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TGFβ as a therapeutic target in cystic fibrosis

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Abstract

Introduction: Cystic fibrosis (CF) is a genetic disease characterized by progressive lung disease. Most CF therapies focus on treating secondary pulmonary complications rather than addressing the underlying processes inducing airway remodeling and ineffective response to infection. Transforming growth factor beta (TGFβ) is a cytokine involved in fibrosis, inflammation, and injury response as well as a genetic modifier and biomarker of CF lung disease. Targeting the TGFβ pathway has been pursued in other diseases, but the mechanism of TGFB effects in CF is less well understood.

Areas Covered: In this review, we discuss CF lung disease pathogenesis with a focus on potential links to TGFβ. TGFβ signaling in lung health and disease is reviewed. Recent studies investigating TGFβ's impact in CF airway epithelial cells are highlighted. Finally, an overview of potential therapies to target TGFβ signaling relevant to CF are addressed.

Expert Opinion: The broad impact of TGFβ signaling on numerous cellular processes in homeostasis and disease is both a strength and a challenge to developing TGFβ dependent therapeutics in CF. We discuss the challenges inherent in developing TGFβ-targeted therapy, identifying appropriate patient populations, and questions regarding the timing of treatment. Future directions for research into TGFβ focused therapeutics are discussed.

Keywords

Cystic Fibrosis; cystic fibrosis transmembrane conductance regulator; genetic modifier; Transforming Growth Factor beta

1. Cystic Fibrosis

Cystic fibrosis (CF) is a recessive genetic disease common in the Caucasian population; as of 2015, CF affects approximately 70,000 individuals worldwide, including nearly 30,000 in the United States, with a predicted median survival of 41.7 years [1, 2]. Mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR) disrupt bicarbonate and chloride transport in epithelia throughout the body, leading to progressive multi-organ

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Declaration of Interest

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disease [3]. Lung disease is the most significant source of morbidity and mortality for patients with CF. More advanced CF disease is characterized by acquisition of chronic pulmonary infections (i.e. *Pseudomonas aeruginosa or Burkholderia cepacia*), recurrent bronchopneumonia, progressive lung injury, and loss of lung function typically measured by decline in the forced expiratory volume in one second (FEV_1) [3]. As we better understand the course of early CF lung disease, it is apparent that FEV1 decline and recurrent pulmonary exacerbations are a late marker of lung disease; a third of children under age 3 years have airway damage (bronchiectasis) visible on chest CT [4]. Pulmonary inflammation and infection are also demonstrable early in life, leading to the proposal that very early childhood is a critical window to intervene with new therapies and change the trajectory of future CF lung disease [5]. New therapies that restore function to mutated CFTR, CFTR modulators, have been shown to benefit patients with certain CFTR mutations but are not curative [6, 7]. Thus, it is critical to understand contributors to CF lung disease and identify other novel therapeutic targets that might improve or prevent CF lung disease.

1.1 CF Lung Disease Pathogenesis

Loss of CFTR function produces a cascade of pathologic changes in the lung. Altered ion transport results from CFTR malfunction, loss of CFTR-dependent chloride and bicarbonate transport, and loss of the normal CFTR regulation of the epithelial sodium channel (ENaC) [8–10]. This results in airway surface liquid (ASL) dehydration, increasing mucus solid concentration and compression of the periciliary compartment [11–13]. Epithelial goblet cell and submucosal gland hyperplasia is also seen in CF, with a higher mucin content within goblet cells [14]. Reduction of pH and altered bicarbonate transport due to absent CFTR function results in reduced bacterial killing and thickened, abnormally adherent mucus [15–18]. These defects result in airway obstruction, poor mucociliary clearance, and increased inflammation and infection. Airways in CF are primed for a more aggressive inflammatory response to infectious and obstructive stimuli, with increased cytokine release resulting in chronic neutrophilic inflammation [19, 20]. Lungs of CF patients are thus vulnerable to infection and the severe downstream responses to the resulting inflammation and lung injury. Although later in life the morbidity associated with CF lung disease is quite apparent, the disease starts early in life when it is often asymptomatic; indeed, significant inflammation and ventilatory defects are detectable in the majority of children with CF [4]. The causative and temporal relationships between inflammation and infection in CF are unclear, although it is recognized that both occur early, prior to the onset of clinically identified respiratory symptoms [21].

Multiple signaling pathways (intra and extracellular) are implicated in CF lung disease pathogenesis, such as activation of hypoxic response pathways, NFκB, IL-1, and IL-8 [5, 22, 23]. Free neutrophil elastase in young infants (as early as 3 months old, typically prior to recognized bronchopulmonary pneumonias) has been found to be significantly associated with bronchiectasis by age 3 years, indicating that abnormal early and potentially sterile inflammation sets the stage for CF lung disease progression [4]. A number of investigators have observed abnormal microRNA (miRNA, small non-coding RNAs involved in gene expression regulation) expression in cystic fibrosis lungs, with downregulation of miR-126, miR-31, and miR-93 potentially allowing increased expression of their targets that cause

inflammatory dysregulation, including Target of Myb1, cathepsin S, and IL-8, respectively [24]. In addition to effects on inflammation, a number of miRNAs downregulate CFTR expression, thus exacerbating the underlying etiology that drives cystic fibrosis [25].

1.2 Current Therapies for CF

Until the recent availability of CFTR modulators, CF pulmonary therapy had focused on alleviating secondary symptoms: thinning and mobilizing mucus, counteracting inflammation, and treating infection. Hypertonic saline (a mucus hydrator) and dornase alfa (a mucolytic) are two commonly used pulmonary therapies in CF, and both are associated with decreased pulmonary exacerbations and small improvements in FEV_1 [26, 27]. Multiple modalities of airway clearance are used in CF patients, including postural drainage and percussion, PEP devices, percussion vests, and variable pressure (e.g.: flutter) devices. Little evidence exists regarding the relative benefits of the different types of airway clearance, or indeed as to the precise benefit of airway clearance in general; however, these tools are strongly recommended as part of the daily routine for CF patients given the high theoretical benefit of mobilizing the thick, inspissated lung secretions typical in CF [28]. Anti-inflammatory therapies such as macrolide antibiotics and ibuprofen have also been shown in several studies to improve lung function and decrease exacerbation frequency, and remain an area of intense drug development [29, 30]. Several studies have demonstrated that acute and chronic antibiotic therapies targeting important CF pathogens including *Pseudomonas aeruginosa* and methicillin-resistant *Staphyloccus aureus* are beneficial to CF patients [31–34]. Together, these symptom-based treatments have led to steady improvements in CF outcomes over the past several decades, including lung function and patient longevity.

CFTR modulators, which improve the function of defective, disease-causing CFTR mutations, are an exciting new avenue of CF therapies. Although more modulators are in the pipeline, currently only two exist that are approved for a subset of CF patients: ivacaftor, approved for patients age 2 years and above with certain gating mutations and a number of partially functional CFTR mutations, and ivacaftor in combination with lumacaftor, approved for patients age 6 years and up homozygous for the F508del mutation [6, 7]. The clinical benefits observed in CF patients treated with CFTR modulators (and indeed all therapies) vary, and understanding the factors responsible for variable responses are an area of growing interest in CF pharmacotherapy that strives for personalized and precise treatment regimens [35, 36].

With the combined improvements in patient diagnosis, care, and treatment, the course of cystic fibrosis has shifted dramatically. Today, the majority of patients with cystic fibrosis are adults (51.6% > 18 years) [1]. Our challenge now is to develop agents that modify and alleviate the fundamental defects in CF, with a focus on early and aggressively treatment, before secondary manifestations and irreversible organ damage occur.

2. TGFβ in CF Lung Disease

CF lung disease severity is not predicted by CFTR genotype alone. Numerous factors beyond CFTR genotype contribute to disease heterogeneity, including socioeconomic

factors, adherence, environment, and other non-CFTR genetic modifiers [37–40]. Among these contributors, identification of genetic modifiers of cystic fibrosis has become an important goal for both disease forecasting and identification of novel therapeutic targets [37, 41]. Transforming Growth Factor β (TGFβ), a cytokine that drives diverse, crucial, and context dependent cellular activities has emerged as a master regulator of pulmonary health and disease that may offer insights into new approaches towards our understanding and novel treatment of CF lung disease [42].

2.1 TGFβ Signaling

The TGFβ super-family is vast, encompassing over 30 members including the TGFβ isoforms, anti-Mullerian hormone, and the bone morphogenic proteins (BMPs); for the purpose of this review, we will focus specifically on TGFβ [42–44]. There are three mammalian isoforms of TGFβ: TGFβ1, TGFβ2, and TGFβ3. These isoforms have similar bioactivities and share some function while also fulfilling some unique roles. They are important regulators of lung development, inflammation, injury, and repair, but their roles depend largely on the context of their expression and other parallel cellular processes. TGFβ1 is involved in pulmonary branching during development; overexpression arrests lung morphogenesis [45]. Knockout mice survive *in utero* but die early after birth secondary to pneumonitis and systemic inflammation [46]. Both TGFβ2 and TGFβ3 are expressed in the bronchiolar epithelium, and knockout of either results in perinatal death secondary to pulmonary defects, likely due to abnormal structural development and failure of appropriate epithelial-mesenchymal interactions [47–49].

TGFβ is secreted in a latent form, which is then activated by one of a number of mechanisms including interactions with integrins or matrix metalloproteinases, acidification, and/or reactive oxygen species [43]. Active TGFβ then binds to a complex consisting of two Type I (TGFβRl) and two Type II TGFβ (TGFβR2) receptors, both Ser/Thr kinases. From here, two general downstream cellular pathways are activated (Figure 1).

Canonical TGFβ signaling operates through phosphorylation of Smad transcription factors. While the majority of Smad proteins are activating, Smads 6 and 7 function to inhibit TGFβ activities. TGFβ signaling via this pathway mainly functions through phosphorylation of Smad2 and Smad3 (termed receptor Smads or R-Smads), which interact with Smad4, the shared R-Smad partner, to regulate transcription of several downstream genes [43]. Activated Smads translocate to the nucleus, interacting with co-factors to facilitate binding to specific DNA sequences [42].

Non-canonical TGFβ signaling through non-Smad dependent pathways is perhaps less well understood and may function to either complement or counteract Smad activity. TGFβR2 can directly phosphorylate proteins such as PAR6, leading to altered cell morphology and differentiation [50]. TGFβ signaling can also activate the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, such as Erk, p38, and c-Jun N-terminal Kinase (JNK), although the timing of activation is variable and dependent upon cellular context [42, 43, 51]. The balance of canonical to non-canonical signaling likely functions to modify the cellular response to TGFβ, along with the availability of other co-factors and signaling pathway partners.

TGFβ signaling is also intertwined, upstream and downstream, with miRNAs. miRNAs both modify TGFβ signaling and regulate downstream TGFβ effects; some miRNAs function in both capacities. A multitude of miRNAs are predicted to target TGFβ signaling pathway member mRNAs, but only a subset have been verified experimentally. miRNA regulators of TGFβ include the miR-200 family, which targets TGFβ2, TGFβR1, and Smad2 in a variety of cell types; the miR20 family, which targets TGFβR2 as shown in lung cancer tissues; miR-1343, which targets TGFβR1 and R2 in lung epithelial cells; and miR-21, which targets the inhibitory Smad7, thereby allowing increased canonical pathway activation and driving pro-fibrogeni(activity in pulmonary fibroblasts [52, 53].

TGFβ signaling also alters miRNA expression levels; activated R-Smads interact with the Drosha miRNA processing complex to promote maturation of discreet miRNAs [54, 55]. TGFβ influences the levels of some of the regulatory miRNAs described above, causing downregulation of the miR-200 family and upregulation of miR-21, allowing for greater TGFβ pathway activation [52]. TGFβ decreases miR-203, which normally functions to inhibit epithelial mesenchymal transition (EMT) and metastasis [56]. miR-155 is upregulated after TGFβtreatment of mammary cells in vitro, and knockdown of miR-155 suppresses EMT and cell migration [57]. The intimate interactions between miRNAs and TGFβ signaling have only begun to be described; miRNAs appear to be important modifiers of TGFβ induced pathology.

TGFβ broadly functions to influence injury responses, inflammation, and differentiation [42]. As described above, studies in transgenic mice show that the expression of TGFβ plays a critical role in pulmonary development and regulation of autoimmunity. TGFβ can also drive inflammation in the appropriate context and causes a shift towards Th2-type responses [58]. Also dependent upon the setting, TGFβ can be both pro- and anti-proliferative; in general, TGFβ promotes a mesenchymal phenotype and cell proliferation while inducing extracellular matrix production [59]. Although TGFβ is essential for wound healing, dysregulation of the pathway can drive remodeling and fibrosis, leading to end organ disease. As many pulmonary diseases are characterized by a cycle of pathologic lung inflammation/injury followed by aberrant repair mechanisms, TGFβ is positioned to be a master regulator of lung disease in a variety of disorders.

2.2 TGFβ in Pulmonary Disease

In humans, TGFβ isoforms continue to be expressed in the healthy lung through adulthood. TGFβ expression has been described in human airway epithelium, alveolar macrophages, and airway smooth muscle cells [60–62]. In various pulmonary diseases, TGFβ signaling has been reported to be increased in the airway epithelium, fibroblasts, macrophages, and smooth muscle cells [60, 63]. Reviewing the proposed role of TGFβ in non-CF pulmonary diseases provides a useful framework for understanding and potentially targeting the TGFβ pathway to impact CF lung disease.

Idiopathic pulmonary fibrosis (IPF) is a devastating interstitial lung disease characterized by injury, remodeling, and ultimately progressive fibrosis; median survival is 2.5–3.5 years after diagnosis [64]. A fundamental driver of fibrosis is TGFβ, and levels are elevated in airway epithelium and fibroblasts from IPF patients [60, 63, 65, 66]. TGFβ also drives

myofibroblast differentiation from fibroblasts, a cell type which is proposed to drive the cycle of increased epithelial injury (especially alveolar type II cells) and aberrant injury response that promotes continued fibrosis in IPF [67–70]. In mice, inhibiting TGFβ signaling results in protection in fibrotic disease models [71, 72]. These findings led to a search for therapeutics targeting the TGFβ pathway that are effective in IPF. The first such drug to receive FDA approval for IPF was pirfenidone, an orally available small molecule inhibitor of TGFβ (as well as other signaling pathways including PDGF and TNF-α) with anti-fibrotic and anti-inflammatory effects; the exact mechanism of pirfenidone action is unknown [73]. A 2014 phase 3 study of pirfenidone in IPF showed a significant reduction in the proportion of patients with decline of forced vital capacity (FVC) of 10% or more in the active treatment group versus placebo (16.5% versus 31.8% respectively, $p<0.001$) [74]. Subsequent meta-analysis also supported the therapeutic benefit of pirfenidone in IPF, with pirfenidone use associated with reduction in lung function decline and improved survival over the course of a year [75].

TGFβ signaling has also been implicated in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD). Both diseases are characterized by airway obstruction (typically considered reversible in asthma but irreversible in COPD), inflammation, and remodeling. TGFβ1 levels were elevated in bronchoalveolar lavage (BAL) fluid obtained from asthmatic patients and in the airway and alveolar epithelium of patients with COPD [76, 77]. Higher producing polymorphisms in TGFβ1 (especially a C-509T variant in the TGFβ1 promotor) are associated with worsening asthma severity [78]. Mechanistically, it is hypothesized that TGFβ produces pathologic effects in these diseases by promoting goblet cell hyperplasia, subepithelial fibrosis, epithelial damage, and airway smooth muscle hypertrophy [79–85]. It is important to note, however, that the effects of TGFβ are not universally clear or consistently detrimental. In fact, TGFβ1 underexpressing mice have a more severe phenotype in the ovalbumin induced murine model of asthma, and overexpression of TGFβ1 in Th2 cells reduces disease severity in this model [86, 87]. These studies illustrate the complex and context dependent importance of TGFβ signaling. While TGFβ has been discussed and studied as a potential therapeutic target in asthma for over a decade, no approved medications targeting TGFβ in asthma have advanced to the clinic [88, 89].

2.3 TGFβ as a Genetic Modifier and Biomarker of CF Lung Disease

CF lung disease severity cannot be predicted simply by CFTR genotype [37, 41]; over 50% of the variation in lung disease severity is attributable to non-CFTR genetic contributors [90]. Two TGFβ1 polymorphisms (C-509T polymorphism in the promotor region and T29C polymorphism in codon 10) have been found in genome-wide association studies to be linked to more severe CF lung disease [41, 91, 92]. These polymorphisms have been linked in vitro and in non-CF populations to higher levels of gene expression and secretion. Transfection of the T29C polymorphism in HeLa cells causes a 2.8-fold increase in TGFβ1 secretion, and having at least a single copy of the T29C allele was associated with increased serum TGFβ1 protein levels in a study of Caucasian and African-American normotensive and hypertensive adults and in groups of healthy or osteoporotic Japanese women [93–95]. The C-509T promotor region polymorphism is associated with higher transcriptional activity

of TGFβ1 in vitro and higher plasma TGFβ1 levels in a group of female UK subjects [96, 97]. However, the relationship between these polymorphisms and blood or BAL levels of TGFβ1 has not been clearly defined in the CF population.

In addition to TGFβ1's role as a genetic modifier of CF lung disease, it is also a biomarker of increased disease severity. Increased TGFβ1 blood (plasma) and BAL levels in CF patients have been associated with pulmonary exacerbations, severity of lung disease, increased neutrophilic inflammation in BAL, and infection with *Pseudomonas aeruginosa* [98, 99]. Taken together, these studies implicate TGFβ1 as a prominent potential therapeutic target in CF. In a separate study, serum concentrations of TGFβ2 were demonstrated to be elevated in young CF patients compared to healthy controls, although linkage to disease severity is unclear [100].

2.4 Mechanism of TGFβ Action in CF

Airway remodeling in cystic fibrosis is complex and progressive. Early disease is characterized by inflammation, mucus obstruction, chronic infection, and goblet cell hyperplasia with a relative preservation of FEV_1; later, bronchiectasis and fibrosis result from the cycles of infection and inflammation, chronic infection with dominant organism(s) may take hold, and FEV_1 ultimately declines [101, 102]. TGFβ signaling is involved in different disease mechanisms at different stages of the disease, and TGFβ pathways should not be considered universally detrimental in CF.

One possible mechanism through which TGFβ could negatively affect lung health in CF is through direct downregulation of chloride transport. Several groups have shown that TGFβ1 exposure in human airway epithelial cells in vitro causes downregulated expression and function of CFTR and an alternative chloride transport pathway, Calcium activated Chloride Conductance (CaCC) [103–105]. TGFβ1 exposure also negates the beneficial effects of treatment with VX- 809, a CFTR corrector, on F508del CFTR function [103, 104]. One potential mechanism for this downregulation is through microRNAs, small (~22 nucleotides) non-coding RNAs that bind to specific mRNAs to decrease expression of target genes. miR-145 is a microRNA upregulated by TGFβ1 which downregulates CFTR expression; preliminary evidence suggests that the TGFβ1 induced decrease in CFTR is mediated at least in part by miR-145 upregulation [106, 107]. Increased TGFβ signaling in CF is predicted to induce other pathologies downstream of miRNA alterations. miR-155, an miRNA upregulated by TGFβ as described in section 1.2, is upregulated in CF epithelial cell lines and increases both IL-8 and Smad phosphorylation through inhibition of regulatory associated protein of mTOR, complex 1 (RPTOR) [108].

TGFβ1 may also promote networks of gene expression that drive pathologic airway remodeling. Goblet cell hyperplasia and increased mucin secretion is a well described feature of CF lung disease [109]. Inhibition of TGFβ1 signaling, specifically through Smad dependent pathways, in mouse models of allergen induced rhinitis and airway remodeling suppresses goblet cell hyperplasia [79, 80]. TGFβ1 has recently been shown to drive airway smooth muscle shortening and hyperresponsiveness in vitro via Smad signaling [110]. As described above, TGFβ is linked to pulmonary fibrosis in multiple animal models as well is in IPF and drives myofibroblast differentiation. Studies of CF patients have identified

activation of TGFβ signaling associated with areas of fibrosis and myofibroblast differentiation, and, furthermore, that constrictive bronchiolitis in lung biopsies was associated with myofibroblast differentiation potentially induced by TGFβ [111, 112].

TGFβ signaling is involved in driving inflammatory responses that may augment injurious inflammatory stimuli in CF, both in young patients prior to acquiring chronic pulmonary infections and in older patients with more progressed lung disease and established infection [113]. Neutrophilic inflammation is a hallmark of CF lung disease, and TGFβ is a potent neutrophil chemoattractant [114]. Pro-inflammatory cytokines including IL-1β, IL-6, and IL-8 are higher in CF BAL, while the anti-inflammatory cytokine IL-10 is reduced [115]. Human neutrophil elastase (NE) is also elevated even in young children with CF and is predictive of bronchiectasis and low BMI [4, 116]. TGFβ signaling has mixed effects on these inflammatory regulators and is likely context dependent. NE appears to induce TGFβ1 secretion and activation, and mice lacking NE are resistant to bleomycin induced lung injury and have a lack of active TGFβ in lung tissue [117, 118]. IL-6 and TGFβ synergistically drive differentiation of proinflammatory T helper 17 cells, while TGFβ and IL-1β work together to enhance IL-6 and IL-8 production in retinal epithelial cells [119, 120]. TGFβ1 inhibits IL-10 expression in immune cells, thus reducing modulation of the immune response by this anti-inflammatory cytokine [121]. Human airway epithelial cells exposed in vitro to TGFβ demonstrate suppression of IL-8 production, but in other cell types such as renal epithelial cells and cancer cell lines TGFβ induces IL-8; the role of TGFβ in vivo on IL-8 production in CF is unclear [122–124].

Aberrant secondary inflammatory responses may be another mechanism of TGFβ mediated pulmonary pathology in CF. TGFβ is expressed by a variety of immune cells, including alveolar macrophages, eosinophils, and T lymphocytes, and is a potent neutrophil chemoattractant [61, 125, 126]. Recent studies have shown that TGFβ1 suppresses innate immune responses and alters host responses to infection, with reduced clearance and augmented inflammation, and promotes viral replication in several epithelial cell models [127–129]. TGFβ signaling may thus enhance susceptibility to viral infections while promoting inflammatory responses that lead to lung injury and damage.

Alterations in TGFβ expression are induced by *P. aeruginosa* quorum-sensing (QS) systems, an intercellular density dependent communication linked to biofilm formation and virulence, and TGFβ may in turn lessen the effectiveness of the resulting inflammatory response [130]. In a murine model of burn infection, QS-capable *P. aeruginosa* induced TGFβ1 expression while a QS-defective mutant did not [131]. Similarly, in a rat model of pulmonary *P. aeroginosa* biofilm infection, wild *type P. aeruginosa* but not QS mutant strains induced pulmonary TGFβ1 expression; the authors hypothesized that the cytokines induced by the QS system may upregulate regulatory T cells and thus decrease the effectiveness of the resulting immune response [132]. Adaptations of quorum signaling occur during the transition of initial to chronic infection in cystic fibrosis patients, and it is unknown how TGFβ production and function is altered during this complex process [133, 134].

TGFβ has a multifactorial and complex role in promoting lung disease in CF, as summarized in Table 1. While the time course of TGFβ dependent effects is unclear, it is possible that

early effects and responses to TGFβ1 signaling contribute to early events in CF lung disease pathology. In this scenario, increased TGFβ signaling in young CF patients may drive goblet cell hyperplasia, immune system dysregulation, and counterproductive responses to infection and fibrosis that ultimately contribute to lung function decline.

3. Targeting TGFβ in CF

Due to its broad relevance across multiple organ systems and diseases, targeting TGFβ and its downstream pathways is an area of intense research and development interest. However, the complex network of pathways activated downstream of TGFβ, as well as TGFβ's diverse involvement in critical cellular functions, makes developing an effective but safe therapy challenging. There are several potential strategies for targeting TGFβ and its signaling pathways: broad TGFβ pathway inhibition at the receptor or ligand level, directed blocking of downstream signaling components, targeting microRNAs, or focusing on modifiers and downstream products (Figure 1, Table 2).

As with all CF therapies, drug delivery poses a unique challenge. Pulmonary disease remains the largest source of morbidity and mortality for CF patients, so inhalation is a logical method of delivery. As TGFβ in BALF is a biomarker of more advance CF lung disease, blockade of TGFβ signaling via an inhaled drug may be sufficient to ameliorate TGFβ induced pathology [98]. However, CF pulmonary disease presents unique barriers to effective inhaled drug delivery, including impaired drug deposition in diseased airways, permeation through thickened mucus, and an environment with sustained infection and inflammation [135]. In addition, evidence is gathering that dysfunction related to loss of CFTR not only affects epithelia throughout the body but also the immune system, smooth muscle cells, and fibroblasts [3, 136, 137]. If TGFβ does further downregulate CFTR in CF, systemic drug delivery may provide enough CFTR restoration in certain CF genotypes to lessen CF induced pancreatic and intestinal dysfunction [103]. While systemic drug delivery, either enteral or parenteral, has the benefit of modulating TGFβ signaling throughout the body, it also exposes the patient to more potential toxicity and off-target effects.

While many types of pharmacologic strategies have been investigated for other disease processes, including neutralizing antibodies, small molecule inhibitors, antisense RNAs, ligand traps, and gene therapy, no TGFβ targeting drugs are currently approved for use in CF. There are currently no reports from clinical trials targeting TGFβ signaling in CF, so strategies to modulate TGFβ signaling to treat CF must be extrapolated from animal or cell studies and patient data from other disease processes. What follows is not a complete list of therapeutics that target the TGFβ pathway; rather, in the remaining sections of this review we seek to identify feasible and/or rational approaches based on the scientific literature.

3.1 Existing Medications with Known TGFβ Effects

Several approved medications have known effects on TGFβ signaling pathways, although the exact mechanism of these effects is often not well understood. Pirfenidone, currently approved for IPF, blocks TGFβ signaling and production by an unclear mechanism; TGFβ1-induced airway surface liquid (ASL) dehydration in CF bronchial epithelial cells has been shown to be abrogated by treatment with pirfenidone in vitro [74, 138]. Losartan, an

angiotensin II type 1 receptor inhibitor, reduces TGFβ signaling and also reduces mucociliary dysfunction in CF bronchial epithelial cells after TGFβ treatment [139]. Corticosteroids have been demonstrated to inhibit TGFβ1 expression in a mouse model of asthma, yet these results have not been replicated in humans, and the utility of using corticosteroids to reduce TGFβ signaling is unclear [88]. Simvastatin, used primarily for its lipid-lowering effects, also inhibits TGFβ1-induced myofibroblast production [140]. Finally, mepacrine, an anti-malarial drug, inhibits TGFβ1 expression and subepithelial fibrosis in a mouse model of asthma [141]. As these medications have known side effect profiles and are approved for other indications in humans, they represent low-hanging fruit in the search for TGFβ modifying medications in CF. However, their utility and direct translation to CF may be limited by off target effects and unclear mechanisms of action.

3.2 Broad TGFβ Pathway Inhibition at Ligands/Receptor Level

TGFβsignaling can be broadly inhibited by targeting TGFβ ligands or receptors. A panoply of monoclonal antibodies have been developed, both ligand-specific and capable of targeting multiple TGFβ ligands [51, 127]. Many of these antibodies have been humanized and proceeded through at least the early stages of preclinical and clinical trials in non-CF diseases where TGFβ ligands are overexpressed. The benefit of these monoclonal antibodies includes the possibility of specificity for select TGFβ ligands (while retaining TGFβ signaling via other ligands) and their pharmacokinetic stability. The TGFβ1 isoform has the most evidence for involvement in CF lung disease, and therefore is a logical initial target for consideration. Animals studies have provided evidence of successful TGFβ pathology inhibition via antibodies. In rodents, anti-TGFβ antibodies have been used to halt nephropathy and suppress tumor metastasis; such antibodies are undergoing clinical development for a variety of disease including cancers and scleroderma [52].

Soluble receptor constructs are another strategy to sequester excess TGFβ ligands. Ligand traps using modified or mimetic TGFβII and TGFβRIII constructs have been developed; these pharmacologics have prevented tumor metastasis in animal models and shown promise in suppression of cell growth of human breast and colon cancer in vitro [52]. These therapeutics have not moved far in clinical development, and there may be challenges to systemic drug delivery [51].

Another method of TGFβ pathway inhibition is RNA targeting. Gene silencing techniques such as antisense oligonucleotides, short interfering RNAs (siRNAs), and promoter targeting pyrrole-imidazole polyamides inhibit mRNA translation and have been successfully used against TGFβ ligands in non-CF cell and animal models, but difficulties with drug delivery and stability persist [51, 88]. Antisense oligonucleotides against TGFβ family members have been used to make antisense-modified tumor cell vaccines to promote antitumor immunity; such techniques have unclear applications in CF [142].

Beyond inhibition of ligands, receptors are also appealing targets; monoclonal antibodies against TGFβR1 and R2 have been developed [51]. These antibodies have been shown to have effects on both canonical and noncanonical signaling and would presumably provide the greatest universal, ligand and TGFβ isoform-independent downregulation of TGFβ signaling. Intracellular signal transduction can be blocked by TGFβ1 receptor kinase

inhibitors, which would also provide broad TGFβ pathway inhibition. In vitro data for such inhibitors are promising, with inhibition of TGFβ induced EMT and fibrosis, and clinical development of some such pharmaceutics for cancers such as hepatocellular carcinoma and glioblastoma is advanced [142].

3.3 Targeting Downstream TGFβ Signaling

Several small molecule inhibitors targeting TGFβR1 to inhibit Smad 2/3 phosphorylation have also been developed and have the benefit of oral availability, but may have off target effects on other activin-like kinases (ALKs) [51]. Upregulation of the inhibitory Smad 7 can also be a tool to decrease Smad dependent signaling [88]. However, it is unclear in CF if the primary pathology downstream of TGFβ is driven by the Smad-dependent canonical pathways and/or the non-canonical pathways. In other words, the necessary balance of canonical and non-canonical signaling has not been established to date. Indeed, TGFβ1 induced reduction of CFTR expression and function appears to be via a p38 MAPK mechanism rather than Smad dependent signaling, indicating that Smad inhibition would, at best, only partially relieve TGFβ induced pathology [103, 104].

Non-canonical pathways are also potential targets for inhibition. In oncology and other diseases, small molecule inhibitors of the Erk pathway have been trialed, but their use is limited by variable responses and development of resistance [143]. JNK and p38 MAPK inhibitors have been investigated for use in IPF and beyond, but side effects and toxicity are continuing challenges [144, 145]. The PI3K pathway is also felt to contribute to dysregulated inflammatory responses, airway hyperresponsiveness, and mucin production [146]. Small molecule inhibitors of this pathway are promising, but further work is needed to avoid dangerous immunosuppression in CF (where chronic infection is common), off target effects, unacceptable side effects; indeed, certain inhibitors of the pathway, especially macrolide mTOR inhibitors such as sirolimus, carry a risk of interstitial pneumonitis [146].

3.4 MicroRNA as Targets

A number of studies have implicated miRNAs as altered in CF and potentially playing a role in mediating cystic fibrosis pathophysiology related to TGFβ signaling [147]. These may be amenable to anti-miR targeting, or in the case of protective miRNAs downregulated by TGFβ or miRNAs that target TGFβ pathway members, overexpression. As outlined above, TGFβ signaling can directly promote maturation of specific microRNAs through interaction with the microRNA processing complex [54, 55]. There is extensive crosstalk between TGFβ signaling and microRNAs, and a number of miRNAs are altered by TGFβ [52]. miR-145 specifically has been shown to be upregulated in response to TGFβ signaling and to decrease CFTR expression and function [106, 107, 148].

Development of therapeutics targeting miRNAs has been an enticing goal, especially in oncologic diseases, with some therapeutics reaching clinical development for non-CF diseases [149]. The most common method of miRNA suppression is utilizing an antisense oligonucleotide (antagomir) that bind miRNA targets to prevent miRNA-mRNA interactions [150]. To target knockdown of a network of miRNAs, miRNA sponges are being developed that contain multiple binding sites for related miRNA targets [52]. A third technique,

miRNA masking, involves utilizing a single-stranded oligoribonucleotide that is complementary to the miRNA binding site on the target mRNA to hinder miRNA binding [52]. For miRNAs that might target and downregulate TGFβ pathway members, therapies include miRNA overexpression or development of artificial double-stranded miRNA mimic [149].

There are several challenges involved in developing miRNA therapeutics. With systemic delivery, there is the potential for cytotoxic effects (related to either the miRNA or the vector) and immunogenic reactions; local treatment may be more feasible, with intranasal delivery previously described [52]. Furthermore, difficulties with drug delivery due to size and charge in the setting of pulmonary disease have slowed durg development, although studies are ongoing [147].

In addition to inhibiting TGFβ-upregulated miRNAs and increasing TGFβ-downregulated miRNAs, antagonizing or expressing miRNAs that influence relevant TGFβ target genes may be a viable strategy. Further research would be needed both to identify miRNA targets and validate their utility in blocking TGFβ induced pathology in CF.

3.5 Modulating TGFβ Signaling through Modifiers, Co-Regulators, and Downstream Products

Given the complex network of pathways and genes influenced by and interacting with TGFβ in a context dependent manner, there are a vast number of targets outside the direct TGFβ signaling pathways that may be amenable to therapeutic modulation. The EGFR pathway has been implicated as a positive regulator of TGFβ induced pulmonary fibrosis and functions through activation of non-canonical pathways including PI3K and ERK [151]. Inhibition EGFR signaling in lung diseases has been an area of intense research, but a clinical benefit has only been seen in lung cancer therapy; difficulties with clinical efficacy and toxicities limit therapeutic development [152]. Interferon-gamma (IFN-γ) is a cytokine that promotes a Th1 type response, perhaps balancing the Th2 cytokine production driven by TGFβ, and also downregulates TGFβ2 [88]. Monoclonal antibodies against integrin β6, a key activator of latent TGFβ, prevent activation of TGFβ and have undergone studies in non-CF patients [51]. Key downstream regulators of TGFβ such as protein tyrosine phosphatase α (PTP-α), which mediates profibrotic signaling, are also potentially amenable to inhibition [42]. The principle challenges involved in inhibiting regulators and downstream products involved in transmitted TGFβ effects include difficulty in target selection and potentially narrow windows of clinical efficacy.

4. Conclusion

TGFβ signaling in certain contexts drives a number of processes involved in CF lung disease pathophysiology, including fibrosis, goblet cell hyperplasia, abnormal inflammatory responses, and dysregulated ion transport. TGFβ1 in particular has been shown to be both a genetic modifier and a biomarker of CF lung disease severity. The complexity and context dependence of TGFβ signaling, however, make it a challenging therapeutic target, as exemplified by the difficulties in developing TGFβ-targeting therapies for diseases such as IPF where TGFβ has long been identified as a driving force of lung disease. Given the

building evidence that TGFβ signaling is involved in CF lung disease and can directly drive further decreases in CFTR expression and function, we postulate that it is time to re-evaluate TGFβ pathways and regulators as potential therapeutic targets in CF. Furthermore, as TGFβ signaling may impact the efficacy of emerging and expensive CFTR modulators that are becoming available for certain CF patients, understanding the implications of TGFβ signaling may allow clinicians to make more educated and personalized choices to better treat CF patients.

5. Expert Opinion

Modulating TGFβ signaling in CF presents a unique opportunity and challenge, as it is a critical regulator of lung development and homeostasis, as well as a tumor suppressor and antiinflammatory cytokine depending on its cellular context. Broad TGFβ suppression may well have undesirable effects upon cellular homeostasis, inflammation, and tumor suppression. Efficacy in CF patients y to be variable and depend upon genetic variations in patients, stage of lung disease, and personal disease characteristics including the pulmonary microbiome. Other co-therapies, such as CFTR modulators, will likely impact the response to TGFβ-targeted therapies, including which aspects of CF pathology are affected. A better understanding of the role of TGFβ in CF lung disease will help researchers test novel therapies that impact rational signaling components and ultimately help clinicians select appropriate drugs to administer to the right patient at the right stage of disease.

5.1 Targeting Appropriate Components of TGFβ Signaling

As described above, the TGFβ signaling network is complex and context-dependent. Pan-inhibition of TGFβ signaling would potentially disrupt a number of critical cellular processes. Choosing appropriate targets will be necessary to developing efficacious, safe therapies in CF. Preliminary work has identified that TGFβ-induced p38 signaling contributes to CFTR downregulation in human airway epithelial cells (AECs), but it is unclear if this is a significant contributor to lung pathology in vivo [103, 105]. Furthermore, miR-145 has been identified as an important mediator of CFTR downregulation by TGFβ1, but the relationship between this mechanism and p38 signaling is currently undefined [106, 148, 153]. TGFβ's influence on goblet cell hyperplasia appears to be through Smad-dependent mechanisms, while profibrotic effects appear to include both canonical and noncanonical signaling pathways. Thus, there may be no single target downstream of TGFβ that will abrogate all, or even the majority, of potential detrimental effects. Further studies are needed to define the role of TGFβ in CF lung disease severity, describe the downstream pathways involved, validate potential targets identified using simple systems in translational models, and elucidate the time course of TGFβ effects.

Drug delivery is another translational challenge. Systemic therapies increase the potential for off target effects in unaffected tissues. Direct intrapulmonary delivery may allow higher pulmonary drug exposures and avoid systemic effects, but penetrance and transepithelial transport of drugs is variable and depends on drug geometry, size, and charge. Furthermore, as is the case with other inhaled therapeutics, delivery may be poor in those pulmonary

regions with greater obstruction and mucous plugging, which may ironically be where TGFβ targeting has the greatest potential impact.

5.2 Determining the Right Patient

Other co-therapies may impact the efficacy of TGFβ modulation in CF, or TGEβ inhibitors may improve the efficacy of existing drugs. In vitro studies have shown that TGFβ treatment of human AECs negates the beneficial effects of VX-809 on F508del CFTR; it stands to reason that certain patients may see more benefit from VX-809 with concurrent TGFβ targeted therapy where excessive TGFβ signaling is manifest. This logic may extend to other CFTR modulators, but preclinical support is currently lacking. Clarifying these important interactions in the context of complex CF treatment regimens will be important, but not straightforward.

A patient with one of the previously described polymorphisms in TGFβ linked to development of more severe lung disease may experience more clinical benefit from therapy targeting TGFβ, but this too is currently only theoretical. Beyond CFTR-mutation specific modulator therapy, we are currently far from effectively harnessing our growing knowledge of CF genetic modifiers to inform treatment plans. As a first step, using patient derived samples to identify excessive TGFβ signaling and to test in vitro responses may help guide the selection of patients for clinical trials. With the growing concept of personalized medicine in the CF community, we feel this general approach may become a valuable tool to choose the most appropriate patient to receive next generation therapies.

5.3 Timing of TGFβ Targeting Therapies

It is becoming clear that effective treatment of CF lung disease needs to begin early in life. Bronchiectasis is already present in many toddlers with CF, and studies in pigs suggest that host-defense defects are present at birth [4, 154]. As TGFβ is a genetic modifier of CF, it is possible that it has an early impact on processes such as innate immune response, airway remodeling, inflammation, and fibrosis. However, we currently do not have an understanding of the expression and role of TGFβ in very young children with CF. It is possible, perhaps even likely, that the later elevation of TGFβ in the BAL and blood of CF patients with more advanced lung disease is not only causative but also a marker of lungs with great infectious, inflammatory, and fibrotic burdens. Further studies are needed to define the pattern of TGFβ expression and correlation with the development of lung disease in children with CF.

Although early therapy has the most potential to change long term CF lung disease trajectories, targeting young (and potentially asymptomatic) children for therapy brings certain ethical and medical considerations. Long term toxicity and impact on lung development must be thoroughly evaluated before these medications could be considered in this vulnerable population. It is highly likely that approval would first be sought for anti-TGFβ therapeutics in adults, and then extended towards younger age groups as efficacy and safety are shown. This requirement of drug development could potentially be a barrier, as the impact of TGFβ-targeted therapies may vary substantially based on the stage of lung disease. Thus, findings in older patients with established disease may not predict or reflect effects in young patients with less lung damage.

5.4 Future Directions in Developing Therapies Directed at the TGFβPathway

As TGFβ inhibiting medications are already approved for other disease processes (i.e.: pirfenidone in IPF, losartan in Marfan syndrome), these would be logical initial therapies to trial in CF based on supportive evidence from preclinical and translational model systems. These preclinical studies are essential to understand how these therapies may influence CF lung disease and to select appropriate biomarkers to demonstrate drug bioactivity in the context of CF. Further study is needed, however, to better understand the mechanism(s) of TGFβ induced pathology in CF, the timeline of these effects, the downstream mediators involved, and the connection between discreet TGFβ signaling pathways and pulmonary pathology. We will also need to consider the personal disease characteristics in our CF patients that may impact the efficacy of TGFβ targeting, including TGFβ polymorphisms, CFTR genotype, CF co-therapies, and disease stage. There is a risk of failing to prove efficacy or provoking unacceptable side effects if patient and drug selection are not carefully considered.

In summary, TGFβ is an attractive target for new CF therapeutics, based on its clear lung disease modifying association and relationship to numerous important and fundamental aspects of CF lung disease. The complexity of TGFβ signaling and the enormous reach of its influence across organ systems and diseases also make it a challenging target, and the development of therapies will require careful mechanistic studies to understand relevant signaling pathways and downstream impact. Accumulating research in CF suggests, however, that it could be an excellent therapeutic target with broad beneficial impact on ion transport, goblet cell hyperplasia/mucus production, inflammation, airway remodeling, and/or fibrosis. Our current research understanding supports studies to identify and advance these therapies in CF, with an eye to balancing the relative roles of TGFβ in both lung health and disease.

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Article Highlights

- TGFβ is a genetic modifier of CF lung disease and a biomarker of more severe pulmonary pathology in patients.
- TGFβ signaling drives airway remodeling, dysfunctional inflammatory responses and epithelial ion channel dysregulation in experimental models.
- The mechanism of CF disease modification by TGFβ, and the utility of targeting its pathway, is unknown.
- Therapeutics targeting TGFβ signaling are under development for a variety of pulmonary and non-pulmonary diseases but have not been used in CF.
- Inhibiting the TGFβ pathway in CF may improve outcomes for certain patients and allow for better efficacy of new CFTR modulating pharmaceutics.

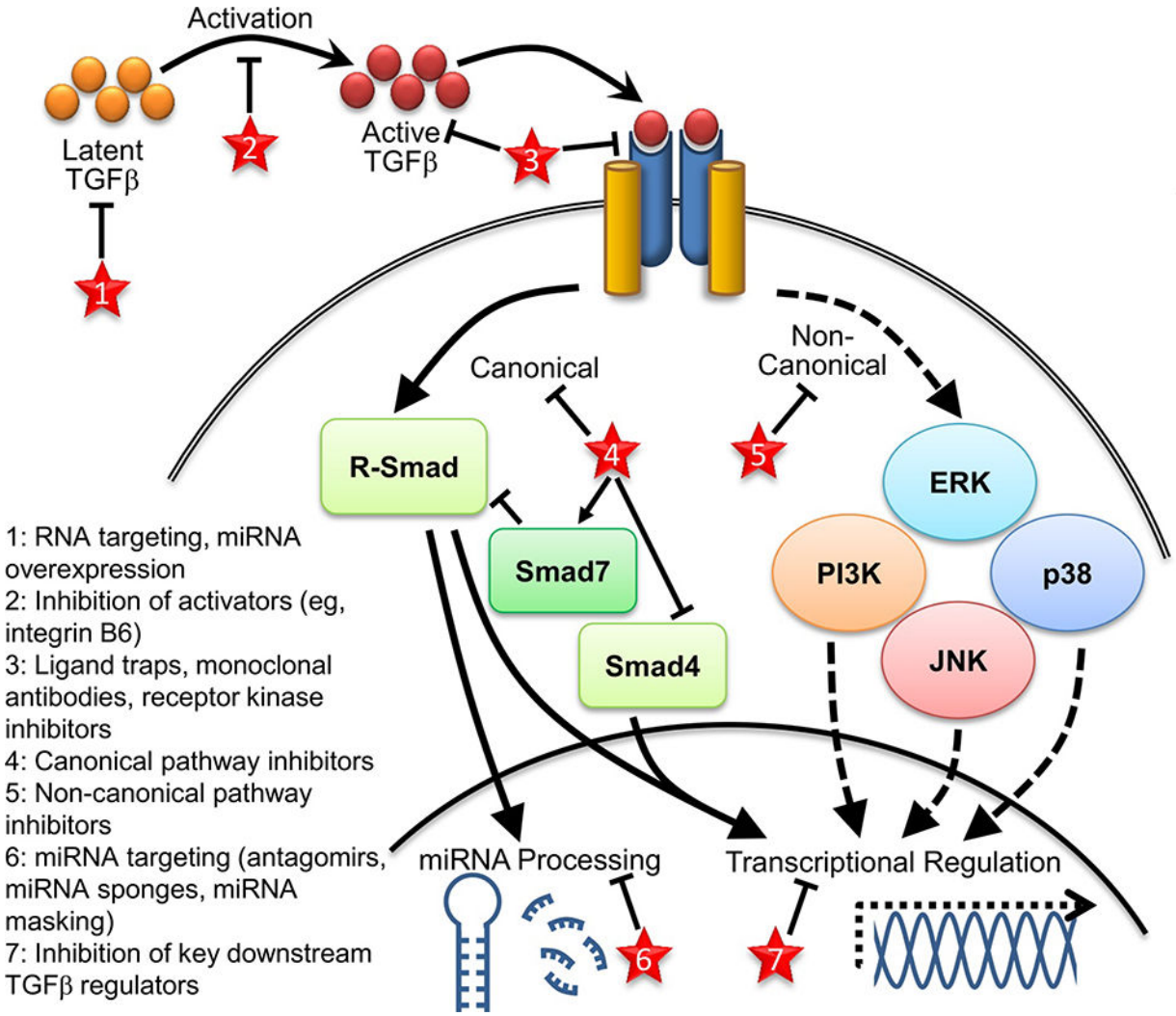

Figure 1. Schematic demonstrating simplified TGFβ signaling and downstream pathways.
Potential sites of therapeutic targeting discussed in text are indicated by red stars.

Table 1.

CF relevant effects and potential mechanisms of pathologic TGFβ actions in CF.

Effect	Suggested Mechanism	Relevance to CF	Refs
Downregulation of epithelial chloride transport	Downregulated CFTR and CaCC expression and function, potentially microRNA mediated	Exacerbates already dysregulated ion transport in epithelia throughout the body in CF	**94, 95, 96**
Driving goblet cell hyperplasia	Potentially via Smad dependent pathways based upon mouse models of allergen induced airway remodeling	Goblet cell hyperplasia and mucin secretion are pathologic features of CF lung disease	**72, 73, 79**
Aberrant inflammatory responses	Suppression of innate immune responses with augmented inflammation	CF lungs are known to be primed for a more aggressive inflammatory response, inability to clear chronic infection, and dysregulated innate immunity	**104, 105, 106**
Enhanced fibrosis	Driving myofibroblast differentiation and aberrant injury response that promotes fibrosis	After cycles of infection and inflammation, fibrotic lung disease significantly contributes to pulmonary decline in CF patients	**56, 100, 101**

CaCC, Calcium activated Chloride Conductance; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulation.

Table 2.

Potential approaches to normalize TGFβ signaling in CF.

Target	Drug Type or Approach
Broad TGFβ pathway inhibition	Monoclonal antibodies targeting ligands or receptors Ligand traps Small molecule inhibitors
TGFβ ligand specific inhibition	Monoclonal antibodies Gene silencing, e. g. siRNAs and antisense oligonucleotides
Smad dependent signaling blockade	Small molecule inhibitors Receptor kinase inhibitors Smad7 upregulation
Non-canonical signaling inhibition	Small molecule inhibitors of relevant pathways, e. g. PI3K and MAPK inhibitors
Pathologic miRNA s	Antagomirs
Modifiers of TGFβ effects:	
Co-regulator inhibition	Small molecule inhibitors, antibodies targeting EGFR pathway
Promotion of Th1 inflammatory response	Upregulation of IFN-γ to balance Th2 response driven by TGFβ
Blockade of TGFβ activation	Monoclonal antibodies targeting integrin β6

IFN-γ, interferon gamma; MAPK, mitogen-activated protein kinase; miRNA, microRNA; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA