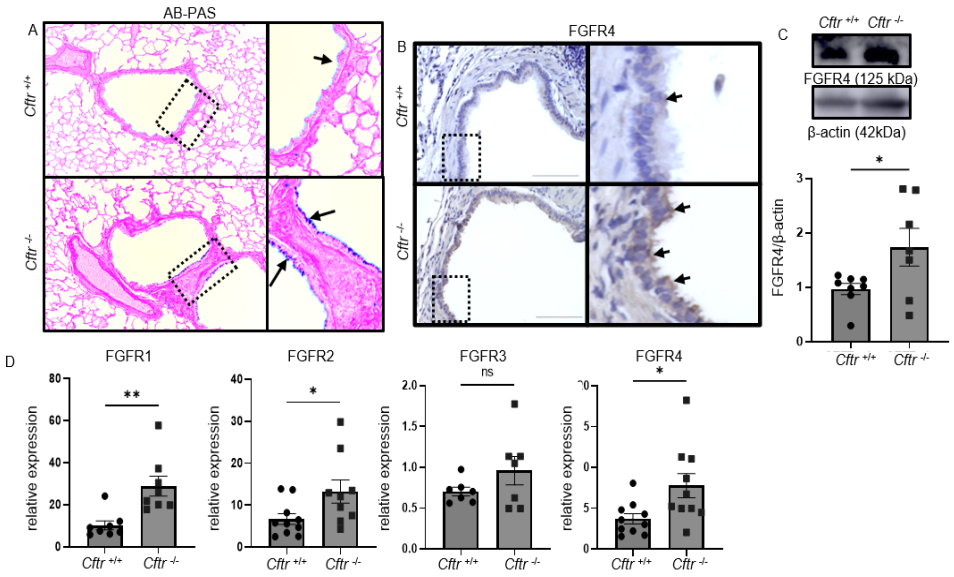

Figure 13. Fibroblast Growth Factor Receptor expression is increased in the lungs of 6-month-old *Cftr* -/- rats.

A) AB PAS staining indicated an increase of intercellular mucus staining in the bronchial epithelium of *Cftr* $^{-/-}$ rats, compared to control rats, arrows show areas stained in blue for intercellular mucus (magnification 20x) B) Immunohistochemical analysis using an isoform specific and validated anti-FGFR4 showed increased staining in the bronchial epithelium of *Cftr* $^{-/-}$ rats, arrows highlight areas of airway epithelium stained for FGFR4 (scale bar= 100uM, magnification 40x). C) Representative images of FGFR4 and β-actin and densitometric analysis demonstrating increased FGFR4 expression in *Cftr*$^{-/-}$ rat airways. D) Relative mRNA levels of FGFR1-4 from *Cftr*$^{-/-}$ total lung tissue, normalized to GAPDH expression. Statistical analysis was done using Student's t-test showing means ± SEM with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ with n = 5-10 rats per group.

Fibroblast Growth Factor Receptor Expression is Increased in the Cftr$^{-/-}$ Rat Lung

Formalin-fixed paraffin embedded sections of total lung tissue from 6-month-old *Cftr*$^{-/-}$ rats were stained with Alcian blue–periodic acid–Schiff (AB-PAS) and recapitulate the previously established muco-obstructive phenotype when compared to controls (Figure 15A) (14). Immunohistochemical analysis using an isoform specific antibody against FGFR4 revealed increased staining of the *Cftr*$^{-/-}$ bronchial epithelium compared to airways from control rats (Figure 15B). FGFR4 protein expression, determined by Western blot analysis, was also significantly increased in *Cftr*$^{-/-}$ rat lungs when compared to controls (Figure 15C). A dearth of validated antibodies limited our ability to assess protein expression of FGFRs 1-3, but we have previously shown that FGFR protein expression correlated with mRNA levels; therefore, qRT-PCR was performed and confirmed a significant increase in mRNA levels of *Fgfr1, Fgfr2* and *Fgfr4* in *Cftr* $^{-/-}$ rat lung tissue (Figure 15D). Transcript levels of the FGFRs have been shown previously to corroborate with protein expression (9). In summary, cell senescence markers as well as FGFRs are upregulated in the lungs of 6-month-old *Cftr-*$^{-/-}$ rats.

Cftr-/- Rats treated with FGFR Inhibition Attenuates Senescence and Improves Mucociliary Clearance

To investigate the effects of FGFR inhibition on reversal of cellular senescence *in vivo*, *Cftr*$^{-/-}$ rats were treated with AZD4547 via oral gavage daily for a total of 5 days. Pharmacological blockade of FGFRs with AZD4547 led to a decrease in p16 and p21 staining in the bronchial epithelium of *Cftr*$^{-/-}$ rats compared to sham treated *Cftr*$^{-/-}$ rats (Figure 16A). Furthermore, total lung protein expression of BCL-xL and p21 from AZD4547-treated *Cftr*$^{-/-}$ rats was significantly decreased when compared to sham treated

rats (Figure 16B). A reduction in phosphorylation of p38 MAPK (Figure 16C) and IL-8 expression (Figure 16D) was also observed. Tracheae of AZD4547 treated *Cftr*$^{-/-}$ rats were analyzed via μOCT (Figure 15E-G) demonstrating significant improvements in air surface liquid (15) depth but no significant differences in ciliary beat frequency (CBF) or periciliary liquid (PCL) depth, suggesting treatment with AZD4547 does not negatively affect the functional microanatomy of the lung epithelium (Figure 16 E, G). Further, there were significant improvements in mucociliary transport (MCT) in the AZD4547 treated *Cftr*$^{-/-}$ rat trachea when compared to the vehicle treatment (Figure 16 F, G). Overall, these data validate our in vitro findings that there is a decrease in cell senescence markers after FGFR inhibition with functional consequences, leading to improved mucociliary clearance without affecting the microanatomy of the CF airway epithelium.

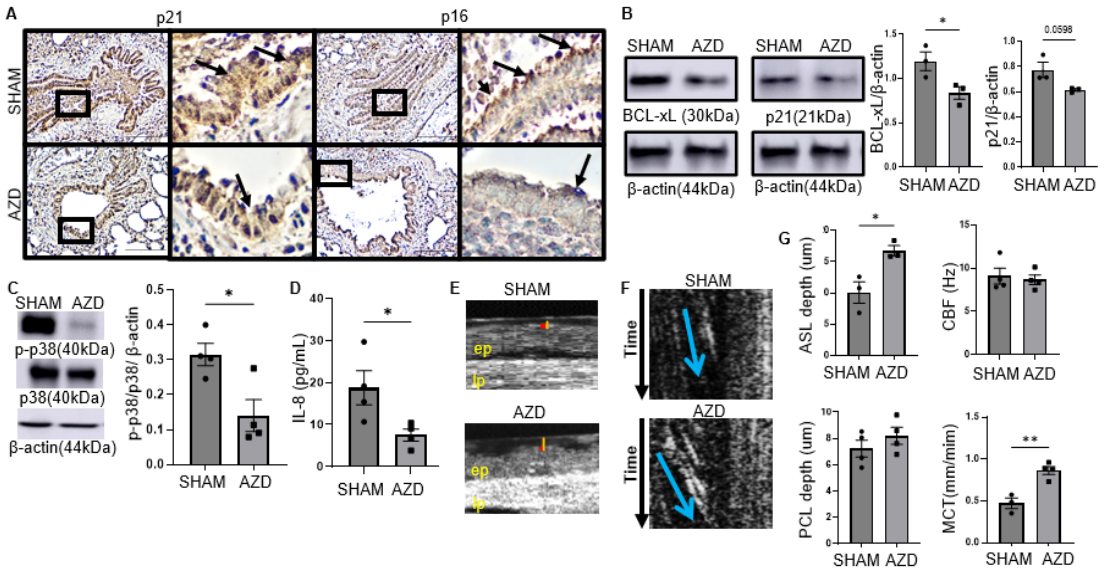

Figure 14. Systemic FGFR inhibition in *Cftr*-/- rats leads to decreases in cellular senescence markers in the lung and improved mucociliary clearance.

A) Representative images of immunohistochemical staining for p16 and p21 in *Cftr*$^{-/-}$ and control rat lungs, arrows present areas of stained airway epithelium (scale bar= 100uM, magnification 20x). B) Representative Western blot images and bar graphs demonstrating densitometric analyses of BCL-xL and p21 protein expression in *Cftr*$^{-/-}$ rat lungs ± AZD4547 treatment. C) Representative immunoblot images of phosphorylated and total p38 MAPK and densitometric analysis. D) IL-8 protein levels in *Cftr*$^{-/-}$ rat lung tissue ± AZD4547 treatment. E) Representative uOCT images of the tracheae of *Cftr*$^{-/-}$ rats (yellow line representing airway surface liquid depth and the red line representing periciliary liquid depth, ep= epithelial layer, lp= lamina propria). F) Representative images showing mucociliary transport (MCT) (cross-sectional arrow in blue indicates the velocity of the mucus particle via the slope) G) bar graphs indicating analysis of uOCT images quantifying airway surface liquid (ASL), ciliary beat frequency (CBF) and periciliary liquid depth (PCL) and mucociliary transport (MCT) from *Cftr*$^{-/-}$ rat tracheae after treatment of 5 days with AZD4547 (12.5 mg/kg) or sham. Statistical analysis was done using Student's t-test showing means ± SEM with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ with n = 3-4 rats per group.

HEMT Does Not Significantly Decrease Cellular Senescence Markers in CF Airways

To investigate whether highly effective modulator therapy could affect cellular senescence, we treated CF primary human bronchial epithelial cells on ALI with VX-661/VX-445/VX-770 (15) and vehicle. First, we demonstrate ETI works to correct *CFTR* dysfunction by μOCT analysis of air surface liquid volume in CF primary bronchial epithelial cells showing that there is a significant increase in air surface liquid volume in CF primary bronchial epithelial cells treated with ETI compared to vehicle treated cells (Figure 17A). Next, we found no significant difference in protein expression of the cellular senescence markers p16, p21 and BCL-xL via immunoblot and densitometric analysis (Figure 17B). To further define the effect of ETI on cellular senescence we examined relative expression of SASPs markers and found that there was no significant difference in relative expression between treated and vehicle groups (Figure 17D). Moreover, we assessed cellular senescence in a hG551D rat model, which is receptive to ivacaftor (VX-770) treatment (16). Immunoblots from hG551D rat lungs treated with VX-770 demonstrated no significant changes in protein levels of cellular senescence markers BCL-xL and p21 (Figure 17C) or SASPs markers (Figure 17E). Taken together, these results show that CFTR correction in CF did not attenuate cellular senescence in our experimental setups.

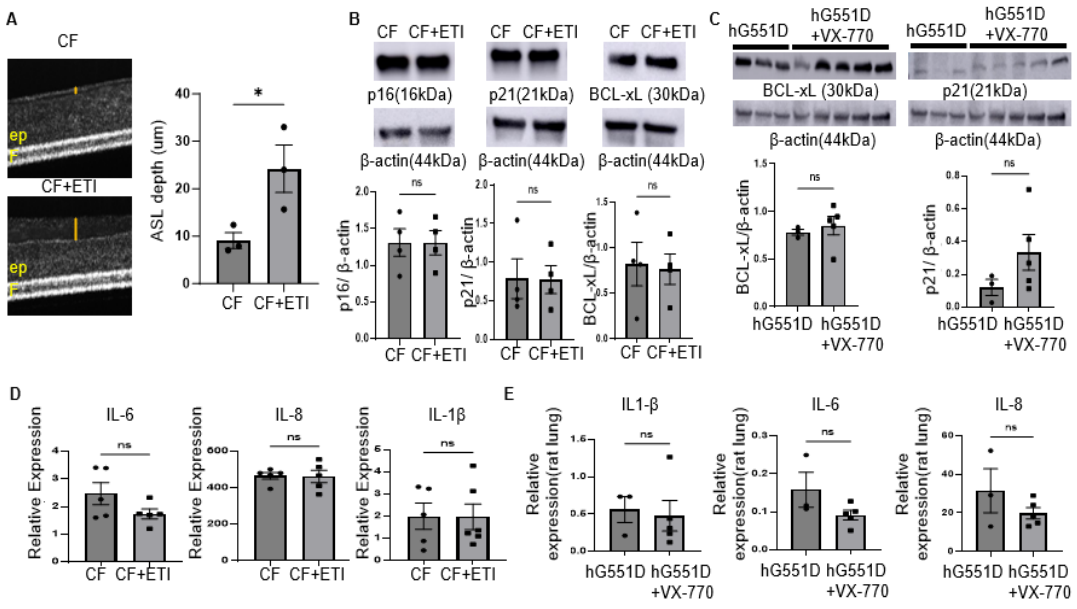

Figure 15. Modular therapy does not significantly decrease cellular senescence markers or expression of FGFRs

A) Representative uOCT images and bar graph of CF primary human bronchial epithelial cells (n=3 donors per group) treated with VX-661/VX-445/VX-770(ETI) for 72 hours showing a significant increase in ASL depth in CF cells treated with ETI, labels: yellow line is ASL depth, ep: epithelial layer and F: filter. B) Representative immunoblots and densitometric analysis showing no significant difference in protein expression of cellular senescence markers p16, p21 and BCL-xL in CF primary human bronchial epithelial cells treated with ETI for 72 hours when compared to untreated CF primary human bronchial epithelial cells (n= 4 donors per group). C) Immunoblots and densitometric analysis of BCL-xL and p21 in hG551D rats and hG551D rats treated with VX-770 for 14 days (hG551d= 3 rats, hG551d+VX-770= 5 rats). D) relative expression of SASPs markers from CF primary human bronchial epithelial cells demonstrates no significant change when compared to untreated CF primary human bronchial epithelial cells. E) Relative expression of SASPs markers IL1-β, IL-6 and IL-8 in hG551D rats and hG551D rats treated with VX-770 for 14 days (hG551d= 3 rats, hG551d+VX-770= 5 rats). Statistical analysis was done using Student's t-test showing means ± SEM with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Methods

Animals

All experiments used male and female SD-*Cftr*^{tm1sage} rats (*Cftr*$^{-/-}$) rats or wild type littermate controls at 6 months old as previously described (14). For experiments involving treatment of *Cftr*$^{-/-}$ rats with AZD4547 (Selleck Chemicals; Houston, TX, USA), we divided *Cftr*$^{-/-}$ rats into two groups (n=8 each): treated with 12.5 mg/kg bodyweight AZD4547 dissolved in DMSO with 1% sodium carboxymethyl cellulose (Selleck Chemicals; Houston, TX, USA) or vehicle once daily for five days. Method of delivery was oral gavage which has been used previously (17). hG551D rats (Envigo) were treated with ivacaftor (VX-770) (Selleck Chemicals; Houston, TX, USA) for 14 days at 30mg/kg/day or 3% methylcellulose vehicle by oral gavage (16).

Cell Culture

Both primary human bronchial epithelial cells and CFBEdelta508 (CFBEs) (18) were used for experiments and cultured on Snap well filters or plates in medium consisting of Minimum Essential Media (MEM) with L-glutamine, Phenol Red then supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals; Fort Collins, CO, USA), 1% L-glutamine, 1% penicillin/streptomycin and 0.2% plasmocin. Human bronchial epithelial cells from cystic fibrosis (ΔF508) and non-CF donors were provided by the Cell Culture Core of the UAB Cystic Fibrosis Research Center and cultured and differentiated on air liquid surface interface as previously described (2).

Inhibitors

CFBEs treated with each inhibitor listed: AZD4547 (Selleck Chemicals; Houston, TX, USA) and BLU9931 (Selleck Chemicals; Houston, TX, USA) at 0.1uM. SB203580 hydrochloride (Tocris Bioscience; Bristol, UK), a selective inhibitor for p38 MAPK was used at 20uM (19). After 24-hour treatment, cells were collected for analysis.

HEMT Treatment

CF primary human bronchial epithelial cells were cultured at the air liquid interface and treated with Tezacaftor (VX-661), Elexacaftor (VX-445), and Ivacaftor (VX-770) (Selleck Chemicals; Houston, TX, USA) for 72 hours at 3uM,1uM and 3uM, respectively. The drugs were refreshed every 24 hours.

Western Blot

Protein lysates were collected using 1x radio immunoprecipitation assay (RIPA) buffer with 1x Halt protease and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were separated on 4-20% precast Ready Gels (Bio-Rad Hercules, CA, USA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Pierce, Thermo Fisher Scientific, Waltham, MA, USA). Membranes were blocked with either 5% BSA or 5% low-fat milk depending on antibody manufacturer recommendations for 30 mins then incubated overnight with the following primary antibodies: rabbit anti-p21, rabbit anti-BCL-xL, rabbit anti-FGFR4, rabbit total and phospho-anti-ERK1/2, rabbit total and phospho-anti-p38 mitogen-activated protein kinase (MAPK), rabbit total and phospho-anti-PLCγ1 (Cell Signaling Technologies, Danvers, MA, USA), mouse anti-β-actin-peroxidase (Sigma, St. Louis, MO, USA) and rabbit anti-p16 (Proteintech, Rosemont,

IL, USA) diluted according to the manufacturer's recommendations. After three washes with TBST, membranes were incubated with goat anti-rabbit peroxidase conjugated (Invitrogen, Carlsbad, CA, USA) at 1:6000 in either 5% low fat milk or 5% BSA depending on primary antibody manufacturer recommendations for one hour. After three washes in TBST, the membranes were imaged by chemiluminescence on a ChemiDoc XRS system (Bio-Rad Hercules, CA, USA) and acquired using Image Lab software (Bio-Rad Hercules, CA, USA). Image J (National Institutes of Health, Bethesda, MD, USA) was used to measure densitometry of positive signals on the membranes.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from rat lungs, primary human cells and CFBEs as previously described (22, 27). Real- time quantitative PCR was performed with the following TaqMan probes: IL-6 Hs00174131, IL1-β Hs01555410, CXCL8 Hs00174103, GAPDH (4333764F), FGFR1 Rn01478647, FGFR2 Rn01269940, FGFR3 Rn00584799, FGFR4 Rn01441815, BCL2 Rn99999125, p21 Rn00589996, p16 Rn00580664, IL-8 Rn00586403, IL1-β Rn00580432. IL-6 Rn01410330, GAPDH Rn01775763 (Invitrogen, Carlsbad, CA, USA).

Immunohistochemistry

Lungs from control and *Cftr*-/- rats were collected and fixed in 10% neutral buffered formalin for 24 hours followed by dehydration in ethanol for 24 hours. The tissue was then embedded in paraffin and cut into 3-5 mm sections and mounted on slides. Lung tissue slides were deparaffinized and stained using rabbit anti-p16, rabbit anti-p21 (Proteintech, Rosemont, IL, USA), anti-rabbit FGFR4 antibody (sc-124; Santa Cruz Biotechnology,

Dallas, TX, USA), rabbit anti-BCL-xL (Cell Signaling Technologies, Danvers, MA, USA) and developed using a rabbit specific HRP/DAB detection IHC kit (Abcam Cambridge, UK) then counter stained with haematoxylin. The lung sections were stained with Alcian blue–periodic acid–Schiff and haematoxylin and eosin by UAB Comparative pathology laboratory core.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs for the quantitative recognition for interleukin-6 and interleukin-8 (Invitrogen, Vienna, Austria) were performed using supernatant from CFBEs after treatment with the different inhibitors as outlined before. ELISA for IL-8 (Abcam Cambridge, UK) was performed on protein lysates normalized to 2mg/mL of total protein in each sample from SHAM and AZD treated *Cftr*-/- rats.

Single-Cell RNA Sequencing Data

Publicly available single-cell RNA sequencing dataset GSE150674 that was previously aligned, filtered, normalized and annotated was used to analyze senescence scores using three separate gene sets (3). From the total dataset of 19 controls and 19 CF donors with end-stage lung disease undergoing lung transplantation. For the analysis, we separated the CF group by CF cells that are ΔF508 homozygous (n=10,131 cells and n=8 donors) with a 34–36-year-old average age for the CF group and compared this CF ΔF508 homozygous group to the healthy donor group (n=23119 cells and n=19) with an average age of 46 years for the control group. Both groups include male and female donors. Using BBrowser 3 software (Bio Turning Inc, San Diego, CA, USA) and three different sets of gene list associated with cellular senescence, we created senescence scores visualized via

UMAP and violin plots. The gene sets used in the senescence scores were: CellAge senescence genes database which was filtered for genes that induce cellular senescence (4), SenoMayo (5) and cellular senescence markers used in this study: *CDKN1A, CDKN2A, BCL2, BCL2L1, IL6, IL1B and GLB1*. A list of genes used from SenoMayo and CellAge senescence genes database is included as a supplemental table. Violin plots were made by extracting the signature score data from BBrowser3 and analysed in Prism9 (GraphPad, San Diego, CA, USA).

Senescence Associated B-Galactosidase Staining

Cytochemical staining for senescence associated β-galactosidase was performed using a Senescence associated β-galactosidase Staining kit from Cell signaling (Cell Signaling Technologies, Danvers, MA, USA) #9860 following the protocol provided. Rat tissue slides were counterstained with DAPI (Vector Laboratories, Newark CA, USA). To quantify the amount of β-galactosidase staining we captured three images from different regions of the cell culture plates and counted all cells and all of β-galactosidase positive cells from the three images and made a ratio of β-galactosidase positive cells to total cells counted. CFBE experiments were done in triplicates and primary cell cultures included 3 different donors from CF and non-CF.

Micro-Optical Coherence Tomography

Measurements of functional microanatomic parameters in CF primary human bronchial epithelial cells on ALI and ex vivo tracheae were performed using micro-optical coherence tomography (μOCT), a high-resolution microscopic reflectance imaging system as previously described (20).

Statistics

Data were analysed with Prism9 (GraphPad, San Diego, CA, USA) as previously described (10) using Student's t test for a minimum three independent experiments in duplicate. Data is shown with individual values from each experiment ±SEM. Statistical significance was accepted at p-value of less than 0.05.

Study Approval

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (UAB) approved all animal protocols.

Data Availability

All data associated with this manuscript are present in the paper. The single-cell RNA dataset is publicly available data (dataset GSE150674) and cited in this manuscript.

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DISCUSSION

Summary of Findings

In my first publication, we demonstrated that FGFR4 deficiency in healthy adult murine lungs caused emphysema, airway inflammation and right ventricular hypertrophy. We showed that serum FGF23 levels are decreased in these mice however FGF23 mRNA levels are increased with no change in FGFR1 mRNA expression. Interestingly, IL1-β and IL-6 mRNA levels are significantly decreased in FGFR4 deficient mice lungs compared to controls. Canonical, proinflammatory signaling mediator p38 is significantly downregulated with no change in PLCγ or ERK in the FGFR4 deficient mice. In the airway epithelium, we observed no differences in mucociliary clearance. However, when we stratified the MTECs into high IL-6 secretor group and low IL-6 secretor group, we found that the IL-6 secretor group had a significant decrease in ASL depth. Interestingly, constitutive FGFR4 activation in the adult murine lung demonstrated a normal lung architecture, decreased FGF23 serum levels, decreased expression of proinflammatory mediators TGF-β, IL1-β and IL-6. Together these results show that FGFR4 deficiency in the healthy murine lung is inflammatory.

To build on these findings, my second manuscript showed that FGFR1-4 contributed to inflammation in CF airways. Moreover, we show that another proinflammatory and pro aging mechanism, cellular senescence is regulated by FGFR1-4/ p38 signaling. We used primary HBEs, CFBEs and CFTR knockout rats to characterize

cellular senescence and FGFR expression in CF. Next, we elucidated the signaling mechanism in CFBEs using FGFR inhibitors AZD4547 and BLU9931. These results showed that FGFR inhibition in CFBEs decreases cellular senescence and p38 phosphorylation. To further confirm these results, a p38 inhibitor in CFBEs demonstrated similar findings to the FGFR inhibitor experiments. Next, we used the CFTR knockout rats and treated them with AZD4547 and found similar results to our in vitro CFBE studies further confirming that FGFRs decreased cellular senescence markers through p38 signaling. Another key finding of this manuscript is that FGFR inhibition not only decreased cellular senescence markers in vivo, but also improved mucociliary clearance, a key functional outcome of CF disease. These results suggest that FGFR inhibition could be an anti-aging, anti-inflammatory treatment for the aging CF population.

Overall, these two manuscripts reveal the complex nature of FGFRs in inflammatory lung diseases. They show that depending on the context or disease state FGFRs can be both pro and anti-inflammatory and multifaceted by regulating several different cellular functions such as inflammation and cellular senescence.

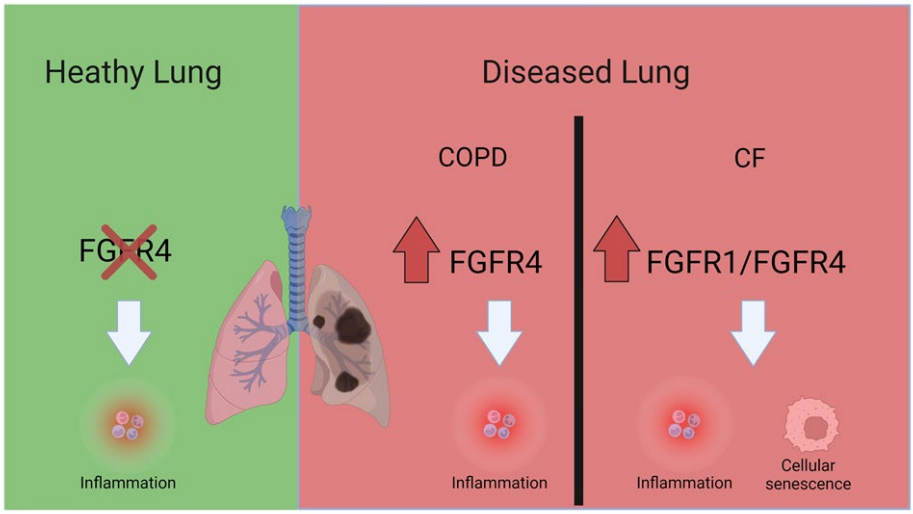

Figure 16. Summary of the role of FGFRs in the healthy lung compared to COPD and CF.

Identifying FGF Ligands in Cellular Senescence

This work highlights the role of FGFRs in regulating cellular senescence in the CF airway epithelium. However, we have not addressed the potential FGF ligand involved in cellular senescence in CF. We explored the endocrine FGFs: FGF19, FGF21 and FGF23. We found no significant differences in levels of FGF21 or FGF23 in our epithelial cells however, we did see significant increases in FGF19 in CF epithelial cells compared to controls.

FGF19 is a hormone secreted by the small intestine (128). Previous studies have shown that FGF19 induces cell cycle arrest at G2 phase via FGFR4-p38/MAPK axis in chondrocytes (129). Moreover, in fibrotic lung diseases like idiopathic pulmonary fibrosis, FGF19 is protective against apoptosis in alveolar epithelial cells by decreasing Bcl-2-like protein 11 expression and reduces myofibroblast differentiation in fibroblast in part by decreasing TGFβ-induced c-Jun N-terminal Kinase phosphorylation (130). These studies highlight FGF19's ability to regulate cellular mechanisms associated with cellular senescence and affect distant tissues including the lung. Interestingly, in our data we demonstrate that FGFR4-p38/MAPK signaling as a mechanism of cellular senescence in CF bronchial epithelial cells which is like the previous mechanism of FGF19 in chondrocytes which induces growth arrest via FGFR4-p38/MAPK. Furthermore, since the first step in cellular senescence is growth arrest, it is possible that FGF19 could play a role in cellular senescence in the CF bronchial epithelium. Given that FGF19 has anti-fibrotic effects in the lungs and that FGF19 is associated with inflammation in the gut, it could be feasible to explore FGF19 in inflammation in the lungs as well as cellular senescence (131).

Interestingly, paracrine FGFs, particularly FGF2, has been shown to delay cellular senescence (119, 132). FGF2 enhances cell proliferation, telomerase activity, and expression of stem cell markers, promoting a more stem like state in cells (133). This has been shown in several cell types including endothelial cells, mesenchymal stem cells, and embryonic stem cells (85, 109, 113). Furthermore, studies have found that FGF2 can upregulate the expression of telomerase and stemness-related genes, contributing to the maintenance of cell function and longevity (133). Additionally, conditioned medium from embryonic stem cells, which contains FGF2, can suppress cellular senescence in fibroblasts and accelerate wound healing in *in vivo* models (134). The effects of FGF2 have yet to be studied in CF. However, in other inflammatory airway diseases like COPD, FGF2 decreases inflammation induced from cigarette smoke exposure and improved regeneration in an elastase-induced emphysema model (113). Moreover, FGF2 plasma levels were significantly decreased in COPD patients (111-113). Together these studies suggest FGF2 plays an anti- senescent and anti-inflammatory role and should be explored as a therapeutic target for inflammation and cellular senescence.

Moreover, fibroblast and epithelial cell cross talk has been shown to drive disease pathogenesis and inflammation in IPF and COPD. Although IPF and COPD are very different diseases, given that IPF is a fibrotic that affects the interstitial of the lung and COPD affects the airways of the lungs, in both diseases' fibroblast and epithelial cell cross talk contributes to their pathogenesis. Interestingly, paracrine signaling between fibroblast and epithelial cells is the mechanism that causes the inflammation and cellular changes seen in fibroblast and epithelial cells. This mechanism is like paracrine senescence in which one senescent cell can secrete mediators that induce senescence in other cells that are near

it. Taken together this concept, with the fact that fibroblast produces a significant amount of paracrine FGFs, this could be a feasible mechanism for FGF ligands to be contributing to cellular senescence in inflammatory airway diseases.

FGF Ligands as Drug Targets

Advances in our understanding of FGF ligand biology have provided opportunities for new drug development. Several recombinant FGFs have been used to treat wounds, diabetes, and hypophosphatemia (135). Both the US Wound Healing Society and European Wound Management Association have recommended FGFs for the treatment of refractory ulcers. The FGFs that have been of interest to pharmaceutical companies for the treatment of wounds include FGF2, FGF7 and FGF10 (136). In 2004, the US Food and Drug Administration approved the use of an intravenous formulation of FGF7 (palifermin) for the treatment of mucositis (137). FGF10 (repifermin) has been tested clinically and has shown conflicting results in patients with chronic venous leg ulcers (138). FGF2, on the other hand, has been more widely used for wound healing in pressure ulcers, diabetic foot ulcers and second-degree burns. Topical use of recombinant FGF2 is approved for use in Japan, China, and the US as trafermin (139).

FGF2 has been tested for the treatment of COPD with positive results such as decreasing inflammation and improving regeneration in preclinical models (113). In human trials, aerosolized FGF2 improved lung function in COPD patients compared to baseline (113). Interestingly, in our work, we found that FGFRs are increased in COPD and CF airways including FGFR1 the target receptor for FGF2. Previous studies have shown that FGF2 and FGFR1 are increased in COPD.

Moreover, other FGFs have shown to have an impact on the lungs, for example FGF7 signaling plays important roles in lung development and has been shown to be important for alveolar self-renewal and growth (140). FGF10 has been shown to be protective and promote lung epithelial regeneration after injury (141). Together these studies demonstrate the current and future possible therapeutics associated with FGF ligands including their important role in lung biology and lung diseases like COPD.

FGF21 regulates glucose and lipid metabolism in obese rodents and primates, acting to reduce blood sugar and triglyceride levels, enhancing sensitivity to insulin, and reducing body weight without causing hypoglycemia (128, 142, 143). Recent studies demonstrate that the FGF21 agonist is a promising therapeutic agent for the treatment of type 2 diabetes and obesity (142). Given the extensive preclinical and clinical studies, FGF21 could be a promising treatment for type 2 diabetes (144). Eli Lilly reported the effects of LY2405319 (LY), a variant of FGF21, that produced significant improvements in proof-of-concept trials in obese and type 2 diabetic patients (145). Additionally, FGF23 neutralizing antibodies have been studied in a chronic kidney disease-mineral and bone disorder (CKD-MBD) rat model which treatment with monoclonal FGF23 antibody demonstrated inhibition of hyperparathyroidism, increased vitamin D and calcium levels, and restored bone mass back to normal levels (146). Overall, these studies suggest that FGF ligands could be effective targets for several diseases. However, one consequence of treatment or inhibition of FGFs is their significant role in many different biological and physiological processes.

Given that FGF23 is increased in both COPD and CF patients' plasma levels, this could be a potential therapeutic target (103, 117). However, it's important to keep in mind

that FGF23 is not the best drug target given that blocking FGF23 in mice and rats leads to their mortality (147). Therefore, targeting FGFRs instead of FGF ligands could be a better method given that blocking them with a drug will have less side effects than blocking FGF ligands.

FGFRs as Drug Targets for Inflammation in COPD and CF

Both studies in this thesis highlight the role of FGFRs in inflammation and cellular senescence in two different inflammatory airway diseases. Additionally, FGFRs expression is increased in both COPD and CF airways with FGFR inhibition decreasing inflammation in both COPD and CF and decreasing cellular senescence in CF (103, 117). Other studies have found that FGFRs play a role in inflammation as well. For example, FGFR1 and FGFR2 have been shown to modulate inflammation and neurodegeneration in multiple sclerosis (148). Furthermore, Studies in mouse models of autoimmune encephalomyelitis found that treatment with infigratinib an FGFR2 inhibitor significantly decreased inflammation (149).

FGFR inhibitors have been well studied in the context of cancers given FGFR abnormalities, like overexpression of FGFRs or constitutively active FGFRs which stem mainly from genetic alterations in the gene sequence or gene amplification, are involved in the development and progression of varies cancers. Given the diversity in FGFR abnormalities each FGFR inhibitor is designed to target a specific type of FGFR abnormalities.

AZD4547 is a pan-FGFR inhibitor with high affinity for isoforms FGFR1-3 that completed phase 2 clinical trial (NCT04439240) in multiple cancers with FGFR

dysfunction, including FGFR1 harboring gatekeeper mutations (V561M) that allows the receptor to adapt and become resistant to FGFR inhibitors (150). FGFR4 inhibitor BLU9931 was the first selective FGFR4 inhibitor for the treatment of hepatocellular carcinomas with aberrant FGFR4 signaling (151). BLU9931 failed to enter clinical stages because of its rapid metabolism in the liver (152). Our studies have demonstrated that FGFR1 and FGFR4 play a role in cellular senescence in CF. In COPD, FGFR4 contributes to airway inflammation.

There are several small molecule inhibitors that target FGFRs and inhibit their activation, which has been shown to significantly reduce several disease pathologies associated with FGFR mutations or aberrant signaling including cancers (135) . Moreover, our data suggests that small molecule inhibitors for FGFRs (AZD4547 and BLU9931, FGFR1-3 and FGFR4 inhibitors respectively) significantly decrease cellular senescence markers, inflammation and improve disease outcomes in CF. Thus, targeting FGFR1 or FGFR4 in COPD and CF airways could be a potential therapeutic for cellular senescence and inflammation. However, more research needs to be done to develop an effective FGFR inhibitor given that there are caveats to using them such as FGFRs developing resistance to inhibitors, side effects in patients and off target effects including binding to other receptors such as vascular endothelial growth factor receptor (VEGFR) (153).

Other therapies for FGFR inhibition like FGFR blocking antibody can be designed to be more effective at targeting FGFRs by tailoring the antibody to confer or remove binding affinity (154). Several studies have shown FGFR blocking antibodies are effective in targeting FGFRs in various diseases and cancers where FGFRs contribute to the pathology (82, 90, 119, 154-158). While FGFR blocking antibodies are highly specific to

their target, there are some limitations to their use such as cost, pharmacokinetics, tissue penetration and potential immune system interference by the blocking antibodies (91, 159). Given this FGFR1, FGFR4 small molecular inhibitors or blocking antibodies are still a feasible treatment for inflammation and cellular senescence in COPD and CF.

Senolytic Therapy for COPD and CF

In recent years, there has been a surge of interest in exploring senolytics as a possible remedy for selectively eliminating senescent cells, a hallmark of aging. This pursuit is driven by the hope of mitigating the chronic inflammation linked to aging and enhancing the reparative capabilities of stem and progenitor cells that are crucial for tissue regeneration (160). Evidence indicates that reducing senescent cells could potentially delay or prevent age-related functional decline and extend lifespan (160, 161). Interestingly, studies have demonstrated the ability to specifically target senescent cells in a mouse model of inducible elimination of p16-positive cells (p16Ink4a-induced apoptosis through targeted activation of caspase) using AP20187 (58). Clearance of p16Ink4a-positive cells delayed tumorigenesis and attenuated age-related deterioration of several organs including the kidney, heart, and adipose tissue without apparent side effects (58). Senescent cells are apoptotic resistance marked by expression of ephrins (EFNB1 or 3), PI3Kd, p21, BCL-xL, and plasminogen-activated inhibitor-2 (162). Previous studies have shown that siRNA to silence expression of these apoptotic resistance markers eradicated senescent cells (162). Compounds like Dasatinib and Quercetin (DQ) have emerged as senolytic agents, demonstrating their ability to diminish senescent cell burden and enhance cardiovascular function in aged mice (150). Clinical trials investigating the efficacy of these compounds in treating age-related diseases such as idiopathic pulmonary fibrosis (IPF) and diabetic

kidney disease demonstrated improvements in disease outcomes warranting further evaluation of DQ in larger clinical trials (163, 164).

COPD is increasingly recognized as a disease of accelerated lung aging with the accumulation of senescent cells in the lungs of patients with COPD compared to aged, matched controls (19, 165, 166). Additionally, COPD is associated with other diseases of accelerated aging such as chronic kidney disease and type 2 diabetes which share common underlying mechanisms including cellular senescence (166). Since COPD disease seems to be partially driven by cellular senescence, senolytic therapy could be a novel approach to treating COPD. Currently, there are no clinical trials of senolytic therapies for patients with COPD but since DQ is in clinical trials for IPF another age-related lung disease its feasible that this DQ treatment could have positive effects on COPD patients and reduce burden of senescence and inflammation in COPD. There are several other senolytic such as Navitoclax, A1331852 and A1155463 (selective BCL-xL inhibitors) and metformin that could be good candidates for targeting senescent cells in COPD. Moreover, since CF patients are aging and several hallmarks of aging including cellular senescence are present in CF lungs, its reasonable that senolytic drugs that work for other age-associated diseases, will be effective for targeting senescent cells in COPD and CF lungs.

Despite these advances, there are caveats and challenges to senolytic therapy. Senescent cells, known for their association with pathology, also have important roles in processes like wound healing, development and pregnancy suggesting that senolytic therapies should be administered intermittently for safety, allowing cessation during critical events like pregnancy, development, or wound repair (167). Moreover, senescent cells exhibit diverse transcriptional, metabolic, and SASP profiles, because of this diversity

senolytic drugs have been shown to demonstrate specificity towards different types of senescent cells (168). Thus, studies are needed to characterize senescent cells and determine the proper senolytic therapy.

Senolytic therapeutics are imperfect in their current state and there are still barriers to clinical application which include bioavailability issues, off-target effects, and side effects associated with their application (169). Moreover, a recent study found that eliminating senescence cells via suicide gene (p16 promoter–driven killer gene construct in p16-ATTAC mice) or senolytic drugs (ABT263 and cell-permeable FOXO4-p53 interfering peptide [FOXO4-DRI]) worsen pulmonary hemodynamics associated with pulmonary arterial hypertension (170). This study further demonstrates the need to understand senolytic drugs in more disease environments. Despite these challenges, senolytic therapy is still a promising therapeutic for age associated diseases including COPD and CF.

FGFR Inhibitors are a Potential Senolytic Drug

Nintedanib and dasatinib are both tyrosine kinase inhibitors (TKIs) that have senolytic effects. Nintedanib is well known for its use to treat IPF and dasatinib was known for treating cancers. The most notable difference between nintedanib and dasatinib and FGFR inhibitors is that they are nonselective TKIs whereas AZD4547 and Blu9931 are highly selective TKIs that target FGFR1-3 and FGFR4 respectively. FGFR inhibitors were able to decrease cellular senescence markers via p38 signaling in CF airways which makes FGFR inhibitors a potential future senolytic.

FUTURE DIRECTIONS

Defining the Role of FGFR4 on Inflammation in the Lung

In these studies, we demonstrate that FGFR4 deficiency leads to increased inflammation in the healthy murine lung. In disease states such as COPD FGFR4 expression is increased and contributes to airway inflammation. In this study we expand on the role of FGFR4 in airway inflammatory diseases and show that FGFR4 contributes to inflammation and cellular senescence in CF airways. Additionally, in other organs such as the liver and the heart FGFR4 also contributes to inflammation (96, 108). Other studies have shown that FGFR4 contributes to inflammation in gastric cancer patients with Helicobacter pylori infection (171). In other inflammatory lung diseases such as asthma, FGF2/FGFR1/MAPK signaling contributes to airway epithelial cell inflammation (172). However, no one has examined if FGFR4 contributes to airway inflammation in asthma, would is feasible given that in CF airways both FGFR1 and FGFR4 contribute to airway inflammation. Additionally, our data on FGFR4 and inflammation is contradictory, we show in a healthy lung FGFR4 deficiency leads to inflammation whereas in COPD airways FGFR4 contributes to inflammation. More studies are needed to further explore FGFR4's role in airway inflammation in the lungs as it could be a potential target to treat inflammation and it could give insight into FGFR4's role in the lung overall.

Do FGFRs Regulate Cellular Senescence in Other Inflammatory Airway Diseases

We demonstrate the FGFRs regulate cellular senescence in CF airways. Since FGFRs are increased in COPD, another inflammatory airway disease that is associated with cellular senescence, it's worth exploring the possibility of FGFRs mediating cellular senescence in COPD airways. Several FGFs are significantly increased in airway brushings from severe asthma patients compared to healthy controls (172). Additionally, pan-FGFR inhibitors decreased inflammation in a mouse model of severe asthma (173). Other studies in asthma have shown that cellular senescence is increased in airway smooth muscle cells from elderly persons with asthma compared to both aged and young controls (174). Given these studies and our results looking at cellular senescence in other inflammatory airway diseases, it's feasible that FGFRs could mediate cellular senescence in asthma.

Defining The Connection Between FGFRs and Mucociliary Clearance in COPD and CF

Mucociliary clearance dysfunction is a common overlaying pathology in both COPD and CF. In COPD, mucociliary clearance dysfunction is mediated by smoke induced CFTR dysfunction (11). Moreover, CF is a disease characterized by CFTR dysfunction which leads to mucociliary clearance dysfunction (51). Mucociliary clearance dysfunction is connected to the progression of disease in both COPD and CF (11). In our studies, we demonstrate that CF rats treated with FGFR inhibitor, AZD4547, there were significant improvements in ASL depth and mucociliary clearance. Demonstrating that FGFRs could play a role in regulating airway hydration, however the mechanism is still unknown. Defining how FGFRs regulate airway hydration will further our understanding of mechanisms of airway hydration and lead to potential therapeutic interventions for airway dehydration in COPD and CF.

OVERALL CONCLUSIONS

Overall, this collective work emphasizes the importance of FGFRs in regulating inflammation and cellular senescence in airway inflammatory diseases. We have presented novel insights into FGFR4's role in regulating inflammation in the healthy lung. We are the first to demonstrate that FGFRs regulate inflammation and cellular senescence in CF. Finally for the first time we show that FGFR inhibitors decrease inflammation and cellular senescence markers while also improving mucociliary clearance in CF airways. Altogether, this work provides impetus to further explore the role of FGFRs in inflammation and cellular senescence not only in airway inflammatory diseases but other inflammatory and age-associated diseases. FGFR inhibition has the potential to be a novel therapeutic treatment for airway inflammatory diseases, chronic inflammatory diseases and age associated diseases.

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APPENDIX A

Institutional Animal Care And Use Committee Approval Forms

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 04-Apr-2023

TO: Krick, Stefanie

1 **FROM:** Shannon M. Bailey

Shannon M. Bailey, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

2 **SUBJECT**: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 04-Apr-2023.

Protocol PI:	Krick, Stefanie
Title:	Linking phosphate and chronic lung disease
Sponsor:	UAB DEPARTMENT
Animal Project Number (APN):	IACUC-21962

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 03-Apr-2026.

1 Institutional Animal Care and Use Committee (IACUC)

403 Community Health on 19th | 933 19th Street South

Mailing Address: CH19 403 | 1720 2nd Ave South | Birmingham AL 35294-2041

phone: 205.934.7692 | fax: 205.934.1188

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 24-Jun-2022

TO: Krick, Stefanie

FROM: 

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 24-Jun-2022.

Protocol PI:	Krick, Stefanie
Title:	Targeting Fibroblast Growth Factor Receptors in Cystic Fibrosis-Associated Airway Inflammation and Mucociliary Dysfunction
Sponsor:	National Heart, Lung, and Blood Institute/NIH/DHHS
Animal Project Number (APN):	IACUC-22370

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 28-Jul-2024.

Mailing
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