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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

DECIPHERING THE MICRORNAOME IN HIV ASSOCIATED LUNG COMORBIDITIES

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMEDICAL SCIENCE

by

Rajib Kumar Dutta

2020

To: Dean Robert Sackstein
College of Medicine

This dissertation, written by Rajib Kumar Dutta, and entitled Deciphering the MicroRNAome in HIV Associated Lung Comorbidities, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Madhavan Nair

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Marisela Agudelo

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Yuan Liu

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Sabita Roy

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Hoshang Unwalla, Major Professor

Date of Defense: June 25, 2020

The dissertation of Rajib Kumar Dutta is approved.

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Dean Robert Sackstein
College of Medicine

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Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2020

DEDICATION

I would like to dedicate this dissertation to my parents: Baba (Father), Maa (Mother) for always inspiring and advising me to be a good human being,

And

To my lovely wife, for bearing with my frustrating stubbornness and for truly supporting me. You have been my strength through everything.

And

To my elder sister, for always motivating and supporting me throughout my life for being successful.

I love you all with my heart.

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A dissertation is not the outcome of the efforts of entirely one individual. Many people have contributed to its development. At this time, I take the opportunity to acknowledge those who have made a tremendous impact on my doctoral journey and accomplishment. First and foremost, I would like to acknowledge my indebtedness and render my warmest thanks to my academic advisor and mentor, Dr. Hoshang Unwalla, for his continuous support, inspiration, and guidance. During my tenure, he contributed to a rewarding graduate school experience by giving me intellectual freedom in my work, supporting my attendance at various conferences, engaging me in new ideas, and demanding a high quality of work in all my endeavors. As my supervisor and mentor, he has taught me more than I could ever give him credit for here.

Next, I would also like to extend my gratitude towards my respected dissertation committee members Dr. Madhavan Nair, Dr. Sabita Roy, Dr. Yuan Liu, and Dr. Marisela Agudelo, for being agreed to be on my committee. It was my privilege to get them as an expert and as my committee member, and I will always be indebted for his support, guidance, and mentorship. Their friendly guidance and expert advice have been invaluable throughout all stages of the work. Their extended discussions and valuable suggestions that have contributed significantly to the improvement of the thesis. My special gratitude to Dr. Nair, who has provided me extensive personal and professional guidance and taught me a great deal about both scientific research and life in general.

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ABSTRACT OF THE DISSERTATION

DECIPHERING THE MICRORNAOME IN HIV ASSOCIATED LUNG COMORBIDITIES

by

Rajib Kumar Dutta

Florida International University, 2020

Miami, Florida

Professor Hoshang Unwalla, Major Professor

Chronic Respiratory diseases like chronic obstructive pulmonary disease (COPD), pulmonary hypertension, asthma, and pneumonia are emerging as significant comorbidities in people living with HIV in the combination antiretroviral therapy (cART) era. HIV is an independent risk factor for these diseases even when accounting for smoking, and cigarette smoking exacerbates outcomes in HIV patients. Mucociliary clearance plays an important role in airway defense. Both tobacco smoke and HIV infection suppress nasal mucociliary clearance (MCC) as well as bronchial MCC, a primary innate defense mechanism in the airway. Optimal MCC depends on airway surface liquid (ASL) lining the airway epithelium that facilitates ciliary beating to clear the mucus. Attenuation of any component of the MCC leads to mucus impaction and microbial colonization leading to lung infections. Cystic fibrosis transmembrane conductance regulator (CFTR) plays a pivotal role in airway MCC by virtue of its ability to regulate ASL levels, ASL pH, and consequently mucus viscosity.

Our studies showed that TGF-β signaling is induced by HIV Tat protein and cigarette smoke. We found that HIV Tat protein and cigarette smoke individually and additively

inhibit CFTR biogenesis and function in normal human bronchial epithelial cells via a common TGF-β signaling pathway. Inhibiting miRNA processing using Aurin-tricarboxylic acid (ATA), a small molecule inhibitor of the pri-miRNA processing enzyme Drosha completely rescues TGF-β -mediated CFTR suppression suggesting an important role for miRNA mediated post-transcriptional gene silencing. We show that TGF-β signaling alters the bronchial epithelial microRNAome. Specifically, TGF-β upregulates miR-145-5p to suppress CFTR and a CFTR modifying gene SLC26A9. We demonstrate that like TGF-β, HIV Tat also alters the bronchial epithelial microRNAome to upregulate miR-145-5p that functions co-operatively with miR-509 to suppress CFTR. A neutralizing aptamer to TGFBR2 and miR-145-5p antagonism rescues TGF-β mediated CFTR suppression. However, given the important role of miR-145-5p as a tumor suppressor, we attempt a novel approach called gene-specific microRNA antagonism to preserve CFTR function in the context of HIV and cigarette smoke without blocking the entire TGF-β signaling pathway or interfering with the broader miRNA-mediated regulation of other genes.

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ABBREVIATIONS AND ACRONYMS

Acquired Immunodeficiency Syndrome	AIDS
Air Liquid Interface	ALI
Cystic Fibrosis Transmembrane Conductance Regulator	CFTR
Combined Antiretroviral Therapy	cART
Chronic Obstructive Pulmonary Disease	COPD
Chromatin Immunoprecipitation	ChIP
Cigarette Smoke	CS
Clustered Regularly Interspaced Short Palindromic Repeats	CRISPR
Cystic Fibrosis	CF
Deoxy-Ribonucleic acid	DNA
Dimethyl Sulfoxide	DMSO
Endoplasmic Reticulum	ER
Glyceraldehyde 3-Phosphate Dehydrogenase	GAPDH
Highly Active Antiretroviral Therapy	HAART
Human Immunodeficiency Virus	HIV
Idiopathic Pulmonary Fibrosis	IPF
Interferon	IFN
Latency-Associated Protein	LAP
Latent TGF-β Binding Protein	LTBP
MicroRNA	miRNA
Normal Human Bronchial Epithelial Cell	NHBE
Nuclear Factor-Kappa B	NF-κB

Phosphate Buffered Saline	PBS
Polymerase Chain Reaction	PCR
Quantitative Real-Time PCR	qRT-PCR
Retinoic Acid	RA
Reactive Oxygen Species	ROS
Ribonucleic Acid	RNA
Small Interfering RNA	siRNA
Solute Carrier Family 26 Member 9	SLC26A9
Standard Deviation	SD
Standard Error of Mean	SEM
Synthetic guide RNAs	sgRNAs
Transcription Factor	TF
Transforming Growth Factor-Beta	TGF-β
TGF-β Type I Receptor	TGF-βRI
TGF-β Type II Receptor	TGF-βRII
Trans-Activator of Transcription	Tat
Tumor Necrosis Factor-Alpha	TNF-α
Protein Kinase A	PKA
Ribonucleic Acid	RNA
micro Molar	μM
3’ Untranslated Region	3’UTR

CHAPTER 1: INTRODUCTION

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1.1. Introduction

Tobacco addiction is one of the leading causes of chronic bronchitis (CB). Cystic fibrosis transmembrane conductance regulator (CFTR) function is compromised in smokers and chronic bronchitis associated with COPD [1]. Effective mucociliary clearance (MCC) requires optimal airway surface liquid depth to allow cilia to beat effectively and hydrate the mucus [2]. MCC is a primary innate defense mechanism of mammalian airways that protects the host from the harmful effects of airborne pathogens, pollutants, and allergens [3]. CFTR plays a pivotal role in MCC by providing the necessary osmotic gradient for serosal to mucosal fluid transport (through its ability to secrete Cl^- and inhibit Na^+ absorption) and enhancing paracellular permeability [4-7]. CFTR activity is attenuated in chronic bronchitis associated with chronic obstructive pulmonary disease and in smokers [8-10]. The system entirely fails with additional insults (such as inflammation, which is more evident during disease exacerbations) when reduced airway surface liquid depth impedes normal clearance, mimicking a mild form of cystic fibrosis. Thus, even in chronic bronchitis, restoring CFTR function is expected to restore adequate airway surface liquid depth consequently restoring MCC.

On the other hand, with the introduction of combination antiretroviral therapy (cART), HIV has become a treatable but long-term disease [11]. People living with HIV live the lifespan equivalent to that of healthy people. However, despite this progress, some of the comorbidities among people living with HIV continue to remain highly prevalent.

COPD and bacterial pneumonia, ware highly prevalent even in HIV positive individuals on cART compared to non–HIV-infected adults [12, 13]. HIV-infected patients with an episode of pneumonia also demonstrate a permanent decline in lung function [14].

Infected alveolar macrophages or other immune cells recruited by persistent inflammation (due to cigarette smoke, recurrent pneumonia, or other chronic airway diseases) can serve as HIV reservoirs in the airway [15]. While cART can control de novo infection and replication, HIV proteins like Tat and gp120 can still be expressed and secreted by these cells. HIV Tat, which has a protein transduction domain, can be secreted from infected cells and is taken up by bystander cells [14, 16]. At the bronchial epithelium, HIV gp120 and Tat can attenuate two primary components of the MCC apparatus, namely ciliary beat frequency (CBF) and CFTR, respectively [17]. In our study, we found that HIV Tat and cigarette smoke individually and additively suppress CFTR mRNA and function via a common pathway involving TGF-β signaling [6, 18, 19]. This is significant, as 60% of HIV patients also smoke tobacco [20-22]. TGF-β signaling downregulates CFTR mRNA and function and, by extension, promotes Air Surface Liquid (ASL) dysregulation, increased in smokers, and chronic diseases [6, 23]. Thus, HIV by itself, or in conjunction with cigarette smoke, can suppress MCC, thereby decreasing microbial clearance from the airway and promoting lung infections.

TGF-β signaling has been shown to promote changes in miRNA homeostasis and upregulate the number of processed miRNAs [24, 25]. CFTR also has a longer (1.5 kb) 3′ untranslated region (UTR) compared to 0.74 kb average for human genes, which strongly correlates with miRNA regulation [26]. TGF-β1 does not suppress transcription from the CFTR promoter [27]. Taken together, this strongly suggests that TGF-β suppresses CFTR

mRNA by miRNA mediated post-transcriptional gene silencing. Identifying the miRNAs involved will assist the long-term goal of developing therapeutics to reverse or bypass this inhibition without inhibiting the entire TGF-β1 signaling cascade.

My dissertation study demonstrated that cigarette smoke and HIV Tat protein decreases CFTR biogenesis and function via TGF-β signaling and blocking TGF-β signaling or the microRNA target sites in the CFTR 3'UTR can restore CFTR activity and enhance MCC in HIV associated chronic bronchitis and smokers. My study also focuses on testing therapeutic leads to rescue CFTR suppression in the context of HIV infection and cigarette smoke with a long-term goal towards preventing recurrent lung infections in HIV patients.

1.2. Mucociliary Clearance (MCC)

MCC is a primary innate defense mechanism of mammalian airways that protects the host from the noxious effects of inhaled pathogens, pollutants, and allergens [3]. MCC works in concert with other antimicrobial substances like lactoperoxidase, lysozyme, and lactoferrin, to protect the host from the noxious effects of airborne pathogens [28]. The MCC apparatus consists of the cilia, a protective mucus layer, and a periciliary (ASL) layer to optimize ciliary beating [29] **(Figure 1)**. Abnormalities in any compartment of the mucociliary system can compromise mucus clearance leading to mucus impaction. The accumulated mucus promotes the colonization of chronic bacteria and fungi and provides a conducive environment for microbial growth [30-32]. Impaired mucociliary clearance (MCC) is a hallmark of chronic airway diseases like chronic obstructive pulmonary disease (COPD), cystic fibrosis, and chronic bronchitis associated with cigarette smoking [8, 10]. A recent study suggests that cigarette smoke induces acquired CFTR dysfunction,

depleting airway surface liquid, and reducing mucociliary clearance (MCC) [33] **(Figure 1)**. The imbalance between salt and water absorption mediated by ENaC and secretion mediated by CFTR leads to the formation of mucus hypersecretion in proximal airways [34]. The height of the ASL layer lining the airway surfaces is crucial for mediating MCC rates [35] and is tightly regulated by CFTR-mediated inhibition of Na^+ absorption and $Cl^−$ efflux, as well as fluid transport from the serosal to mucosal side [4]. While bronchial epithelial cells express aquaporins, deletion mutants, and inhibitors of aquaporins only have a modest effect on ASL depth, pointing to a paracellular component for fluid transport [36]. Moreover, Claudin 2, known to form paracellular water channels [37], is a component of airway epithelial tight junctions [38]. Water follows through the transcellular/paracellular pathway maintaining ASL height leading to efficient MCC [39, 40]. We have shown that CFTR activation also enhances epithelial permeability [41]. Thus, CFTR plays a critical role in maintaining ASL depth as it regulates the osmotic gradient and the resultant water transport.

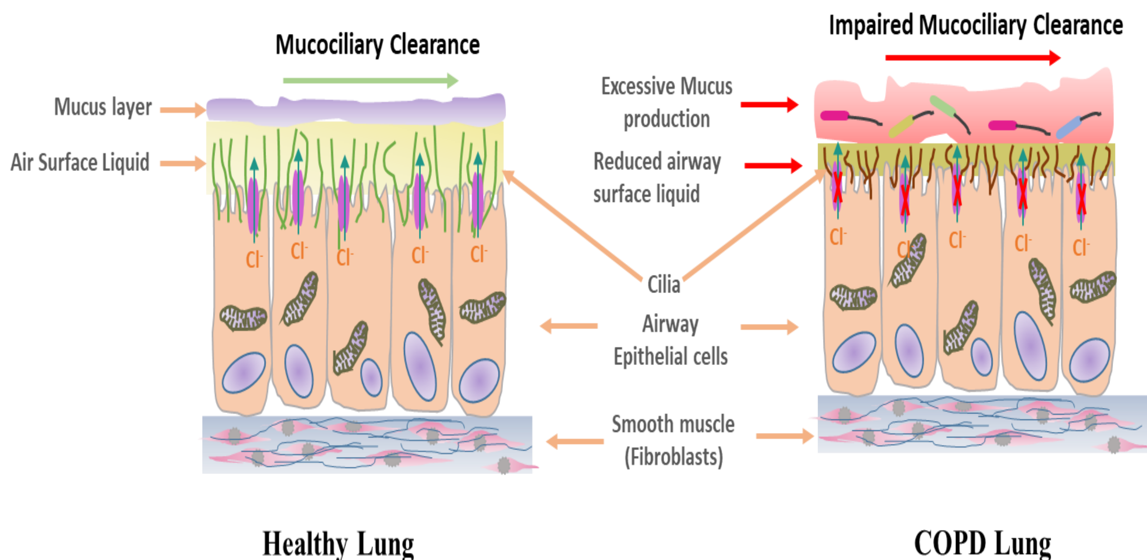

Figure 1: Mucociliary clearance (MCC) and its components.
Effective mucociliary clearance (MCC) consists of the coordinated beating of cilia, optimal airway surface liquid, and the correct composition and quantity of mucus. Mucociliary clearance (MCC) is a principal component of the respiratory tract and functions as a potential physical barrier and innate immune response of the airway. The MCC system is integrated, and alteration in any component leads to the compromised activity of MCC. Airway surface liquid (ASL), a vital component of MCC, is regulated by the coordinated action of Chloride secretion (Cl-) and Sodium (Na+) absorption channels. The combination of Cl- secretion and reduced reabsorption of Na+ favors normal ASL hydration and efficient mucociliary clearance. Proper ciliary beating involved in clearing inhaled environmental irritants and pathogens. Overproduction of very viscous mucus impairs the ciliary beating. Misfolding and impaired trafficking of CFTR to the plasma membrane lead to the reduced expression and activity of CFTR protein, which ultimately results in diminished anion secretion and formation of mucus dehydration. These properties attributed to reduced mucociliary clearance, microbial deposition, and eventually, chronic airways infection and inflammation. In COPD patients, the impaired mucociliary function may be due to a combination of excessive mucus production, increased viscosity of mucus due to acquired dysfunction of CFTR, and reduced ciliary beating.

1.3. COPD development and exacerbation

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality and results in an increasing economic and social burden worldwide. According to the Global Burden of Disease Study, COPD will become the third leading cause of death worldwide by 2020 [42]. Developing novel therapeutics for this disease will account for over $40 billion in annual healthcare costs [43]. Cigarette smoking is the principal cause of COPD development along with environmental factors like heavy metals and toxic fumes such as pollution or diesel particulates [44]. COPD is regarded as a disease of accelerated lung aging and manifests as chronic bronchitis and/or emphysema. Accelerated lung aging in COPD is associated with significant loss of pulmonary function [45, 46]. The progressive airflow limitation in patients with COPD is a consequence of small airway remodeling and narrowing due to the persistent chronic inflammation, which is a hallmark of COPD (**Figure 2**). Pathologically, COPD patients have been classified into two

categories: Emphysema and chronic bronchitis (CB), based on clinical and epidemiological phenotypes. Emphysema is characterized by abnormal enlargement of airspace within the lung and alveolar destruction. On the other hand, clinical manifestations of chronic bronchitis (CB) include chronic mucus hypersecretion and impaired mucus clearance with chronic inflammation [47]. Acquired CFTR dysfunction in chronic bronchitis and smokers results in excessive mucus production, increased mucus viscosity due to acquired dysfunction of CFTR, and reduced ciliary beating [48].

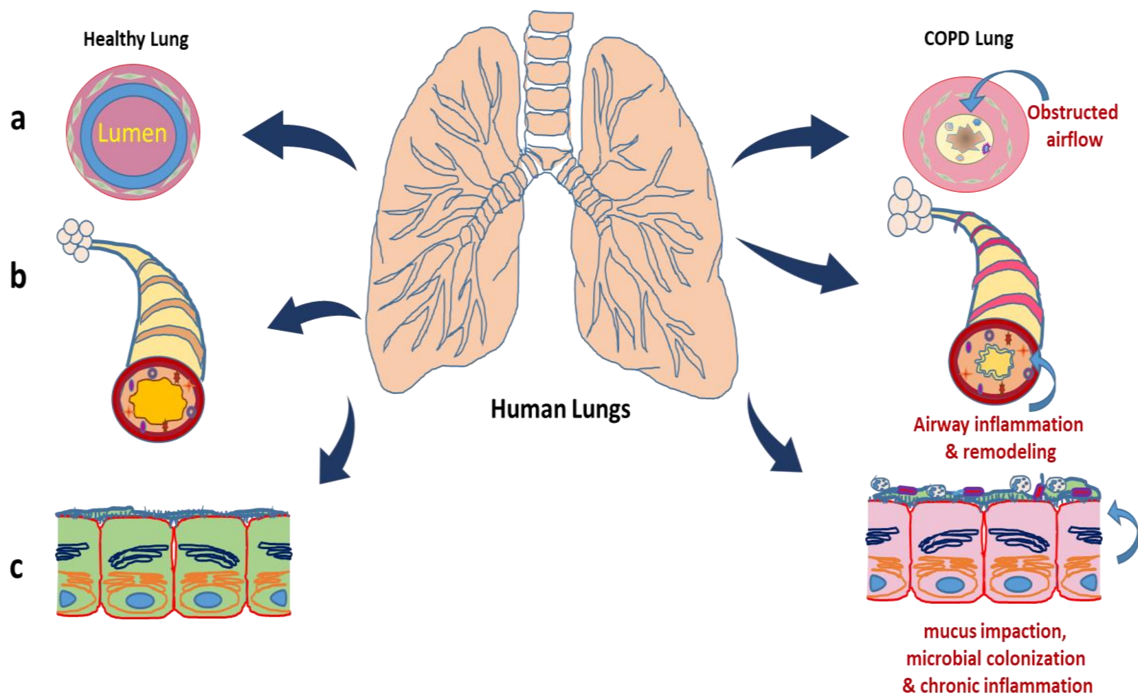

Figure 2: Schematic of airway obstruction and lung pathophysiology in COPD. Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory lung disease that causes obstructed airflow from the lungs and attenuates mucociliary clearance (MCC), leading to mucous obstruction, and provides a nutrient-rich environment for bacterial reproduction, leading to pulmonary infections and chronic inflammation.

Clinical manifestations revealed that smokers and people exposed to environmental pollutants have mucus hypersecretion and ciliary dysfunction, which play independent roles in emphysema and small airway fibrosis in airflow obstruction. Chronic inflammation is also associated with the epithelial cellular senescence-associated secretory phenotype (SASP) plays an important role in the pathogenesis of COPD [49-51]. Cigarette smoke exposure, TGF-β signaling (upregulated in COPD and smokers), and increased oxidative stress have been implicated in promoting cellular senescence [52, 53] in airway epithelial cells. More recently, multiple prospective studies have established that acquired CFTR dysfunction in smokers and COPD patients correlate with disease severity and clinical symptoms.

1.4. CFTR dysfunction in COPD

Small airways account for the most substantial portion of the total surface area of the airways and are a major site of pathology in chronic airway diseases, like asthma, chronic bronchitis, and cystic fibrosis. Cigarette smokers and COPD patients exhibit reduced CFTR function by potential nasal difference (NPD) measurements, and these differences were accompanied by reduced CFTR mRNA levels in the nares as well as the lower airways [54, 55]. Chronic pulmonary infection and the associated inflammation contribute to the decline in respiratory function in patients with COPD [56]. CFTR dysfunction was tightly related to symptoms of chronic bronchitis and positively correlated with reduced forced expiratory volume in one second (FEV1) in the lungs of patients with COPD [57]. Due to smoking, COPD patients experienced reduced activity of CFTR protein, excessive mucus production, and impaired mucociliary clearance, resulting in a unique phenotype property of 'acquired CFTR dysfunction.' Given the pivotal role of CFTR

in MCC, a primary innate defense mechanism of the airways, several reports have implicated acquired CFTR dysfunction to impaired mucociliary clearance, recurrent lung infections, and consequently inflammation, setting up a vicious cycle of infection, inflammation, and injury [58-60]. CFTR also conducts reduced glutathione (GSH), a key redox buffer in cells [61]. Hence, acquired CFTR dysfunction can also alter the redox balance in the airways, directly contributing to inflammation. In addition to CFTR, there are additional ion channels and transporters like solute carrier 26A (SLC26A) family of anion exchangers, sodium channel (ENaC), and calcium-activated chloride channels in the apical airway epithelium which play an important role in maintaining ASL homeostasis [62]. In our study, we found that cigarette smoke induces transforming growth factor- β1, which modulates CFTR biogenesis and function in primary bronchial epithelium ex vivo and in non-CF A/J mouse models in vivo [6, 63].

1.5. Transforming Growth Factor

Transforming growth factor-β (TGF-β) is a multifunctional protein that plays a critical role in cellular responses, such as development, proliferation, and differentiation [64, 65]. In 1978, de Larco and Todaro first discovered and isolated a group of secreted polypeptides from sarcoma virus-infected cells [66]. Assoian et al. (1987) reported that activated alveolar macrophages and peripheral blood monocytes secrete TGF-β and regulate the growth factor response [67]. TGF-β is considered as the prototype of a TGF-β family comprised of secreted growth factors and cytokines, which is encoded in mammals by 33 genes [68]. TGF-β family proteins are now known to be critically involved in embryonic development, cell and tissue differentiation, tissue homeostasis in the adult, and are also involved as modifiers in many disease states. Like other growth factors,

members of the TGF-β family have pleiotropic effects in the formation and development of tissues and organs via autocrine, paracrine, and endocrine mechanism [69].

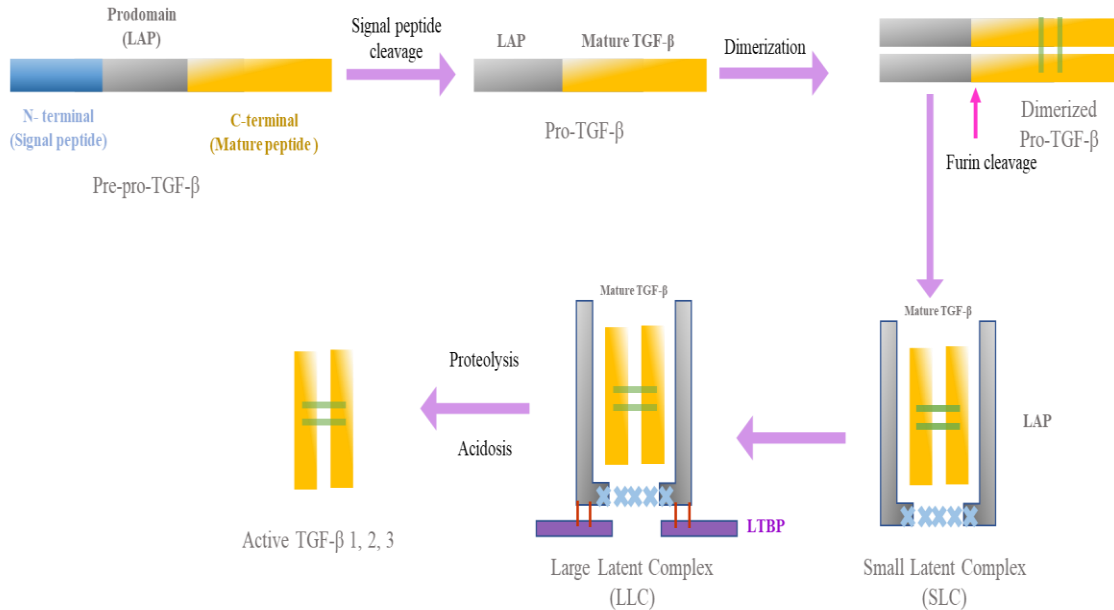

Figure 3: Schematized illustration of the sequential steps in the activation and secretion of TGF-β from the latent complex.
TGF-β isoforms are first synthesized as a pre-pro-TGF-β precursor molecules. The precursor molecule contains a signal peptide, and proform of TGF-β includes Latency-Associated peptide (LAP), and the mature domain of TGF-β. Following dimerization, pro-TGF-β homodimer is now targeted and cleaved in the Golgi apparatus by a furin-like peptidase to form mature TGF-β. A homodimer of this new protein, called the latency-associated protein (LAP), is noncovalently associated with a homodimer of mature TGF-β and form the small latent TGF-β complex (SLC). Latent TGF-β complex (SLC) can associate through disulfide bonding with latent TGF-β binding protein (LTBP) into a large latent complex (LLC). Latent-TGF-β-binding protein (LTBP) plays an important role in targeting TGF-β to the extracellular matrix but does not confer latency. After its secretion, LLC is directed to the extracellular matrix (ECM) and stored through LTBP-1 binding to fibrillin and ECM. Upon activation, active TGF-β mediates its biological functions by binding to TGF-β type I (TGF-βRI) and type II (TGF-βRII) receptors, both of which are serine/threonine kinases.

The precursor polypeptides for the TGF-β family are composed of three distinct domains: an amino-terminal signal peptide, a large precursor segment, and the carboxy-

terminal [70]. The TGF-β family is characterized by numerous vital functions, including the bone healing process and the associated inflammatory response, proliferation, and differentiation of mesenchymal stem cells (MSCs), and the production of an extracellular substance in bone and cartilage tissues [71, 72]. The TGF-β family comprises the three isoforms in mammalian tissues: TGF-β1, TGF-β2, and TGF-β3 [73]. Although each isoform is encoded by distinct genes located on different chromosomes, they share highly conserved regions (around 70-80% homology) within their sequence [74, 75]. Nine cysteines are conserved in their position in three TGF-β isoforms. In most cases, these isoforms are expressed together and act through the same receptor signaling mechanism for similar biological functions [76, 77].

TGF-β is secreted in a biologically inactive (latent) form in association with the latency-associated peptide (LAP) and stored in the extracellular matrix (ECM) [78]. In latent TGF-β complex, LAP and mature TGF-β are linked together by non-covalent bonds, which later form a large latent complex with latent TGF-β binding protein (LTBP) through covalent linking [73, 79]. Specific cellular proteolytic enzymes like proteases, plasmin, furin, calpain, and mechanical force cleave the latent TGF-β and release the mature, active TGF-β [80-82]. Mature TGF-β is a 25-kD disulfide-linked dimeric molecule, composed of 112 amino acids per unit [83, 84] **(Figure 3)**. TGF-β2 is basically secreted by neurons and astroglial cells in the embryonic nervous system [85]. TGF-β3 plays a critical role in epithelial-mesenchymal interaction and is suggested to have anti-fibrotic activity during wound repair [86, 87]. The 65-kD type I receptor (TGFβRI), the 85-kD type II receptor (TGFβRII), and the 280-kD type III receptor (TGFβRIII) or beta glycan are widely expressed in all tissues [88, 89]. TGFβRI and TGFβRII are transmembrane

serine/threonine kinases that regulate signal transduction [90]. TGFβRIII largest (250–350 kDa) and the most abundant binding molecule is expressed on both fetal and adult tissues and most cell types [91].

1.6. TGF-β signaling

Optimal TGF-β signaling plays a crucial role in many circumstances of cell and tissue physiology in humans. Dysregulation of TGF-β signaling promotes developmental anomalies like cancer development or excessive fibrosis [73]. TGF-β acts on multiple cell types through both canonical and non-canonical pathways [92]. TGFβ associates with receptors expressed on the plasma membrane and begin the subsequent signal transduction cascade on the target cells [93]. TGFβ superfamily members work through specific sets of hetero-tetrameric receptor complex with two types I and two types II receptor [94]. In humans, seven types I receptors (also termed activin-like kinase or ALK receptors) in combination with one of five types II receptors (ActRIIA, ActRIIB, TGFβRII, BMPRII, and AMHRII) are expressed and serve for TGF-β ligands [95]. For example, binding of TGF-β1 with the type II receptor (TGFβRII) induces the recruitment and phosphorylation of the type I receptor (TGFβRI) and form heterotetrameric receptor complexes [96]. Heterotetrameric combination recruits and phosphorylates the downstream intracellular signaling proteins SMADs, especially Smad2 or Smad3, which are specific transcriptional effectors of TGFβ superfamily members. Then, Smad2 or Smad3 protein binds with Smad4 and forms an intracellular complex Smad2/4 or Smad3/4, which later translocates into the nucleus from the cytoplasm [97]. In the nucleus, the Smad3 component of the complex binds directly to the promoter regions of the genes and positively or negatively regulates the transcription of specific TGF-β target genes [98] **(Figure 4)**.

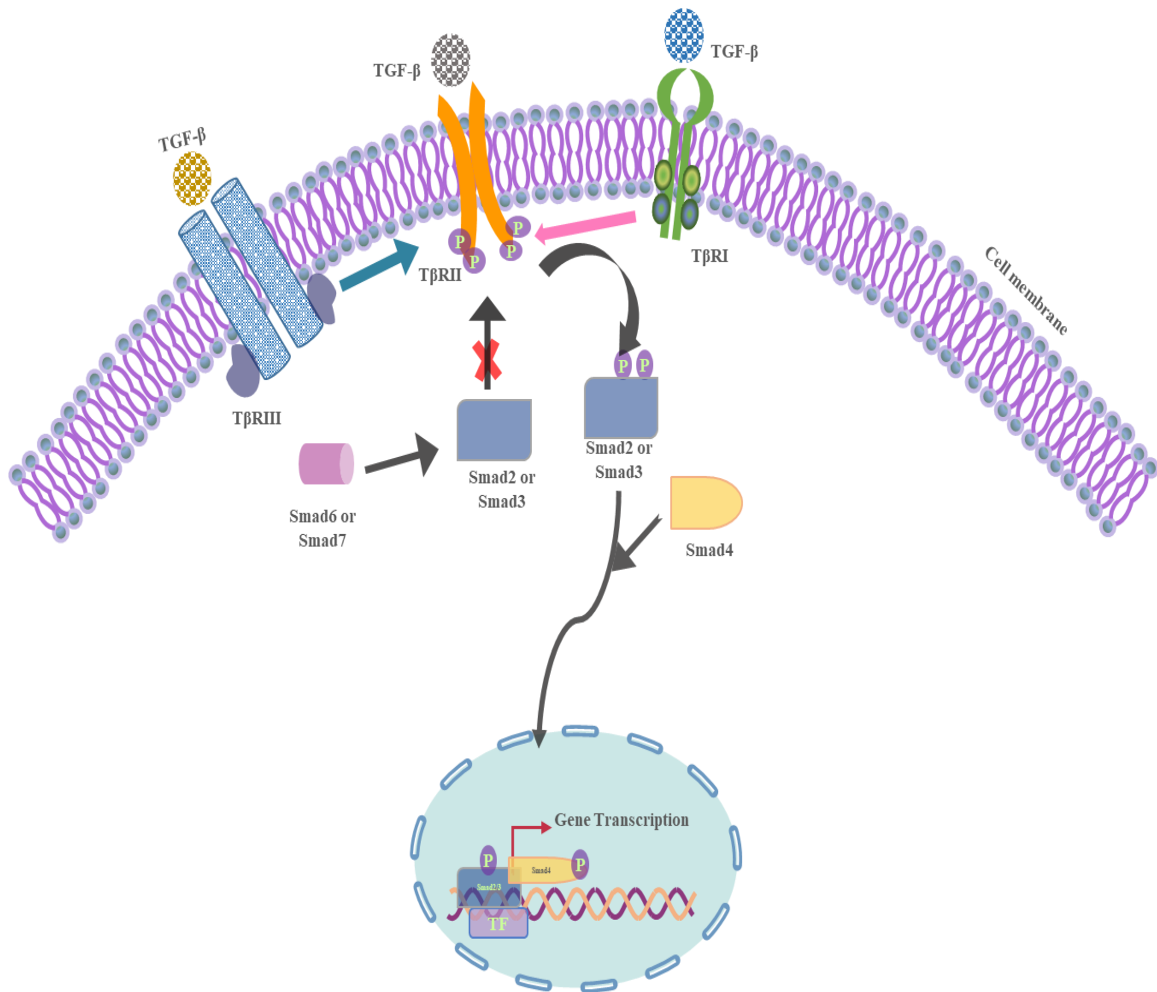

Figure 4: The TGF-β/SMAD canonical signaling pathway.
In normal cellular condition, TGF-β ligand in extracellular space binds either to the type III TGF-β receptor (TβRIII), which presents to the type II receptor (TβRII), or directly to the type II receptor (TβRII) on the cell membrane. Binding of the ligands to type II receptors recruits and activates the type I receptor via the interaction between type II and type I receptors. Thus activated, the type I receptor, in turn, phosphorylates and activates the receptor-activated SMADs (R-SMADs), SMAD2 and SMAD3 allowing to form a complex with the common mediator SMAD4. SMAD7 competes with R-SMADs for interacting with the type I receptor, thus preventing R-SMAD activation and proper propagation of the signaling. The trimeric complex translocates to the nucleus, where it associates with high-affinity DNA binding transcription factors (TF) in order to positively or negatively regulate the transcription of target genes.

On the other hand, bone morphogenetic proteins (BMPs), members of TGF-β superfamily inhibit the TGF-β mediated fibrosis through activating inhibitory Smad proteins Smad-1/5/8 [99]. TGF-β family receptors also activate other non-Smad signaling pathways like ERK signaling, mitogen-activated protein kinase (MAPK) pathways, PI3K-AKT–mTOR signaling, and JAK-STAT activation pathways [100]. For example, autophosphorylation of tyrosine molecule in the TGFβ type I receptor binds to the ShcA and activates the Ras and the ERK1/2 mitogen-activated protein kinase (MAPK) pathway [101]. Developmental control of Epithelial-mesenchymal transition (EMT) is modulated by TGF-β signaling and related proteins [102]. Characterization of TGF-β signaling and related proteins reveals that crosstalk between Smad pathways and the non-Smad pathway controls cell differentiation and growth during development and in cancer.

1.7. TGF-β signaling in HIV patients and smokers with COPD

TGF-β1 is ubiquitously expressed and secreted by several cell types, including airway epithelia, smooth muscle, fibroblasts, and most cells of the immune system. TGF-β isoforms are synthesized intracellularly in association with the latency-associated peptide, and this complex is secreted, thereby providing a TGF-β reservoir in the extracellular matrix [103]. For active signaling, TGF-β dissociates from the complex [104, 105]. TGF-β signaling is increased in chronic airway diseases like chronic bronchitis and COPD [106-109], and TGF-β levels correlate with the severity of obstruction [109, 110]. TGF-β1 expression in epithelial cells from patients with chronic bronchitis correlated with features of airway remodeling, including basal membrane thickness and the number of peribronchiolar fibroblasts [107, 108]. There is also clear evidence of increased TGF-beta activity in chronic bronchitis associated COPD, where it plays a predominant role in airway

remodeling [106, 108]. Interventions targeting TGF-beta signaling or its intermediates may represent suitable option therapeutic options in COPD [111]. Cigarette smoke and TGF-β1 signaling can suppress CFTR mRNA levels and, consequently, its function, and blocking TGF-β1 signaling in cigarette smoke-exposed cells can restore this [6].

HIV patients show abnormalities in their nasal MCC apparatus [112, 113]. However, nasal Cl^- efflux, and CBF are often measured as a barometer of overall airway MCC health [8, 114, 115]. Tat is an immediate-early gene of HIV, and its expression cannot be suppressed by antiretrovirals [116-119]. HIV Tat has a protein transduction domain that allows its secretion by infected cells and uptake by bystander cells where it mediates pleiotropic effects [120-123]. HIV Tat induces TGF-β1 expression in a number of cell types [124-126], including NHBE cells, possibly by binding to a Tat responsive element in the TGF-β1 promoter [126, 127]. This translates to a decrease in CFTR mRNA, which can impact CFTR availability and, by extension, ASL depth. TGF-β1 is known to activate multiple cell signaling pathways that can exert transcriptional and post-transcriptional control of protein expression. Transcriptional suppression by TGF-β could either involve direct suppression of transcriptional initiation or via post-transcriptional gene silencing. Although TGF-β1 signaling most often leads to transcriptional activation, one report demonstrates transcriptional suppression via Smad binding to a TGF-β inhibitory element (TIE) [128]. Alternately, TGF-β signaling has been shown to upregulate the Snail family of transcriptional repressors that play a role in epithelial to mesenchymal transition [129]. Our study rules out the role of transcriptional suppression from the CFTR promoter by

TGF-β [27]. Our study supports the hypothesis that TGF-β suppresses CFTR via miRNA-mediated post-transcriptional gene silencing.

1.8. Genomic Structure of CFTR and polymorphism

The CFTR gene was first discovered and published in 1989 by several groups [130-132]. Initially, it was thought that the CFTR gene comprises 24 exons, but subsequent analysis revealed that the CFTR gene is composed of 27 exons [133]. The CFTR gene is located on human chromosome 7 and is approximately 250 kB in length [134] (**Figure 5**). CFTR gene transcribes into a 6.2 kb long mRNA, including an open reading frame of 4,440 coding bases and translates into a protein of 1,480 amino acids with a molecular weight approximately 150-170 kDa [135, 136]. More than 1900 mutations have been reported in the CFTR gene [137, 138]. 82% of ∼1900 reported mutations to have deleterious effects, whereas others have been found either having any no potential effect or unknown function.

Moreover, most of the mutations (97.5%) involve one or a few nucleotides (missense or nonsense mutations) in CFTR, and other mutations (2.5%) include deletions of entire exons or multiple exons of the gene. The most frequent mutation is the deletion of three nucleotides resulting in phenylalanine deletion at position 508 (ΔF508) is responsible for 90% of CF alleles [139]. ΔF508 impairs the ability of the CFTR protein to fold in the endoplasmic reticulum (ER), thereby enhancing the rapid degradation of the protein during ER processing. Heterologous expression of ΔF508CFTR-cDNA has revealed that mutant CFTR protein undergoes only one cycle of glycosylation. It fails to complex with oligosaccharide chains and as a result of failing to be transported to the cell surface [140, 141]. Moreover, ΔF508CFTR causes the misfolding of CFTR protein and

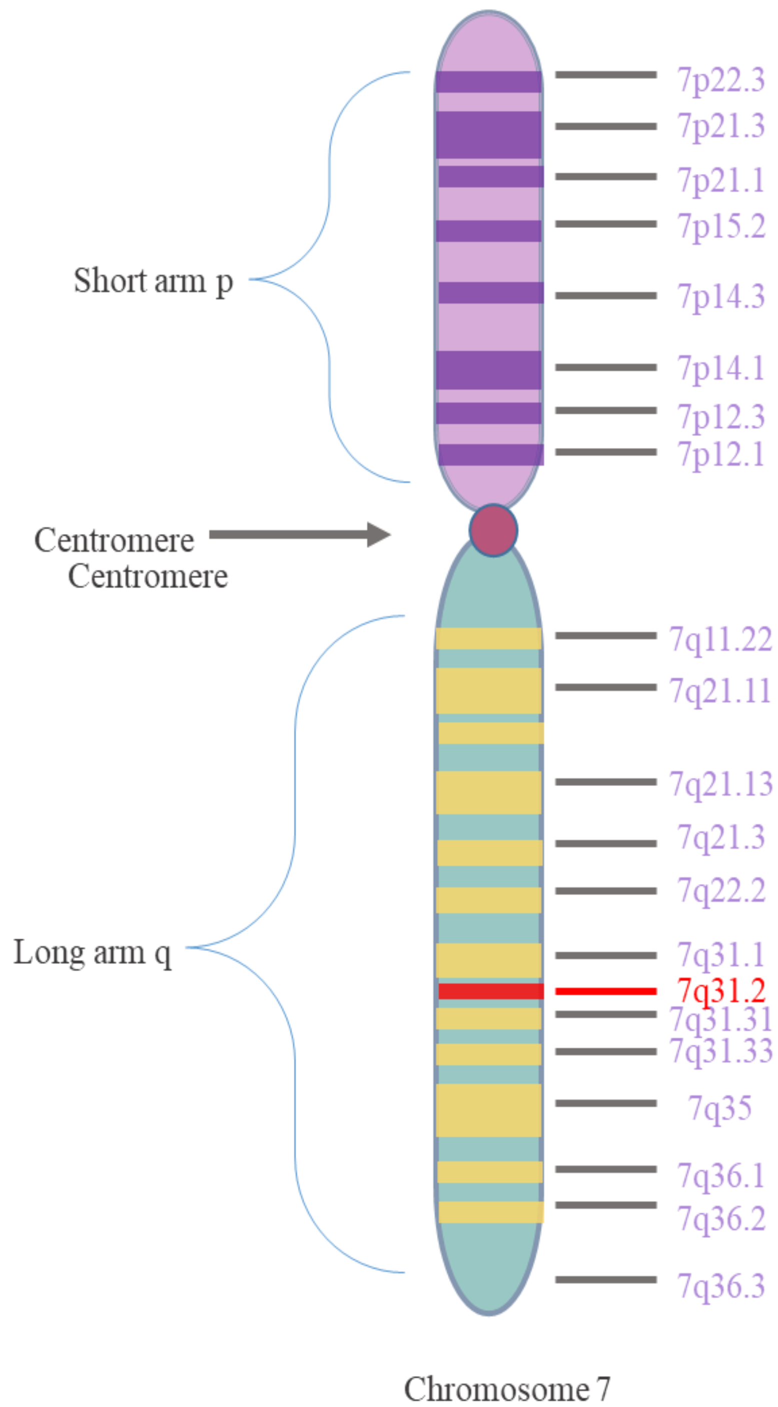

Figure 5: Genomic location of the Human CFTR gene.
It is located on the long arm (q arm) of chromosome 7 and consists of 27 exons. The CFTR gene produces the CFTR protein, consist of 1,480 amino acids, which regulates the chloride ion content of epithelial cells that line the nasal cavity, lungs, and stomach.

Also conformationally masks di-acidic motif that seems necessary for ER export [142, 143]. The existence of deleted transcripts with alternative splicing certain polymorphisms alter the amount of functional CF gene product. The well-studied example is the thymidine polymorphism in intron eight situated near the acceptor site of splicing [144]. Three different polymorphism exists as a 5-, 7-, or 9-thymidine (T) variant. The 5T variant predisposes to the alternative splicing, which leads to mRNA lacking exon nine and lowers the amount of normal CFTR transcript [145]. Recently, it has been reported that 5T polymorphism in association with the mutation M470V, and another polymorphism is found in bronchiectasis [146].

1.9. CFTR protein structure and function

The CFTR gene encodes a protein named Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein, a large transmembrane glycoprotein, and a cyclic AMP-dependent ion channel which modulates the chloride ion levels across the epithelial cells in the nasal cavity, lungs, and stomach [147, 148]. In addition, CFTR was reported to involve in intracellular membrane trafficking and endo- and exocytosis [149]. CFTR protein is a member of the ATP-binding cassette (ABC) gene family and composed of 1480 amino acids [150]. CFTR protein and members of membrane protein families, the family of ABC transporters (ATP-binding cassette) have the homologies between them and responsible for the active transport of substrates across the cell membrane, where ATP hydrolysis serves as the source of energy. Collins et al. (1990) first proposed a putative model for the CFTR protein structure, which was later modified by the Morales group [151, 152]. They both proposed that two homologous transmembrane domains (TMD1 & TMD2) provide the CFTR protein structure where each domain contains six membrane-

spanning domains (MSDs) followed by two nucleotide-binding domains (NBD1 & 2) which catalyze ATP. Both transmembrane domains are connected by a cytoplasmic regulatory domain (R) with several potential phosphorylation sites [153] This ion channel protein is mostly cytoplasmic where 77% of the CFTR protein is present in the cytoplasm, and only 19% is membrane-spanning domains, and 4% is extracellular loops **(Figure 6).** CFTR channel activity is regulated by phosphorylation of its R domain at multiple sites by p cAMP-dependent protein kinase A (PKA) and PKC [154]. R region regulates most of CFTR's regulatory intramolecular and intermolecular protein-protein interactions. Diverse binding elements of the R region can interact with more than one partner at a time [155]. Moreover, there are several cytosolic proteins involved in maintaining CFTR channel conductance via G protein-coupled signaling mechanisms and modulate CFTR activity to maintain the ion balance in tissues [156]. A recent study found that PKA-independent activation of CFTR by Ca2+ elevation. Bozoky et al. (2017[157]) demonstrated that calcium-loaded calmodulin binds directly with the R domain of CFTR in a Ca2+-, and phosphorylation-dependent manner and leads to the activation of the ion channel.

CFTR, an anionic channel which is responsible for maintaining trans-epithelial secretion of chloride (Cl^-) and bicarbonate (HCO_3^-) in multiple exocrine organs including the lungs, pancreas, liver, and intestine [158]. In normal conditions, apical nucleotides (ATP and its metabolites) are important for hydrating airway surfaces [159]. ATP binds to purinergic G-protein coupled receptors leading to activation of Ca^{2+} dependent Cl^- channels but also CFTR. The normal mucociliary function fails when the capabilities of the cAMP and ATP/Ca^{2+}-mediated apical Cl^- efflux fail, leading to airway surface dehydration [160]. Notably, however, progressive lung disease and respiratory failure are the major cause of

morbidity and mortality for most patients. Dysregulation of CFTR expression, as well as their function, leads to the development of severe obstructive pulmonary diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) [161]. The loss of function of CFTR in the airway system results in viscous secretions, which is the major characteristic of airway diseases [162]. CFTR plays a pivotal role in maintaining a thin layer of airway surface liquid (ASL) to facilitate ciliary beating [6]. In chronic pulmonary diseases, reduced secretion of chloride ion is suggested to be an ultimate consequence of defective CFTR [163].

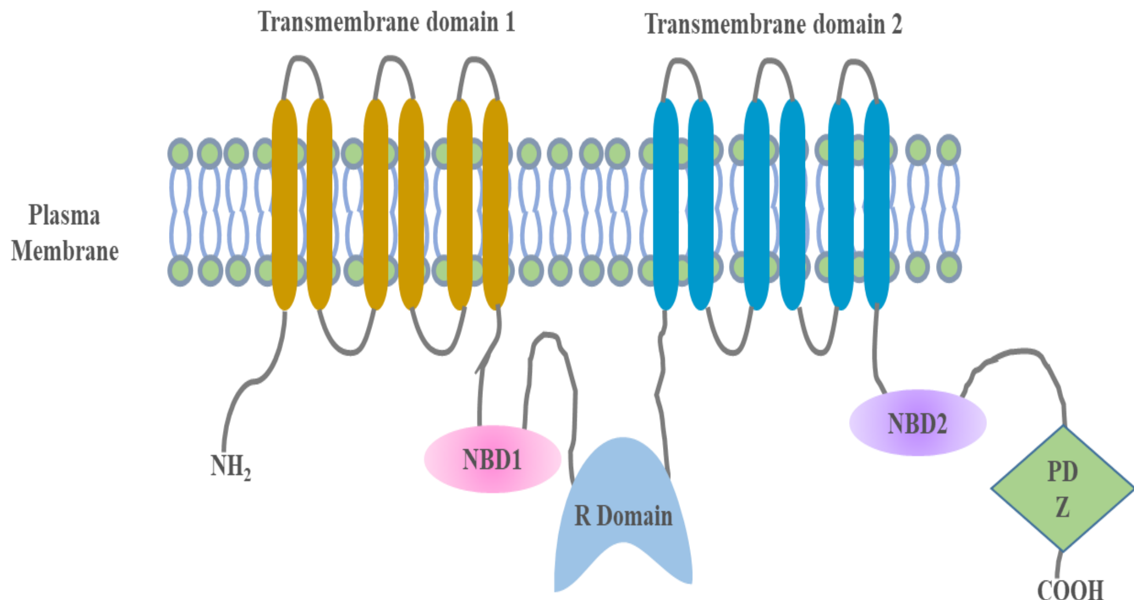

Figure 6: Putative domain structure of the CFTR protein.
CFTR protein is composed of two transmembrane domains (TM1 and TM2, each composed of 6 spanning regions), and two nucleotide-binding domains (NBD1 and NBD2) contains sequence predicted to interact with ATP. The two MSD–NBD motifs are linked by a unique cytoplasmic regulatory domain (R), which contains multiple consensus phosphorylation sites. The carboxy-terminal part of the protein makes contact with numerous other proteins (PDZ) significantly affect the function of the CFTR, the conductance, the localization, and the mediation of other ion channels. NH2: amino-terminal tail; COOH: carboxyl-terminal tail.

CFTR modulates a set of diverse mechanisms in a various range of epithelial tissues in humans. In the normal situation, combined effects of CFTR mediated Cl^- efflux and reciprocal inhibition of Na^+ absorption increase the paracellular permeability and the osmotic driving force with paracellular water flow to maintain optimal airway surface liquid (ASL) depth on the mucosal side [158]. This leads to optimal MCC and clearance of pathogens and allergens in the airway. CFTR also plays a pivotal role in regulating H_2O_2 levels in the ASL by a feedback mechanism in which ASL H_2O_2 activates CFTR [164-166]. CFTR secretes the SCN(-) that reacts with H_2O_2 in a reaction catalyzed by lactoperoxidase to form hypothiocyanite ion, which mediates antibacterial action [167, 168]. CFTR also secretes reduced glutathione, which plays a key role in the removal of H_2O_2. Hence the combined effects of SCN (-) and GSH secretion by CFTR regulate H_2O_2 levels in the airway. A decreased availability of active CFTR (in Cystic fibrosis or due to acquired CFTR dysfunction) can disrupt this feedback mechanism increasing the levels of H_2O_2 in the ASL [169] and increasing oxidative stress.

1.10. Transcriptional and post-transcriptional regulation of CFTR gene expression and activity

CFTR protein is exclusively expressed in specialized epithelial cells (ciliated cells and goblet cells) within the respiratory and digestive systems [170, 171]. Over the past few decades, CFTR protein expression has been reported in epithelial cells of the kidney, smooth muscle cells, immune cells, as well as in neurons of the central nervous system (CNS) [172]. But other studies also demonstrated that CFTR could be expressed in non-epithelial cells [173]. Very little information was available about the role of CFTR in humans until the discovery of the CF gene in 1989. That time three research groups

demonstrated that defects in the CFTR gene and poor function of CFTR protein involved in cystic fibrosis disorder [174].

CFTR gene expression is spatially and temporally regulated both during development and within different tissue types [175, 176]. In airway epithelial cells, CFTR is reported to express during the second trimester of human development and progressively increases up to the 24th week of pregnancy [177, 178]. In contrast, CFTR expression significantly repressed at birth and remains well-maintained throughout adulthood [179]. Surprisingly, transcription of the CFTR gene was detected at a higher level in the mid-trimester pancreas, small intestine, colon, genital ducts [180]. Few studies have demonstrated that several transcriptional activators and repressors are involved in the regulation of CFTR transcription [181, 182]. Analysis of 3.8 kb of genomic sequence upstream of exon 1 of the CFTR gene contains binding sites for Sp1 and AP-1 protein [183]. CFTR promoter has multiple transcriptions start sites that are positively controlled by a set of transcription factors, including CCAAT enhancer-binding protein (C/EBP) proteins and Forkhead Box A (FOXA) factors [184]. C/EBPβ binds to a DNase I hypersensitive site (DHS) that facilitates the long-range interactions between various regulatory elements cluster specifically to the CFTR promoter [185]. The CFTR promoter resembles the characteristics of a "housekeeping" gene and contains multiple transcriptions start sites that exhibit time and tissue-specific CFTR expression [173]. Several cis-regulatory elements (CREs) in the CFTR promoter region controls the cell-specific and temporal coordination of CFTR transcription [186]. The three-dimensional (3D) structure of chromatin revealed that CFTR locus is located into a unique topologically associating domains (TADs) which position starts at −80.1 kb 5′ to the translational start site and +48.9 kb from the translational stop

site [187]. CCCTC-binding factor (CTCF), an architectural protein also observed at the boundary (−80.1 kb and +48.9 kb sites) and associated with chromatin organization [188, 189].

Moreover, using primary bronchial epithelial cells in vitro or from bronchial brushings ex vivo, different groups reported that CFTR expression is post-transcriptionally regulated by microRNAs (miRNAs) [190-194]. Although cis-acting genomic elements are likely to be the primary mediators of CFTR expression, post-transcriptional regulation of the CFTR mRNA may also be important. A growing number of studies have demonstrated that miRNAs function as regulatory factors in most cellular functions and involved in the development and progression of lung diseases [175]. There are 255 microRNAs reported that target the CFTR gene. By analyzing miRNA expression profiles of adult and fetal lungs, miR-145, miR-150, and miR-451 were reported to have a temporal effect in the adult lung [195].

CFTR has a long (1.5 kb) 3′ UTR in comparison with the 740 bp average for human genes, and there is evidence that 3′ UTR length strongly correlates with miRNA regulation [26]. In 2011, Gillen et al. first identified two microRNAs (miR-145 and miR-494) control CFTR expression by directly binding to the discrete sites in the CFTR 3′ UTR [196]. Hassan et al. (2012) reported that air pollutants and cigarette smoke induce the expression of miR-101 and miR-144, which target the CFTR biogenesis [190]. At the same time, another group found that the synergistic effect of miR-101 and miR-494 modulates the post-transcriptional expression of the CFTR gene [197]. CFTR is also indirectly regulated by miR-138, which targets the transcriptional regulatory protein SIN3A. This action was mediated through knockdown of the transcriptional regulatory protein SIN3A and allowed

the expression of wild type and a mutant form of CFTR restoring Cl− transport in CF epithelia [198].

TGF-β1 treatment leads to changes in levels of a number of miRNAs [24] and upregulation of processed miRNAs [25]. In airway epithelial cells, CFTR can be regulated by at least three different microRNAs [196]. Specifically, TGF-β1 has been shown to induce expression of at least one of these microRNAs, hsa-mir-494, in a different cell type [199]. Thus, it becomes essential to study the effect of miRNA mediated silencing on CFTR mRNA in the context of TGF-β signaling. Cross-talk between TGF-β1 signaling and the miRNA machinery is very important for lung homeostasis, while its dysregulation leads to lung disease [200].

1.11. CFTR isoforms and processing

The overall biogenesis of CFTR protein involves the interaction of multiple proteins, followed by several cellular compartments inside the cell. Under normal conditions, the cellular signals promote the CFTR gene to transcribe into the mRNA. Then, the transcribed mRNA moves across the nuclear pores and translocate into the endoplasmic reticulum inside the cytoplasm [201]. Translation of the CFTR protein and most of their modification occurs within the endoplasmic reticulum lumen in an association of ER membrane-associated and other chaperone proteins [202]. During the co-translational transport, the CFTR polypeptide is targeted to the endoplasmic reticulum (ER) membrane and undergoes N-glycosylation through the insertion of two additional glycosyl groups on its fourth extracellular loop [203]. This glycosylation process allows the changes in the molecular weight of the CFTR protein from 130kDa to 150kDa. With the help of calnexin and cytosolic chaperones (e.g., Hsc70/Hsp70, Hdj1/2, Hsp40, and the Hsp90 complex), the

CFTR polypeptides are properly folded, which makes them protease-resistant, and to transport to the Golgi apparatus [203, 204]. In 2003, Guggino reported that PDZ domain proteins were found to bind to the C-terminal motif of CFTR and involved in regulating the trafficking and processing of CFTR protein [205]. CFTR associated ligand (CAL) is ubiquitously expressed in human tissues primarily in the perinuclear region of the cell and found to bind with CFTR in Golgi and move with CFTR to the plasma membrane [206].

CFTR polypeptide is further subject to another cycle of glycosylation and forms a mature CFTR protein of 170kDa in the Golgi compartment. With the help of clathrin-coated vesicles, this mature form of CFTR protein is now transported to the plasma membrane of the cells, where it can serve as a chloride channel [206]. In contrast, the mutant variant of CFTR (ΔF508) unable to fold properly during the processing and later is subjected to degradation by cellular machinery [207]. Basically, ER-associated folding mechanisms concerts with ER-associated with degradation where misfolded proteins are being subjected to proteolytic degradation in the cytoplasm [208]. Wang et al. (2006) demonstrated that Hsp90 cochaperones regulate the CFTR folding in the endoplasmic reticulum and siRNA targeting the Hsp90 cochaperone ATPase regulator Aha1 rescues trafficking of ΔF508 to the cell surface and restores channel function [209].

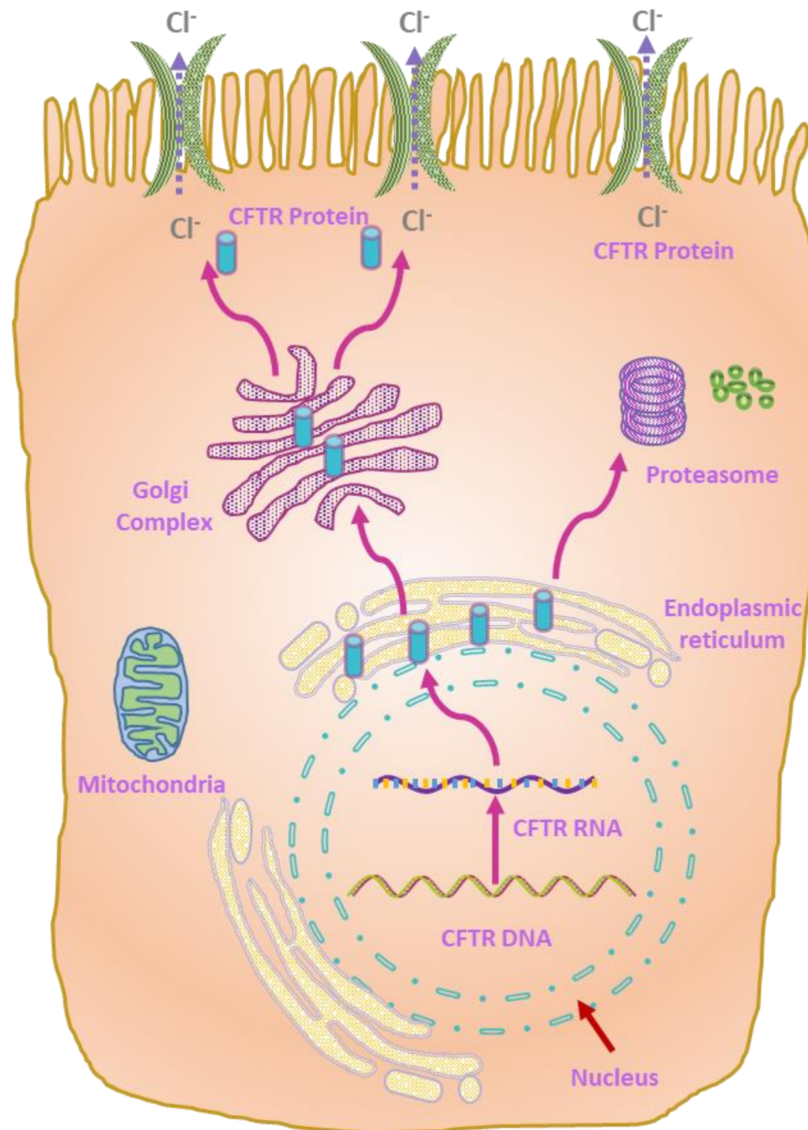

Figure 7: Model for CFTR biogenesis and function in the airway epithelial cells.
In the normal cell, signals from the external environment stimulate the CFTR gene to transcribe into CFTR mRNA. The resultant transcript then translocates into the endoplasmic reticulum (ER) through nuclear pores. With the help of transfer RNA, the CFTR transcript translated into immature CFTR polypeptide within the lipid bilayer of the rough endoplasmic reticulum (RER). Following further maturation steps in the endoplasmic reticulum, CFTR protein is then moved into the Golgi apparatus for post-translational modification and loaded into transport vesicles. At last, the mature CFTR protein is transported or "trafficked" to the cell surface on the apical membrane of epithelial cells to function as a cAMP-regulated Cl^- channel and regulator of other channels.

There are more than 1700 mutations in the CFTR gene that result in mild to severe degrees of CF phenotype (see CF Foundation https://www.cff.org/What-is-CF/Genetics/CF-Mutations-Video-Series/). These mutations can be homozygous or heterozygous (for two different mutations). CFTR protein levels are tightly controlled at the post-translational level by ER based degradation and by calpain based processing of the channel on the plasma membrane surface [210]. Approximately 70% of the wild type CFTR is degraded within the ER itself, with only one in three CFTR molecules reaching the mature form [211, 212]. At the surface, CFTR turnover is a function of balancing the effects of HSP90 versus calpain [213, 214]. Mature CFTR is cleaved by calpain at the surface into two fragments of 100kd and 70kd, leading to internalization of the CFTR channel [214]. The split CFTR is then internalized into endocytic vesicles and slowly digested by lysosomes. As a result of this slow turnover, significant amounts of split CFTR are detectable using antibodies specific to C-terminal and N-terminal domains of CFTR, indicating that calpain is involved in regulating the turnover of mature CFTR [213, 214]. Increased calpain expression is associated with increased CFTR turnover, and calpain inhibitors have been shown to rescue CFTR function in wild type as well as mutant CFTR [215]. CFTR expression is also regulated by alternate splicing events. These alternative splicing events have been shown to lead to an altered intron 23, resulting in a shorter C-terminus [216] or an omission of exon 9, thereby translating to a smaller CFTR protein called CFTR isoform-2 [217]. Another CFTR isoform (CFTR isoform 3) resulting from alternative splicing yields a ~69kd CFTR protein. Hence, different cell types demonstrate different CFTR levels, different isoforms, and glycosylated forms of CFTR with varying levels of CFTR

function, and this depends to a great extent on the regulation of CFTR biogenesis, transport, and turnover.

1.12. Biogenesis of microRNAs

MicroRNAs (miRNAs) are a class of short (20–23-nucleotide), endogenous, single-stranded non-coding RNAs that play important roles in regulating the expression of target genes by directly binding to their mRNAs [218, 219]. Initial studies reported that miRNAs were located in intergenic regions distinct from known transcription units [220]. Early annotation of the genomic position of miRNAs indicated that most miRNA loci are found only in the noncoding transcription units (TU) or in unidentified coding TUs [221, 222]. Later Ambros et al. (2003) and other groups reported that few miRNAs are also encoded from either within the intronic regions of either coding or noncoding TUs [223, 224]. However, the significant differences between intergenic and intronic miRNAs biogenesis remain in the procedure for transcriptional regulation. Intergenic miRNAs can be monocistronic with their own promoters or polycistronic with a shared promoter. On the other hand, Intronic miRNAs are being transcribed from the same promoter as their host genes [225]. Transcription of miRNA genes is regulated by RNA polymerase II or RNA polymerase III [226, 227]. MiRNAs transcription starts from the DNA sequences and converted into primary miRNAs (pri-miRNAs), which later processed into precursor miRNAs (pre-miRNAs) and mature miRNAs [228]. The whole miRNAs biogenesis and processing involve two distinct steps involving both nuclear and cytoplasmic events and carried out by two RNase III enzymes: DROSHA in the nucleus and DICER in the cytoplasm [229, 230].

MiRNAs are initially expressed as a long (~80-nt), single-stranded RNA which in turn, folds over on itself to create a single-stranded loop which consists of about 20 base-pairs and with a 5′ 7-methylguanosine cap and a 3′ poly(A) tail known as pri-miRNA, i.e., a primary transcript [231, 232]. The long primary (pri) miRNA transcripts are now subjected by two cleavage events: first, the microprocessor (Drosha/DGCR8) complex excise the upper part of the stem-loop structure into a hairpin-shaped structure, about 70nt long, known as precursor-miRNA (pre-miRNA) [233]. Pre-miRNAs are characterized by double-stranded (ds) RNA short RNA hairpins containing a 2-nt 3′ overhang, which is subsequently recognized and targeted by the RNA export factor Exportin 5 (XPO5) [234]. XPO5 and its cofactor, RanGTP, regulate the transportation of the pre-miRNA from the nucleus to the cytoplasm with the help of Ran-GTP [235, 236]. In the cytoplasm, a second RNase III enzyme termed Dicer associates with transactivation-responsive RNA-binding protein (TRBP) and cleaves off the terminal loop of pre-miRNA hairpins to create ~20-22 bp mature miRNA duplexes with 2-nt 3′ overhangs [237, 238] (**Figure 8**). The miRNA duplex is then incorporated into an Argonaute (Ago) proteins ((AGO1, AGO2, AGO3, or AGO4) to form a miRNA-induced silencing complex (miRISC) [239, 240].

The double-stranded miRNA molecule is composed of a passenger strand, and a guide strand (also termed as mature miRNA) commonly denoted miRNA:miRNA* [240, 241]. Ultimately, the RNA duplex is unwound, and the single strand mature miRNA is bound by an Argonaute protein and retained in the miRISC complex together with members of the GW182 family of proteins to function as a guide, directing towards target mRNA for post-transcriptional gene silencing [242, 243] (**Figure 8**). The silencing mechanism is characterized by degradation, translational suppression, or breaking down of

the target mRNAs depending on the complementarity between miRNA and mRNA [244]. The resulting passenger strands of miRNA (designated as miRNA*) are cleaved by AGO2 and degraded by cellular machinery, which indicates a strong strand bias [237, 245].

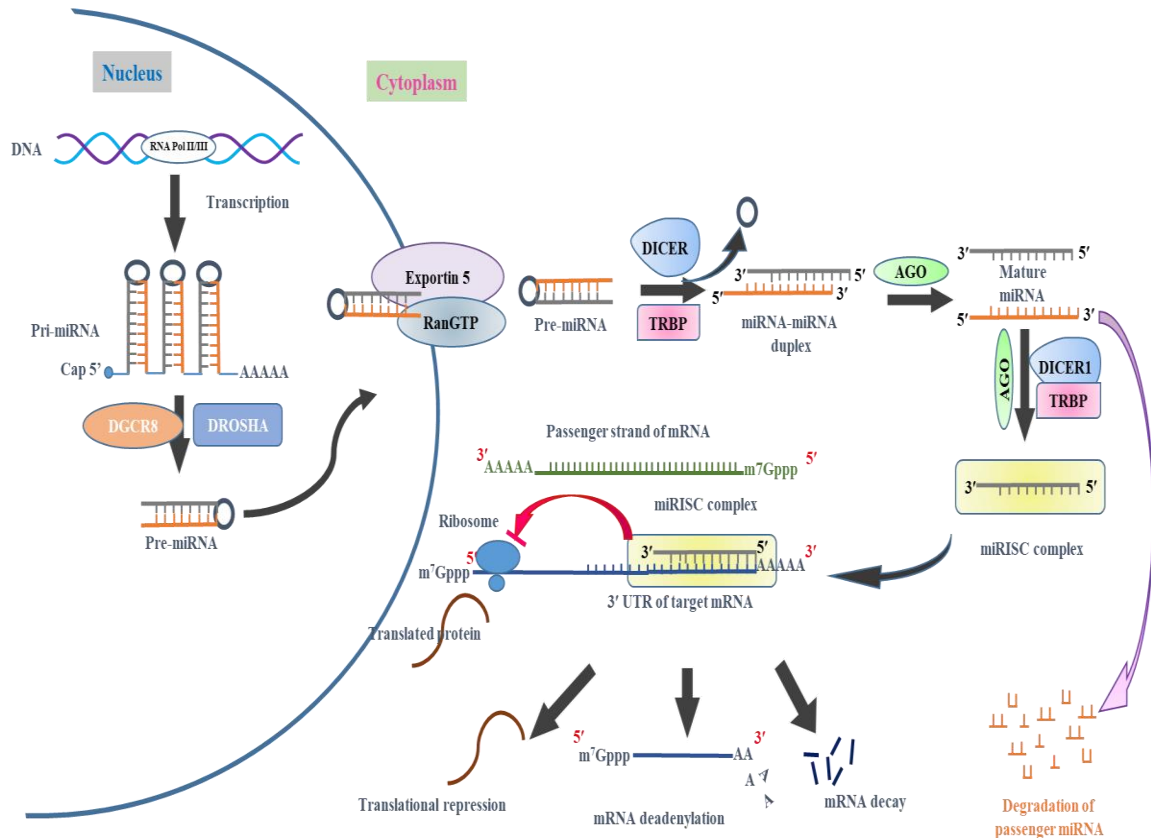

Figure 8: Mechanism of MicroRNA Processing and Their Inhibitory Mechanism. The microRNA (miRNA) processing pathway begins with transcription of their genes with the help of RNA polymerase II (Pol II) or polymerase III (Pol III) to produce pri-miRNAs in the nucleus. Then a microprocessor complex, composed of RNA-binding protein DGCR8 and type III RNase Drosha, cleaves pri-miRNA into an ~85-nt stem-loop structure called pre-miRNA. The exportin 5-RAN/GTP complex mediates the transport of pre-miRNA from the nucleus into the cytoplasm. The RNase DICER in complex with double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to a ~20- to 22-nt miRNA/miRNA duplex. After the duplex is unwound, the functional strand of the mature miRNA (the guide strand) is loaded into the miRISC-containing DICER1, TRBP, and Argonaute (AGO) proteins. This miRISC silences/inhibits the target mRNAs expression/function through mRNA cleavage, translational repression, or deadenylation.

The passenger strand of the miRNA is degraded. AGO, Argonaute proteins; DGCR8, DiGeorge syndrome critical region gene 8; m7G cap, 7-methylguanosine; miRISC, miRNA-induced silencing complex; miRNA, microRNA; pre-miRNA, miRNA precursor; pri-miRNA, primary miRNA; RAN-GTP, Ras-related nuclear protein-coupled with guanosine-5′-triphosphate; TRBP, transactivating response RNA-binding protein.

1.13. Function and Mechanism of miRNA

A single miRNA can possibly target/regulate multiple target mRNAs, and conversely, one target mRNA can be regulated by multiple miRNAs [246]. Several studies demonstrated that to pairs fully and accurately to the target sequence within mRNA is crucial for target recognition for miRNAs [243]. For that purpose, miRNAs use a 2- to 8-nt long “seed sequence,” which is located at the 5′ region of the miRNA and binds to miRNA responsive elements (MREs) at the 3′ untranslated region (UTR) of the target mRNA [247]. Moreover, other studies also reported that MREs are also distributed in 5′ UTR sequences as well as in coding regions of mRNAs [248, 249]. Binding at the seed sequences is a vital step for the thermal stability of the miRNA-target mRNA interaction, which determines miRNA specificity and activity [250]. Several other factors are controlling to the miRNA-mRNA binding strength and repressive effect of a potential target site which include the number and possible positions of target sites for the same miRNA, accessibility to the target sites, secondary structure of target RNA, flanking sequences of the target site, and their context [251, 252]. However, the silencing of the target genes is regulated at both the transcriptional and post-transcriptional stage and affects the overall translation of the target gene [253]. In general, miRNAs function as multi-protein complexes termed micro-ribonucleoproteins (miRNPs), which regulate the functional outcome [254].

Khraiwesh et al. (2020) reported that miRNAs are loaded to a specialized RNA-induced transcriptional silencing (RITS) complex, which consists of Ago1, interact with target mRNAs, and downregulates their expression chromatin remodeling [255]. PABP directly binds to GW182 proteins and interacts with deadenylase complex PAN2–PAN3, which in turn recruits CCR4–NOT to the target mRNAs and enhances miRNA-mediated deadenylation and resulting in silencing of mRNAs [256]. Decapping activator, DDX6 directly associates with the MIF4G (middle of eIF4G) domain of CNOT1, the catalytic subunit of the CCR4–NOT complex, CNOT7, which enhances the miRNA-mediated translational repression [257]. Decapping enzyme DCP1–DCP2 complex promotes decapping followed by deadenylation allows the target mRNA susceptible to rapid degradation by 5′–3′ exoribonuclease 1 (XRN1) [258]. Several initial studies also showed that miRNAs cause the deadenylation and degradation of mRNAs [259, 260]. For example, in *C. elegans* development, let7 and lin-4 appear to enhance the degradation of its lin-41, and lin-14 and lin-28 target mRNA, respectively [259]. On the other hand, during the early development of zebrafish embryos, expression of miR-430 directly control the several hundred maternally expressed mRNAs through accelerating the deadenylation of target mRNAs [261]. Ago2 protein assembled in miRISC competes eIF4E, an eukaryotic translation initiation factor, and prevents the binding of 40S ribosomal subunits from targeting mRNAs and resulting in translational inhibition [262, 263]. Chendrimada et al. (2007) study also suggested that miRNAs inhibit an early translation step of target mRNA [264]. By associating with Ago2 protein and large ribosomal subunits, elF6 inhibits the binding of the small ribosomal subunit to the complex and resulting in translational inhibition. Fukaya et al. (2014) reported that the endogenous let-7 dissociate elF4A from

the initiation complex and play a role in deadenylation and decay of the target mRNAs in *Drosophila* [265]. Bhattacharyya et al. demonstrated that in human hepatoma cells, CAT1 mRNA is translationally repressed by miR-122 and concentrated in P bodies [266]. Moreover, expression of c-Myc is reduced by the let-7 miRNA, and its repression is increased by the HuR protein binding to adjacent AU-rich element (ARE)-which is next to a miRNA let-7-binding site [267]. Recently, The GRB10-interacting GYF protein 2 (GIGYF2) was discovered, which directly interacts with the miRNA-induced silencing complex (miRISC) component GW182 and plays a role in miRNA-mediated translational repression [268].

1.14. Regulation of microRNA expression

Most of the miRNA genes are found in intergenic locations or antisense orientation to the annotated gene, implying that they have their transcription machinery.[269, 270] Lee et al. (2004) first demonstrated that miRNAs are transcribed by RNA polymerase II (Pol II), although recently, other studies found that miRNA transcription is also mediated by RNA polymerase III. [226, 227] Saito et al. (2006) first showed that chromatin remodeling and epigenetic alterations by DNA methylation and histone tail modifications could regulate the expression of several miRNAs with consequent effects on cellular functions.[271] To examine miRNA expression in human cancer cells, they treated the cells with a DNA-demethylating drug named 5-Aza-CdR and histone deacetylase inhibitor named 4-phenyl butyric acid and found that miR-127 was up-regulated significantly among other miRNAs targeting the proto-oncogene BCL-6 that is upregulated in cancer cells.[272, 273] another study supported this study showed that by using the HDAC inhibitor named LAQ824 in breast cancer cell line SKBr3 causes a significant change in 40% of the

different miRNA species.[274] Most of the miRNAs are intragenic or found in the introns of the protein-coding genes and are reasonable to demonstrate that transcription of miRNAs is cooperatively regulated with the host genome. As miRNAs have their promoter, it is believed that CpG islands of host promoters also found within the same intron and transcription of both protein-coding genes and miRNAs are regulated by DNA methylation.[275, 276]

Apart from the epigenetic modulation, several other nuclear proteins or factors are responsible for regulating the miRNA expression. Fukuda et al. (2007) demonstrated that DEAD-box RNA helicases p68 and p72, component of large Drosha-mediated processing complex could interact with several transcription factors like Smads, p53 and estrogen receptor to correctly recognize and bind to a subset of pri-mRNAs and initiate the cleavage to form pre-miRNAs.[277] Guil et al. (2007) reported that heterogeneous nuclear ribonucleoproteins (hnRNP proteins), RNA-binding proteins, play a role in the processing of endogenous pri-miR-18a, which is context-dependent and regulating the activity of miR-18a.[278] Importantly, they found that depletion of hnRNP A1 affects able processing miR-18a, which increases cell proliferation and promotes the anchorage-independent growth of cancer cells.[279] KH-type splicing regulatory protein (KSRP), multifunctional single-strand RNA-binding protein binds to the conserved G-rich elements in the terminal loop (TL) of a cohort of miRNA precursors and interacts with both Drosha and Dicer to promote miRNA maturation.[280] KSRP interacts with heterogeneous nuclear RNA-binding proteins (hnRNPs), which is involved in mRNA maturation as well as acts as an auxiliary factor for the Drosha-mediated processing of a microRNA precursor by binding to the terminal loop (TL) of a group of pri-miRNA.[278, 281] High Mobility Group A

(HMGA) proteins, extensively synthesized during the early embryonic stage as well as growth and development and also involved in regulating miRNA expression by regulating chromatin structure and gene expression.[282, 283].

1.15. Role of microRNAs in lung development

Lung development and maturation is a complex and vital morphogenetic process that is temporally and spatially regulated by a defined set of genes.[284] In the fetus, lung development goes through six defined stages: embryonic, glandular, canalicular, saccular, alveolar, and vascular expansion.[285] Several cytokines and their signaling pathways like transforming growth factor- β (TGF-β), fibroblast growth factors (FGFs), sonic hedgehog (Shh) and WNT/β-CATENIN are involved in lung development.[286-288] Stage-specific and tissue-specific miRNA expression are crucial for lung development and in maintaining lung homeostasis.[289]

For instance, members of the miR-17-92 cluster (miR-17, -18a, -19a, -20a, 19b-1, and 92-1) are highly expressed in embryonic lungs.[290] Expression of these same microRNAs decreases during lung maturation. Conversely, the let-7 family microRNAs are elevated in the adult lung compared with early embryonic stages.[291] Hayashi et al. (2011) reported that the expression of miR-21 is required and has a crucial role in branching morphogenesis, a primary developmental process in the lung.[292] Furthermore, expression of miR-142-3p and miR-326 regulates the proper differentiation and proliferation of mesenchymal cells by wingless-type MMTV integration site family (WNT) signaling and Sonic Hedgehog (Shh) signaling pathway, respectively.[293, 294] In the development of vascular smooth muscle cells (vSMC), the role of miRNAs and proteins involved in the miRNAs pathway were studied extensively. The co-operative role of both

miRNAs (miR-145 and miR-143) was reported in maintaining proper SMC phenotype, whereas miR-133 and miR-206 play crucial roles in the proliferation, migration, and development of vSMC by targeting transacting transcription factor-1 and Notch3, respectively.[295] Inhibition of miRNA processing by conditional inactivation of DICER, during the embryonic stage, resulted in deformed lung development and excessive epithelial cell death was reported.[296] At the embryonic stage, reduced expression of Ago1 and Ago2 in the distal epithelium and mesenchyme respectively suggesting that miRNA-regulated gene expression involved in the lung developmental processes.[297, 298]

1.16. Role of microRNAs in lung health and disease

MicroRNAs play an important role in lung health and diseases. The dysregulation of miRNAs plays an important role in the pathological hallmarks of several lung diseases. Several studies identified altered miRNA expression profiles, which may be associated with pathological processes within the lung and lead to the development of several respiratory diseases, ranging from inflammatory diseases (chronic airway diseases like COPD, asthma, and cystic fibrosis) to lung cancers.[299, 300] A group of microRNAs has been identified to play a role in inflammatory responses in chronic airway diseases like COPD, asthma, and cystic fibrosis. Likewise, other groups of both pro-fibrotic and anti-fibrotic miRNAs been identified to play a role in interstitial pulmonary fibrosis. Lung diseases are the leading cause of morbidity and mortality worldwide.[301] According to the WHO, Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide and predicted to become the third leading cause by 2030.[302] On the other hand, Asthma, a complex, heritable disease affects more than 300 million people

globally and IPF, a chronic fibrotic lung disease affects approximately 3 million people worldwide, with the incidence increasing with age.[303, 304] The GLOBOCAN 2018 database reports 2.09 million new cases and 1.76 million deaths from lung cancers.[305] Hence, identifying the molecular mechanisms involved in the development and progression of these diseases is important to public health. Many reports are now investigating microRNA mediated post-transcriptional gene silencing in lung diseases. Much attention and research remain to be conducted to explore the function and pathological role of miRNAs in respiratory diseases. In the following sections, we will be focusing on aberrant miRNA expression, their target sites, and findings in the five most common lung diseases.

1.17. MicroRNAs in COPD

Chronic obstructive pulmonary disease (COPD), a common airway complication that comprises chronic obstructive bronchitis and lung emphysema.[306] COPD is a multifactorial disease represents the leading cause of higher morbidity and mortality globally. It is also expected that COPD will become the third leading cause of death worldwide by 2020 due to increased prevalence with older age, environmental risk factors, excessive cigarette smoking, and noxious gases.[307, 308] The hallmarks of COPD are characterized by chronic inflammation in the lungs, a shorter interval between breathing, severe cough, and repetitive impediment across the tracheal wall during inhalation.[309] Several miRNAs have been implicated in the pathobiology of COPD.[310-314]

In COPD patients, increased secretion of Prostaglandin E2 (PGE2) results in collagen overproduction, ultimately reducing lung capacity and accelerating COPD.[299] COPD patients demonstrate decreased miR-146a expression and increased expression of its target,

Cox-2, with a consequent increase in PGE2 levels.[310] Matrix metalloproteases (MMPs) play a major role in respiratory inflammation and structural remodeling in COPD patients. During the early stage of COPD, cigarette smoke induces macrophages, lymphocytes, and neutrophils to be deposited in the walls of bronchioles, alveolar ducts, and alveoli.[315, 316] Macrophage-derived MMPs, including MMP-2, MMP-9, and MMP-12 degrade and solubilize extracellular matrix proteins, collagen, and elastin.[317, 318]. MMP-12 is overexpressed in the lungs of COPD patients.[319] Graff et al. demonstrated that miR-452, an MMP12 targeting microRNA, is significantly downregulated in COPD patients resulting in overexpression of MMP12.[320]

Shen at al. (2017) have shown that levels of miR-149-3p play a protective role in COPD by suppressing the TLR4/ NF-κB pathway by targeting two distinct signaling intermediates, namely TLR- 4 and MyD88.[321-323] miR-149-3p levels are progressively suppressed in non-COPD smokers, followed by stable COPD smokers with maximal suppression observed in smokers with acute exacerbation COPD.[321] Dysregulation of TLR4 expression has multiple downstream effects by increasing the expression of proinflammatory cytokines IL-1β, IL-6, IL-8, IL-10, TNF-α, and IFN-γ.[324-326] Persistent activation of the TLR-4 signaling and MyD88 dysregulation also induces matrix metalloproteinase 1 (MMP-1) via MyD88 and IRAK1 pathway, which plays an important role in COPD.

Another microRNA, miR-145-5p, is significantly upregulated in COPD and smokers and can serve as a promising biomarker of COPD.[327] Tobacco smoking is the principal risk factor for COPD. Cigarette smoke and COPD patients demonstrate the chronic induction of TGF-β signaling.[6, 27, 328-330] We have demonstrated that TGF-β

upregulates miR-145-5p in bronchial epithelial cells.[27] miR-145-5p dysregulation can have multiple downstream effects, which can lead to a progressive decline in lung function. For instance, miR-145-5p is involved in TH2 response activation, macrophage differentiation, and recruitment of eosinophils.[331, 332] Likewise, TGF-β mediated miR-145-5p induction plays an important role in the regulation of airway smooth muscle (ASM) function in COPD patients by targeting SMAD3 that negatively regulates the release of pro-inflammatory cytokines.[333] Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) functions as a chloride channel involved in maintaining airway fluid homeostasis as well as regulating innate immune responses in the airway. Smokers and COPD patients demonstrate an acquired CFTR dysfunction even though they have regular copies of the CFTR gene.[55, 334] Acquired CFTR dysfunction results in impaired mucociliary clearance and dysfunctional airway innate immune responses, which result in chronic microbial infection and lung inflammation.[59, 60, 167, 335-339] In COPD patients, expression of CFTR targeting miRNAs miR-101 and miR-144 is upregulated with consequent CFTR suppression.[190] We have recently demonstrated that TGF-β signaling and cigarette smoke (via TGF-β signaling) upregulates miR-145-5p to suppress CFTR as well as an important CFTR modifier SLC26A9 which also functions as a backup Cl- channel.[27]

MiR-144, along with miR-15b, are potential mediators of the TGF-β signaling cascade and genes that are functionally associated with TGF-β superfamily involved in the development and progression of COPD, inflammatory response, and airway epithelial repair after injury.[329, 340] The miR-15b expression is higher in COPD patients with a concomitant decrease in the inhibitory SMAD7.[341] Expression of another microRNA,

miR-199a-5p, is diminished in COPD patients due to hypermethylation of CpG sites in the miR-199a-5p promoter.[342] Decreased expression of miR-199a-5p leads to an intensification of the unfolded protein responses (UPR) and contributes to lung cell apoptosis and lung inflammation.[342]

1.18. MiRNA-targeted interventions as therapy in respiratory diseases

They are identifying clinically relevant miRNAs, which are important for exploiting their therapeutic potential. Given that miRNAs expression profiles are similar for both human and mouse lung, in most cases, mouse models can be used to study the effects of aberrant microRNAomes in lung diseases while also identifying therapeutic leads to reverse the downstream effects of the dysregulated miRNAs **(Figure 9)** [289]. By using nucleic acid-based inhibitors like siRNAs, miRNA mimics, and miRNA inhibitors, researchers are trying to restore the normal microRNAome and improve clinical outcomes. The mechanism of cellular uptake of antisense oligonucleotide (ASO) depends on the structure of ASO and the cell type.[343] Various energy-dependent and non-energy dependent entry pathways are believed to be involved in oligonucleotide internalization.[344] However, effective delivery of the oligonucleotides to their intracellular site of action remains a major challenge, and therapeutic applications can be limited due to problems associated with in vivo delivery of these therapeutic oligonucleotides and possible off-target effects.[345] The airway system uniquely consists of pulmonary surfactants, which are zwitterionic lipids that possess cationic properties at the pH of the respiratory tract.[346] Moschos et al. (2011) demonstrated that anionic oligonucleotides are designed in a way to be absorbed by the respiratory surfactant and efficiently taken up by the cells.[347] Moreover, the miRNA mimics, siRNAs, or

antagomiRs can stimulate the immune system or saturate the post-transcriptional gene silencing mechanism.[348] Several strategies like single nucleotide polymorphisms (SNPs) in the miRNA gene, miRNA 3' tailing, editing, methylation are being designed to minimize off-target effects and enhance uptake and increase their stability.[349]

1.19. Therapies using mimics to restore microRNA levels

Earlier efforts for delivery of mimics focused on direct intratumoral injections (in case of cancers) or by viral vectors. Unfortunately, using modified viral vectors as therapeutic vehicles has some limitations and is considered controversial due to the risk of integration of viral DNA into transcriptionally active sites in the host genome, possibly dysregulating the expression of oncogenes, or imparting excessive immunogenicity.[350] Lately, liposome and nanoparticle-based drugs have been used to facilitate the delivery and uptake of miRNA mimics or inhibitors and siRNAs. Trang et al. explored the therapeutic delivery of lipid-based let-7, and miR-34 formulations to show tumor-suppressive effects in KRAS mouse model for lung cancer and Rai et al. (2011) showed that a miR-7 expressing plasmid has anti-proliferative effects against EGFR oncogene addicted lung cancer cells using liposomal delivery.[351, 352] Also, Chen et al. (2010) found that GC4 single-chain variable fragment (scFv) targeted nanoparticles containing miR-34a actively reduce the tumor size as well as survivin expression, an inhibitor-of-apoptosis protein by targeting MAPK pathway in lung metastasis.[353] MRX34 is the first microRNA (miRNA) mimic encapsulated in a liposomal nanoparticle system to facilitate target cellular uptake to be tested in a clinical setting.[354] However, researchers are trying to overcome the liposome-based therapies due to charged molecules in liposomes and low pH sensitivity.[355] On the other hand, Xiao et al. (2014) identified one small molecule

activator of miR-34a called Rubone, which can upregulate the miR-34a expression in hepatocellular carcinoma.[356] Young et al. (2010) reported that small molecule activator induces the expression of miR-122 in liver cancer cells and promotes apoptosis through caspase activation.[357] Chen et al. (2012) tested a small molecule activator from photoreaction of naphthalene-1, 4-dione with acetylenes that has the regulatory activities on muscle-specific miRNA (miR-1), is frequently downregulated in various types of cancers.[358, 359] For the treatment of pulmonary diseases, miRNA-based therapeutics can be formulated as aerosols and delivered through inhalation that might decrease systemic exposure and reduce the possible toxicity and off-target effects.[360]

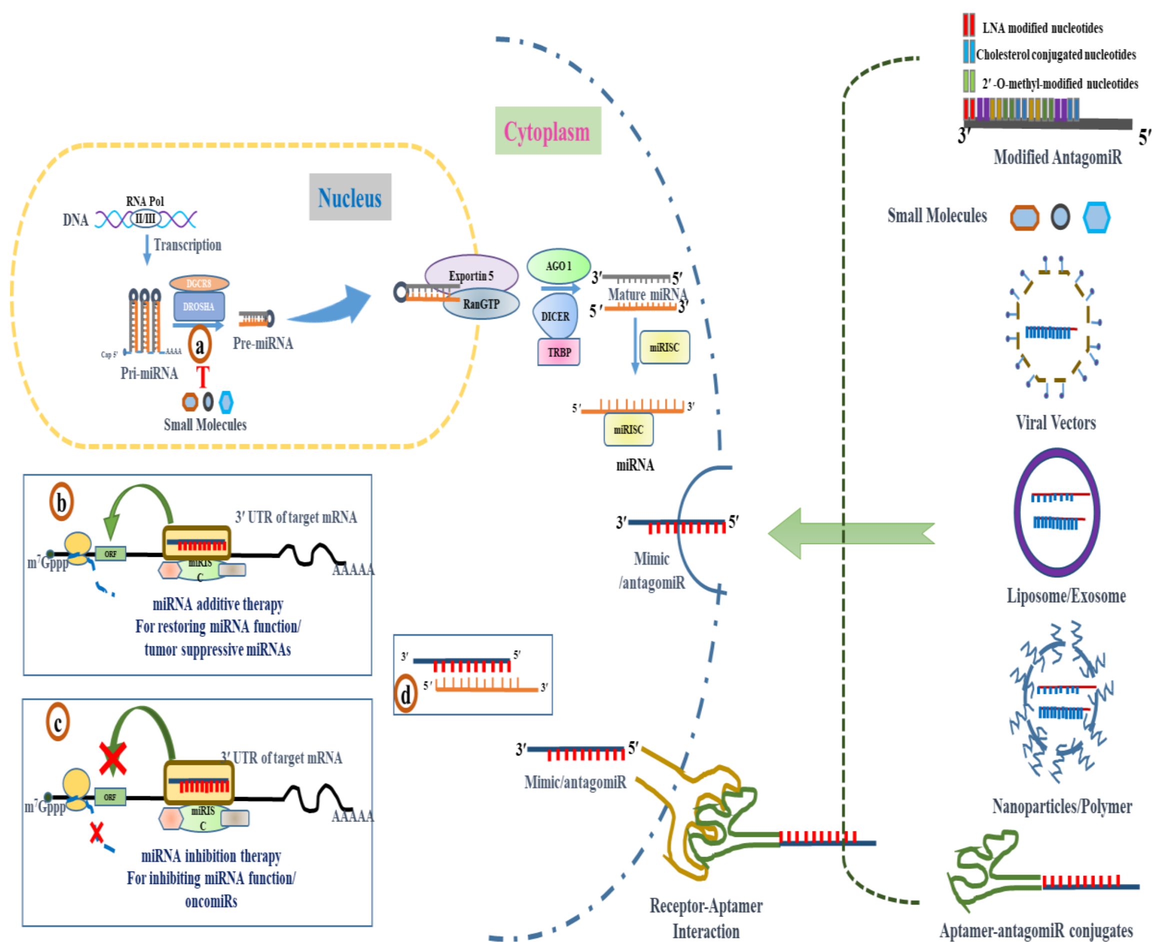

Figure 9: Therapeutic approaches to rescue miRNA dysfunction.
Exosome/liposome, viral vectors (LV, AAV, Adeno, and plasmid), Nanoparticles/polymers, and Aptamer mediated antagomiR, miRNA mimics delivery into the pulmonary cells. a) Small molecules bind to Drosha and Dicer processing sites of human miRNAs that are disease-associated and inhibit their biogenesis. b) miRNA mimics function like endogenous miRNAs restoring the activity of a miRNA. c) Binding of single-stranded antagomiRs having complementary sequences to the target endogenous miRNA genome sequence and inhibit the synthesis of disease-causing miRNAs, and d) antagomiRs having seed sequence sequesters the endogenous free miRNA target inhibiting the activity. TRBP: transactivating response RNA-binding protein; miRNA: microRNA; pre-miRNA, miRNA precursor; pri-miRNA, primary miRNA; AGO: Argonaute proteins; miRISC: miRNA-induced silencing complex; RAN-GTP: Ras-related nuclear protein-coupled with Guanosine-5'-triphosphate; DGCR8 = DiGeorge syndrome critical region gene 8; m7G cap: 7- Methylguanosine. T = inhibitory effect.

1.20. Therapies targeting miRNAs

Anti-sense oligonucleotide-based techniques (antagomirs, locked nucleic acid, and miRNA sponges) have also been designed to inhibit onco-miRs in lung cancer.[360] Chemical modifications like 2'-O-methyl group in antagomir give the required stability against nucleases, and insertion of cholesterol moiety into the passenger strand facilitates cellular uptake. Antagomirs, also known as anti-miRs, are chemically synthesized oligonucleotides complementary to the microRNAs and designed to bind to and interfere with their function (**Figure 9**).[361] We have shown that CFTR and SLC26A9 suppression in primary human bronchial epithelium redifferentiated ex vivo can be rescued by miR-145 antagonism with the consequent restoration of chloride efflux.[27] An antagomir targeting miR-9 rescues protein phosphatase 2A (PP2A) activity with the consequent restoration of dexamethasone (DEX)-induced GR nuclear translocation and restores steroid sensitivity in airway hyperresponsiveness (AHR).[362] Use of Locked Nucleic Acid (LNA) based anti-miRs in which ribose sugar ring in each nucleotide is "locked" with

Methylene Bridge between 2'-O and the 4'-C groups confers high affinity to target miRNA sequence and improves resistance to nucleases. "Miravirsen," an LNA based drug effectively inhibits miR-122, which plays a crucial role in hepatitis C virus (HCV) replication.[363] Of note multiple miRNA "miRNA sponges," considered as transgenes have been suggested which encode RNA transcripts consists of several tandem repeats of miRNA target sequence serve as decoys to compete with native mRNA targets for miRISC binding, thereby lowering sequestering the miRNA to prevent it from binding to its cellular target sites.[364]

On the other hand, high-throughput screening and reported based assays had identified several small molecules from a small molecule drug library that act by either inhibiting the formation of active RNA-induced silencing complex (RISC) or preventing the expression of miRNA genes from pri-miRNA **(Figure 9)**.[365, 366] Gumireddy et al. (2008) reported that azobenzene inhibits the expression of miR-21, an anti-apoptotic factor that is elevated in various cancers like breast, ovarian, and lung cancer as well as glioblastomas.[367, 368] Later on, several studies subsequently identified other diverse small molecule modifiers that can act as activators or inhibitors of miRNA mediated post-transcriptional gene silencing. Shi et al. (2013) reported that AC1MMYR2, a potent and selective inhibitor of miR-21, reverses epithelial-mesenchymal transition, and suppresses tumor growth and progression.[369] Young et al. (2010) also discovered one small molecule inhibitor that suppresses the hepatitis C virus (HCV) replication in the liver cells by targeting miR-122 and thereby function as a novel treatment approach in HCV infection.[357]

1.21. Aptamers: an emerging class of therapeutics

Aptamers, an emerging class of therapeutics, are high affinity single-stranded nucleic acid ligands that exhibit specificity and avidity comparable to, or exceeding that of antibodies, and can be generated against most targets.[370-372] Unlike antibodies, aptamers, can be synthesized chemically and hence offer significant advantages in terms of production cost, more straightforward regulatory approval and lower immunogenicity when administered in preclinical doses 1000-fold higher than those used for animal and human therapeutic application.[373, 374] Aptamers are highly specific and can discriminate between related proteins that share common sets of structural domains.[375, 376] Nucleic acid aptamers are already approved for use in humans (e.g., macugen).[377, 378] Different strategies have been employed to develop cell-specific aptamers for the delivery of oligonucleotide-based therapies.[379, 380] Upon receptor-mediated uptake, miRNA cargo is processed by DICER and incorporated in the RNA induced silencing complex and, finally, binds to the target of interest (**Figure 9**).[355] MUC1 aptamer-functionalized as nanoparticles and coupled with miR-29b has demonstrated selective delivery of miRNA-29b to lung tumor cells and tissues.[381] Likewise, aptamers conjugated to miR-34c and miR-212 have been shown to suppress the proliferation of NSCLC or promote susceptibility of NSCLC cells to TNF-related apoptosis-inducing ligand (TRAIL) mediated-apoptosis.[382, 383] Also, Esposito et al. (2014) characterized a selective RNA-based aptamer (GL21.T), which is conjugated with tumor suppressor let-7g miRNA sequence and binds with high affinity to the oncogenic tyrosine kinase receptor, Axl.[384] They found that specific delivery of this multifunctional conjugate complex to the Axl-expressing cancer cells and suppress the let-7g targeting gene expression resulted

in the inhibition of cancer cell progression and invasion as well as reduction of tumor growth in a xenograft model of lung adenocarcinoma.[385] Hence, aptamer-miRNA conjugates can function as novel tools with the therapeutic potential to inhibit cancer cell survival and migration in vitro and in vivo in lung cancer.

CHAPTER 2: SIGNIFICANCE OF THE STUDY

The advent of cART has led to a dramatic decline in morbidity and mortality from HIV/AIDS [386]. Lung-related comorbidities like chronic obstructive pulmonary disease (COPD) remain highly prevalent in HIV patients when compared to non–HIV-infected adults. [387, 388] HIV is an independent risk factor for COPD, even when accounted for smoking. [389] Tobacco smoke and inhalation of particulate matter are the main causes of chronic bronchitis associated with COPD [390]. Chronic inflammation is a hallmark of chronic bronchitis and COPD in HIV patients and presents a significant health problem associated with increased morbidity and even mortality. Chronic inflammation consequently promotes COPD and lung function decline, also inducing immune cell recruitment and activation, leading to de novo infection and enhanced HIV replication. Hence, COPD can also contribute to HIV progression. It is estimated that the annual healthcare expenditures for chronic bronchitis associated with COPD exceed $11 billion, with hospitalizations accounting for more than half of this cost. [391] Effective mucociliary clearance depends, at least in part, on maintaining adequate ASL depth and CBF [29, 159, 392]. CFTR activity is a critical determinant of ASL depth by helping to create an osmotic gradient and to regulate paracellular permeability for serosal to mucosal vectorial fluid flux by Cl- secretion [35]. CFTR activity is attenuated in the airways of patients with chronic bronchitis and HIV [334, 338, 393]. Increased TGF-β signaling associated with COPD and HIV smokers, leads to a state of acquired CFTR dysfunction and the system fully fails with repeated insults including during exacerbations mimicking cystic fibrosis. Understanding the pathophysiological mechanisms that lead to acquired CFTR dysfunction and its downstream effects on inflammation in chronic bronchitis associated COPD and HIV

smokers is therefore important to public health. Especially, identifying new treatment regimens for preventing acquired CFTR dysfunction and decreasing airway inflammation and lung function decline in chronic bronchitis and HIV smokers is the ultimate goal.

CHAPTER 3: AIMS OF THE STUDY

Aim 1: To elucidate the role of miRNA in TGF-β-mediated suppression of CFTR and SLC26A9 mRNA and function.

Hypothesis: Given that TGF-β signaling does not suppress transcription from the CFTR promoter, we hypothesize that TGF-β signaling, directly, or via another intermediate induces anti-CFTR miRNA leading to miRNA-mediated post-transcriptional gene silencing of CFTR.

This aim was accomplished by identifying miRNAs induced upon TGF-β1 treatment having the ability to silence CFTR mRNA and whether blocking these miRNAs restores CFTR function in TGF-β1 treated cells. We compared these observations in non-CF A/J mice exposed to cigarette smoke.

Aim 2: To investigate therapeutic leads targeting TGF-β signaling for restoring cigarette smoke-induced CFTR dysfunction in vitro and in vivo.

Hypothesis: Suppressing TGF-β signaling will restore CFTR mRNA and its activation by β_2-agonists.

A neutralizing aptamer targeting Transforming growth factor-beta receptor 2 and miRNA targeting antagomir tested in primary bronchial epithelial cells and secondary cell lines restore CFTR function following exposure to cigarette smoke. The combined action of the aptamer and antagomir suppress the receptor involved in TGF-β signaling and target the miRNAs and restrict the inhibition of TGF-β signaling to the bronchial epithelium.

Aim 3: To study the effect of HIV Tat on CFTR activity and Mucociliary clearance.

Hypothesis: HIV Tat suppresses CFTR mRNA levels and MCC via TGF-β signaling. TGF-β1 suppresses CFTR mRNA and function, and this will translate into a reduced mucociliary clearance in our ex vivo model of primary bronchial epithelium. Since TGF-β does not suppress transcription, and inhibiting Drosha rescues its effect on CFTR function, we hypothesize that TGF-β suppresses mucociliary clearance by miRNA-mediated post-transcriptional gene silencing. We will first determine if HIV tat suppresses CFTR and then look at the HIV Tat altered microRNAome to identify the role of altered microRNAomics in HIV mediated CFTR suppression.

Aim 4: To test the CRISPR-mediated CFTR-specific microRNA antagonism prevents acquired CFTR dysfunction in the context of HIV Tat.

Hypothesis: HIV Tat suppresses MCC via a common pathway involving TGF-β signaling. TGF-β signaling induces miR-145-5p and miR-509-3p to suppress CFTR. Disrupting the target site in the CFTR 3'UTR using CRISPR-based gene editing prevents TGF-β mediated CFTR suppression. Thereby allowing normal MCC, mitophagy, cellular senescence, and consequently preventing airway inflammation. We will design gRNAs targeting in close proximity to the miRNA target sites in the CFTR 3'UTR and determine if CRISPR based editing rescues CFTR mRNA and function using qRT-PCR techniques and Ussing chamber respectively.

CHAPTER 4: TGF- β SUPPRESSES CFTR BIOGENESIS AND FUNCTION BY POST-TRANSCRIPTIONAL GENE SILENCING

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4.1. Introduction

Mucociliary clearance (MCC) is a primary innate defense mechanism of mammalian airways that works in concert with other antimicrobial substances like lactoperoxidase, lysozyme, and lactoferrin, to protect the host from the noxious effects of airborne pathogens, pollutants and allergens [3, 394]. The mucociliary apparatus consists of cilia, a protective mucus layer, and a periciliary ASL layer to optimize ciliary beating [29]. Abnormalities in any compartment of the mucociliary system can compromise mucus clearance leading to mucus impaction. The accumulated mucus entraps bacteria and promotes chronic bacterial infection [30-32]. The ASL layer lining the airway surfaces is crucial for mediating MCC rates [35] and is tightly regulated under normal conditions [4]. Cystic fibrosis transmembrane conductance regulator (CFTR) plays a pivotal role in MCC and other mechanisms of airway innate immunity like the lactoperoxidase thiocyanate defense mechanism of the airways [167, 168, 395]. CFTR regulates airway surface liquid (ASL) due to Cl- efflux and reciprocal inhibition of Na^+ absorption [396]. Water follows through the transcellular/paracellular pathway maintaining ASL height leading to efficient MCC [39, 40]. We have demonstrated that CFTR also regulates paracellular space and, consequently, water transport by increasing paracellular permeability [6]. Hence CFTR plays a critical role in regulating the ASL depth. CFTR dysfunction leads to decreased ASL

depth [39], ASL acidification [397, 398], and increased mucus viscoelasticity [399], which in turn impairs MCC and cough clearance mechanisms.

An extensive body of evidence has established that smoking also induces an acquired state of CFTR dysfunction in patients with normal copies of the CFTR gene [54, 55]. We have shown that acquired CFTR dysfunction due to smoke exposure rapidly and severely inhibits the mucociliary clearance apparatus of the airways [400]. In our earlier report, we have demonstrated that transforming growth factor β (TGF-β) signaling and cigarette smoke (CS) (via TGF-β signaling) suppress CFTR mRNA, which translates to a concomitant and proportional suppression of CFTR function [6]. TGF-β1 is ubiquitously expressed and secreted by several cell types, including airway epithelial, smooth muscle, fibroblast, and most immune cells. Airway epithelia of smokers, as well as patients with chronic bronchitis or chronic obstructive pulmonary disease (COPD), show increased TGF-β1 expression [106-109].

In this report, we determine the mechanism by which cigarette smoke and TGF-β suppresses CFTR. We demonstrate that miR-145-5p plays an important role in cigarette smoke (and TGF-β)-mediated CFTR suppression in primary bronchial epithelial cells re-differentiated at the air-liquid interface, small animal models and human lung samples from smokers. We also demonstrate that miR-145-5p also modulates another important chloride channel, SLC26A9, which physically interacts with CFTR and plays an important role in CFTR biogenesis and activation [401-403]. We demonstrate that antagonizing miR-145-5p rescues CFTR and SLC26A9. Finally, we show that a neutralizing aptamer to TGFBR2 can rescue CFTR mRNA and function in primary bronchial epithelial cells exposed to cigarette smoke.

4.2. Methods and Materials

4.2.1. Cell line and Cell Culture

Primary human bronchial epithelial cells were isolated and re-differentiated at the air-liquid interface (ALI) cultures, as described by Fulcher and Randall [404, 405] adapted by us [6, 406]. Cells were obtained from properly consented donors whose lungs were not suitable for transplantation for the causes unrelated to airway complications and supplied by the University of Miami Life Alliance Organ Recovery Agency. Since the material was obtained from deceased individuals with minor, de-identified information, its use does not constitute human subjects research as defined by CFR 46.102. A signed and well-documented consent of each individual or legal healthcare proxy for the donation of lungs for research purposes is on file with the Life Alliance Organ Recovery Organization allows the research purpose of this material.

Unless otherwise mentioned, experiments will use cells from non-smokers to not confound the findings in unknown ways. These primary cultures undergo mucociliary differentiation at the ALI, reproducing both the in vivo morphology and key physiologic processes to recapitulate the native bronchial epithelium ex vivo [404, 405]. All experiments with NHBE used cultures re-differentiated at the Air-liquid interface. Except for Ussing chamber experiments, NHBE cultures were re-differentiated at the ALI in 10mm transwell filers (Corning # 3460). For Ussing chamber experiments, NHBE cells were re-differentiated on snap well filters (Corning # 3801). The immortalized normal human bronchial epithelial cell line BEAS-2B (ATCC CRL-9609) was purchased from the American Type Culture Collection (Manassas, VA, USA). BEAS-2B cells were cultured in BioLite 75 cm^2 flasks (Cat. #130190, Thermo Scientific) containing Bronchial Epithelial

Cell Growth Medium (BEGM). BEGM media was supplemented with 0.1% (v/v) human recombinant epidermal growth factor, 0.1% (v/v) insulin, 0.1% (v/v) hydrocortisone, 0.1% (v/v) ethanolamine, 0.1% phosphoryl ethanolamine, 0.1% (v/v) retinoic acid, 0.1% (v/v) epinephrine, 0.24% (v/v) transferrin, 1% (v/v) penicillin/streptomycin and 0.1% (v/v) bovine pituitary extract as published by Fulcher et al [404]. The cells were cultured in 95% air and 5% CO2 at 37 °C and maintained free of mycoplasma contamination.

4.2.2. TGF-β1 treatment of NHBE ALI cultures

Recombinant TGF-β1 (R & D Cat # 240-B-002) was dissolved according to the manufacturer's instruction at a stock concentration of 10ngs/μL. A working dilution was prepared at 10ngs/ml in ALI media. Treated media (TGF-β or vehicle) was added basolaterally and apically (50μl added apically) to mimic physiological conditions. While one report has demonstrated the expression of TGFBR1 on the basolateral side [407], a number of reports have demonstrated TGF-β1 on the mucosal (apical) side under physiological conditions [408]. Likewise, TGF-β has been shown in the BAL fluid in both healthy and CF subjects, suggesting that at least a fraction of TGF-β signaling also occurs from the apical side [409]. The Concentration of TGF-β used is within the mean physiological range (2-20ngs/ml) suggested by Sun et al. [410].

4..2.3. Cigarette Smoke Exposure

Normal human bronchial epithelial (NHBE) ALI cultures were exposed to air or CS using a SCIREQ smoke robot (Montreal, Canada). Four research-grade cigarettes (University of Kentucky, Kentucky) were smoked with a puff volume of 35 ml for 2 seconds every 60 seconds and blown over cell culture filter at a rate of 5mL/min according

to ISO 3308. Control cells were similarly treated without smoke (air control). We have used this regimen before, and the regimen was not found to affect viability or trans-epithelial electrical resistance of NHBE ALI cultures (data not shown) [6]. Cells were allowed to recover for 16 hours before initiating experiments.

4.2.4. Electrophysiology Experiments

Ussing chambers were used to determine CFTR activation, as reported by our lab previously [338]. Briefly, NHBE cultures re-differentiated at the ALI on snap wells were mounted in EasyMount Chambers (Physiologic Instruments, San Diego, CA) with Krebs Henseliet (K.H.) in apical and basolateral chambers. KH consisted of 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 1.2 mM $CaCl_2$, and 5.5 mM glucose, pH 7.35 when gassed with 95% O_2/5% CO_2. Solutions were maintained at 37°C by heated water jackets and were continuously bubbled with a 95% O_2/5% CO_2 mixture. To monitor the short-circuit current (I_{SC}), the transepithelial membrane potential was clamped at 0 mV with a single-channel voltage-current clamp (Model #VCC600, Physiologic Instruments), using Ag/AgCl electrodes in agar bridges. Signals were digitized and recorded with DAQplot software (Acquire and Analyze ver. 2.3.300, Physiologic Instruments). Amiloride (10 μM) was added apically to inhibit epithelial sodium channel influences. CFTR activation was measured by the addition of albuterol (10μM) as described by us before [338]. Albuterol was used for CFTR activation to maintain conformity with our earlier publication [6] and for potential therapeutic use in CFTR dysfunction given that β2-agonists are already prescribed in these diseases. Change in short circuit current (ΔI_{SC}) in response to albuterol was determined as an indicator of CFTR activity. All additions were prepared at the 1000X stock solution. Albuterol

hemisulfate was dissolved in sterile water. Amiloride, CFTR inhibitors CFTRinh172 and GlyH101 were dissolved in DMSO to make a 1000X stock. The total DMSO concentration at the end of the experiments was 0.003%.

4.2.5. Chemical and Reagents

5-aza-2′-deoxycytidine (5-aza-CdR) and Aurintricarboxylic acid (ATA) were purchased from Sigma Aldrich (St. Louis, MO, USA). TGF-β1 (Recombinant Human Protein) from Life Technologies. High-capacity cDNA reverse transcription kit from Applied Biosystems. Taqman Fast Advanced Master Mix from Life Technologies. Antibody against large subunit of RNA polymerase, RPB1 from Santa Cruz, (sc-899). Lipofectamine RNAiMAX Transfection Reagent and Opti-MEM^{TM} Reduced-Serum Medium were purchased from Thermo Fisher Scientific (Cat. No. 13778150 and 31985062). Mimics of hsa-miR- 145-5p, 22-5p, 449b, 33b-3p, were purchased from Sigma Aldrich (Mission miRNA mimics; Sigma St. Louis, MO, USA). AntagomiR to miR-145-5p was purchased from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). Likewise, the TGFBR2 aptamer was custom synthesized by IDT. Amiloride (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich) to make concentration 10 mM. Albuterol hemisulfate (also known as salbutamol, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile water to make a concentration 10 mM. CFTR (inh)-172 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO to make Concentration 20 nM.

4.2.6. Treatment with 5-aza-2'-deoxycytidine (5-aza-CdR)

NHBE ALI cultures were pre-treated with 5-aza-2'-deoxycytidine for 24 hours and retained for the remainder of the experiment. Recombinant TGF-β1 (10ng/ml) was added

apically and basolaterally. 16 hours post-treatment of TGF-β1, total RNA was isolated, and CFTR mRNA levels determined qRT-PCR.

4.2.7. ChIP assay

Chromatin immunoprecipitation (ChIP) was performed using a commercially available ChIP kit (Chroma Flash high sensitivity ChIP kit, Epigentek), where NHBE ALI cultures were treated with recombinant TGF-β1 (10ng/ml) or vehicle. 16 hours post-treatment of TGF-β1, chromatin from the vehicle and TGF beta treated NHBE cells precipitated using an antibody against the large subunit of RNA polymerase, RPB1 (Santa Cruz, sc-899) according to the manufacture instructions. The amount of immunoprecipitated DNA was quantified by qPCR (discussed separately) by using primers designed to span the CFTR transcription start site.

4.2.8. ATA treatment in NHBE ALI cultures

NHBE ALI cultures grown on snapwells were treated with TGF-β1 and separately cells were treated with ATA (25 uL/mL) three hours prior to TGF-β1 treatment and was retained for the remainder of the experiment. 16 hrs post-treatment, cells were mounted in Ussing chambers, and Cl- efflux in response to albuterol addition was determined as an index of CFTR function, and CFTR mRNA from these cells was analyzed by qRT-PCR. n = 3 different lungs.

4.2.9. Microarray screening of cellular miRNAs

Exiqon miRCURY Ready-to-Use PCR Human panel I and II, V4 (Exiqon, Woburn, MA, U.S.) was used to detect differential expression profile of miRNA between vehicle as control and TGF-β1 as treatment. 5 ng/uL of each template RNA from NHBE cells was

reverse transcribed using the miRCURY LNA™ Universal R.T. cDNA Synthesis Kit (Exiqon, Cat. No. 203301). The cDNA template was then amplified using the microRNA Ready-to-Use PCR, Human Panel I + II (Exiqon, Cat. No. 203615) in 384 well plates according to the manufacturer's instruction. The qPCR reactions were run on a CFX384 (Bio-Rad) using the thermal-cycling parameters recommended by Exiqon (Denaturation at 95 °C 10 min, 40 amplification cycles at 95°C for 10s and 60°C for 1 min). The relative quantification of miRNA expression levels was performed using the delta Cq method. Ct value < 37 and assays detected with 5 Ct's less than the negative control (No Template Control, NTC) were allowed for analysis. The amplification curves were analyzed by GenEX software (Exiqon). From the 768 wells, 742 miRNA primer sets were used for miRNA expression profiling; the remaining wells contained interplate calibrator oligonucleotides, spike-in control oligonucleotides for quality controls. Targeted MiRNAs that were identified from the initial screen phase were subjected to validation through R.T. and qRT-PCR according to the manufacturer protocol (Taqman™ MicroRNA Reverse Transcription Kit, Cat. No. PN 4366596 and PN 4366597).

4.2.10. Transfection of miRNA mimics and antagomiR in BEAS-2B Cells

BEAS2B cells were transfected with miRNA mimics or antagomirs using lipofectamine RNAiMAX according to the manufacturer's instruction. 20 nM of each mimic individually or as pools were transfected in BEAS2B cells. Cells were harvested 48 h post-transfection for RNA isolation and RT-qPCR. In the case of TGF-β treatment, cells were treated with 10 ng/mL TGF-β and harvested 24 h after treatment for RT-qPCR analysis.

4.2.11. mRNA extraction and Quantitative Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from NHBE cells or BEAS-2B cells following the termination of experiments using an RNeasy mini kit (Qiagen Inc. Valencia, CA). The Concentration and integrity of the extracted RNA were analyzed by measurement of the OD260/280 (Synergy™ HTX Multi-Mode Microplate Reader, Winooski, VT, USA). Complementary DNA (cDNA) was reverse transcribed using the Applied Biosystems High-performance kit (Applied Biosystem, Carlsbad, CA). Reverse transcription of 2 μg of total cellular RNA was performed in a final volume of 20μl containing 10 μl RNA, 2 μl 10X R.T. buffer, 0.8 μl dNTP Mix (100mM), 2.0μl 10X R.T. random hexamer primers, 1.0μl MultiScribe™ reverse transcriptase, 1μl RNase inhibitor and 3.2 μl nuclease-free water. The reverse transcription reaction conditions were 25°C for 10 minutes, 37°C for 120 minutes, and 85 °C for 5 seconds. cDNA samples were stored at −20 °C. qPCR for reverse-transcribed cDNA was performed on the Bio-Rad CFX96 real-time system (BioRad, Hercules, CA, USA) using validated TaqMan probes (GAPDH, cat #Hs02758991_g1, CFTR, cat # HS00357011-m1, hsa-miR-145-5P, cat # PM11480) using cycling conditions specified by the manufacturer. Gene-specific or miRNA expression was normalized to GAPDH. The results were determined using the delta-delta method and expressed as fold change.

4.2.12. Animal experiments

All animal protocols were reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. A/J mice aged 6-8 week-old and expressing wild type CFTR (+/+) were evenly divided along with gender for all studies.

As previously reported [411], mice were exposed in whole-body chambers (28” x 19” x 15”) to diluted mainstream cigarette smoke (up to 200 μg/l of total particulate matter, 35-ml puffs of 2-s duration at a rate of 3 L/sec each minute for 40 min) from 3R4F reference cigarettes (University of Kentucky, Lexington, KY) 2 sessions/day for five weeks using a computer-controlled cigarette smoke generator (SCIREQ, *InExpose* model, Toronto, Canada). Control mice were exposed to room air in same-sized chambers. Characterizations of whole cigarette smoke exposures (e.g., volumetric flow rate calibration, aerosol concentration, particle size distribution) were previously reported [63]. Animals humanely euthanized and freshly isolated lung sections were flash-frozen in liquid nitrogen for expression analyses.

4.2.13. Statistical analysis

Unless otherwise mentioned**,** data were expressed as mean ± SEM from NHBE ALI cultures from at least three lungs or three different experiments. The data were subjected to statistical analysis using unpaired t-tests for single comparisons or ANOVA followed by Tukey Kramer honestly significant difference test for multiple comparisons as appropriate. Graphpad prism was used for analysis. The significance was considered at the level of $p<0.05$.

4.3. Results

4.3.1. TGF-β1 mediated CFTR mRNA suppression is due to miRNA-mediated post-transcriptional gene silencing

Given reports that TGF-β can regulate transcription of several genes, we tried to determine if any effects of TGF-β mediated CFTR suppression were due to a suppression of transcription from the CFTR promoter. To determine if CFTR mRNA suppression is a

result of transcriptional suppression, we first tested whether TGF-β induces CFTR promoter hypermethylation given reports that CFTR promoter is silenced by hypermethylation in several cancers [412]. NHBE cultures re-differentiated at the ALI were preincubated with 5-aza-2'-deoxycytidine, an inhibitor of Histone methylation prior to treatment with TGF-β1. 5-aza-2'-deoxycytidine can reactivate epigenetic silencing established due to histone methyltransferases [412]. **Figure 1A** demonstrates that pretreatment with 5-azacytidine does not rescue TGF-β mediated CFTR mRNA suppression. To further confirm or rule out transcriptional suppression, we performed a CHIP experiment on NHBE ALI cultures treated with TGF-β1 or vehicle. Chromatin was immunoprecipitated using an antibody against the large subunit of RNA polymerase II and probed with primers designed to hybridize 150 bp downstream of the transcription start site. **Figure 1B** demonstrates that TGF-β1 treatment does not affect transcription initiation from the CFTR promoter.

To determine if TGF-β mediated CFTR mRNA suppression is due to post-transcriptional gene silencing, NHBE ALI cultures were grown on snapwells were treated with TGF-β1. Separately another subset of cells was treated with Aurintricarboxylic acid (ATA) three hours prior to TGF-β1 treatment. ATA is a small molecule inhibitor of DROSHA [365], the enzyme involved in pri-miRNA processing. Hence blocking DROSHA will block the entire miRNA pathway. Snapwells were mounted in Ussing chambers, and CFTR activity was determined with albuterol, as reported earlier by us [6]. **Figure 1C** shows that blocking the miRNA pathway rescues TGF-β mediated CFTR suppression. Total RNA was isolated from these cells and analyzed for CFTR mRNA levels. **Figure 1D** shows that the rescue of CFTR function by ATA is a consequence of the

rescue of CFTR mRNA levels in NHBE ALI cultures treated with TGF-β. Together these data demonstrate that TGF-β mediates CFTR suppression by miRNA mediated post-transcriptional gene silencing.

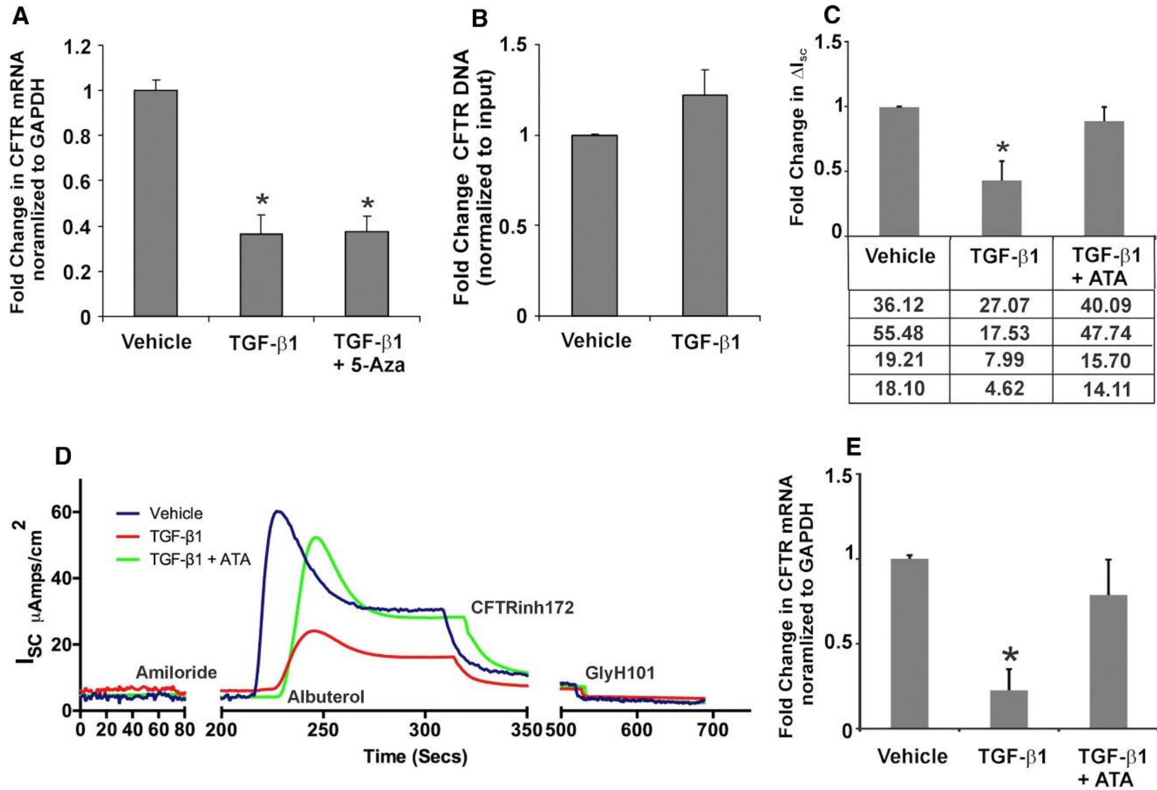


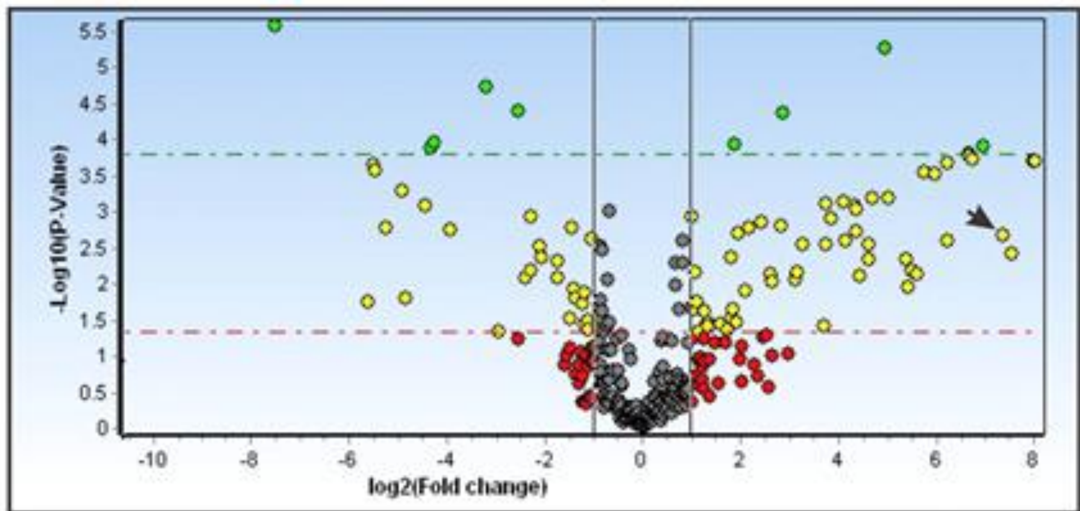
Figure 10: TGF-β1 Signaling Does Not Suppress Transcription from the CFTR Promoter.

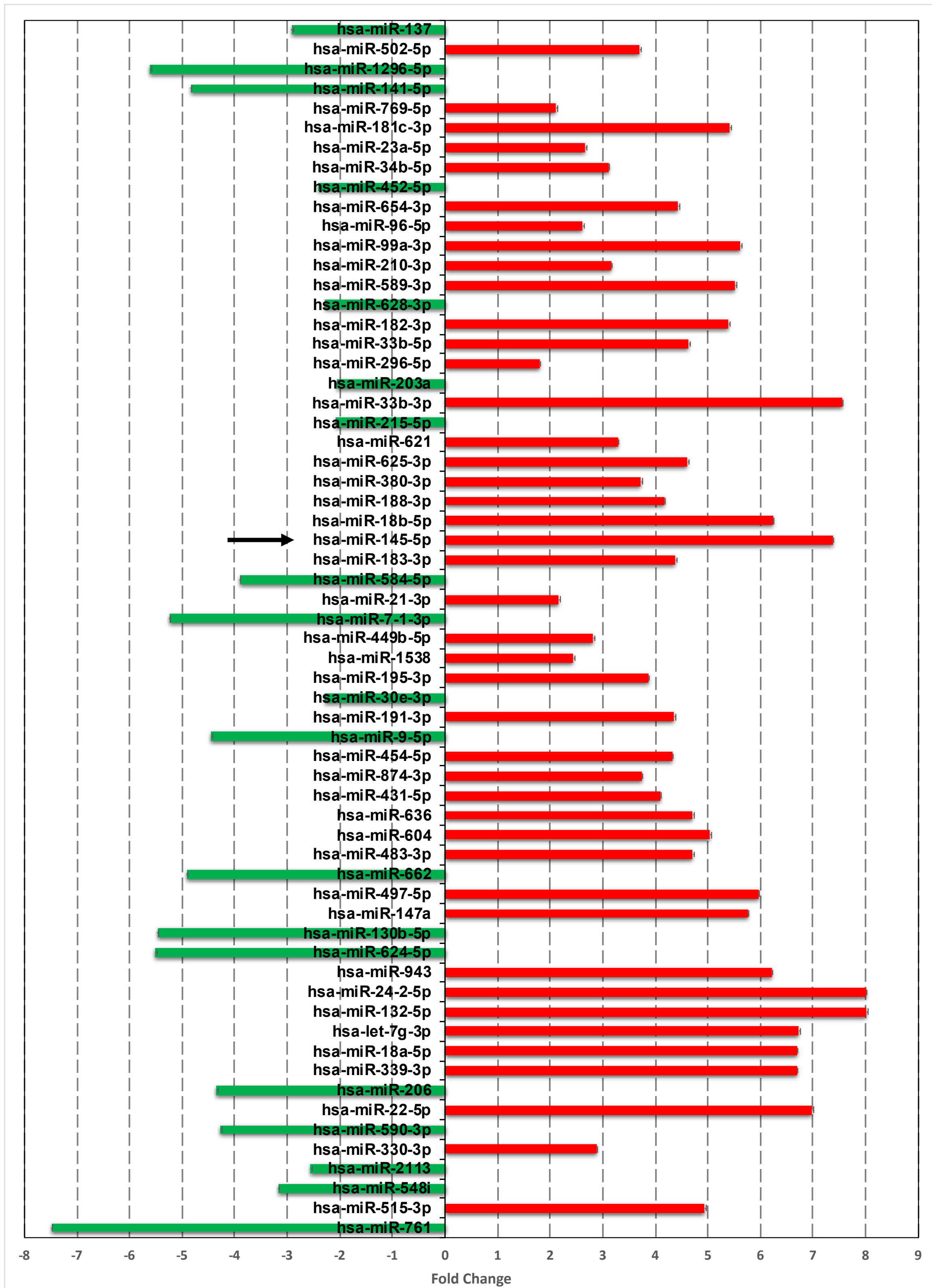
(A) 5-aza-CdR did not block TGF-β1-mediated CFTR mRNA suppression, suggesting that non-coding RNA-mediated transcriptional gene silencing is not involved. N = 3 experiments from different lungs. **(B)** NHBE ALI cultures were treated with recombinant TGF-β1 (10 ng/mL) or vehicle (as control). Transcriptional initiation from the CFTR promoter was determined in real-time by ChIP. The amount of immunoprecipitated DNA was quantified by qPCR using primers designed to hybridize 150 bp downstream of the CFTR promoter. TGF-β1 treatment does not affect transcription initiation from the CFTR promoter. n = 3 experiments from different lungs. **(C)** CFTR function was determined in Ussing chambers (ΔISC), and CFTR mRNA from TGF-β1- or TGF-β1 plus ATA-treated cells was analyzed by qRT-PCR. TGF-b suppresses CFTR function (p = 0.0194) ATA completely rescued the TGF-β-mediated CFTR function (p = 0.0464). The table below the graph shows actual ΔISC values for the experiment. **(D)** Ussing chamber trace for TGF-β1 treatment and its rescue by ATA. **(E)** Total RNA from these cells shows the concomitant

rescue of CFTR RNA (Vehicle v/s TGf-β1 $p = 0.0145$; TGF-β1 v/s TGF-β1 + ATA $p = 0.0483$). $n = 4$ experiments from different lungs. *Significant ($p < 0.05$) from control.

4.3.2. TGF-β alters the bronchial epithelial microRNAome affecting diverse cellular pathways

NHBE ALI cultures were treated with TGF-β1 as reported by us earlier [6]. Change in miRNA expression profile was determined using the Exiqon microRNA Human panel I and II, V4 (Exiqon, Woburn, MA, U.S.). This allows us to assay 739 mature human microRNAs. Data were analyzed using the GenEx software (Qiagen). **Figure 2A** shows the volcano plot of the TGF-β miRNA array in NHBE ALI cultures. TGF-β significantly alters the microRNAome of NHBE ALI cultures with a statistically significant change ($p < 0.05$) in the expression of 70 different miRNAs, including our previously reported miR-141-5p [413]. Analysis of the altered microRNAome shows that TGF-β upregulates the expression of forty-nine different miRNAs (~2-fold or higher) and downregulates the expression of twenty-one miRNAs (~2-fold or higher). Thirty-five miRNAs demonstrate a 2 to 5-fold change in expression, and twenty-two miRNAs show over a 5-fold change in expression (**Figure 2B**). Gene network analysis using Ingenuity Systems Pathway Analysis web-based software application (Qiagen) on the effects of dysregulation of these miRNAs shows that TGF-β1 treatment can alter signaling of several different pathways involved in lung diseases.


A
-Log10(P-Value)
log2(Fold change)
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-10
-8
-6
-4
-2
0
2
4
6
8



hsa-miR-137
hsa-miR-502-5p
hsa-miR-1296-5p
hsa-miR-141-5p
hsa-miR-769-5p
hsa-miR-181c-3p
hsa-miR-23a-5p
hsa-miR-34b-5p
hsa-miR-452-5p
hsa-miR-654-3p
hsa-miR-96-5p
hsa-miR-99a-3p
hsa-miR-210-3p
hsa-miR-589-3p
hsa-miR-628-3p
hsa-miR-182-3p
hsa-miR-33b-5p
hsa-miR-296-5p
hsa-miR-203a
hsa-miR-33b-3p
hsa-miR-215-5p
hsa-miR-621
hsa-miR-625-3p
hsa-miR-380-3p
hsa-miR-188-3p
hsa-miR-18b-5p
hsa-miR-145-5p
hsa-miR-183-3p
hsa-miR-584-5p
hsa-miR-21-3p
hsa-miR-7-1-3p
hsa-miR-449b-5p
hsa-miR-1538
hsa-miR-195-3p
hsa-miR-30e-3p
hsa-miR-191-3p
hsa-miR-9-5p
hsa-miR-454-5p
hsa-miR-874-3p
hsa-miR-431-5p
hsa-miR-636
hsa-miR-604
hsa-miR-483-3p
hsa-miR-662
hsa-miR-497-5p
hsa-miR-147a
hsa-miR-130b-5p
hsa-miR-624-5p
hsa-miR-943
hsa-miR-24-2-5p
hsa-miR-132-5p
hsa-let-7g-3p
hsa-miR-18a-5p
hsa-miR-339-3p
hsa-miR-206
hsa-miR-22-5p
hsa-miR-590-3p
hsa-miR-330-3p
hsa-miR-2113
hsa-miR-548i
hsa-miR-515-3p
hsa-miR-761
-8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9
Fold Change

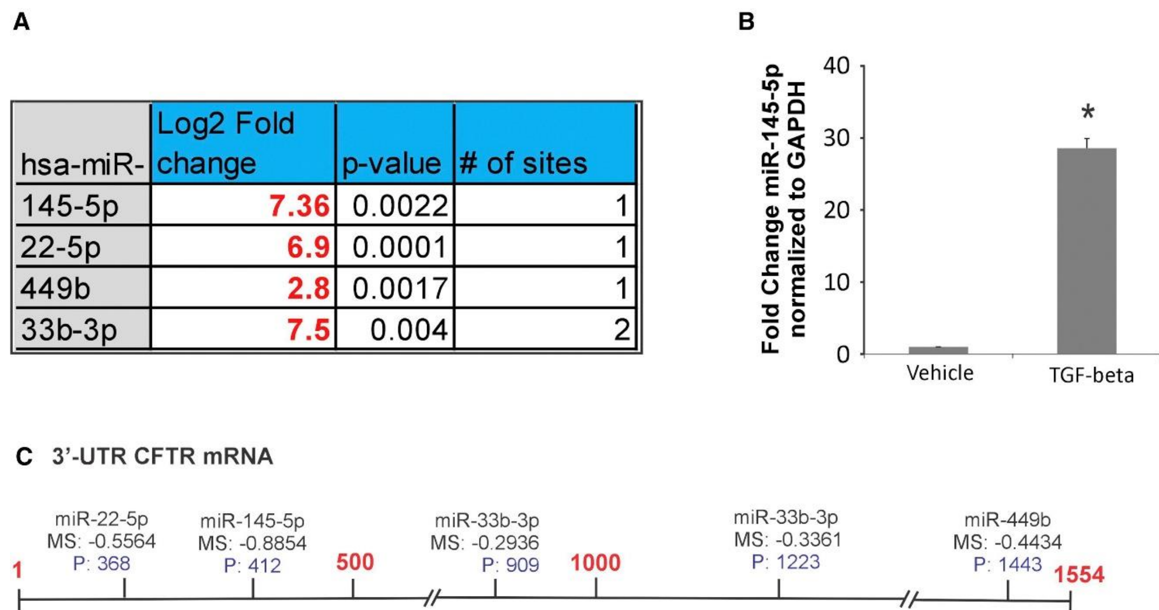
Figure 11: TGF-β1 Alters the Bronchial Epithelial MicroRNAome.
(A) Volcano plot of the altered miRNA expression profile of primary NHBE ALI cultures treated with TGF-β1. Primary bronchial epithelial cultures redifferentiated at the ALI were treated with TGF-β1 (10 ng/mL) or vehicle (as control) as described by us earlier [6]. TGF-β-mediated changes in miRNA expression profile in NHBE ALI cultures were determined using Exiqon microRNA Human panel I and II, v.4 (Exiqon, Woburn, MA, USA). Data were analyzed using the GenEX software. **(B)** TGF-β1 shows a statistically significant ($p < 0.05$) Log2-fold change in the expression of 62 miRNAs. Green, downregulated miRNAs; red, upregulated miRNAs. $n = 3$ different lungs. Arrow indicates the position of miR-145-5p.

4.3.3. TGF-β suppresses CFTR via miR145-5p mediated silencing of CFTR

In airway epithelial cells, CFTR can be regulated by at least three different microRNAs [196]. MiR-145 has been experimentally validated to suppress CFTR function, and its upregulation by TGF-β signaling has been demonstrated in lung myofibroblasts [414]. More recently, Lutful et al. have demonstrated that miR-145 antagonism reverses TGF-β mediated Inhibition of F508del CFTR Correction in Airway Epithelia [415]. We first validated our array data to determine the upregulation of miR-145-5p. NHBE cultures re-differentiated at the ALI were treated with TGF-β and expression of miR-145-5p was validated using specific probes (Taqman). **Figure 3A** shows that TGF-β treatment upregulates miR-145-5p expression. Using a combination of Target site prediction algorithm miRanda, miRSVR scores; (< -0.5), and miRTARbase (a database of experimentally validated miRNAs), we identified 4 different miRNAs known to suppress CFTR function **(Figure 3B)**. **Figure 3C** shows the schematic of the putative target sites of the identified miRNAs on the 3’ UTR of CFTR mRNA.

In order to determine if the miRNAs identified by us in **Figure 3B** suppress CFTR mRNA, we transfected miRNA mimics in BEAS2B airway epithelial cells and looked for

CFTR mRNA suppression. We preferred BEAS2B cells for transfection experiments because BEAS2B cells are easier to transfect while also demonstrating CFTR expression. Moreover, the RNAi machinery is identical for all cells, including NHBE ALI cultures, and hence using BEAS-2B cell lines will not affect our observations. BEAS2B cells were transfected with respective miRNA mimics (20nM) (Mission microRNA mimics; Sigma) of miRNAs identified in **Figure 3B**. Lipofectamine alone was used as control. TGF-β treatment was used for comparison.



hsa-miR-	Log2 Fold change	p-value	# of sites
145-5p	7.36	0.0022	1
22-5p	6.9	0.0001	1
449b	2.8	0.0017	1
33b-3p	7.5	0.004	2

Figure 12: Identification of Potential CFTR-Targeting miRNAs Induced by TGF-β1
(A) miRNAs targeting CFTR were identified by a combination of target site prediction algorithm miRanda (miRSVR scores <−0.5) and miRTARbase (database of experimentally validated miRNAs). miRNAs are listed in order of their scores, starting with the best. **(B)** Array validation for miR-145-5p. NHBE ALI cultures were treated with TGF-β1 (10 ng/mL) or vehicle (as control). Total RNA was analyzed for miR-145-5p expression using qRT-PCR and normalized to GAPDH. TGF-β1 treatment upregulates miR-145-5p in NHBE ALI cultures. n = 3 different lungs. *Significant ($p < 0.05$) from control. **(C)** Schematic of the putative target sites of the identified miRNAs on the 3′ UTR of CFTR mRNA.

As seen in **Figure 4A**, only miR-145-5p mimic suppressed CFTR mRNA. Since no suppression is observed by other miRNAs, we tried to determine if any miRNAs work in a cooperative manner with miR-145-5p. We made four pools of three miRNAs each. BEAS2B cells were transfected with these miRNA pools to determine if increased or synergistic suppression is observed by the pooled miRNAs. miR-145-5p was transfected for comparison. The total miRNA mimic concentration for each pool was 20nM. As seen in **Figure 4B**, only the pool that had miR-145-5p showed a small suppression of CFTR, suggesting that the suppression by the pool was because of miR-145-5p and not any other miRNA. The pool in which miR-145-5p was excluded did not show any CFTR suppression. We further tried to confirm the role of miR-145-5p in CFTR suppression in attempting to determine if antagonizing miR-145-5p can rescue TGF-β mediated CFTR suppression. BEAS2B cells were transfected with an antagomiR to miR-145-5p (IDT technologies). Separately, another set of antagomiR transfected cells was treated with TGF-β1. Lipofectamine alone and TGF-β plus lipofectamine alone treated cells were used for comparison. As seen in **Figure 4C**, antagomiR to miR-145-5p rescues TGF-β mediated CFTR mRNA suppression confirming its role in TGF-β mediated CFTR suppression.

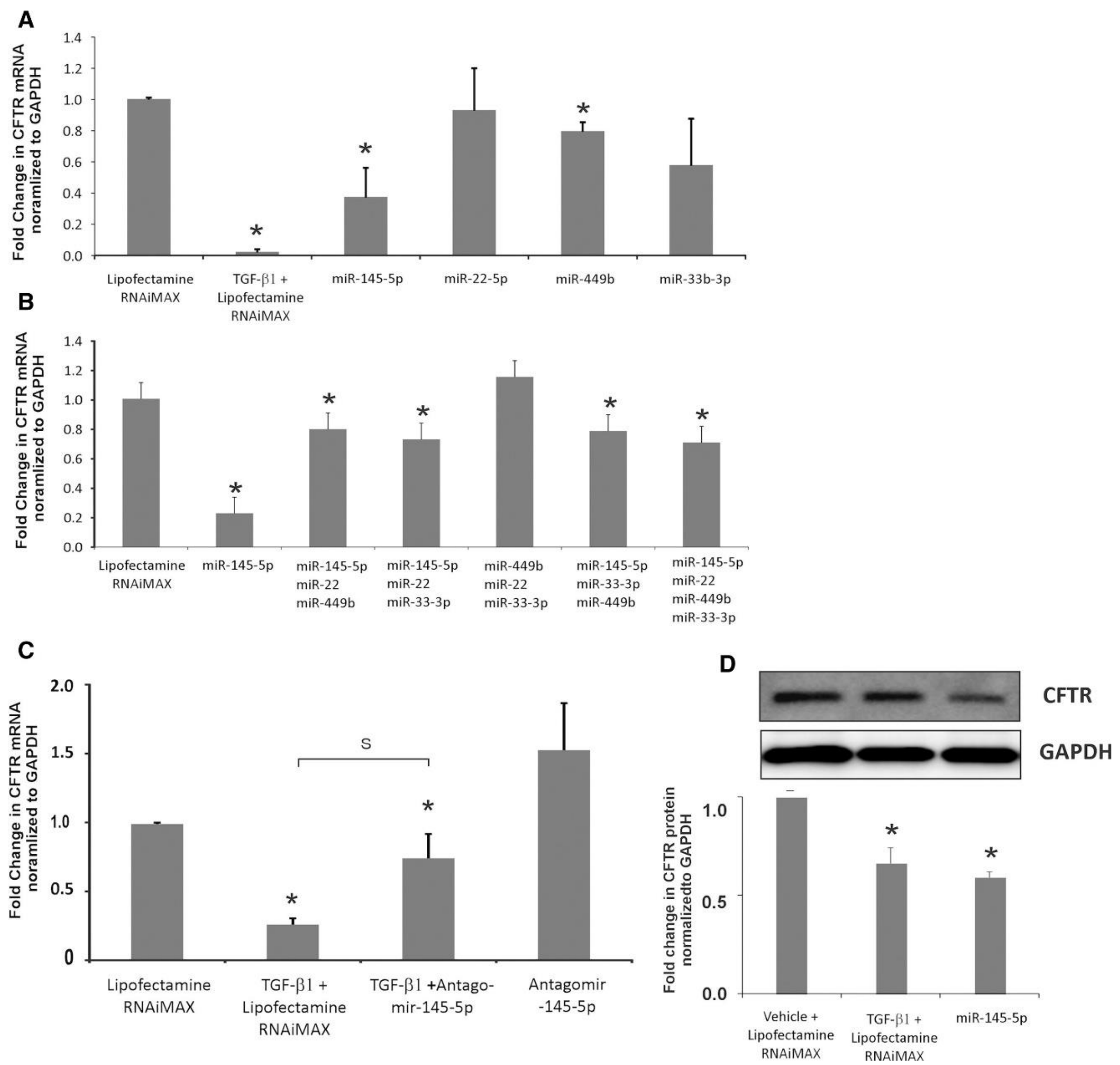


Figure 13: miRNA Validation.
(A) miRNA mimics for the 4 miRNAs identified in Figure 3B were tested for their ability to suppress CFTR mRNA by transient transfection in the airway epithelial cell line BEAS-2B. Lipofectamine RNAiMAX plus the vehicle was used as the control, and lipofectamine RNAiMAX plus TGF-β1 treatment was used for comparison. Only miR-145-5p mimic suppressed CFTR mRNA ($p = 0.005$). A minimal suppression (~10%) was observed with miR-449b ($p = 0.0001$). Some suppression was also observed with miR-33b-3p, but this was not found to be statistically significant. $n = 5$ different experiments. **(B)** To check for the cooperative effects of miRNA, the miRNA mimics were pooled into 4 pools of 3 miRNAs each (Figure 3B). BEAS-2B cells were transfected with each of the pools. Lipofectamine RNAiMAX alone was used as the control, and lipofectamine RNAiMAX and TGF-β1-treated cells and miR-145-5p-transfected cells were used for comparison.

Only the pools containing miR-145-5p showed some suppression of CFTR mRNA ($p = 0.0233$), and the suppression was not comparable to that observed for miR-145-5p, suggesting that the suppression by the pool was due to the presence of miR-145-5p and not any cooperative effects. $n = 5$ different experiments. (C) BEAS-2B cells were transfected with antagomir to miR-145-5p. Lipofectamine RNAiMAX plus the vehicle was used as the control, and lipofectamine RNAiMAX plus TGF-β1 was used for comparison. At 24 hr post-transfection, cells were treated with TGF-β1 (lipofectamine RNAiMAX and TGF-β alone with lipofectamine RNAiMAX for comparison). Antagomir-145-5p rescues TGF-β1-mediated CFTR suppression ($p = 0.0157$), confirming its role in TGF-β-mediated CFTR mRNA suppression. $n = 5$ different experiments. (D) BEAS-2B cells were transfected with miR-145-5p, as described. TGF-β1 and lipofectamine RNAiMAX-treated cells were used for comparison. miR-145-5p transfection suppresses CFTR protein levels comparable to that observed with TGF-β1-treated cells ($p = 0.0179$). We observed a ~70kd CFTR band that corresponds to either the isoform 3 generated as a result of alternative splicing or as a consequence of Calpain based processing of CFTR at the membrane [214]. *Significant ($p < 0.05$) from control. S, significant from each other ($p < 0.05$).

4.3.4. TGF-β suppresses SLC26A9 via miR-145-5p mediated gene silencing

miR-145-5p is an extensively studied miRNA and plays an important role as a tumor suppressor. Its expression is regulated by multiple signaling pathways [416]. We tried to determine if miR-145-5p can alter the expression of other genes involved in CFTR expression and function. Using miRanda and miRSVR algorithms for target identification, we identified SLC26A9, a constitutively active Cl- channel [417] as a potential target of miR-145-5p (miRSVR score of -0.99). SLC26A9 plays an important role in CFTR biogenesis and function. It stimulates CFTR expression and function [401] and also modulates airway response to CFTR-directed therapeutics [403]. Likewise, defects in CFTR protein inhibit the function of SLC26A9 [418]. The mechanism of SLC26A9 regulation is poorly understood, with one report suggesting that WNK kinases inhibit expression of SLC26A9 [419]. However, none of the reports have demonstrated TGF-β mediated suppression of the role of miRNA in the regulation of SLC26A9. Since TGF-β

upregulates miR-145-5p, we tried to determine if TGF-β also suppresses SLC26A9. NHBE ALI cultures were treated with TGF-β and total RNA was analyzed for SLC26A9 expression by qRT-PCR. As seen in **Figure 5A**, TGF-β suppresses expression of SLC26A9. Next, we tried to determine if SLC26A9 is a target of miR-145-5p. BEAS2B cells were transfected with mimic or an antagomiR to miR-145-5p (IDT technologies). Twenty-four hours post-transfection, antagomiR transfected cells was treated with TGF-β1. Lipofectamine alone and TGF-β plus lipofectamine alone treated cells were used for comparison. Experiments were terminated after a further 16 hours and total RNA was analyzed for SLC26A9 expression using specific Taqman probes. As seen in **Figure 5B**, transfection of miR-145-5p mimic suppressed SLC26A9. miR-145-5p antagonism also rescues SLC26A9 in TGF-β treated BEAS2B cells confirming the TGF-β ⇒ miR145-5p ⇒ SLC26A9 pathway (**Figure 5C**).

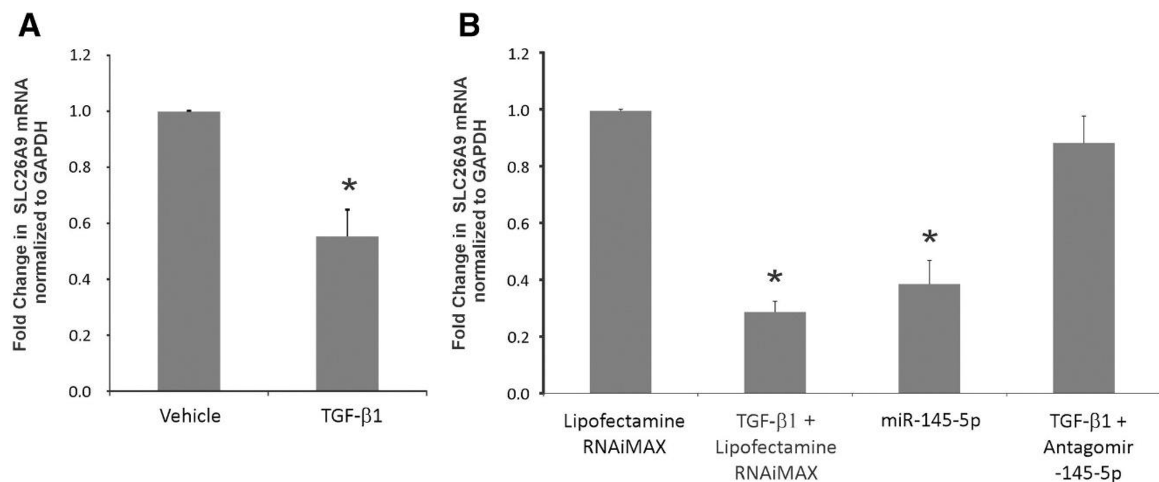


Figure 14: TGF-β suppresses the Expression of SLC26A9 by miR-145-5p-Mediated Silencing
(A) NHBE cultures redifferentiated at the ALI were treated with TGF-β (10 ng/mL) apically and basolaterally. 16 hr post-treatment, total RNA was isolated, and SLC26A9 mRNA levels were determined. TGF-β signaling suppresses the expression of SLC26A9

($p = 0.0015$). n = 3 different lungs. **(B)** To determine the role of miR-145-5p in this suppression, BEAS-2B cells were transfected with miR-145-5p mimic. Lipofectamine RNAiMAX alone was used as the control. Another subset of cells was transfected with antagomir to miR-145-5p. 24 hr post-transfection, cells were treated with TGF-β1 (lipofectamine RNAiMAX and TGF-β alone for comparison). miR-145-5p mimic suppresses SLC26A9 expression ($p = 0.0362$) while antagomir-145-5p rescues TGF-β-mediated SLC26A9 suppression, confirming the role of miR-145-5p in TGF-β-mediated SLC26A9 mRNA suppression. n = 5 experiments. *Significant ($p < 0.05$) from control.

4.3.5. Non-CF A/J mice exposed to C.S. suppress CFTR mRNA with a concomitant increase in the miR-145-5p expression

C.S. exposure leads to an acquired state of CFTR dysfunction in smokers with normal copies of the CFTR gene [54, 55]. A number of reports have demonstrated that C.S. upregulates TGF-β signaling in airway epithelial cells [6, 420, 421]. We have demonstrated that C.S. suppresses CFTR expression and function via TGF-β signaling [6]. We have also shown that chronic smoke exposure leads to CFTR suppression in non-CF A/J [63]. We tried to determine if CS also upregulates miR-145-5p as a means to suppression of CFTR and SLC26A9. Non-CF A/J mice were exposed to air or cigarette smoke. Following 5 weeks of smoke exposure, mice were sacrificed, and total RNA was isolated from lungs and analyzed for CFTR, SLC26A9, and miR-145-5p expression. As seen in **Figure 6**, cigarette some upregulate miR-145-5p **(Figure 6A)** with concomitant suppression of CFTR **(Figure 6B)**. The upregulation of miR-145-5p also translates to suppression of SLC26A9 in smoke-exposed mice **(Figure 6C)**.

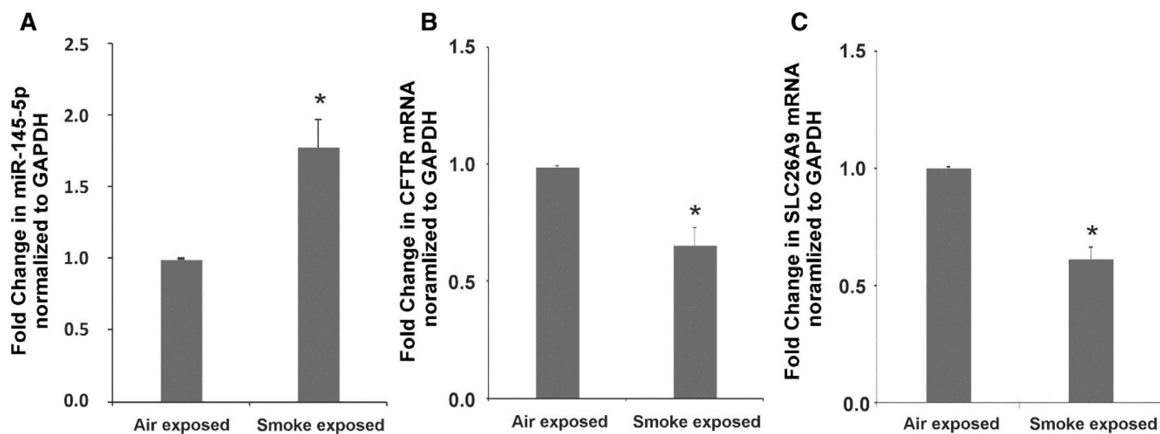


Figure 15: Cigarette Smoke Upregulates miR-145-5p with Concomitant Suppression of CFTR and SLC26A9 mRNA in Mouse Models of Acquired CFTR Dysfunction. **(A)** Non-CF A/J mice were exposed to cigarette smoke for 5 weeks. Mice exposed to room air were used as controls. Mice were sacrificed, and lungs were obtained. Total RNA was isolated from the lungs and analyzed for miR-145-5p. Smoke exposure upregulates miR-145-5p in mouse lung samples ($p = 0.0013$). **(B and C)** Total RNA was also analyzed for CFTR and SLC26A9 expression using specific TaqMan probes designed to amplify mouse genes. Cigarette smoke exposure decreases mRNA levels of CFTR ($p = 0.0009$) (B) and SLC26A9 ($p = 0.0001$) (C). Values were plotted as fold change normalized to GAPDH. n = 8 mice per group. *Significant ($p < 0.05$) from air-exposed mice.

4.3.6. Neutralizing aptamer to Transforming growth factor-beta receptor-2 (TGFBR2) rescues CFTR suppression in NHBE ALI cultures exposed to cigarette smoke or treated with TGF-β1

Transforming growth factor-beta receptor-2 (TGFBR2) is one of the experimentally validated targets of miR-145-5p [422, 423]. Hence upregulation of miR-145-5p should suppress TGFBR2. To determine if this is the case, NHBE cultures redifferentiated at the ALI were treated with TGF-β1 (10 ng/mL) apically and basolaterally. At 16 hr post-treatment, total RNA was isolated, and TGFBR2 mRNA levels determined by qRT-PCR. As seen in **Figure 7A**, TGF-β1 suppresses TGFBR2 levels in NHBE ALI cultures.

To determine if TGFBR2 is a target of miR-145-5p mimic, BEAS-2B airway epithelial cell lines were transfected with miR-145-5p mimic (lipofectamine RNAiMAX as the control). Transfection with miR-145-5p mimics likewise suppressed TGFBR2 mRNA in BEAS-2B cells **(Figure 7B)**. However, western blot analyses showed that significant levels of TGFBR2 protein were still available to mediate TGF-β signaling **(Figure 7C)**. This could explain continued CFTR suppression even when TGFBR2 levels were downregulated in the context of increased miR-145-5p. Hence, neutralizing TGFBR2 at the surface can be considered a good strategy in airway diseases where TGF-β signaling (and miR-145-5p) is upregulated, as this can synergize with miR-145-5p-mediated suppression of TGFBR2 to maximize CFTR rescue.

In our previous work, we have demonstrated that a TGFBR2 neutralizing antibody rescues CFTR function in NHBE ALI cultures [6]. We tried to rescue CFTR mRNA suppression in TGF-β treated and smoke-exposed cells using a neutralizing aptamer reported by Zhu et al. [424]. Aptamers are high affinity single-stranded nucleic acid ligands that exhibit specificity and avidity comparable to, or exceeding that of antibodies, and can be generated against most targets [370, 371]. NHBE cultures re-differentiated at the ALI were pre-treated with recombinant TGF-β or vehicle as described by us [6]. Separately, another subset of cells was pretreated with anti-TGFBR2 neutralizing aptamer reported by Zhu et al. [424] (scrambled aptamer (SCR) as control). TGFBR2 neutralizing antibody was used for comparison. Sixteen hours following TGF-β1 treatment, Total RNA was analyzed for CFTR mRNA levels by qRT-PCR. As seen in **Figure 7D**, anti-TGFBR2 aptamer rescues TGF-β mediated CFTR mRNA suppression comparable to that observed by TGFBR2 neutralizing antibody.

To determine if the aptamer can rescue the effects of cigarette smoke on CFTR expression, NHBE ALI cultures were exposed to cigarette smoke as described by us earlier [6]. Separately another set was pre-treated with anti-TGFBR2 neutralizing aptamer (or scrambled aptamer (SCR) as control) three hours prior to smoke exposure and was retained for the remainder of the experiment. Sixteen hours following smoke exposure, Total RNA was analyzed for CFTR mRNA levels by qRT-PCR. As seen in **Figure 7E**, TGFBR2 aptamer rescues cigarette smoke mediated suppression on CFTR mRNA comparable to that observed with the TGFBR2 neutralizing antibody.

Finally, we tried to determine if mRNA rescue by the aptamer translates to a functional rescue, NHBE ALI cultures grown on snapwells were similarly exposed to cigarette smoke in presence or absence of anti-TGFBR2 aptamer. Cells were mounted in Ussing chamber, and CFTR function was determined by the addition of albuterol as reported by us earlier [6, 338]. As seen in **Figure 7F**, anti-TGFBR2 aptamer significantly rescues the effects of cigarette smoke on CFTR function. **Figure 7G** shows an Ussing chamber trace of the rescue of CFTR function by anti-TGFBR2 aptamer in smoke-exposed NHBE ALI cultures grown on snapwells.

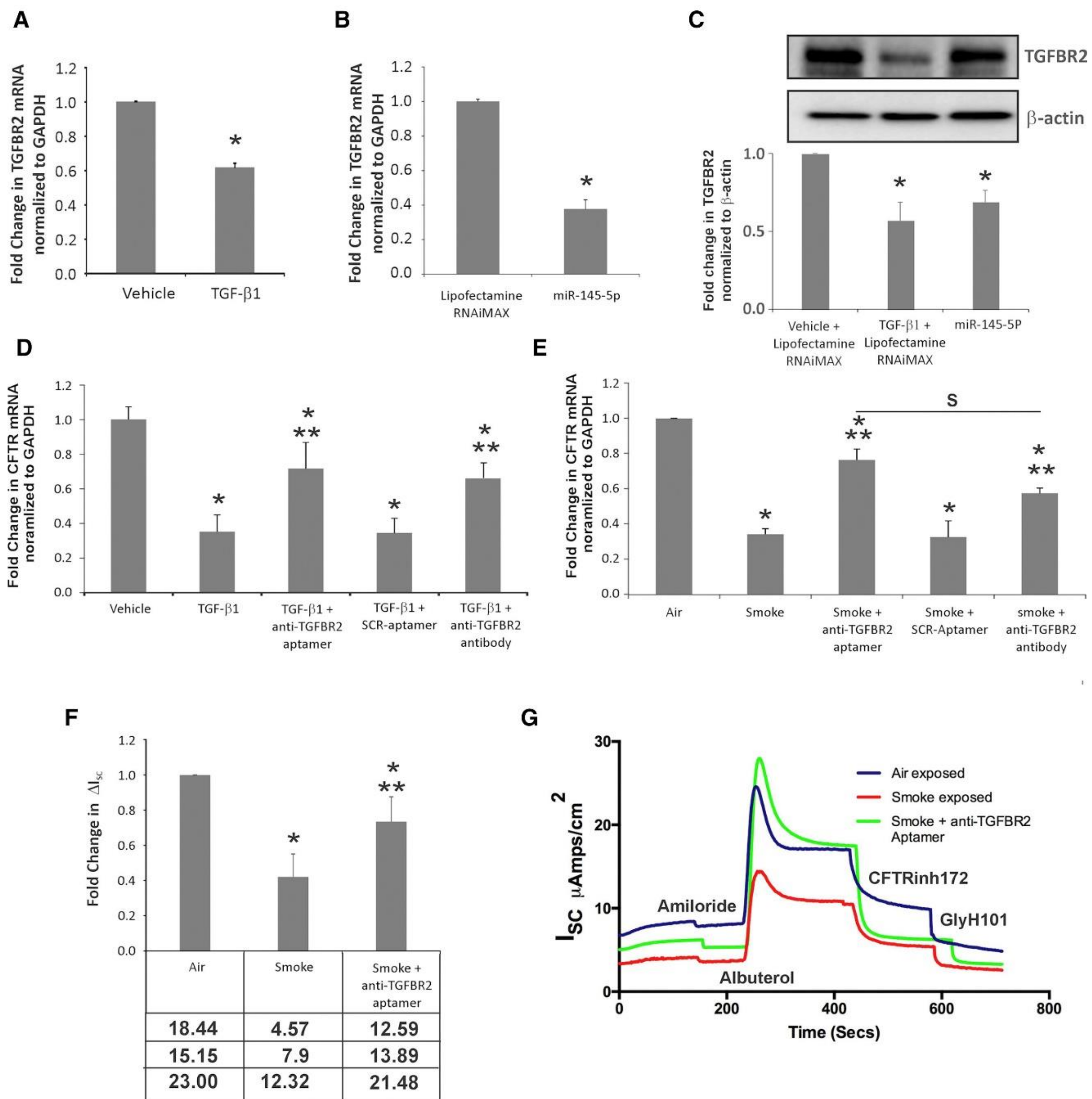


Figure 16: Anti-TGFBR2 Aptamer Rescues TGF-β- and Cigarette Smoke-Mediated CFTR Suppression.

(A) TGF-β signaling suppresses TGFBR2 expression in NHBE ALI cultures. 16 hr post-TGF-β1 treatment, total RNA was isolated, and TGFBR2 mRNA levels were determined. TGF-β1 signaling suppresses the expression of TGFBR2. **(B)** BEAS-2B cells were transfected with miR-145-5p mimic (lipofectamine RNAiMAX as control). BEAS-2B cells transfected with miR-145-5p mimic show suppression of TGFBR2 mRNA ($p = 0.0004$). **(C)** BEAS-2B cells were transfected with miR-145-5p mimic (lipofectamine RNAiMAX as control; TGF-β1 plus lipofectamine RNAiMAX for comparison). miR-145-5p suppresses TGFBR2 protein levels, as observed by western blot analyses ($p = 0.0179$). **(D)**

TGFBR2 aptamer rescues TGF-β-mediated suppression of CFTR function. NHBE ALI cultures were treated with TGF-β1 or vehicle. Separately, a subset of cells treated with TGF-β1 was pretreated with anti-TGFBR2 neutralizing aptamer reported by Zhu et al. [424], at a concentration of 2nmole (or scrambled aptamer [425] as the control) three hours prior to smoke exposure and was retained for the remainder of the experiment. TGFBR2-neutralizing antibody was used for comparison (25μg/mL based on [6]). Anti-TGFBR2 aptamer rescues TGF-β signaling-mediated CFTR mRNA suppression ($p = 0.0307$), possibly by blocking the receptor and preventing TGF-β signaling. **(E)** NHBE ALI cultures were exposed to cigarette smoke (or air as the control) using a smoke regimen described by us [413] Separately, a subset of cigarette smoke-exposed cells was treated with anti-TGFBR2 aptamer (or scrambled aptamer [425] as the control). TGFBR2-neutralizing antibody was used for comparison. Cigarette smoke suppresses CFTR ($p < 0.0001$). TGFBR2 aptamer rescued cigarette smoke effects on CFTR mRNA ($p = 0.0018$) comparable to that observed with the TGFBR2-neutralizing antibody. **(F and G)** NHBE ALI cultures grown on snapwells were exposed to cigarette smoke (or air as the control) using the smoke regimen described by us [413]. Separately, a subset of cigarette smoke-exposed cells was treated with anti-TGFBR2 aptamer. **(F)** CFTR function was determined in Ussing chambers (ΔISC). TGFBR2 aptamer rescues smoke-induced suppression of CFTR function ($p = 0.0225$), possibly by rescuing CFTR mRNA suppression. The table below the graph shows actual ΔISC values for the experiment. **(G)** An Ussing chamber trace for the anti-TGFBR2 aptamer-mediated rescue of CFTR function. n = 3 lungs *Significant from vehicle; **significant from TGF-β1 and/or CS exposed.

4.4. Discussion

Lung infections are significant comorbidity in smokers and COPD patients [33, 426], and tobacco smoking is the principal risk factor of streptococcal pneumonia in healthy young adults [427]. COPD and recurrent lung infections also increase the risk of lung cancer [428]. Cigarette smoke and COPD lead to impaired mucociliary clearance (MCC), thereby promoting microbial colonization and lung infections. The etiology of pneumonia associated with COPD and tobacco smoking is similar to that seen in cystic fibrosis [429, 430]. CFTR dysfunction (and MCC dysfunction) plays an important role in the early pathogenesis of these chronic airway diseases [8, 10]. CFTR tightly regulates the ASL height, which is crucial for mediating ciliary beating and MCC rates [4, 35], and its

dysfunction can compromise ASL and CBF, decreasing MCC rates [e.g., 160]. CFTR mediated $HCO3^-$ secretion maintains ASL pH [397, 398] and mucus viscosity [399] and regulates airway antimicrobial host defenses [431, 432]. CFTR plays an important antibacterial role in the innate immune response mediated by the lactoperoxidase-thiocyanate-H_2O_2 antibacterial system by secreting thiocyanate (SCN(-)[167, 168]). Decreased SCN(-) secretion in cystic fibrosis patients has been implicated in lung infections [433-435]. Hence CFTR suppression can have global effects on airway innate immunity by suppressing mucus clearance and antimicrobial responses, setting up a vicious cycle of infection, inflammation, and injury [58-60].

TGF-β signaling is increased in chronic airway diseases like chronic bronchitis, asthma, and COPD [106-109], and TGF-β levels correlate with the severity of obstruction [109, 110]. We have shown that TGF-β signaling is also induced by HIV Tat. TGF-β increases HIV infection of bronchial epithelial cells and also leads to CFTR suppression [338, 413]. TGF-β isoforms are expressed and secreted by several cell types in the airway, including epithelia [103]. We have already demonstrated that TGF-β and cigarette smoke (via TGF-β) signaling suppresses CFTR mRNA expression, which translates to a functional suppression.

In this study, we tried to determine the mechanism by which TGF-β suppresses CFTR mRNA and if targeting TGF-β signaling or the intermediates can rescue CFTR function in the context of cigarette smoke or TGF-β signaling. We first tried to determine if CFTR mRNA suppression is due to a decreased transcription from the CFTR promoter (transcriptional suppression) and the contribution of epigenetic silencing if any. Our data

demonstrated that TGF-β does not affect transcriptional initiation suggesting that the effect was not due to transcriptional suppression further positing the role of post-transcriptional silencing mechanisms. TGF-β signaling is known to alter miRNA homeostasis by directly acting on components of the miRNA processing pathway [24, 25]. CFTR also has a longer (1.5 kb) than average 3′ untranslated region (UTR), which strongly correlates with miRNA regulation [26]. Hence, we determine if TGF-β suppresses CFTR mRNA by miRNA mediated silencing. Our data demonstrate that Aurintricarboxylic acid, a small molecule inhibitor of the miRNA processing enzyme DROSHA, completely reverses the effects of TGF-β on CFTR mRNA with a concomitant rescue of CFTR function. We used the β_2-agonist albuterol to study the impact on CFTR activation as β_2-agonists are routinely prescribed during exacerbations or used as maintenance medications in chronic airway diseases. β_2-adrenergic receptor physically interacts with CFTR [436] and has been shown to activate mucociliary clearance [437] by activating CFTR as well as ciliary beat frequency [6, 438] suggesting that β_2-agonists can also serve in a dual role of increasing CFTR activity and mucociliary clearance.

Next, we performed a miRNA array to determine mature miRNAs dysregulated by TGF-β signaling. A miRNA array of NHBE ALI cultures treated with TGF-β demonstrates that TGF-β signaling significantly alters the microRNAome by dysregulating the expression of 62 different miRNAs (2-fold change) which can impact signaling of several different pathways involved in lung diseases. Specifically, we observed altered expression of four different miRNAs that can potentially regulate CFTR, including miR-145-5p reported by Gillen et al. [415] and [439]. Using multiple experiments involving mimics

individually and in pools, we confirmed that of the four, only miR-145-5p suppresses CFTR. We further confirmed the role of miR-145-5p using antagomirs to rescue TGF-β mediated CFTR mRNA suppression. We further confirmed the role of miR-145-5p using antagomir to miR-145-5p to rescue TGF-β-mediated CFTR mRNA suppression. Using miRNA-target algorithms, we predicted and then confirmed that miR-145-5p could also suppress SLC26A9.

None of the reports to date have identified miRNA mediated suppression of this very important CFTR modifying chloride channel. The deleterious effects of miR-145-5p on SLC26A9 can be 2-fold, considering that miR-145-5p directly regulates SLC26A9 while CFTR suppression by miR-145-5p could affect its localization to the surface [418]. Our experiments validate the role of miR-145-5p, and they also suggest a potential therapeutic approach to rescue CFTR and SLC26A9 suppression. Not surprisingly, our data confirm observations of other groups that miR-145-5p mimic (and TGF-β1 treatment in NHBE ALI cultures) suppresses TGFBR2 mRNA and protein levels. However, we were not able to detect this suppression in smoke-exposed mice (data not shown). This could be due to other signaling mechanisms induced by CS that may counter this suppression. However, most COPD patients have already quit smoking, but the chronic inflammation in the airways and the resultant TGF-β signaling would manifest as decreased TGFBR2 levels in airway epithelial cells.

Lutful et al., have suggested that antagomir to miR-145 rescues TGF-β mediated inhibition of F508del CFTR Correction in Airway Epithelia [415]. However, this approach can have several limitations in clinical application as miR-145-5p is a potent tumor suppressor in multiple cancers (for review see [416]), and inhibition of miR-145-5p can

have adverse effects. Moreover, any therapeutic effects of microRNA antagonism will require an uptake of antagomir by airway bronchial cells, which is difficult with current delivery mechanisms. A neutralizing antibody or an aptamer, on the other hand, acts on the cell surface to modulate TGF-β signaling in the airway is more clinically feasible. We have already demonstrated that the anti-TGFBR2 antibody reverses the effects of cigarette smoke on CFTR in NHBE ALI cultures. Our western blot data show that significant levels of receptor remain even after TGF-β1 treatment of miR-145-5p transfection.

We tested a nucleic acid-based aptamer to determine if this can have effects similar to that of the antibody. Aptamers are high affinity single-stranded nucleic acid ligands that exhibit specificity and avidity comparable to, or exceeding that of antibodies, and can be generated against most targets [370, 371]. Unlike antibodies, aptamers can be synthesized chemically, and hence offer significant advantages in terms of production cost, more straightforward regulatory approval and lower immunogenicity even when administered in preclinical doses 1000-fold higher than those necessary for animal and human therapeutic application [373, 374]. TGFBR2 neutralizing aptamer can cooperate with miR-145-5p mediated suppression of TGFBR2 expression by neutralizing the diminished receptor levels at the surface and synergistically rescue CFTR and SLC26A9 in smokers and chronic airway diseases. Our data show that the anti-TGFBR2 aptamer rescues the effects of TGF-β signaling and cigarette smoke mediated decrease in CFTR mRNA, and this rescues CFTR function in NHBE ALI cultures exposed to cigarette smoke. While we did not get a complete rescue of CFTR function, this could be due to the transient effects of cigarette smoke on surface CFTR independent of TGF-β signaling [10]. Aptamer-based therapeutics have several advantages over small molecule TGF-β inhibitors as the size and cation

selectivity of airway epithelial tight junctions [440, 441] will prevent transmigration across the epithelium and restrict TGF-β inhibition to the airway.

In conclusion, this study demonstrates that miR-145-5p plays a crucial role in TGF-β mediated suppression of CFTR and SLC26A9 mRNA. A neutralizing aptamer to TGFBR2 can reverse these effects to rescue the CFTR function. Rescue of CFTR function can restore mucociliary clearance and innate airway immunity, thereby decreasing the incidence of recurrent lung infections and disrupting the cycle of infection, inflammation, and MCC dysfunction.

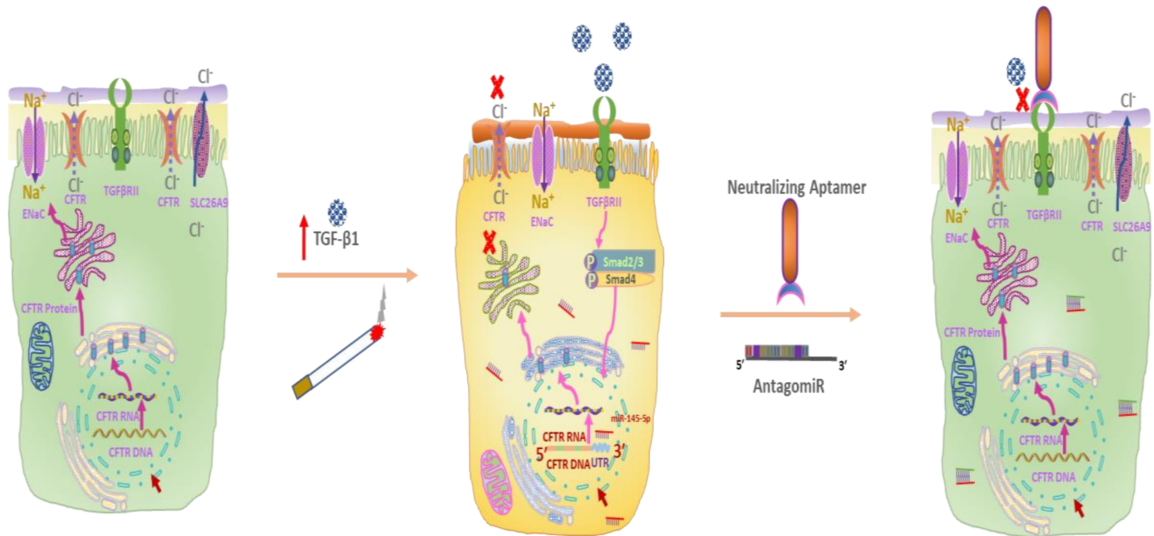


Figure 17: A Neutralizing Aptamer to TGFBR2 and miR-145 Antagonism Rescue Cigarette Smoke- And TGF-β-Mediated CFTR Expression.

CHAPTER 5: HIV TAT PROTEIN-INDUCED TGF-BETA SUPPRESSES CFTR BIOGENESIS AND ACTIVITY BY MICRORNA MEDIATED GENE SILENCING

5.1. Introduction

In aging HIV-infected populations, comorbid diseases are important determinants of morbidity and mortality. The advent of combination antiretroviral therapy (cART) has led to a dramatic decline in morbidity and mortality from HIV associated AIDS [442]. However, despite having progress on effective cART, the life expectancy of HIV patients is lower than the non-HIV patients, and non-AIDS associated comorbidities have continued to remain highly prevalent among people living with HIV [386]. The HIV care Continuum published by the CD shows that even with improved access to cART, that ~50% of people living with HIV are not virally suppressed [443]. Lungs can serve as anatomical reservoirs of HIV, and this has been demonstrated by the recovery of HIV from cell-free bronchoalveolar lavage fluid, alveolar macrophages, and intrapulmonary lymphocytes and suggesting that lungs constitute an anatomical HIV reservoir [444-446]. Lung diseases such as chronic obstructive pulmonary disease (COPD), pulmonary hypertension, pneumonia, Asthma, and Asthma COPD overlap syndrome (ACOS) are emerging as significant comorbidities in the HIV-infected population [447, 448]. COPD remains highly prevalent among these HIV associated lung comorbidities remains highly prevalent in HIV patients when compared to non-HIV infected adults [388, 449]. HIV infection act as a driving and independent risk factor for the development and progression of COPD, even when compensated for smoking [389, 450]. However, the molecular mechanism by which HIV infection promotes chronic inflammation predisposing to COPD is not clear [451]. In

our earlier work, we have shown that HIV infects the bronchial epithelium and suppresses components of the MCC apparatus [338].

Impaired mucociliary clearance (MCC) is a hallmark of chronic airway diseases like chronic obstructive pulmonary disease (COPD), cystic fibrosis, and chronic bronchitis associated with cigarette smoking [452, 453]. MCC is a primary innate defense mechanism of the airways and protects the host from airborne pathogens, pollutants, and allergens [2]. Optimal MCC requires mucus, cilia, and a thin layer of airway surface liquid (ASL) to facilitate ciliary beating [454]. Abnormalities in any of these components lead to MCC dysfunction promoting microbial colonization and chronic inflammation [455, 456]. The height of the ASL layer lining the airway surfaces is crucial for mediating MCC rates [35] and is tightly regulated by Cystic Fibrosis Transmembrane conductance regulator (CFTR) [4]. CFTR dysfunction can compromise ASL depth and CBF decreasing MCC rates [e.g., 39, 160]. HIV patients show abnormalities in their nasal MCC apparatus [112, 113]. Nasal epithelium can serve as a surrogate for tracheobronchial epithelium and has been used as a barometer of overall MCC health [8, 114, 115].

We have shown that HIV Tat and Cigarette Smoke (CS) suppresses CFTR via a common pathway involving TGF-β signaling [6, 338], and the suppression is additive [413]. This is especially important, given that 60% of HIV patients are addicted to nicotine and smoke tobacco [20]. While cART controls de novo infection and replication, Tat being an immediate-early gene of HIV continues to be expressed, and its expression is not suppressed by antiretroviral drugs [457, 458]. HIV Tat contains a protein transduction domain that can be secreted from infected cells and is taken up by bystander cells [459, 460]. Tat is released extracellularly and accumulates in tissues having pleiotropic effects

[120-122, 461]. In our earlier report, we demonstrated that TGF-β signaling alters the bronchial epithelial microRNAome with consequent suppression of CFTR. Specifically, TGF-β upregulates miR-145-5p to suppress CFTR. In this study, we show that HIV Tat also alters the bronchial epithelial microRNAome to upregulate miR-145-5p that functions co-operatively with miR-509 to suppress CFTR. We have demonstrated that a neutralizing aptamer to TGFBR2 and miR-145-5p antagonism rescues TGF-β mediated CFTR suppression. Given that miR-145-5p plays an important role as a tumor suppressor [416], we will explore a novel approach called gene-specific microRNA antagonism to preserve CFTR function in the context of HIV and cigarette smoke without blocking the entire TGF-β signaling pathway or interfering with the broader miRNA-mediated regulation of other genes.

5.2. Methods and materials

5.2.1. Chemicals, Reagents, and Materials

CFTR (inh)-172 (Sigma-Aldrich, Catalog# C2992) was dissolved in DMSO (Sigma-Aldrich, Catalog# D4540) to make a stock concentration of 20 mM. GlyH-101 (Sigma-Aldrich, Catalog# 219671) was dissolved in DMSO) to make a stock concentration of 50 mM. Amiloride hydrochloride hydrate (Sigma-Aldrich, Catalog# A7410) was dissolved in distilled H_2O (Invitrogen™, Catalog# 10977015) to make a stock concentration of 10 mM. Salbutamol hemisulfate salt (Sigma-Aldrich, Catalog# S5013) was dissolved in distilled H_2O to make a stock concentration of 10 mM. HEPES (Sigma-Aldrich, Catalog# H4034) was dissolved in Hank's Balanced Salt Solution (HBSS) (Gibco™, Catalog# 14025092) to make a stock concentration of 10 mM. Recombinant TGF-β1 (R&D Systems, Catalog# 240-B-002) was dissolved in sterile 4 mM HCl (Fisher

Chemical, Catalog# SA49) containing 1 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, Catalog# A7638) to prepare a stock concentration of 10 µg/µL. HIV-1 IIIB Tat Recombinant Protein (Catalog # 2222) was received from NIH AIDS Reagent Program and reconstituted in PBS containing 1 mg/ml BSA and 0.1 mM DTT to make the stock concentration to 10 µM. mirVana® miRNA mimics hsa-miR-145-5p (ThermoFisher Scientific, Assay ID: MC11480) and has-miR-509-3p (ThermoFisher Scientific, Assay ID: MC12984). mirVana® miRNA inhibitor: hsa-miR-145-5p (ThermoFisher Scientific, Assay ID: MH11480) and hsa-miR-509-3p (ThermoFisher Scientific, Assay ID: MC12984). Radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher Scientific, Catalog # 89901), Protease inhibitor cocktail (ThermoFisher Scientific, Catalog # 78410), Bio-Rad protein assay dye reagent (Bio-Rad, Catalog # 5000006), 4–20% Precast Bis-Tris acrylamide Gels (Bio-Rad, Catalog # 4561094), Immuno-Blot PVDF Membrane (Bio-Rad, Catalog #1620177), Blotting-Grade Blocker (Bio-Rad, catalog #1706404). Mouse primary monoclonal antibody against the R Domain of CFTR (the University of North Carolina, Cystic Fibrosis Center, Chapel Hill, and Catalog #570), Rabbit Anti-GAPDH primary antibody (Sigma Aldrich, Catalog #G9545); HRP conjugated anti-mouse IgG secondary antibody (Promega, Catalog#W4021) and anti-rabbit IgG secondary antibody (Promega, Catalog #W4011), SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, catalog # 34094). TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, catalog# 4366596). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, catalog# 4368814). TaqMan™ Fast Advanced Master Mix (Applied Biosystems, catalog# 4444964). Edit-R Cas9 expression plasmid (Dharmacon # U-005600-120). DharmaFECT Duo Transfection Reagent (Dharmacon, Catalog# T-2010-

02). PolyMag CRISPR Transfection Reagent (OZ Biosciences, Catalog# PNC40200), Super Magnetic plate 8 x 12 cm (OZ Biosciences, Catalog# MF10000), Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen™, Catalog# 13778075); Opti-MEM™ Reduced-Serum Medium (Gibco™, catalog# 31985062). BEAS-2B cells were cultured in BioLite 75 cm2 flasks (Thermo Scientific, Catalog# 12-556-010); BioLite 12-well plate (Thermo Scientific, Cat. #12-556-005). Cells were harvested using 0.25% Trypsin-EDTA (Gibco™, Catalog# 25200114) and Trypsin neutralizer solution (Gibco™, Catalog# R002100). NHBE cells were cultured on Snapwell™ Culture Inserts (Corning™, Catalog# 07-200-708) and Transwell™ Membrane Inserts (Corning™, Catalog# 07-200-261). Inserts were coated with Type IV collagen from the human placenta (Sigma-Aldrich, Catalog# C5533). microRNA Ready-to-Use PCR, Human panel I+II, V4.M (Exiqon, Catalog# 203615); Universal cDNA Synthesis Kit II (Exiqon, Catalog# 203301); ExiLENT SYBR® Green master mix (Exiqon, Catalog# 203421).

5.2.2. Cell culture and cell line

Human small airway epithelial cells will be obtained from the lungs provided by the University of Miami Life Alliance Organ Recovery Agency (LAORA). Lungs from organ donors that are compatible for transplant but released by the donor's family for research was obtained with minor de-identified information like age, sex, smoking status, HIV/HCV/EBV/CMV infection, COPD status (if any), pack-years (in case of smoker's lungs), etc. Primary bronchial epithelial cells were isolated as described by [404, 405] and adapted by us [6, 338, 406, 413]. The cells were obtained under an MTA where the University of Miami Pulmonary division obtains the lungs and then dissects and isolates primary human bronchial epithelial cells and provides to us for a nominal fee. The primary

cultures undergo mucociliary differentiation at the ALI, reproducing both the in vivo morphology and key physiologic processes to regenerate the native bronchial epithelium ex vivo [404, 405]. Since the cells were obtained from the deceased individual's lung with minor, de-identified information, its use does not constitute human subjects research as defined by CFR 46.102. Unless otherwise mentioned, we used lung cultures from the lungs of non-HIV non-smokers to not confound the findings in unknown ways. Normal human bronchial epithelial (NHBE) cells were expanded in T25 cell culture flasks coated with collagen I in bronchial epithelial growth medium (BEGM). 7-10 days later, the 350,000 NHBE cells per well were differentiated in collagen I-coated 12 mm Transwell or snap well clear permeable supports in an air-liquid interface (ALI) culture system. The ALI medium was supplemented with bovine pituitary extract (BPE, 0.1 mg/ml), recombinant epidermal growth factor (EGF, 0.5 ng/mL), insulin (5 µg/mL), hydrocortisone (0.5 µg/mL), epinephrine (0.6 µg/mL), penicillin (100 units/ml) and streptomycin (100 µg/ml), ethanolamine (500 nM), phosphoethanolamine (500 nM), 3,3,5-triiodothyronine (T_3, 6.5 ng/ml), transferrin (10 µg/ml) and 0.1 ng/ml retinoic acid. The ALI medium in the apical side was removed once the cells form a monolayer, and the medium in the basolateral area was changed every 2–3 days. The NHBE cells in Air-liquid interface (ALI) cultures were allowed to differentiate fully for at least 3 weeks prior to experiments.

The transformed bronchial epithelial cell line, BEAS-2B, expressing endogenous CFTR was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in BEGM basal medium. The following chemicals and reagents were dissolved in LHC media to prepare BEGM media; bovine pituitary extract (BPE, 0.1 mg/ml), recombinant epidermal growth factor (EGF, 25 ng/mL), insulin (5 µg/mL),

hydrocortisone (0.5 µg/mL), epinephrine (0.6 µg/mL), penicillin (100 units/ml) and streptomycin (100 µg/ml), Gentamicin (50 µg/mL), Amphotericin B (2.5 µg/mL), ethanolamine (500 nM), phosphoethanolamine (500 nM), 3,3,5-triiodothyronine (T3, 6.5 ng/ml), transferrin (10 µg/ml) and 0.1 ng/ml retinoic acid. For all experiments, BEAS-2B cells were grown in monolayer culture for 48 hr before treatment, a time at which CFTR expression is close to highest. All cells were grown at 37°C in a humidified 5% CO2 atmosphere.

5.2.3. miRNA array analysis

The Tat MicroRNAome was determined by Exiqon miRCURY-Ready-to-Use PCR-human-panel-I+II-V1.M (Exiqon miRNA qPCR panel). According to the manufacturer's protocol, total 20 to25 ng RNA extracted from heat-inactivated HIV Tat (as control) and biologically active HIV Tat (as treatment) treated NHBE cells were subjected for reverse transcription and converted to cDNA by using the miRCURY Locked Nucleic Acid (LNA) Universal Reverse Transcription (RT) microRNA PCR, polyadenylation, and cDNA Synthesis Kit II. Then, the resulting cDNA used for real-time PCR mixed with ExiLENT SYBR® Green master mix and loaded into Exiqon miRCURY-Ready-to-Use PCR-Human-panel-I + II-V1.M which could identify total 742 miRNAs in HIV Tat treated NHBE cells which were differentially expressed compared to the heat-inactivated HIV Tat (as control). The thermal cycling protocol involved denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 seconds, and 60 °C for 60 seconds. Each 384-well PCR plates were scanned on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Only the miRNAs with cycle threshold (Ct) value < 37 and5 Ct's less than the negative control (No Template Control, NTC) in the panel were processed using

the Exiqon GenEx qPCR analysis software version 6. The Ct values obtained from the different panels were adjusted by an Inter Plate Calibrator (IPC). An RNA spike-in control (UniSp6) was used as technical controls to evaluate if the technical performance of all samples was similar.

5.2.4. Western Blotting Analysis of CFTR Protein Expression

Proteins were extracted from BEAS-2B cells treated/transfected with HIV Tat protein (10 nM), mimics of miR-145-5p (25 nM), miR-509-3p (25 nM), antagomirs of miR-145-5p, miR-509-3p and synthetic guide RNA to target the miR-145-5p and miR-509-3p target site in the CFTR 3'UTR (25 nM). 48 hours post-treatment or post-transfection, cells were washed with PBS for two times and resuspended in radio-immunoprecipitation assay (RIPA) lysis and extraction buffer containing Halt proteinase inhibitor cocktail and centrifuged at 10,000 revolutions/min for 15 min at 4 °C. The concentration of protein in the total cell lysate was quantified with the Bio-Rad protein assay using GENESYS 10 Spectrophotometer. Equal volumes of whole-cell lysates (50 μg) were denatured at 100 °C for 5 minutes and loaded onto 4–20% precast polyacrylamide gel and ran at 100 V. Proteins were electro-transferred to PVDF membrane at 100 V for 60 min. Membranes were blocked with 10% non-fat dry milk for 1 h at room temperature. Then, the Membranes were probed with mouse monoclonal anti-CFTR antibody (at a dilution of 1:2500) and anti-GAPDH antibody (at a dilution of 1:2500) in 5% milk for overnight at 4 °C. Following day, the blots were washed with TBS-T and incubated with horseradish peroxidase (HRP) conjugated anti-rabbit (1:2500) and anti-mouse (1:2500) secondary antibodies for 1 hour at room temperature. The membrane blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate following

the manufacturer's protocol and were detected with ChemiDoc XRS+ System (Bio-Rad Laboratories, USA). Membranes were analyzed with densitometry using the Image Lab software, and the signal intensity of each band was normalized with GAPDH densitometry values.

5.2.5. Quantification of miRNA and mRNA expression by Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNAs were isolated from NHBE cells and BAES-2B cells treated/transfected with different treatments using an RNeasy mini kit (Qiagen Inc. Valencia, CA) according to the manufacturer's protocol. The purity and concentration of the extracted RNA were assessed (O.D. 260/280 nm absorbance ratio of at least 2.0) by a microspot RNA reader (Synergy HT Multi-Mode Microplate Reader from BioTek). 0.5 µg of total RNA was reverse transcribed (RT) to synthesize cDNA using high capacity cDNA reverse transcription kit as per the manufacturer's protocol after eliminating genomic DNA. Then, the relative amount of mRNA was quantified by using TaqMan Fast Advanced Master Mix as per the manufacturer's protocol in 20 µL real-time PCR reactions with gene-specific TaqMan primers using a BioRad CFX96 real-time system. All data were normalized to GAPDH mRNA and calculated as mean fold change in expression of the target gene using the comparative CT method.

For analyzing the expression of individual miRNA, the total RNA was subjected for reverse transcription by using the TaqMan MicroRNA Reverse Transcription Kit and the respective TaqMan primer according to the manufacturer's instructions through a two-step protocol to ensure high specificity and sensitivity. Each reverse transcription reaction contains 5-10 ng of total RNA, 10X reverse transcription buffer, 100 mM dNTPs, 20U/µL

of RNase inhibitor, 50U/µL of MultiScribe Reverse Transcriptase, and 3 µL of 5X miRNA-specific stem-loop primers (has-miR-145-5p and has-miR-509-3p), in a final volume of 15 µL, adjusted with nuclease-free water. The reaction parameter for reverse transcription was: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, followed by a holding step at 4°C. After that, each RT product was amplified using the TaqMan Universal Master Mix and the specific 20x TaqMan MicroRNA Assays to validate miRNA expression on the 7500 Real-Time PCR System (Software 2.01, Applied Biosystems, Foster City, CA, USA). Each qPCR reaction contains 1.33 µL of the RT product, 10 µL of TaqMan Universal Master Mix and 7.67 µL of Nuclease-free water up to a final volume of 20 µL. Reactions are run in a qPCR thermocycler set to perform the following protocol: 10 min at 95°C, 40 two-step cycles of 15 s at 95°C, and 1 min at 60°C.

Primers used for amplification were acquired from Applied Biosystems as follows GAPDH (Catalog# 4331182, Assay ID: Hs02786624_g1), CFTR (Catalog# 4331182, Assay ID: Hs00357011_m1), hsa-miR-509-3p (Catalog# 4427975, Assay ID: 002236), hsa-miR-145 (Catalog# 4427975, Assay ID: 002278).

5.2.6. Electrophysiological experiments by Ussing chamber

Changes in short circuit currents (ΔI_{SC}) in NHBE cells were measured with Ussing chambers (Physiologic Instruments, San Diego, CA) as previously described. Electrode offset and bath solution resistances were adjusted before starting the experiments. The apical and basolateral compartment was perfused in standard buffer solution (SBS) comprising Hank's Balanced Salt Solution (HBSS, pH 7.4) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Chambers and bathing solutions were kept at 37°C using heated water jackets and continuously gassed with a 95%

O_2 / 5% CO_2 mixture. Inserts with NHBE cells grown in the growth or differentiation medium were washed twice with HBSS buffer.

Fully differentiated NHBE cells cultured on the filters of snapwells were mounted in Ussing chambers (EasyMount chamber) connected to a VCC MC6 voltage-clamp unit (Physiologic Instruments, San Diego, CA). To monitor the short-circuit current (I_{SC}), the transepithelial membrane potential was clamped at 0 mV with a single-channel voltage-current clamp using Ag-AgCl electrodes in agar bridges. Following a short equilibration period, the baseline I_{SC} was recorded, followed by the addition of 10 μM of Amiloride apically to block epithelial sodium ion (Na^+) channel activity through ENaC and only keep the chloride ion (Cl^{-}) channel active. Subsequently, Albuterol ((10 μM) was added at the basolateral side to activate CFTR, and change in short circuit current (ΔI_{SC}) was determined as an indicator of CFTR activity. CFTR specificity was confirmed by the sequential addition of CFTR inhibitor $CFTR_{inh}$-172 (20 μM) and GlyH-101 (50 μM) to the apical bath solution. Signals were acquired with DAQplot software (Acquire and Analyze v. 2.3.300, Physiologic Instruments), and data were expressed by subtracting the Isc after CFTRinh-172 addition from the peak of albuterol-stimulated Isc calculated as the $CFTR_{inh}$-172 sensitive Isc.

5.2.7. Transfection of microRNA mimics and antagomirs in BEAS-2B cells

BEAS-2B cells were plated on collagen-coated 12- well tissue culture plates at a 70% confluence and allowed to adhere overnight. 24 hr following plating, BEAS-2B cells were transfected with miRNA mimics or antagomir to miR-145-5p and miR-509-3p using lipofectamine RNAiMAX. Before transfection, 25 nM of each mimic or antagomir individually or as pools mixed with Lipofectamine RNAiMax in Opti-MEM medium. The

mixtures were gently vortexed and kept at room temperature for 20 minutes before adding to the BEAS-2B cells to allow the formation of a better complex. Separately, another set of BEAS-2B cells were treated with equivalent amounts of lipofectamine RNAiMAX in Opti-MEM used as transfection control. Each mimic or antagomir specific complex was added directly to the cells in the 12-well plates in triplicate and overlaid with OptiMEM media. Furthermore, 4–6 h post-transfection, the media were removed and replaced with BEGM media containing antibiotic supplements. Cells were incubated at 37°C, 5% CO_2 for 24–48 h prior to harvesting.

5.2.8. Bioinformatics tools used for miRNA target sites in the CFTR gene

The role of miRNAs in the regulation of CFTR expression was performed using a computational approach, which predicted the putative seed regions in the 3′UTR of CFTR mRNA that are identified by a variety of miRNAs. The putative target sites for the miRNAs were predicted for CFTR mRNA using the online target prediction algorithms miRANDA (http://www.microrna.org/microrna/), TargetScan (http://targetscan.org), and miRTARbase, a database of experimentally validated miRNAs (http://mirtarbase.mbc.nctu.edu.tw/php/). The NCBI database https://www.ncbi.nlm.nih.gov was used to get information on the human CFTR gene, transcript (NCBI Reference Sequence: NM_000492.4). The miRBase database (http://www.mirbase.org) were used to get information on the location and sequence of has-miR-145-5p (MIMAT0000437) and has-miR-509-3p (MIMAT0002881).

5.2.9. CRISPR guide RNA design and screening

Two sets of gRNAs were designed as follows to target the 3' UTR of the CFTR gene using the specificity check feature of Dharmacon (Horizon inspired cell solutions;

CO) CRISPR design tool (https://dharmacon.horizondiscovery.com/gene-editing/crispr-cas9/crispr-design-tool/). sgRNA-410 to target position 410 (for the miR-145 site) and sgRNA-1281 to target position 1281 such that both miR-509-3p sites are within ~200 bp of the cleavage site. The specificity check feature excludes any sequences with two or fewer mismatch alignments anywhere else in the genome, thereby increasing the specificity of the gRNA and preventing off-target effects. The gRNAs were custom synthesized by Dharmacon.

5.2.10. CRISPR/Cas9 transfection in NHBE cells

In preparation for CRISPR/Cas9 transfection, NHBE cells were grown in a 6-well plate insert as a monolayer for three weeks for re-differentiation. The re-differentiated NHBE cells were transfected with Edit-R Cas9 expression plasmid (Dharmacon) with puromycin resistance using PolyMag CRISPR Transfection Reagent (OZ Biosciences), and DharmaFECT Duo Transfection Reagent recommended by the manufacturer protocols. Briefly, 200 nM of the synthetic guide RNAs (sgRNA-410/ sgRNA-1281 resuspended in 10 mM Tris–HCl pH7.5) was mixed with Dharmafect Duo Transfection Reagent (2 mg/mL) and diluted in 200 µL of the serum-free medium in one tube. In another tube, Edit-R Cas9 expression plasmid (4 µg/well) was diluted in 200 µL of serum-free medium and mixed with PolyMag CRISPR transfection reagent (2 µL/mL). The tube contained Cas9 expression plasmid, and PolyMag CRISPR transfection reagent was combined with the tube provided of sgRNAs and Dharmafect Duo Transfection Reagent. The transfection mixer was gently vortexed and incubated for 20 min at room temperature. Then, the transfection reagent mixture was added dropwise onto the cells in a single well of a six-well cell culture plate containing 1.6 mL of ALI media. The plate was then set on

the top of a plate magnet (Oz Biosciences) for 2 hours in the incubator. 48 hours following transfection, cells were treated with TGF- β1/HIV Tat. After an additional 16 hours, experiments were terminated, cells were trypsinized, and total RNA was analyzed for CFTR mRNA levels.

5.2.11. CRISPR/Cas9 transfection in BEAS-2B cells

In preparation for CRISPR/Cas9 transfection, BEAS-2B cells (~$0.5x10^6$/well) were seeded into a 12-well plate and incubated cells at 37°C with 5% CO_2 overnight. 250 ng/ µL of working solution Cas9 plasmid was prepared by adding Tris buffer. 25 µM synthetic sgRNA were prepared for Tris buffer. The plated BEAS-2B cells were transfected with synthetic guide RNAs (sgRNA-410/ sgRNA-1281; Dharmacon), and Edit-R Cas9 expression plasmid (Dharmacon) with puromycin resistance using DharmaFECT Duo Transfection Reagent (Dharmafect) recommended by the manufacturer protocols. Briefly, 100 nM of the synthetic guide RNAs were mixed with Dharmafect Duo Transfection Reagent (2 mg/mL) and diluted in 100 µL of the serum-free medium in one tube. In another tube, Edit-R Cas9 expression plasmid (2 µg/well) was diluted in 100 µL of serum-free medium. The tube contained Cas9 expression plasmid was mixed with the tube provided of sgRNAs and Dharmafect Duo Transfection Reagent. The transfection mixer was gently vortexed and incubated for 20 min at room temperature. Then, the transfection reagent mixture was added dropwise onto the cells in a single well of a twelve-well cell culture plate containing 800 µL of BEGM media. 48 hours following transfection, cells were treated with TGF- β1/HIV Tat. After an additional 16 hours, experiments were terminated, cells were trypsinized, and total RNA was analyzed for CFTR mRNA levels.

5.2.12. Statistical analysis

Unless otherwise mentioned**,** data were expressed as mean ± SEM from NHBE ALI cultures from at least three lungs. The data were subjected to statistical analysis using unpaired t-tests or ANOVA followed by Tukey Kramer honestly significant difference test for multiple comparisons as appropriate. The significance was considered at the level of $p<0.05$.

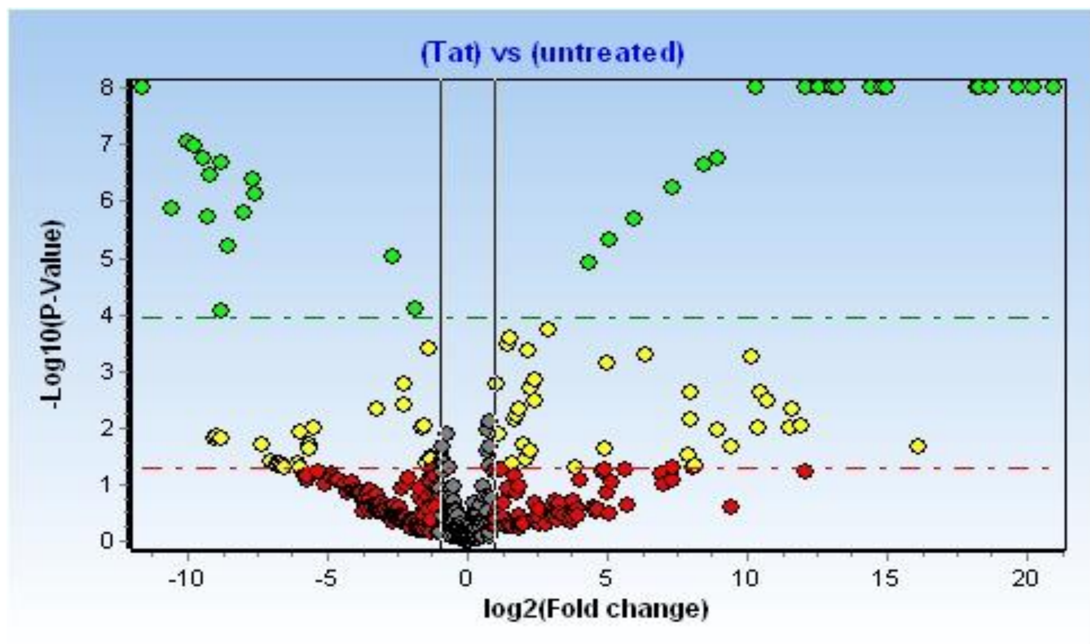
5.3. Results

5.3.1. HIV Tat alters the bronchial epithelial microRNAome to suppress CFTR

In our earlier study, we have shown that HIV Tat suppresses CFTR biogenesis and function via TGF-β signaling [17]. We have also demonstrated that TGF-β alters the bronchial epithelial microRNAome [27]. Specifically, TGF-β upregulates miR-145-5p to suppress CFTR [27]. We tried to determine the effects of Tat on the bronchial epithelial microRNAome. NHBE ALI cultures were treated with HIV Tat, as reported by us earlier [6]. Change in miRNA expression profile was determined using the Exiqon microRNA Human panel I and II, V4, and data were analyzed using the GenEX software. This allows us to assay 754 mature human microRNAs. We found that HIV Tat substantially alters the bronchial epithelial microRNAome. HIV Tat treatment shows statistically significant Log2-fold change ($p < 0.05$) in the expression of over 83 different miRNAs. Next, we compared these changes to those observed in TGF-β treated NHBE ALI cultures. Our data show that Tat and TGF-β1 upregulated or downregulated at least 8 common miRNAs, albeit to different magnitudes. An additional 5 microRNAs were either upregulated by Tat and suppressed by TGF-β or vice versa, thereby showing effects opposite to TGF-β. Tat

and TGF-β are both potent signaling molecules that can drive transcriptional activation of multiple genes [462, 463]. It is important to remember that while HIV Tat can upregulate TGF-β signaling possibly by binding to the TGF-β1 promoter and increasing its transcription, Tat is a transcriptional activator in its own right altering gene expressions [462]. Tat also directly interacts with components of the RNA interference apparatus, thereby altering mature miRNA processing [464]. Moreover, Tat can also alter or initiate cellular signaling pathways, which may alter miRNA expression and counter some of the activations or suppressions by TGF-β. Surprisingly, we found that while Tat, like TGF-β, upregulated miR145-5p, in addition, Tat also upregulated miR-509-3p known to modulate CFTR expression [465] which we had not observed with our TGF-β studies [27].

A



B

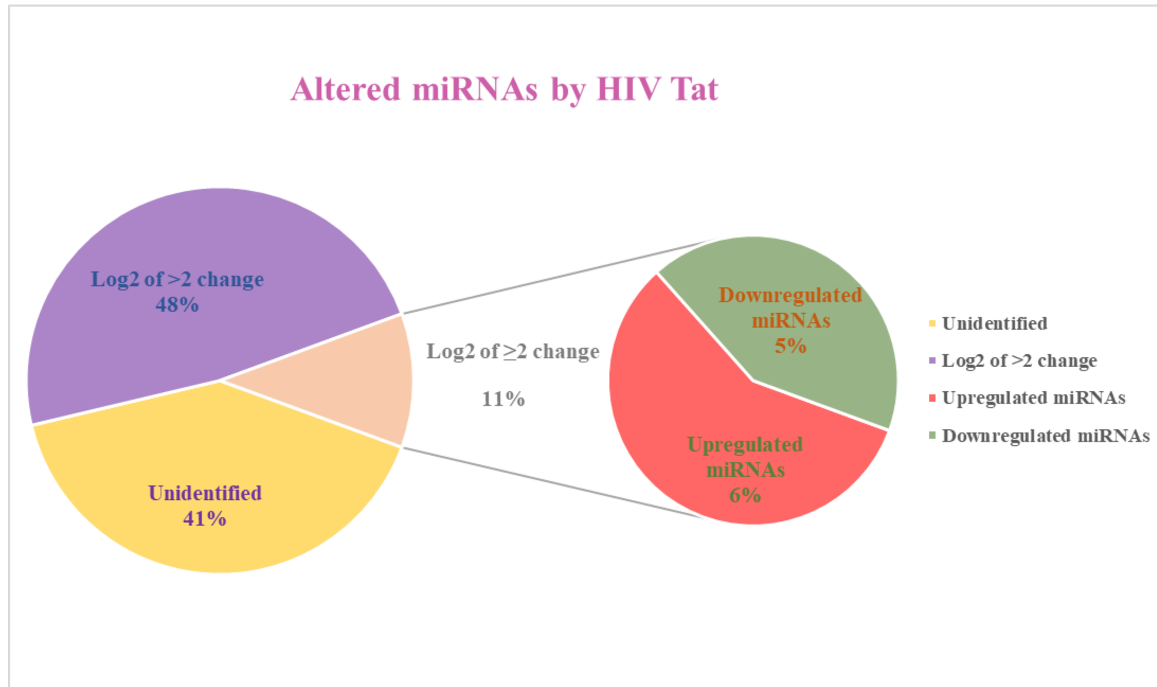
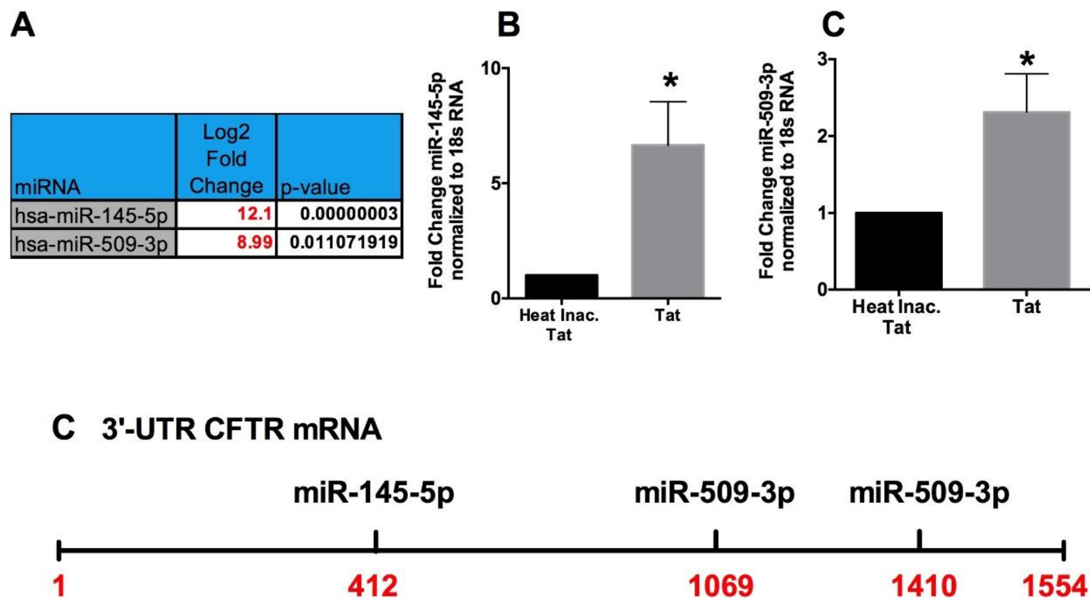


Figure 18: HIV Tat protein alters the microRNAome in bronchial epithelial cells. **(A)** Volcano plot of the altered microRNAome in primary bronchial epithelial cultures treated with HIV tat protein. Primary bronchial epithelial cultures redifferentiated at the ALI were treated with HIV tat protein (10 nM) or Vehicle (as control) as described by us earlier [338]. Total RNA was analyzed for miRNA expression using Exiqon microRNA Human panels I and II, V4 (Exiqon, Woburn, MA, US). This allows us to assay 739 mature human microRNAs. Data were analyzed using the GenEx software (Exiqon; Now Qiagen). **(B)** Pie chart of altered microRNAs in HIV Tat treated NHBE cells. Out of 742 mature microRNAs, 440 microRNAs were being dysregulated by HIV Tat protein in NHBE cells compared to the normal NHBE cells. Tat treatment leads to an altered expression of 83 different miRNAs out of 440 dysregulated miRNAs, whereas 48 miRNAs were being upregulated, and 35 miRNAs were being downregulated with statistically significant Log2-fold change ($p < 0.05$). Green, downregulated miRNAs; red, upregulated miRNAs. n = 3 different experiments (3 different lungs for NHBE cells).

5.3.2. Expression of miR-145-5p and miR-509-3p in HIV Tat protein treated Airway epithelial cells

The transcriptional regulation of CFTR activity is tightly controlled by transcription factors (TFs) and microRNAs [466], and reports including ours have shown the ability of both miR-145 and miR-509-3p to regulate CFTR [27, 439, 465, 467, 468]. **Figure 2A** shows that microRNA array data from HIV tat treated NHBE ALI cultures demonstrates that HIV Tat upregulates miR-145-5p and miR-509-3p. Next, we validated our miRNA array results to determine the upregulation of miR-145-5p and miR-509-3p in NHBE ALI cultures treated with HIV Tat. NHBE ALI cultures were treated with HIV Tat (heat-inactivated Tat as control) as described by us [338]. 48-hours following Tat treatment, total RNA was analyzed for miR-145-5p and miR-509-3p using specific Taqman probes. HIV tat upregulates both CFTR targeting RNAs with ~6-fold upregulation of miR-145-5p **(Figure 2B)** and a ~2.5-fold upregulation of miR-509-3p **(Figure 2C)**. **Figure 2D** shows the predicted target sites of miR-145-5p and miR-509-3p on the CFTR 3'UTR.



miRNA	Log2 Fold Change	p-value
hsa-miR-145-5p	12.1	0.00000003
hsa-miR-509-3p	8.99	0.011071919

Figure 19: Identification of Potential CFTR-Targeting miRNAs Induced by HIV Tat protein.

(A) miRNAs targeting CFTR were identified by a combination of target site prediction algorithm miRanda (miRSVR scores <−0.5) and miRTARbase (database of experimentally validated miRNAs). miRNAs are listed in order of their scores, starting with the best. (**B & C**) Array validation for miR-145-5p and miR-509-3p. NHBE ALI cultures were treated with HIV Tat (10nM) and heat-inactivated Tat as controls. Total RNA was analyzed for miR-145-5p and miR-509-3p expression using qRT-PCR and normalized to GAPDH. n = 3 different experiments (3 different lungs for NHBE cells). *Significant ($p < 0.05$) vs control. **(D)** Schematic of the putative target sites of the identified miRNAs on the 3′ UTR of CFTR mRNA.

5.3.3. miR-145-5p and miR-509-3p cooperatively suppress CFTR expression in HIV Tat treated cells

To test whether miR-145-5p and miR-509-3p directly target the 3′-UTR of the human CFTR mRNA, we transfected BEAS-2B cells with miR-145-5p and miR-509-3p mimics using lipofectamine RNAiMAX and looked for CFTR mRNA suppression. Transfection with lipofectamine RNAiMAX was used as a control. 48 hours post-

transfection, experiments were terminated, and total mRNA was isolated and analyzed for CFTR suppression. We have already shown that miR-145-5p suppresses CFTR. **Figure 3A** reproduces these results, and **Figure 3B** shows that miR-509-3p also suppresses CFTR mRNA compared to lipofectamine RNAiMAX control. Surprisingly, we noticed a synergistic suppression of CFTR when both mimics were transfected simultaneously, such that the total mimic concentration remained the same as individually transfected mimics. To determine if mRNA suppression translates to protein suppression, an identical experimental set was analyzed for CFTR protein levels by western blot analysis. **Figure 3B** shows that CFTR protein suppression is reflective of CFTR mRNA suppression, and in cells co-transfected with both mimics, a synergistic suppression was observed. These data suggest that both miRNAs may be acting to co-operatively to suppress CFTR. Several miRNAs have been shown to act independently as well as cooperatively to regulate target genes [469-471].

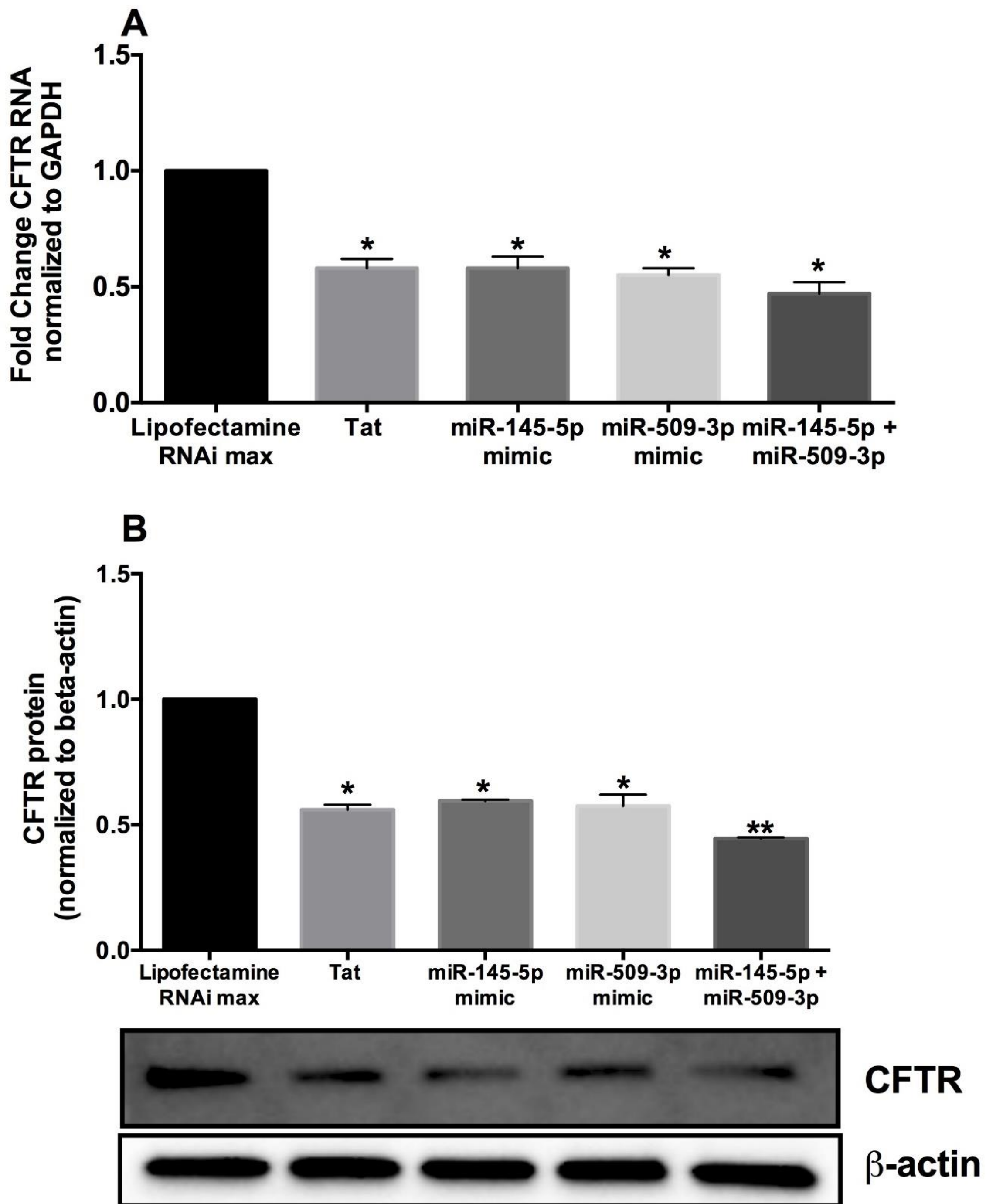


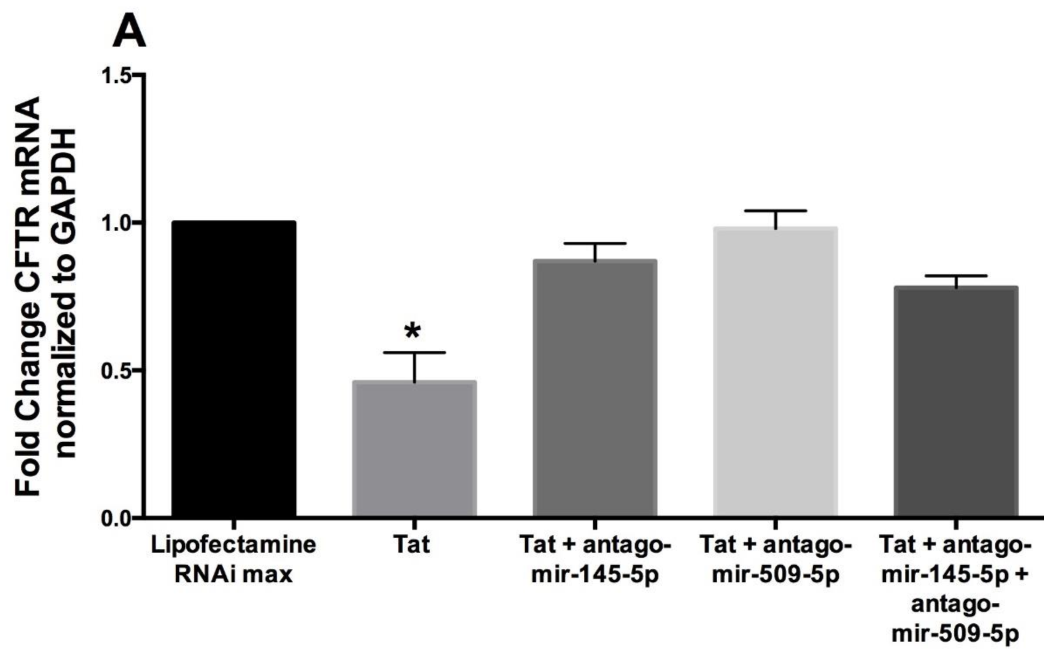
Figure 20: HIV Tat protein upregulates miR-145-5p and miR-509-3p to suppress CFTR biogenesis and function.

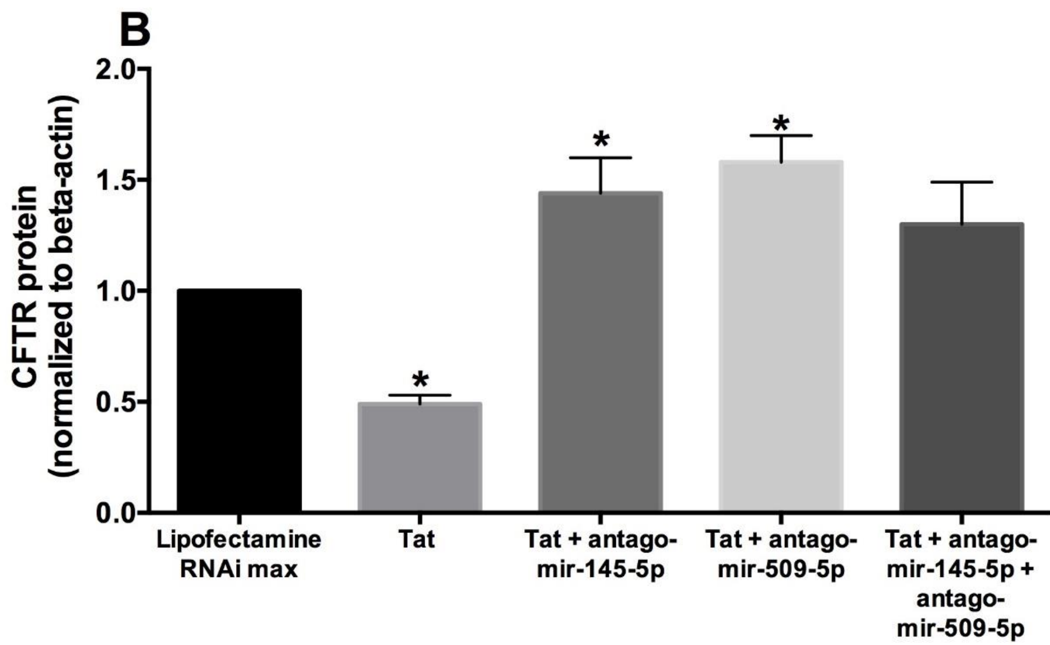
(A) To test the ability of miR-145-5p and miR-509-3p identified in **figure 2B** to suppress the CFTR mRNA, transient transfection of hsa-miR-145-5p mimic and hsa-miR-509-3p mimics in BEAS2B airway epithelial cell line was performed. Lipofectamine RNAiMAX

was used as the control, and lipofectamine RNAiMAX plus HIV Tat protein treatment was used for comparative analysis. Each miRNA suppresses CFTR mRNA expression confirming that both miRNAs have target sites in 3′ UTR of CFTR mRNA. Cooperative effects in CFTR mRNA suppression was overserved when both microRNA mimics were transfected simultaneously, such that the total mimic concentration remained the same as individually transfected mimics. n = 10 experiments, *Significant ($p < 0.05$) from control. **(B)** To further confirm miR-145-5p and miR-509-3p suppress CFTR protein, we performed western blot experiments with these miRNAs as described above. HIV Tat protein and lipofectamine RNAiMAX-treated cells were used for comparison. Similar results were observed in CFTR protein when both miRNAs were used for transient transfection in BEAS-2B cells, suggesting that both miRNAs function cooperatively. We observed a ~70kd CFTR band that corresponds to either the isoform-3 generated as a result of alternative splicing or as a consequence of Calpain based processing of CFTR. n=3 independent experiments for BEAS-2B cells; *Significant ($p < 0.05$) from control.

We further tried to validate the role of miR-145-5p and mir-509-3p in HIV Tat-mediated CFTR suppression. We also tried to determine if the suppression is co-operative and requires both miRNAs. For these purposes, we transfected BEAS2B airway epithelial cells with respective antagomirs. An additional set was co-transfected with a combination of both antagomir-145-5p and antagomir-509-3p. Separately, another set of BEAS-2B cells was treated with HIV Tat and Lipofectamine RNAiMAX. Lipofectamine RNAiMAX alone was used for comparison. If suppression is co-operative and requires both miRNAs to be present, then either one antagomir will completely rescue CFTR suppression, and the rescue will not be additive in cells co-transfected with a combination of both antagomirs. As seen in **Figure 4A**, antagomir's targeting to miR-145-5p and miR-509-3p rescued Tat-mediated CFTR mRNA suppression comparable to that seen in cells transfected with a combination of both antagomirs. An identically treated experimental set was used to determine if CFTR mRNA rescue translates to the rescue of CFTR protein levels. **Figure 4B** shows that protein levels exactly represent the mRNA data observed in **Figure 4A**.

Together these data demonstrate that both miR-145-5p and miR-509-3p are required to suppress CFTR. Indeed Ramachandran et al. have demonstrated that miR-509-3p acts cooperatively with another CFTR suppressing miRNA, in their case miR-494, to suppress CFTR expression [465].


A
Fold Change CFTR mRNA normalized to GAPDH
1.5
1.0
0.5
0.0
*
Lipofectamine RNAi max
Tat
Tat + antago-mir-145-5p
Tat + antago-mir-509-5p
Tat + antago-mir-145-5p + antago-mir-509-5p


B
CFTR protein (normalized to beta-actin)
2.0
1.5
1.0
0.5
0.0
*
*
*
Lipofectamine RNAi max
Tat
Tat + antago-mir-145-5p
Tat + antago-mir-509-5p
Tat + antago-mir-145-5p + antago-mir-509-5p

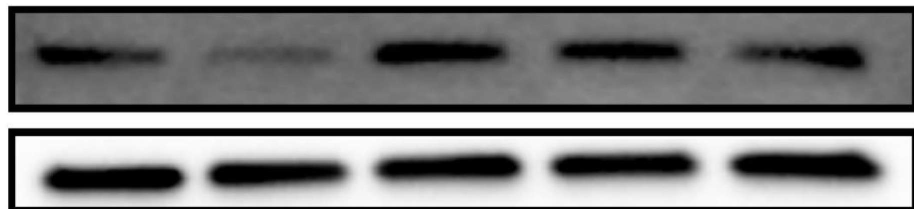


Figure 21: MicroRNA antagonism reverses HIV Tat-mediated inhibition of CFTR in airway epithelium cells.
(**A**) To determine if the suppression is co-operative and requires both miRNAs, BEAS-2B cells were transfected with antagomirs to miR-145-5p and miR-509-3p. An additional set was co-transfected with a combination of both antagomir-145-5p and antagomir-509-3p. Separately, another set of BEAS-2B cells was treated with HIV Tat and Lipofectamine RNAiMAX. Lipofectamine RNAiMAX alone was used for comparison. Antagomir's targeting to miR-145-5p and miR-509-3p rescued Tat-mediated CFTR mRNA suppression ($p = 0.0003$ and $p < 0.0001$) comparable to that seen in cells transfected with a combination of both antagomirs ($p = 0.0064$). n = 8 experiments. *Significant ($p < 0.05$) from control. n = 10 experiments. (**B**) An identically treated experimental set was used to determine if CFTR mRNA rescue translates to the rescue of CFTR protein levels. Targeting miR-145-5p and miR-509-3p with respective independent antagomiRs ($p < 0.0001$ for both antagomir-145 and antagomir-509-3p), and combination antagomirs ($p = 0.0005$), restores the CFTR protein levels and exactly represents the mRNA data observed in CFTR mRNA, suggesting that both miR-145-5p and miR-509-3p are required to suppress CFTR. We observed a ~70kd CFTR band that corresponds to either the isoform-3 generated as a result of alternative splicing or as a consequence of Calpain based processing of CFTR at the membrane. n=3 independent experiments for BEAS-2B cells; *Significant ($p < 0.05$) from control.

5.3.4. Gene-specific microRNA antagonism to rescue CFTR suppression in the context of HIV Tat

In our earlier reports, We have shown that blocking TGF-β signaling rescues HIV Tat, cigarette smoke, and TGF-β mediated CFTR suppression [27, 338]. In this manuscript, as well as our earlier report, we have shown that miR-145-5p antagonism rescues TGF-β mediated CFTR suppression. However, TGF-β and miR-145-5p play important cellular roles, and a global suppression of TGF-β signaling and miR-145 mediated gene regulation can have deleterious effects. Specifically, miR-145-5p is a tumor suppressor, and its downregulation relates to tumor progression and metastasis [416, 472-474]. Given these important roles of TGF-β signaling and miR-145, it is necessary to restrict microRNA antagonism to the gene of interest. We propose a novel CFTR-specific microRNA

antagonism approach using CRISPR that edits the miR-145-5p or miR-509-3p target site within CFTR 3' UTR region.

CRISPR-based gene editing has found significant applications in several preclinical studies [475-477]. CRISPR introduces targeted double-stranded breaks in the DNA, which are repaired primarily by the NHEJ (non-homologous end joining) DNA repair. CRISPR based gene-specific microRNA antagonism is attractive in that, even though one miRNA can regulate multiple genes, its target sequences vary significantly between the genes, with only a 6-8 nucleotide seed sequence required for gene silencing. For instance, the reported target site of miR-145-5p on EGFR is 5'-CCTTCCTGGGCAAAGAAGAAA-3' while that on NRAS gene is 5'-TGTTTAAAAAATAAAAACTGGAA-3'. Hence a gRNA designed to edit the miR-145 site on NRAS will not edit the miR-145 site on EGFR restricting miR-145 antagonism to a specific gene of interest. We exploited these differences in target sites to design gRNAs to target the miR-145-5p target site (position 422 in the CFTR 3'UTR) and miR-509-3p (predicted positions 1069 and 1410 in the CFTR 3'UTR).

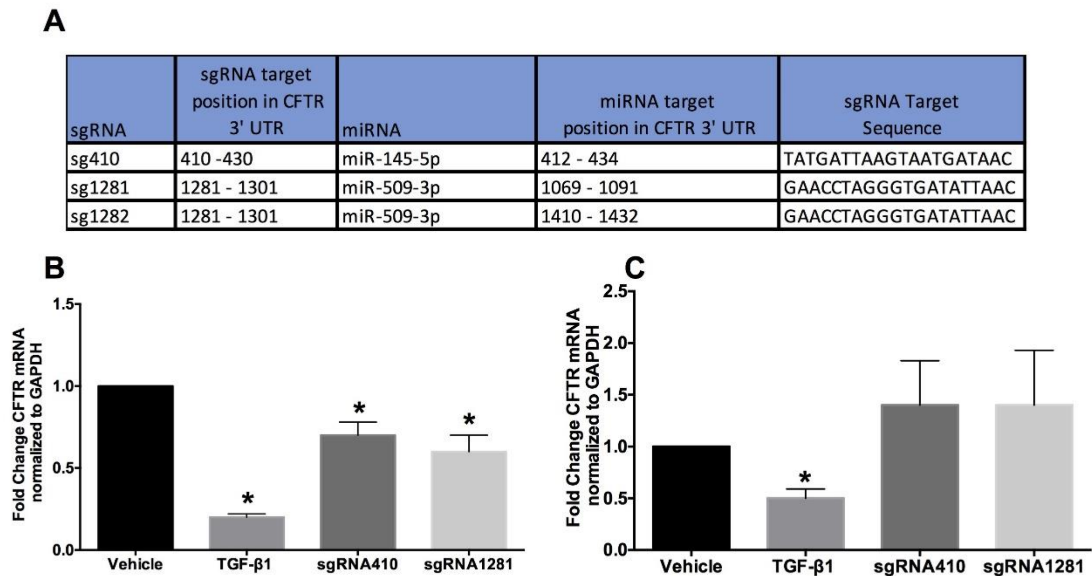
One of the limitations of the CRISPR/Cas9 system is that apart from requiring a 20bp guide sequence that perfectly hybridizes with the target DNA, it also requires a to direct sequence-specific double-strand break protospacer adjacent motif (PAM) by the Cas9 complex [478]. Moreover, it is also necessary to avoid regions that have significant homology to other genes to prevent non-specific targeting. Hence CRISPR introduces some limitations in targeting the exact site. However, Hollywood et al. have shown that CRISPR repair tracts of insertions and deletions can be bidirectional and can span > 100 bp [479] and can even extend over 200 bp [479]. Hence, it should still be possible to edit a

microRNA target site efficiently by using a PAM ~ 200bp away. We designed sgRNA410 to target position 410 (for the miR-145 site) and sgRNA1281 to target position 1281 such that both the miR-509-3p sites are within ~200 bp of the cleavage site. The sites were selected using the specificity check feature of Dharmacon (Horizon inspired cell solutions; CO) CRISPR design tool (https://dharmacon.horizondiscovery.com/gene-editing/crispr-cas9/crispr-design-tool/). The specificity check feature excludes any sequences with two or fewer mismatch alignments anywhere else in the genome, thereby increasing gRNA specificity and preventing off-target effects. The sgRNAs were custom synthesized and obtained from Dharmacon. The DNA targets are shown in **Figure 5A**.

5.3.5. CRISPR-mediated CFTR specific microRNA antagonism restores TGF-β1-mediated CFTR suppression

The two sgRNAs were tested for their ability to block TGF-β1-mediated CFTR suppression in BEAS-2B cells. Briefly, BEAS2B cells were co-transfected with either sgRNA410 or sgRNA1281 along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo reagent according to the manufacturer's instructions. BEAS-2B cells treated with Dharmafect Duo alone was used as control and with TGF-β1 treatment were used for comparative analysis. 48 hours post-transfection, 10ng TGF-β1 was added. After an additional 16 hours, experiments were terminated, and total RNA was analyzed for CFTR mRNA levels. **Figure 5B** shows that TGF-β suppresses CFTR mRNA. However, CFTR mRNA levels are rescued to a similar extent in cells transfected with either sgRNA410 or sgRNA1281. These data confirm our previous observations with antagomirs that both sites are necessary for CFTR suppression and targeting even one miRNA

completely rescues CFTR suppression. Next, we tried to determine the rescue of CFTR mRNA translates to the rescue of CFTR protein. Next, we tried to determine if sg410 and sg1281 can preserve CFTR mRNA levels in TGF-β treated NHBE ALI cultures. Successful transient transfection of NHBE cells has been shown to be very difficult and challenging [480, 481]. To address this challenge, we developed an in-house magnetofection protocol to get a robust and better transfection efficiency in NHBE cells. In this protocol, the NHBE cells redifferentiated at the ALI on snap wells were co-transfected with either sgRNA410 or sgRNA1281 along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo and Polymag CRISPR transfection reagent (OZ Biosciences) according to the respective manufacturer's instructions. One set of NHBE cells was treated with Cas9 alone and Dharmafect Duo and Polymag CRISPR transfection reagent. TGF-β treatment was used for comparison. 48 hours following Magnetofections, 10 ng of TGF-β1 was added to the co-transfected NHBE cells. After an additional 16 hours, total RNA was isolated and analyzed for CFTR mRNA. **Figure 5C** shows that TGF-β1 suppresses CFTR mRNA in NHBE ALI cultures, and magnetofection with our sg410 and sg1281 significantly preserves the CFTR mRNA levels. Together, these data suggest that both miR-145-5p and miR-509-3p act cooperatively to suppress CFTR and antagonism of either of these miRNAs preserves CFTR expression and function in the context of TGF-β signaling.



A

sgRNA	sgRNA target position in CFTR 3' UTR	miRNA	miRNA target position in CFTR 3' UTR	sgRNA Target Sequence
sg410	410 -430	miR-145-5p	412 - 434	TATGATTAAGTAATGATAAC
sg1281	1281 - 1301	miR-509-3p	1069 - 1091	GAACCTAGGGTGATATTAAC
sg1282	1281 - 1301	miR-509-3p	1410 - 1432	GAACCTAGGGTGATATTAAC

Figure 22: CRISPR-mediated gene editing of miR-145-5p and miR-509-3p target sites on the CFTR 3'UTR protects against TGF-β mediated CFTR suppression.
(A) sgRNAs were designed and custom synthesized against the three predicted target sites for miR-145-5p and miR-509-3p on the 3'UTR of CFTR. sgRNAs and miRNAs target position; sequence of the target DNA, position in the 3' UTR are shown. (**B**) BEAS-2B cells were co-transfected with either sgRNA410 or sgRNA1281 along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo reagent. BEAS-2B cells treated with Dharmafect Duo alone was used as control and with TGF-β1 treatment were used for comparative analysis. 48 hours post-transfection, 10ng TGF-β1 was added apically. After an additional 16 hours, experiments were terminated, and total RNA was analyzed for CFTR mRNA levels. TGF-β suppresses CFTR mRNA. Both sgRNA410 and sgRNA1281 ($p < 0.0001$ for both gRNAs) preserve CFTR mRNA levels in the context of TGF-β signaling. **(C)** NHBE cells redifferentiated at the ALI on snap wells were co-transfected with either sgRNA410 or sgRNA1281 along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo (Dharmafect) and Polymag CRISPR transfection reagent (OZ biosciences). One set of NHBE cells was treated with Cas9 alone and Dharmafect Duo and Polymag CRISPR transfection reagent. TGF-β treatment was used for comparison. 48 hours following Magnetofections, 10 ng of TGF-β1 was added to the co-transfected NHBE cells. After an additional 16 hours, total RNA was isolated and analyzed for CFTR mRNA. TGF-β1 suppresses CFTR mRNA in NHBE ALI cultures, and magnetofection with our sg410 and sg1281 preserves the CFTR mRNA levels. n = 3 different experiments (3 different lungs for NHBE cells); n=3 independent experiments for BEAS-2B cells. * = significant ($p < 0.05$) from control.

5.3.6. CRISPR-mediated CFTR specific microRNA antagonism restores HIV Tat-mediated CFTR suppression

We have shown that HIV Tat protein induces TGF β1 signaling to suppress CFTR in bronchial epithelial cells [17]. Hence, we tested the ability of our CRISPR-based gene-specific miRNA antagonism approach to preserve CFTR function first, in airway epithelial cells lines and then, in primary bronchial epithelial cells, redifferentiated at the ALI. BEAS2B cells were transfected with the sgRNA409, or sgRNA1281 along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo transfection reagent according to the manufacturer's instructions. 48 hours the following co-transfection, BEAS-2B cells were treated with recombinant HIV Tat. 16 hours of post-Tat treatment, total RNA was analyzed for CFTR expression by qRT-PCR. **Figure 6A** shows that both sgRNAs preserve CFTR mRNA expression in Tat treated cells. CRISPR based targeting can insert mutations that, while preserving the mRNA, can disrupt the ORF leading to dysfunctional proteins that can be eliminated in the endoplasmic reticulum. Hence, we tried to determine whether sgRNAs while preserving mRNA levels do not affect protein structure and stability. An identically treated set was used to determine if CFTR protein levels. **Figure 6B** shows that CFTR protein levels mimic the CFTR mRNA restoration suggesting that CRISPR based editing of CFTR 3' UTR region does not affect protein stability. Next, we magnetofected the NHBE ALI cultures redifferentiated at the ALI on snapwells with either sgRNA410 or sgRNA1281 gRNAs along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo and Polymag CRISPR transfection reagent according to the protocol described in methods. 48 hours post magnetofection, NHBE ALI cultures were mounted in Ussing chambers, and CFTR function was

determined by albuterol mediated activation as described by us [27, 338, 406]. **Figure 6C** shows that magnetofection with either sgRNA preserves CFTR mRNA levels in NHBE ALI cultures in HIV Tat treated cells. To determine that CRISPR-based editing of the microRNA target sites in the CFTR 3'UTR does not affect CFTR trafficking and activity, NHBE ALI cultures grown on were magnetofected with sgRNA410 or sgRNA1281 gRNAs as described in methods, 48-hours post-transfection, cultures were mounted in Ussing Chambers, and CFTR activity was determined using albuterol as described by us [6, 27, 338].

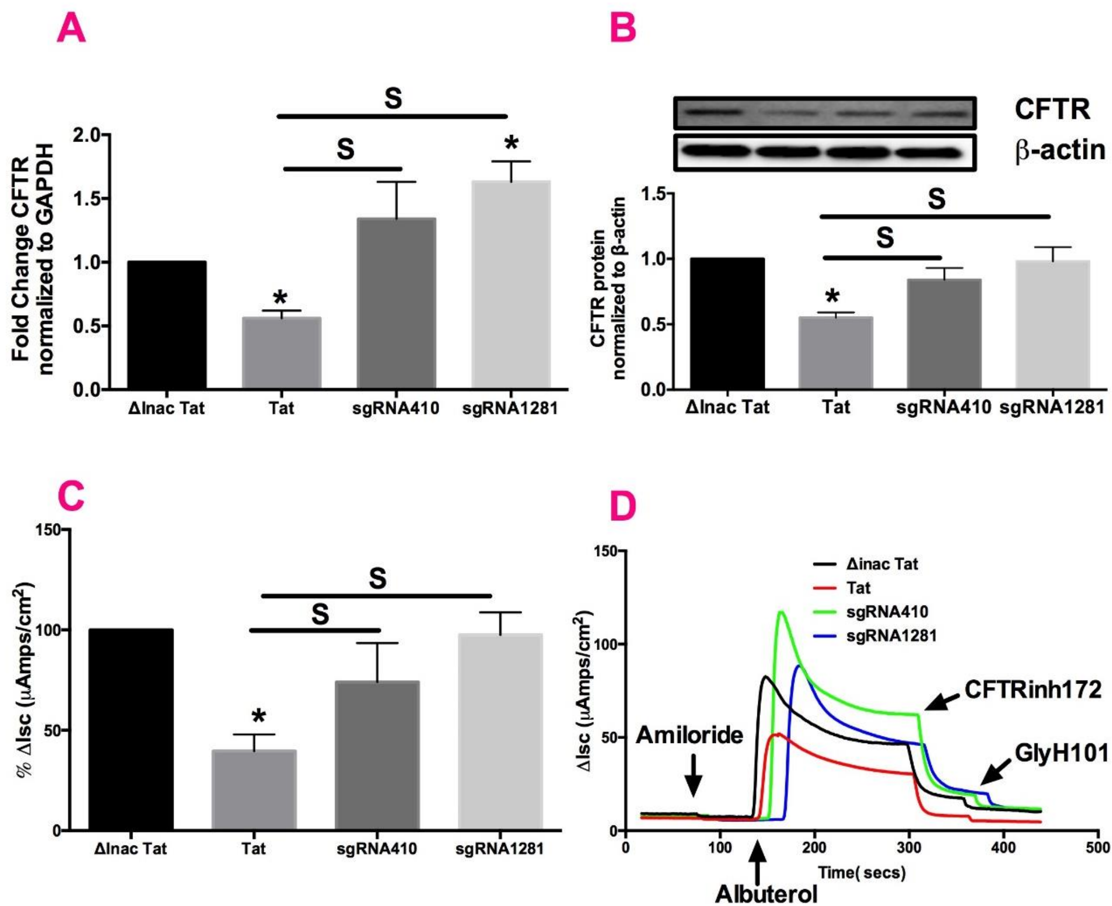


Figure 23: CRISPR-mediated gene editing of miR-145-5p and miR-509-3p target sites on the CFTR 3'UTR protects against HIV Tat-mediated CFTR suppression.

(A) BEAS2B cells were transfected with the sgRNA409, or sgRNA1281 along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo transfection reagent. 48 hours the following co-transfection, BEAS-2B cells were treated with recombinant HIV Tat. 16 hours of post-Tat treatment, total RNA was analyzed for CFTR expression by qRT-PCR. **Figure 6A** shows that sgRNA410 and sgRNA1281 preserve CFTR mRNA expression in Tat treated cells ($p < 0.0001$ for both gRNAs). n = 11 experiments. **(B)** To determine if sgRNAs preserve CFTR protein expression, BEAS-2B cells were co-transfected with either sgRNA410 or sgRNA1281 along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo reagent. BEAS-2B cells treated with Dharmafect Duo alone were used as a control, and with HIV, Tat treatment was used for comparative analysis. 48 hours post-transfection, HIV Tat was added apically. After an additional 16 hours, experiments were terminated, and total protein was analyzed for CFTR protein levels. CFTR protein levels mimic the CFTR mRNA restoration suggesting that CRISPR based editing of CFTR 3' UTR region does not affect protein stability ($p = 0.0262$ for sgRNA410 and $p = 0.0098$ for sgRNA1281). n = 3 experiments. **(C)** To determine the CFTR function, we magnetofected the NHBE ALI cultures redifferentiated at the ALI on snapwells with either sgRNA410 or sgRNA1281 gRNAs along with the Edit-R Cas9 Nuclease expression plasmid using the Dharmafect Duo and Polymag CRISPR transfection reagent. 48 hours post magnetofection, NHBE ALI cultures were mounted in Ussing chambers, and CFTR function was determined. magnetofection with either sgRNA preserves CFTR mRNA levels in NHBE ALI cultures in HIV Tat treated cells ($p = 0.0441$ for sgRNA410 and $p = 0.0001$ for sgRNA1281). n = 8 experiments. **(D)** Ussing trace to determine that CRISPR-based editing of the microRNA target sites in the CFTR 3'UTR does not affect CFTR trafficking and activity, NHBE ALI cultures grown on were magnetofected with sgRNA410 or sgRNA1281 gRNAs as described in methods. 48-hours post-transfection, cultures were mounted in Ussing Chambers, and CFTR activity was determined. Amiloride is added to inhibit the epithelial sodium channel. Albuterol (10 µM) was added to activate CFTR. CFTR inh172 was added to confirm CFTR currents. GlyH101 another inhibitor of CFTR was added to inhibit any residual CFTR current, further confirming their CFTR inhibitory functions. n = 3 different experiments (3 different lungs for NHBE cells); n=3 independent experiments for BEAS-2B cells. * = significant ($p < 0.05$) from control. S = significant from each other ($p < 0.05$). S* is significant from each other ($p < 0.1$).

5.4. Discussion

HIV infection was found to be an independent risk factor for COPD in the cART era, even when accounting for smoking status [389]. HIV-infected cells in the airway

(including bronchial epithelial cells [338]) can serve as a source of HIV proteins like Tat. HIV Tat is an immediate-early gene of HIV, and its expression is not suppressed by antiretrovirals [116-119]. The protein transduction domain of Tat allows its secretion by infected cells and uptake by bystander cells, where it mediates pleiotropic effects [120-123]. Hence HIV Tat can have deleterious effects not only on infected cells but also on uninfected cells. WE have shown that HIV Tat induces TGF-β signaling [338], and in our recent report, we have demonstrated that TGF-β signaling alters the bronchial epithelial microRNAome [27]. Since the first report of RNA interference, multiple reports have established that miRNAs play an important role in temporal control of gene regulation [247]. A single gene is controlled by multiple miRNAs, and a single miRNA can control multiple genes [482]. In this study, we tried to determine if HIV Tat alters the bronchial epithelial microRNAome. Our miRNA array analysis of mature microRNAs demonstrates that HIV Tat significantly alters the bronchial epithelial microRNAome. While there were some miRNAs common to both TGF-β and HIV Tat, HIV Tat also altered miRNAs distinct from those altered by TGF-β. This could be because HIV Tat can itself activate the expression of genes that can have further downstream effects on miRNA expression. Not surprisingly, we did find that like TGF-β, HIV also upregulates miR-145-5p. We have shown that TGF-β also upregulates miR-145-5p to suppress CFTR. However, our array showed that HIV Tat also upregulates miR-509-3p, another CFTR targeting miRNA [465], which had not been detected by our TGF-β array. Using miRNA mimics and antagomirs for both miRNAs, we were able to demonstrate that the suppression is co-operative and requires the presence of both miRNAs as antagomir to either one microRNA completely

rescues Tat-mediated CFTR suppression. It is possible that even though TGF-β did not alter the expression of miR-509-3p, the upregulated miR-145-5p works in tandem with constitutive levels of miR-590-3p to suppress CFTR. Our data demonstrate that CFTR mRNA suppression by mimics and rescue by antagomirs mirrors the levels of CFTR protein determined by western blot analysis. Ramachandran et al. have also reported that miR-509-3p works cooperatively with other miRNAs to regulate CFTR expression [465]. Together these experiments suggest that both miR-145-5p and miR-509-3p are required to suppress CFTR. Such cooperativity in miRNA based regulate may be more common than reported given that a single miRNA can regulate multiple genes. For instance, miR-145-5p has been experimentally validated to regulate over 50 genes. There would be other targets that have not been reported. Indeed, We recently reported the first miRNA based regulation of SLC26A9 and demonstrated the ability of miR-145-5p to suppress this important CFTR modulator [27]. In these circumstances, counter-regulation or cooperative regulation by multiple miRNAs can still maintain mRNA homeostasis of certain miR-145-5p targets when it is upregulated.

TGF-β isoforms are expressed and secreted by several cell types in the airway and regulate a wide range of biological processes, including cell proliferation, differentiation, extracellular matrix (ECM) synthesis, and apoptosis [483-485]. Likewise, miR-145-5p is considered a major tumor suppressor tumor progression and metastasis [416, 472-474]. We and others have previously reported efficient rescue of CFTR by modulating TGF-β signaling or by miR-145 antagonism [27, 415]. However, suppressing TGF-β signaling, or interfering with miR-145 regulation can have non-specific effects. Under those circumstances, modulating the miRNA target site on the CFTR target site can preserve

CFTR function without suppressing TGF-β signaling or miR-145-5p mediated regulation of other genes. While this manuscript was in preparation, A very recent study has shown that masking the 412 sites with peptide nucleic acids (PNA) augments CFTR availability [486]. This report confirmed previous predictions and identification of the miR-412 target site for miR-145 on the CFTR 3' UTR. However, the study had certain limitations in that the authors have used Calu-3 cell lines and not primary cells and have not shown apical localization nor CFTR function. This is important since masking by PNAs may protect the RNAs but leave them incapable of translation, and the differences would be more pronounced in primary cells, which have much lower levels of cellular transcription and translation compared to cell lines. Also, PNAs are primarily used in an anti-sense role for gene inhibition instead of gene augmentation [487-491], and they have to be designed specifically for the target sequence. Hence, their effects on mRNA stability or any off-target effects due to sequence complementarity with other genes can limit their application in a clinical setting. However, this report does confirm the 412 positions in the CFTR 3' UTR as a miR-145 target site. We developed a novel gene-specific microRNA antagonism approach to preserve CFTR function in the context of increased TGF-β signaling. CRISPR based editing of miRNA target sites has two distinct advantages in that it works at the DNA levels requiring fewer administrations and the ability of NHEJ recombination to create repair tracts almost 200 bp away from the target sites allows flexibility in the selection of targets with more specificity to the 3' UTR of interest. We used the specificity check feature of Dharmacon to design two sgRNAs. sgRNA409 was designed to edit the 412 sites of miR-145-5p. sgRNA1281, on the other hand, was selected to target two different predicted miR-509-3p sites at position 1069 and 1410 in the CFTR 3'UTR. While for initial

studies with cell lines, we used sgRNAs and Cas9 plasmid to effect CFTR rescue, in subsequent experiments with primary NHBE ALI cultures, we used sgRNAs with Cas9 protein. This is because delivering Cas9 expressing plasmid can result in the insertion of plasmid fragments, including Cas9, into the host genome due to recombination. Delivering the Cas9 protein would be more clinically palatable as the effects of Cas9 activity are transient. In our lab, we had shown that magnetofection using PolyMag could efficiently deliver plasmid DNA to cells (data not shown). We developed an in house protocol using reagents from Dharmacon and OZ Biosciences to deliver our sgRNA and Cas9 protein to redifferentiated NHBE ALI cultures. Our data demonstrate that CRISPR based CFTR specific microRNA antagonism preserves CFTR mRNA in BEAS2B airway epithelial cells as well as NHBE ALI cultures. In line with our observations that both miRNAs are involved in CFTR suppression, targeting either the miR-145-5p target site or the miR-509-3p site preserves CFTR mRNA in both BEAS2B and NHBE ALI cultures. Moreover, editing the 3'UTR does not interfere with apical CFTR localization and function, as seen in out Ussing chamber experiments. Together with our reports show that HIV Tat upregulates miR-145-5p and miR-509-3p to suppress CFTR a cooperative regulation and demonstrate that antagonizing either site rescues CFTR suppression by HIV Tat. We demonstrate that gene-specific microRNA antagonism can preserve CFTR biogenesis and function in the context of HIV Tat. Our approach exploits the differences in target sites for the same miRNA for different genes to ensure that only the gene of interest is exempted from miRNA regulation without interfering with the normal functions of miRNA. This can be applicable to many diseases that are characterized by an aberrant microRNAome. Our

approach has the potential to revolutionize therapy in diseases with inherited or acquired CFTR dysfunction, thereby arresting or possibly, even reversing lung function decline.

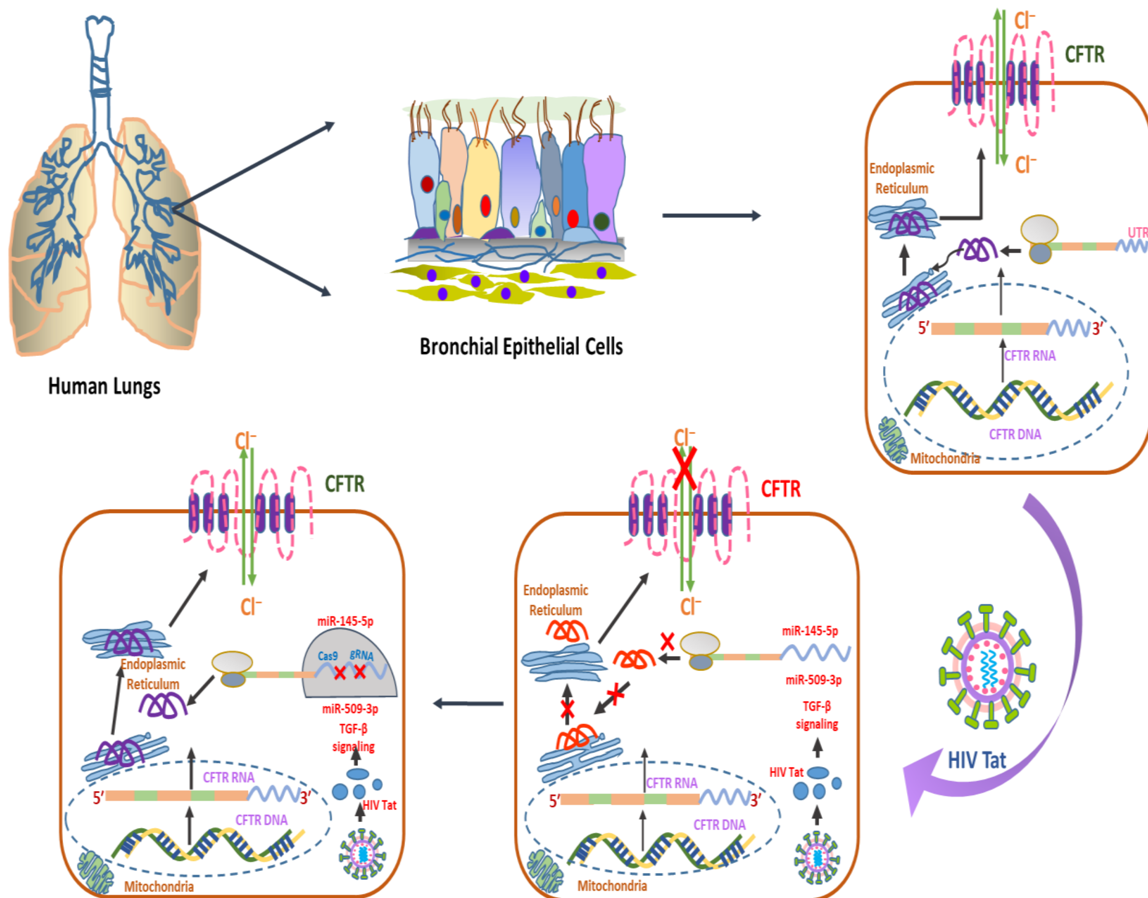


Figure 24: Gene-specific microRNA antagonism to preserve CFTR function in the context of HIV without blocking the entire TGF- β signaling pathway or interfering with the broader miRNA-mediated regulation of other genes.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

With the advent of cART, there is a dramatic decline in morbidity and mortality from HIV/AIDS [442]. Nevertheless, HIV patients die of non-AIDS comorbidities almost a decade earlier than their non-HIV counterparts [386]. Lung diseases such as COPD, pulmonary hypertension, and pneumonia are emerging as significant comorbidities in the HIV-infected population [492]. Absolute rates of pneumonia increase with age, although the relative differences between those with and without HIV infection are greatest in younger people [450]. HIV patients are six times more likely to contract pneumonia, with four times higher mortality from an episode of pneumonia compared to non-infected age-matched controls [493-495]. Recurrent pneumonia can lead to pulmonary arterial vasoconstriction, shunting the blood to the normally aerated segments of the lung, which can lead to pulmonary arterial hypertension (PAH). Pulmonary infection also induces recruitment and activation of immune cells leading to de novo infection of target cells and enhanced HIV replication. Thus, bacterial pneumonia can also contribute to HIV progression. Understanding the pathophysiological mechanisms that lead to microbial colonization of the airways in HIV-infected patients is, therefore, important to public health. HIV proteins Tat and gp120 can suppress two major components of the MCC system, which can lead to depressed MCC and consequent bacterial colonization. Restoring tracheobronchial MCC to increase microbial clearance and decreasing incidence of bacterial pneumonia in HIV patients is the long-term goal of this study.

Transforming growth factor β (TGF-β), signaling induced by cigarette smoke (CS), plays an important role in the progression of airway diseases, like chronic bronchitis associated with chronic obstructive pulmonary disease (COPD), and in smokers. Chronic

bronchitis is characterized by reduced mucociliary clearance (MCC). Cystic fibrosis transmembrane conductance regulator (CFTR) plays an important role in normal MCC. TGF-β and CS (via TGF-β) promote acquired CFTR dysfunction by suppressing CFTR biogenesis and function. Understanding the mechanism by which CS promotes CFTR dysfunction can identify therapeutic leads to reverse CFTR suppression and rescue MCC. TGF-β alters the microRNAome of primary human bronchial epithelium. TGF-β and CS upregulate miR-145-5p expression to suppress CFTR and the CFTR modifier, SLC26A9. miR-145-5p upregulation with a concomitant CFTR and SLC26A9 suppression was validated in CS-exposed mouse models. While miR-145-5p antagonism rescued the effects of TGF-β in bronchial epithelial cells following transfection, an aptamer to block TGF-β signaling rescues CS- and TGF-β-mediated suppression of CFTR biogenesis and function in the absence of any transfection reagent. These results demonstrate that miR-145-5p plays a significant role in acquired CFTR dysfunction by CS, and they validate a clinically feasible strategy for delivery by inhalation to locally modulate TGF-β signaling in the airway and rescue CFTR biogenesis and function.

In this study, we show that HIV Tat also alters the bronchial epithelial microRNAome to upregulate miR-145-5p that functions co-operatively with miR-509 to suppress CFTR. We have shown that a neutralizing aptamer to TGFBR2 and miR-145-5p antagonism rescues TGF-β mediated CFTR suppression. Given that miR-145-5p plays an important role as a tumor suppressor [416], we explored a novel approach called gene-specific microRNA antagonism to preserve CFTR function in the context of HIV and cigarette smoke without blocking the entire TGF-β signaling pathway or interfering with the broader miRNA-mediated regulation of other genes.

At the conceptual level, we provide a new framework to understand the role of MCC dysfunction in lung comorbidities in COPD and HIV patients. This is a novel study where we determined the pathophysiology behind one of the principal comorbidities in HIV patients in the cART era. Our discovery that (1) HIV Tat and Cigarette Smoke suppress CFTR function via a common pathway involving TGF-β signaling; (2) TGF-β signaling suppresses CFTR biogenesis and function via miRNA mediated post-transcriptional gene silencing, and [41] suggesting that HIV Tat and Cigarette Smoke inhibit a critical component of the MCC apparatus. 3) In view of the well-described roles of TGF-β signaling during development, tissue regeneration, and homeostasis, the focus on developing aptamer-antagomir chimeras as inhaled therapeutics to restrict TGF-β signaling modulation to the airway and neutralize the available microRNAs. Nucleic acid aptamers are already approved for use in humans (e.g., macugen [377, 378]). The cation and size selectivity of tight junctions in airway epithelia serves to inhibit paracellular transport of the negatively charged nucleic acid macromolecules (in our case, aptamer chimeras), thereby restricting TGF-β inhibition to the airway epithelium. The therapeutic approach exploits the differences in target site sequences of the same miRNA for different genes to propose specific editing of the miRNA target site only on the gene of interest using a CRISPR-based approach. Such gene-specific microRNA antagonism can find application in miRNA target site identification as well as therapeutics. Our approach limits miR-145-5p antagonism to the CFTR gene without compromising the broader TGF-β signaling or regulation of other genes by the miR-145-5p tumor suppressor. Moreover, the therapeutic could be delivered using non-lentiviral vector-based delivery, and fewer administrations as it would effect changes at the DNA level. This gene-specific approach has never been

explored or implemented and can revolutionize treatment in lung associated comorbidities in HIV patients that are a consequence of an aberrant microRNAome.

At the technical level, this study innovates by combining 1) miRNA arrays in our ex vivo model of fully differentiated airway epithelium to determine changes in microRNA profile upon TGF-β1, CS and HIV Tat exposure, 2) Electrophysiology experiments measuring ion transport using Ussing chamber techniques, 3) manipulation of RNA interference (RNAi) machinery and aptamer mediated inhibition of TGF-β signaling; 4) The use of whole CS (not extracts) for treatment of the surface of fully differentiated primary human bronchial epithelia ex vivo; 5) Use of CRISPR-mediated manipulation of miR-145-5p and miR-509-3p target sites to prevent CFTR suppression in the context of increased TGF-β signaling. Whole CS exposure mimics exposure of airway epithelium to CS observed under physiological conditions in smokers.

At the translational level, this study identified miRNAs as biomarkers or as therapeutic targets to rescue HIV Tat, and CS mediated MCC suppression. We tested nucleic acid-based therapeutics with the established potential to modulate TGF-β signaling and to restore MCC. In our study, we showed that TGF-β1 and CS (via TGF-β) suppresses expression of CFTR mRNA and, consequently, CFTR function. In addition, we found that HIV Tat increases TGF-β1 expression with a concomitant suppression in CFTR biogenesis and function via a common pathway involving TGF-β signaling in primary bronchial epithelial cells and blocking TGF-β signaling rescues this completely [6].

HIV-infected patients demonstrate that HIV proteins Tat and gp120 can potentially suppress two different components of the MCC apparatus, namely, CFTR function, and

CBF. In the future, we will seek to test clinically feasible intervention strategies in HIV Tat transgenic mice [496] and human subjects that can restore CFTR function and CBF with the ultimate goal of restoring MCC and decreasing the incidence of bacterial pneumonia in HIV-infected patients. Bronchial brushings from HIV smokers/non-smokers patients with impaired MCC will demonstrate decreased CFTR mRNA, and this will be reflected as increased microbial colonization despite treatment with cART.

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