# Novel therapeutic strategies for idiopathic pulmonary fibrosis: targeting epithelial repair and regeneration

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Bachelor Research Project 2021 (period IIa)
06-04-21

## Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive respiratory disease in which excessive scarring and impaired epithelial regeneration declines lung function. Because currently used drugs do not cure IPF, the need for more effective and safe drugs is still urgent. Opportunities may lie at specific epithelial targeting by interfering with growth factor signaling, or by using gene therapy. Despite extensive performed research on fibrotic pathways, current knowledge regarding treatment strategies which target epithelial repair in IPF is still limited. We therefore summarized the most potent findings of 4 strategies (TGF-β, FGF, miRNA, TERT) with differently working mechanisms on the lung epithelium. In this review, we will introduce IPF and elaborate its pathophysiology. We then discuss the individual involvement of the four mediators in IPF and evaluate studies which performed interventions or investigated treatment options, after which recommendations for future research are provided in order to address the faced challenges regarding pleiotropic mediator functions as well as development of drug and model design. Addressing these remarks will provide better or even curing treatment options in the near future for this severe and pathologically complex disease.

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a severe respiratory disease characterized by dysregulation of tissue repair mechanisms and an inability to restore the epithelial barrier after repeated injury. This eventually leads to excessive scarring of the alveoli and bronchioles (1)(2). A typical characteristic of fibrotic lung tissue is thickening of the alveolar wall and the loss of its structural integrity, leading to limited gas exchange **(Figure 1)**. Patients experience shortness of breath, coughing, fatigue, or ,in end-stage disease, respiratory failure (3). The cause of IPF is unknown but gender (male>female), age, genetic predisposition, and environmental factors (e.g. exposure to silica and cigarette-smoke) have been shown to be risk factors (3,4). Patients aged above 60 years (494,5 per 100.000 cases per year) with a smoking history (75 %) or who express more susceptible genetic polymorphisms (20%) are generally more likely to develop IPF (3)(5). Examples of higher genetic susceptibility are polymorphisms in the *MUC5B* gene (6). Other contributing factors include the use of certain drugs (e.g. anti-cancer, immunosuppressants), chronic viral infections (e.g. Epstein-Bar virus), or underlying diseases (e.g. lung carcinoma)(6)(7).

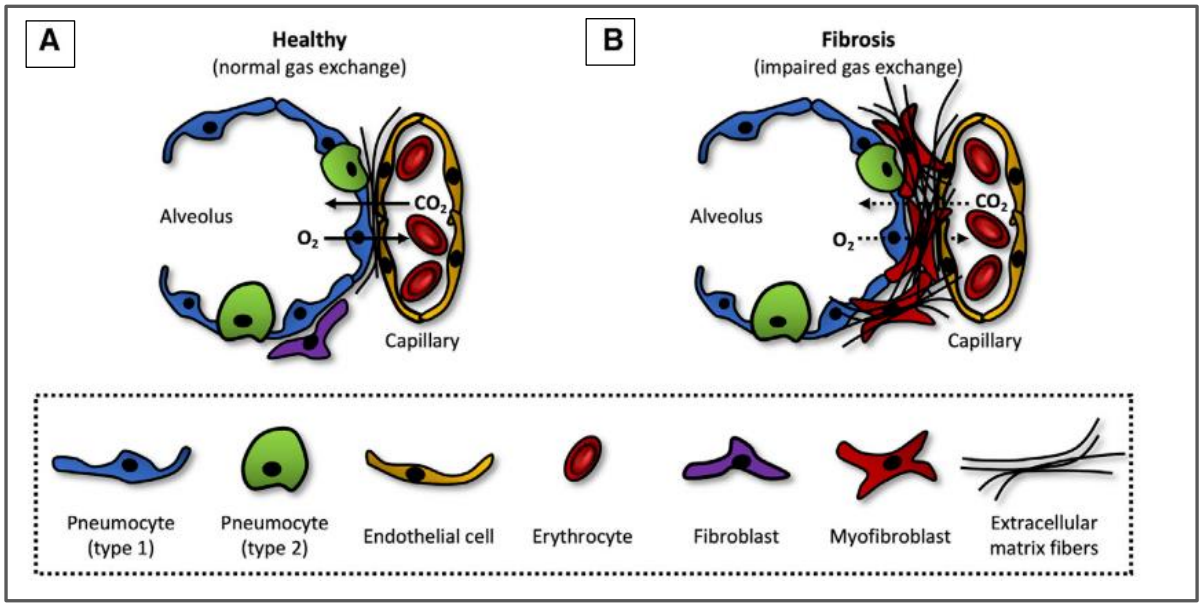


*Figure 1: Gas exchange of $O_2$ and $CO_2$ between the alveolus and capillary barrier. (A) Fast gas exchange in healthy alveolus. (B) Impaired gas exchange in a fibrotic alveolus, caused by a thickened barrier as a result of excessive ECM deposition* (6).

Currently, two drugs are available which both slow down the progression of the disease. Both are given orally and are called nintedanib (Ofev®) and pirfenidone (Esbriet®). Nintedanib acts on a broad spectrum of tyrosine kinases (e.g. PDGF-R, VEGF-R, FGF-R1), which are strongly involved in fibrosis related gene expression(8). In patients, nintedanib has shown to reduce the forced vital capacity (FCV) by approximately 50% compared to placebo (8). Occasionally, a number of gastro-intestinal adverse effects (e.g. nausea, diarrhea) may be observed, forcing ~5% of the patients to suspend treatment. With respect to pirfenidone, the mechanism of action is still unknown. However, it has been shown to be effective by reducing the number of patients with disease progression by 10% and death by 43,8% (9). Secondly, the number of patients with no disease progression increased by 59,3%. In addition, the numbers of observed skin and gastro-intestinal side effects (e.g. nausea, diarrhea) were low and only led to suspension of the treatment in a small number of patients. However, the side effects that did occur by both drugs can be considered as unpleasant and must certainly be taken into account. Even though these treatments effectively slow disease progression, the prognosis remains poor as the progression is only halted and side effects may occur. Considering this, the need for new curing and safe drugs is still of great importance.

Cell signaling pathways are at the heart of fibrosis, providing a vast number of treatment possibilities. Aberrant scarring in IPF could possibly be prevented or ameliorated by interfering with gene expression or specific signaling messengers involved in fibroblast recruitment, collagen deposition and architectural remodeling. Further progression of the disease could be diminished by inhibiting apoptosis in epithelial cells to maintain their recovery function. A different approach could be via initiation of epithelial cell proliferation in order to re-epithelialize the alveolar barrier and initiate a regenerative response. Reducing senescence may be another approach to reduce fibrosis and ameliorate lung function. All processes are highly

interconnected, which can be used in favor but possibly bring challenges as well. Once a better understanding of the disturbed fibrotic process and epithelial repair is obtained, more effective and specific targeting can be achieved.

Therefore, the aim of this review was to provide an overview of the current knowledge and developments regarding epithelial repair and its connection with fibrotic dysregulation in IPF. Furthermore, potential treatment targets will be evaluated, in order to provide directions for future studies. Molecular signaling mechanisms in epithelial repair and progenitor cell differentiation will be discussed in order to find out if targeting of epithelial repair mechanisms can reduce fibrosis, resolve progenitor cell dysfunction and/or restore regenerative epithelial repair. In addition, current challenges that are faced, such as clinical translation, drug targeting and used models will be covered, accompanied by assessed recommendations for this severe respiratory disease. Altogether this review will create a comprehensive insight in IPF pathology regarding epithelial repair and the fibrotic interplay.

## Pathophysiology of IPF

In order to comprehend the concepts of epithelial repair and progenitor cell dysfunction in IPF, the basic processes of tissue repair in normal lungs and its molecular signaling are described first. In brief, repair of damaged tissue is distinguished in four consecutive stages, which show overlap upon transitioning (6). This well-organized program begins with a coagulation stage, followed by an inflammatory stage, a fibroblast recruitment/proliferation stage and finishes with a remodeling stage (6). Upon injury, epithelial and endothelial cells rapidly secrete anti-fibrinolytic cytokines (e.g. Tissue Factor Xa, fibrinogen, thrombin) in order to create a temporary matrix consisting out of fibrin and fibronectin (FN)(10). During the formation of this network, multiple inflammatory cells (e.g. macrophages, lymphocytes, eosinophils,

neutrophils) are attracted to the affected tissue to phagocytose and neutralize pathogens. Secondly, they remove debris present as a result of (induced apoptotic or necrotic) cellular damage. The now present immune cells set off the third stage by secreting various profibrotic cytokines. These are responsible for the recruitment of fibroblasts, which differentiate into myofibroblasts when activated. Expression of the pro-fibrotic cytokine transforming growth factor β (TGF-β) is upregulated upon injury and responsible for this activation (11). The final differentiated cellular form produces and secretes a wide variety of extra cellular matrix (ECM) proteins (e.g. laminin, FN, collagen), with collagen type-1 as main contributor to this network. Now the alveolar wall can safely be re-shaped as executed in the final stage. Myofibroblasts locally contract the damaged tissue site, due to presence of contractile smooth muscle α- actin (α-SMA) bundles, so endothelial and epithelial cells are able to cover the temporary ECM. Complete remodeling of the damaged tissue is reached when the matrix composition and organization has been normalized, the fresh produced matrix has been broken down, the endothelial/epithelial wall has been fully enclosed and the remaining myofibroblasts have undergone apoptosis or entered a senescent cellular stage (6).

With the scope on epithelial repair in this review, understanding the reparative ability of these cells is of great importance. Compared to other tissues like the intestine or epidermis, the lungs in a healthy condition display a low rate of cell turnover and are less delineated with stem cells. Yet, lung tissue is well capable of recovering upon damage (12). This capability is highly interesting because induced epithelial repair mechanisms could have the potential to reverse or minimize pulmonary fibrotic damage as a result of, for example IPF. The epithelium which covers the inside of our lungs, is developed from a small region of anterior ventral foregut endoderm during morphogenesis of the premature lung. This process can be described by two stages which are branching and alveologenesis, both regulated by transcription factor Nkx2-1

(12). Branching of the lung endoderm naturally occurs between week 7 and 16 of gestation, whereas alveologenesis happens between week 16 to 24. Compared to the alveoli, the formed larger airways contain different epithelial cell types including ciliated, undifferentiated columnar, secretory and basal cells. When emerging to the smaller airways, submucosal glands make place for Clara cells and the cartilage cell population decreases (13). Differentiation and saccular formation of the alveoli eventually gives rise to functional gas exchange surfaces, squamous and cuboidal epithelium and vascularization (3). Once fully maturated, alveolar epithelial cells in the lung can be distinguished into two types: the alveolar epithelial type 1 cell and the alveolar type 2 cell (AEC1/2). AEC1 cells occupy most of the alveolar cell surface and are characterized by their squamous morphology, allowing them to facilitate gas exchange. AEC2 cells exhibit progenitor cell characteristics due to their ability to differentiate into AEC1 cells and produce, surface tension reducing, surfactant proteins which keep the alveolar wall flexible (3). In steady state, differentiation into AEC1 cells is low but becomes enhanced upon cellular injury (3,12). Discovery of the cellular mechanisms involved in this upregulation may be key for a better understanding of epithelial dysregulation in IPF. Ultimately, this may provide new developments in prevention or recovery treatments of the scarred epithelial wall architecture.

In IPF patients, repair mechanisms are strongly disrupted, resulting in excessive ECM deposition **(Figure 2)**. Other histopathological characteristics of IPF lungs include AEC2 hyperplasia and aberrant proliferation of mesenchymal cells (14). In addition, the lungs are not able to restore the normal alveolar structure. This is caused by the development of a profibrotic milieu due to altered matrix composition, cellular senescence or/and epigenetic rearrangements, which can ultimately lead to irreversibility of disease progression (6). The characteristic excess of ECM deposition in IPF could be caused by decreased myofibroblast apoptosis or reduced

differentiation and proliferation of epithelial cells, but this is still to be confirmed. Besides these possible direct causes, supportive mechanisms like angiogenesis and oxidative stress are observed in IPF patients as well (6). These effects could be a consequence of the imbalance in alveolar environment and may indirectly cause additional damage. With regard to lung development and differentiation, several morphogenic pathways are associated with IPF pathology including TGF- β, FGF, Wnt, hedgehog and Notch signaling. Most of the time, these mechanisms remain dormant but are activated upon injury (3). Mesenchymal cells play a large role in these differentiation steps and are able to transition into several important cells which are essential for a functional pulmonary system such as: vascular-and-endothelial smooth muscle cells, myofibroblasts, lipofibroblast and pericytes. The lung mesenchyme has shown to be a supporting resource in regenerative repair of epithelial progenitor cells (11). With respect to mesenchymal cells in pulmonary fibrosis, studies have reported an increased presence due to epithelial-to-mesenchymal transition (EMT) from AEC2 cells, possibly causing more myofibroblast differentiation and ultimately fibrosis as a result (15). Though multiple present mechanisms are proven contributors to the excessively scarred tissue or progenitor dysregulation, their interconnection and origin remains a gray area and a point of attention for further research.

In the following sections, promising highlights of the current state of knowledge of four signaling pathways will be discussed, all making use of unique working mechanisms. By elucidating these different routes, the complexity and diversity of IPF will be illustrated, with the goal to show multiple target opportunities for treating IPF.

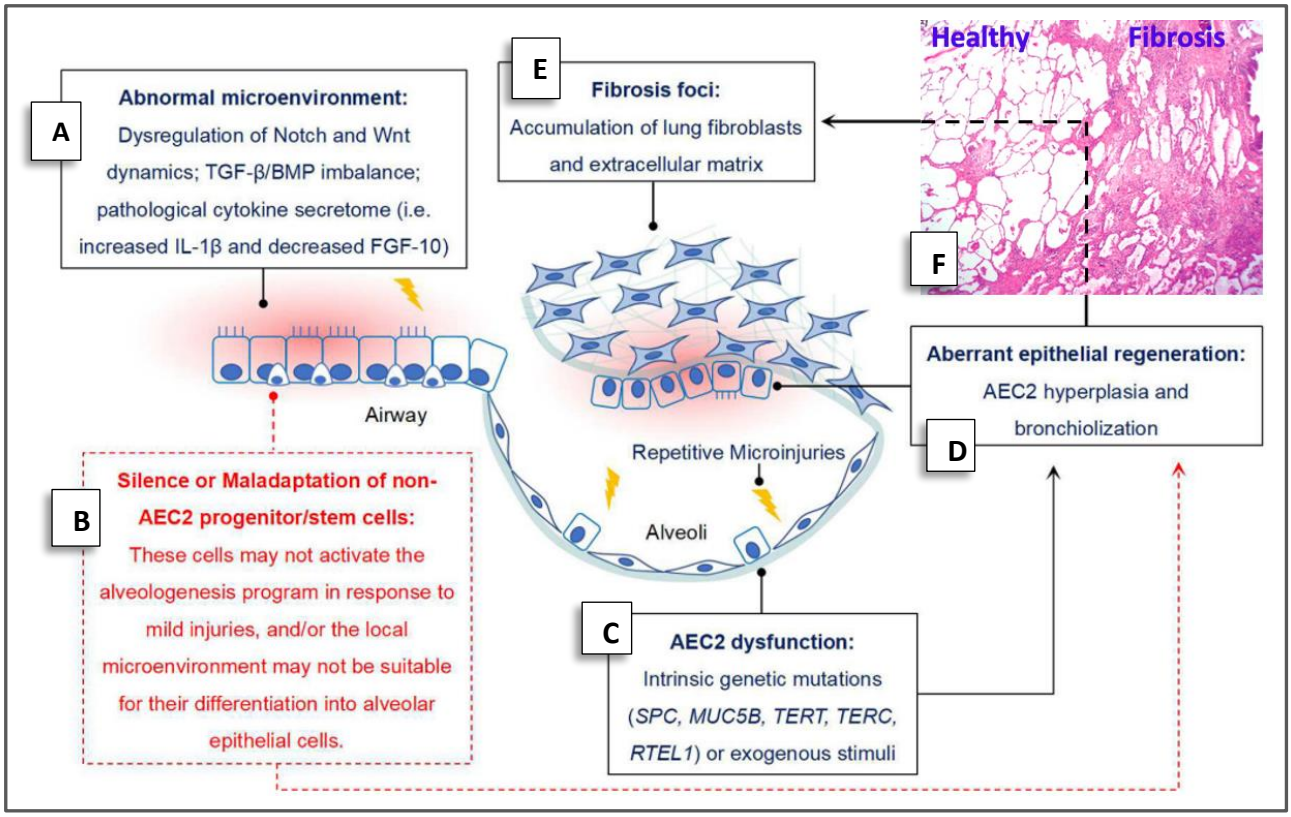


***Figure 2:*** *IPF pathogenesis; (A) Abnormal presence of risk factors in the microenvironment contributes to disease progression. (B, C) AEC2 (progenitor) dysfunction contributes to disease initiation/progression due to loss of their repairing ability. (D) Impaired repair results in an aberrant epithelial regenerative response, including AEC2 hyperplasia and bronchiolization. (E) Progressive fibrotic end state of IPF. (F) Histological images of healthy and typical IPF (fibrotic) alveoli* (16,17).

## Potential therapeutic strategies

Based on the elaborated complexity of IPF, not all main contributors involved can be discussed. Hence in this review, four therapeutic targeting strategies will be evaluated which all influence epithelial repair in IPF in their own particular manner. First, TGF-β will be addressed which is to this day a highly investigated mediator. Secondly FGF-signaling will be discussed, followed by reviewing of miRNA and TERT as potential therapeutic strategies. Lastly, this review will partly illustrate the current knowledge on potential IPF treatment strategies, by taking corresponding advantages and disadvantages into account.

## TGF-β

Transforming growth factor β (isoforms TGF-β1, TGF-β2, TGF-β3) is a pleiotropic cytokine from a large polypeptide family which is involved in numerous homeostatic events by regulating gene transcription, cellular differentiation, proliferation and apoptosis (3). Its functional effect is initiated upon binding to the TGF-βI/II/III receptor which consequently leads to phosphorylation and nuclear translocation of signal transducing Smad-proteins **(Figure 3)** (11). In normal circumstances, TGF-β acts as an anti-inflammatory cytokine, activates fibroblasts to differentiate into myofibroblasts, and decreases ECM degradation by inhibition of matrix metalloproteinases (MMPs) (3,11). TGF-β is highly involved in tissue repair mechanisms and homeostatic dysregulation, and is often associated with various chronic diseases like IPF, where it is overexpressed (3).

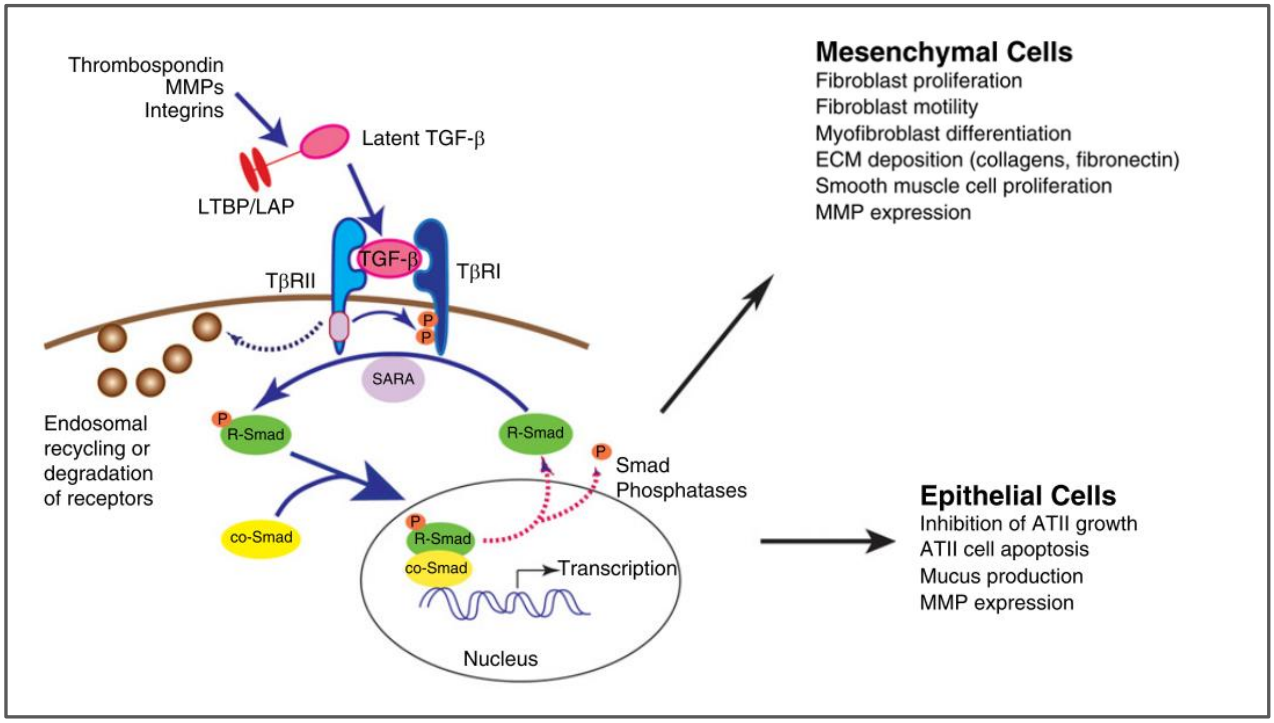


***Figure 3**: Canonical TGF-β signaling cascade; After activation from latent form, TGF-β binds and forms a receptor complex which subsequently phosphorylates R-Smads. Combined with co-Smads, the signal is translocated into the nucleus where it exerts its transcriptional effects on many cell types, including epithelial and mesenchymal cells. Several of these effects are implicated in IPF pathology* (18).

For instance, excessive TGF-β1 concentrations in alveoli have been shown to result in thickening of interstitial membranes, induced fibroblast differentiation, increased ECM production and AEC2 hyperplasia (3). Furthermore, transcriptome analysis of TGF-β induced myofibroblast differentiation in mice lung epithelial organoids showed a dysregulation in Wnt/β-catenin signaling and impairment of the ability of fibroblasts to support epithelial repair and organoid formation (11). This decreased ability to form organoids gives reason to suspect that TGF- β mediated mesenchymal activation may be a direct contributor to repair deficiencies in pulmonary fibrosis. According to follow-up transcriptome analysis, TGF-β mediates up and downregulation of multiple genes, including downregulation of hepatic growth factor (HGF), fibroblast growth factor-7 and -10 (FGF-7/10). All of these factors are involved in inducing transcription of lung repair factors which are produced by mesenchymal cells. Addition of HGF and FGF-7 to the pre-treated TGF- β organoid cultures restored organoid growth, therefore implying that reduced epithelial organoid formation is related to decreased expression of these growth factors (11).

In order to elucidate the importance of the TGF-βII receptor on embryonic lung morphogenesis and TGF-β mediated epithelial response, bleomycin-induced injury receptor knockout mice were used to establish whether its signaling is crucial and involved in IPF (19). In TGF-βII receptor deficient mice without bleomycin administration, functional and viable pulmonary morphogenesis together with normal epithelial differentiation was observed after three weeks. Subsequently in week eight, signs of alveolar enlargement and emphysema were reported, implying TGF-βII receptor necessity for healthy alveolar morphogenesis. After bleomycin-induced injury, receptor knockout mice showed significant higher resistance towards injury and proved to be better protected against fibrotic consequences as compared to wild type mice (no

mortality versus almost 100% mortality). This suggests that the TGF-βII receptor is specifically involved in fibrotic disease progression after repeated injury (19).

Besides fibrotic related pathways of TGF-β, its role in branching morphogenesis has been investigated for a while. Studies have reported inhibition of embryonic mouse branching morphogenesis by TGF-β1 and TGF-β2 overexpression, with increased effects under increased concentrations (18). Similarly, *in vitro* upregulation of TGF-βII receptor and SMADs-2/3/4/6, resulted in inhibited murine lung branching as well (3). In contrast, promotion of murine branching was observed by upregulation of the inhibitory SMAD-7 *in vitro* (3). Next to this, overexpressed TGF-β1 impeded epithelial differentiation, followed by impaired phospholipid and surfactant protein production (18). With regard to alveologenesis, postnatal deletion of Smad-3 between day 7 and 28 disturbs mice alveolar maturation which leads to bronchopulmonary dysplasia (20). Together, all evidence suggests a direct regulatory role of TGF-β and its isoforms on functional branching and alveolar morphogenesis, depending on the targeted receptor and/or downstream signal. Considering the performed research on the developing, healthy and IPF lungs, TGF-β remains a highly potent treatment target. However, attenuation of TGF-β signaling should take place in affected regions only to avoid adverse effects in otherwise healthy tissue.

## FGF

A different approach could be via fibroblast growth factor (FGF) signaling. FGFs originate from a large signaling protein family, are paired with different cofactors, and activate the tyrosine kinase receptors FGFR1, FGFR2, FGFR3, and FGFR4. Phosphorylation of tyrosine residues upon canonical FGFR binding gives rise to signal transduction via intracellular mechanisms, such as PI3K-AKT, PLCγ/DAG/IP3/PKC, JAK-STAT, and RAS-MAPK (3,21). Consequently, the signal is transduced by nuclear translocation, leading to transcriptional activation. Canonical FGFs (e.g., FGF1, FGF4, FGF7, FGF8, and FGF9) bind FGFRs paired to heparin sulfate proteoglycans (HSPGs) **(Figure 4-A)**, whereas endocrine FGFs (e.g., FGF15/19) bind to Klothoα/β co-factors **(Figure 4-B)**. Intracellular FGFs (subfamily FGF11) bind voltage-gated sodium channels (not shown), which activate intracellular FGFs (iFGFs)(21). FGFs are found in nearly all tissues and fulfill, in early life stages, a crucial role in embryonic development and organ morphogenesis (e.g. lungs, liver) by controlling progenitor cell activity, mediating cellular growth and patterning (21). In adults, they regulate tissue repair, regeneration, cellular homeostasis and metabolism. Specifically, canonical FGF signaling is involved in positively or negatively controlling cell proliferation, differentiation and survival **(Figure 4-C)**(21). Their reparative ability, by reactivation of developmental signaling mechanisms, and involvement in wound healing make them highly attractive for regeneration-oriented research and could provide new treatment possibilities. In addition, some FGFs exert pro- and anti-fibrotic effects depending on the targeted cell type (22). Because of their overall regulatory importance, dysregulations in FGF pathways are implicated in several diseases such as disrupted organ morphogenesis, IPF, COPD and cancer(21,23).

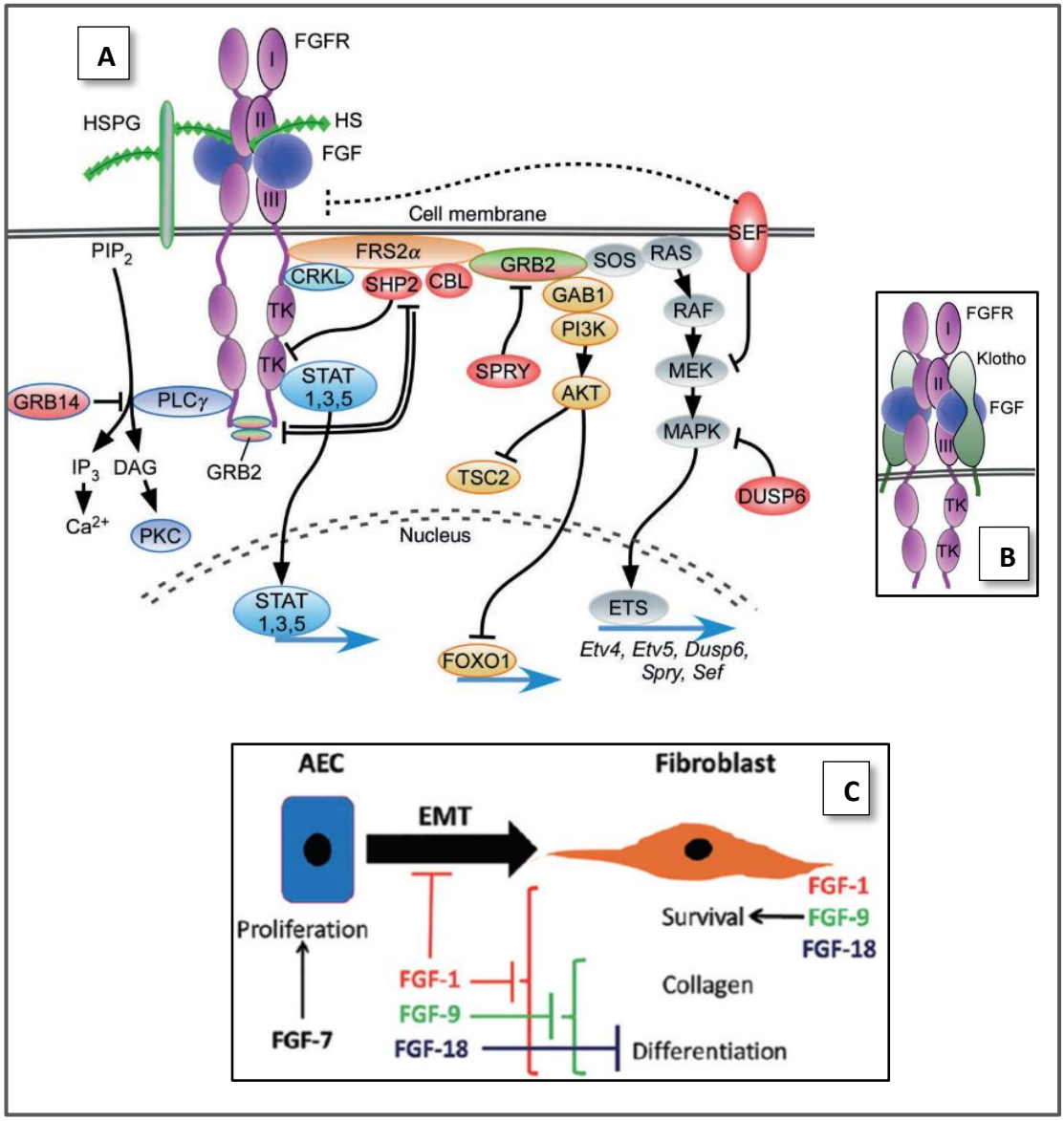


*Figure 4: FGF signaling cascade; (A) After canonical receptor binding and consequent complex formation with HSPGs, multiple signaling pathways (PI3K-AKT, PLCγ/DAG/IP3/PKC, JAK-STAT, RAS-MAPK) are triggered by intracellular tyrosine kinases activation as a result from phosphorylation of specific tyrosine residues, leading to nuclear translocation and subsequently transcriptional events. (B) Endocrine FGF receptor binding with Klotho α/β co-factors, ultimately leading to complex formation and subsequent tyrosine kinase activation, followed by gene transcriptional events. (C) Cellular homeostatic effects on AECs and fibroblast as a result of induced canonical FGF transcription* (21,22).

In fibrotic conditions, several FGFs serve a complex role in regulation and communication between AECs, fibroblasts, and myofibroblasts through EMT **(Figure 4-C)**(22). For instance, in an *in vitro* study with murine TGF-β-induced fibrosis lung models, the effect of FGF1 on fibroblasts and alveolar epithelial cells was studied (22,24). This study showed that FGF1 inhibited EMT due to decreased TGF-β signaling, caused by increased caveolin-1-dependent

proteasomal degradation of the TGF-βI receptor, therefore marking it as an anti-fibrotic mediator. Furthermore, TGF-β mediated signaling in fibroblasts and differentiation into myofibroblast was decreased due to comparable diminished transcription and increased proteasomal degradation of the TGF-βI receptor by FGF1 (22). Next to this, raised levels of FGF1 in serum levels and in alveolar epithelial cells of IPF patients were found, but in contrast not in myofibroblasts. This absence could possibly contribute to maintenance of the fibrotic environment and failure of initiating a regenerative response in IPF patients (22). Other evidence of anti-fibrotic events in *in vivo* studies were observed as well, with a reported reduction of TGF-β-induced fibrosis in FGF1 overexpressing rats, probably caused by enhanced proliferation and hyperplasia of rat AECs, in which FGF1 expression was upregulated (22)(24). This gives reason to suspect that FGF1 plays a crucial role in epithelial regeneration, limiting fibrosis and conferring protection against sustained fibrotic injury.

After FGF2 administration, rabbit *in vitro* wound models reported similar inhibition of TGF-β mediated signaling via TGF-βI/II receptor depletion and lowered TGF-β transcription in fibroblasts (25). Though *in vitro* FGF-2 administration resulted in increased myofibroblast differentiation and proliferation, FGF-2 knockout mice showed no alterations in fibrotic development in response to *in vivo* bleomycin-induced fibrosis, but interestingly resulted in deficient recovery of epithelial wall integrity (26). Besides, FGF2 overexpression did lead to attenuation of bleomycin-induced fibrosis (27). Depletion of the FGFR2 in knockout mice in AEC2s upon tamoxifen-induced injury, showed worsened alveolar homeostasis and lung injury compared to controls **(Figure 5)**(28). Altogether, implying FGF2 and its receptor not to be pro-fibrotic but to be a crucial ligand or target for epithelial regeneration and fibrotic protection. Overexpressed FGF2 for 5 months did not show histological alterations, suggesting good tolerance for administration (27).

In IPF patients, several ligands are upregulated with FGF1, FGF7 and FGF10 being of particular interest, based on their high affinity for the FGFR2 (isoform IIIb) (28). Via this receptor, FGF10 has been shown to be regulate and induce lipofibroblast (LFB) proliferation which are thought to serve as AEC2 niche cells by governing homeostatic AEC2 maintenance and repair (28). On top of this, LFBs serve a supportive role to the epithelium by supplying triglycerides to close AEC2s, necessary for surfactant protein synthesis. Upregulation op LFP-derived FGF10 in IPF context may therefore suggest an endogenous attempt of the lungs to support AEC2s in order to protect against fibrotic injury.

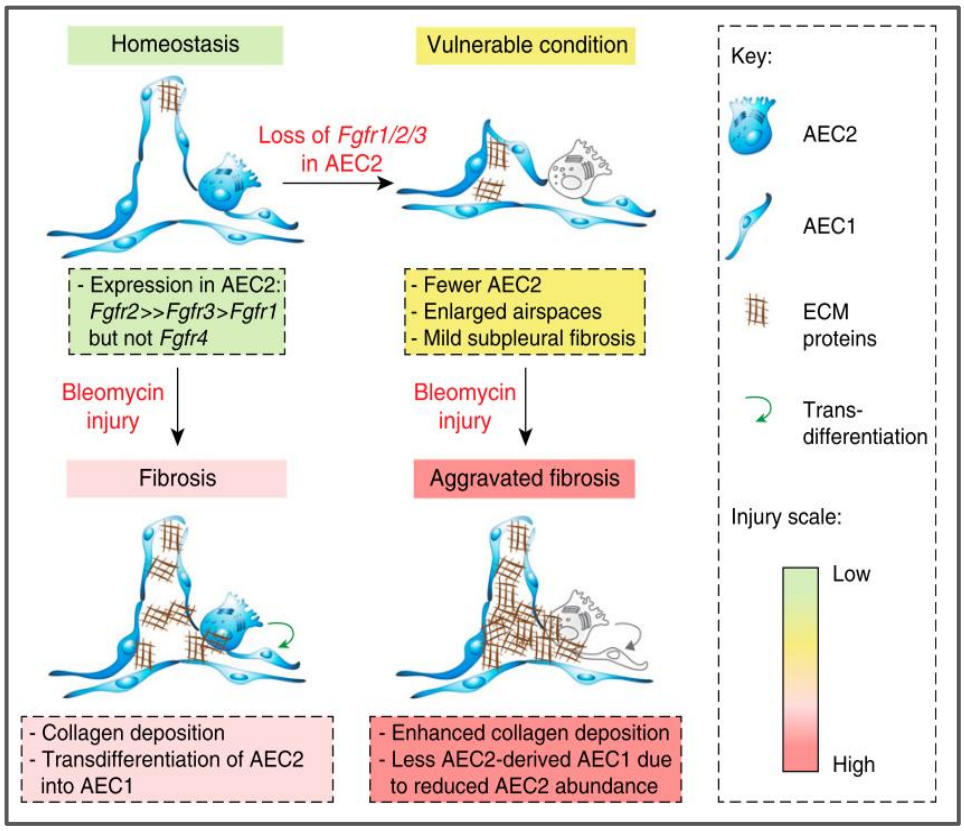


***Figure 5**: The importance of FGFR2 in AEC2 homeostasis after injury; Bleomycin-induced injury causes fibrosis but epithelial wall integrity in restored due to AEC2 to AEC1 transdifferentiation. Depletion of FGFR1/2/3 in AEC2s (KO-mice) creates a more susceptible pulmonary condition, resulting in aggravated pulmonary fibrosis after bleomycin administration due to a loss of epithelial regeneration and enhanced collagen deposition*(28).

Another recent study investigated the interaction between FGFR2b and FGF10 upon bleomycin-induced injury in order to maintain epithelial wall integrity (29). By creating a progenitor cell niche, caused by induced FGF10 production from myofibroblasts and airway smooth muscle cells after injury, distal airway club cells dedifferentiate directly into AEC1/2s or indirectly into AEC1/2s with neo-basal cells (BC) as intermediate. Besides results showing

increased neo-BC populations, FGFR2b depletion resulted in solely AEC1 formation, indicating its importance in AEC2 progenitor maintenance. Because IPF is an age-related disease and FGF10 expression diminishes over the course of life, a link between the impaired regeneration of bronchial epithelial cells into alveolar epithelial cells may be suggested by this FGFR2-FGF10 interaction.

Important to realize is that in IPF patients compared to control donors, even though multiple ligands are upregulated, epithelial FGFR1/2 (isoform IIIb) are downregulated and the FGFR1/2/3 (isoform IIIc) are upregulated in the mesenchyme (28). With FGF1 being receptor unselective, FGF2 being FGFR1/2/(IIIc) selective and FGF7/10 being selective for FGFR2 (IIIb), mainly epithelial signaling in AECs appears to serve a protective and injury repairing role when exposed to injury and fibrosis (28). As current IPF treatment options include Nintedanib, a broad-spectrum tyrosine kinase inhibitor binding FGFR1/2/3, more precise targeting via epithelial FGF-receptor isoforms could increase drug efficiency and reduce limiting interactions from counteractive signaling, originating from other FGF ligand-receptor binding (22).

In summary, FGF-signaling in normal and IPF lungs is highly heterogenic and implicated in anti-fibrotic responses, as well as epithelial regeneration and fibrotic protection. It is this heterogenicity which complicates the quest for new treatment options. FGF1 seems a potent mediator based on its proliferative effects on AECS and anti-fibrotic function. Yet, this factor may form a risk when used in treatment due to lack of receptor selectivity which could target other counteracting pathways simultaneously. FGF2 showed similar anti-fibrotic and epithelial regenerative functions but is only selective for the mesenchymal FGFR, which possibly makes it a good mediator for fibrotic protection, but less potent for epithelial regeneration. FGF10

appears promising due to its direct effect on epithelial progenitor niche cells, followed directly by proliferation into AEC1/2s and basal cells. In addition, its receptor selectivity for the epithelial FGFR isoform has been proven to support the epithelium via homeostatic AEC2 maintenance and repair as a result of induced LFBs proliferation. Hence, FGF10 can be designated as most prominent mediator in epithelial repair and regeneration. However, considering FGF heterogeneity, complete knowledge of all involved FGF signaling is required in order to give a full representation of their modulating activities and to propose new intervening treatment possibilities which exclude unwanted side effects. Despite potent findings from the above-mentioned studies, other opportunities may lie at less heterogenic signaling mechanisms which directly control transcriptional events and are less homeostatically involved.

## miRNA

Directly approaching gene translational events involved in pathological processes to inhibit protein synthesis, could offer opportunities for new treatment possibilities which tackle pathways directly at the source. After transcription from DNA, mRNA can be regulated in the cytosol by non-coding microRNA (miRNA) consisting out of 21 to 24 nucleotides (30). Their role is to repress gene expression in multiple events including differentiation, cell proliferation, cell development and metabolism, and are downregulated in some diseases including IPF (30). One or a combination of miRNA can target up to 100 genes making it a broad and complex regulatory network (6). Therefore, multiple studies increasingly suggest the use of miRNAs as possible therapeutic agent or biomarker (30). Initially, miRNA forms an RNA-induced silencing complex (RISC) followed by stable complementary binding to a small part of the mRNA **(Figure 6-A)**(6). Subsequent protein synthesis suppressing actions include destabilizing nucleotide sequences in a deadenylating manner which leads to decapping, sterically hindering

ribosomal translation or cleavage of the targeted mRNA (6). Currently, two RNA-interfering drugs are approved which both use small interfering RNA (siRNA), an alternative but comparable method used to silence genes (31). This represents the feasibility of therapeutic use of miRNA applications in the future.

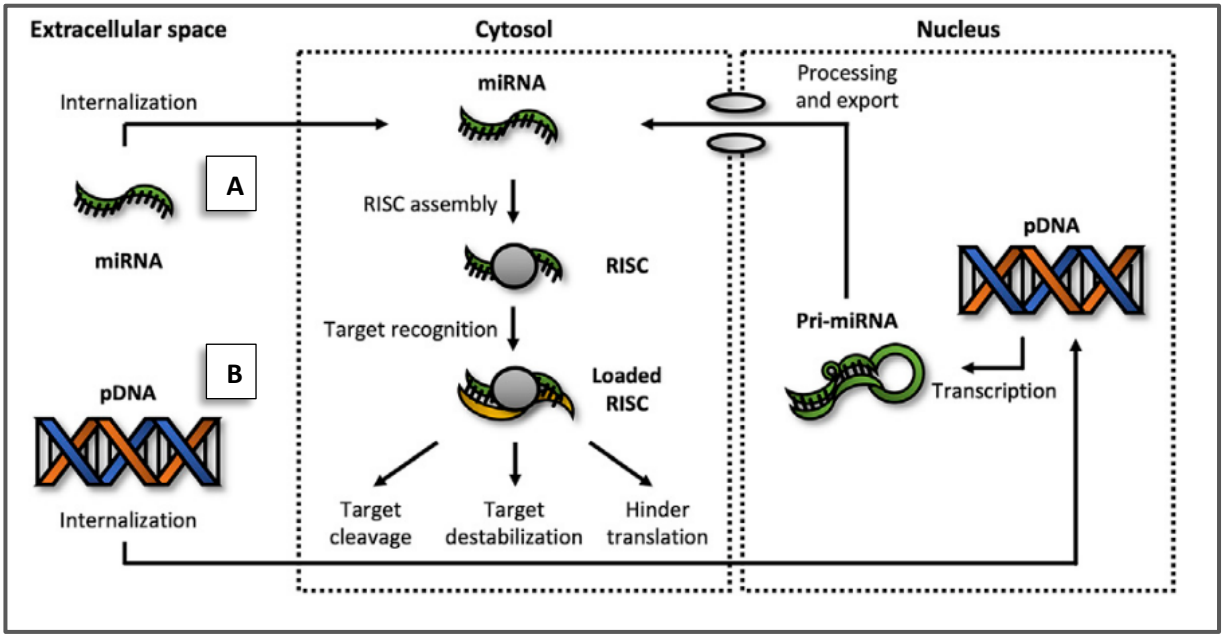


*Figure 6: miRNA mediated repression of gene expression; (A) After cytosolic entrance of miRNA, miRNA machinery is activated leading to RISC assembly and loading, consequently exerting its variable functional effects. (B) Internalization of pDNA initiates a more permanent transcriptional response from which pri-miRNA is formed. After processing, miRNA is formed and subsequently exported into the cytosol, where the initial miRNA machinery starts* (6).

Altered miRNA expression in fibrotic mechanisms (TGF-β1, EMT, apoptosis) plays a large role in IPF with various known variants being up or downregulated (30). Nevertheless, its involvement in epithelial repair is of interest here, with a small number of currently discovered variants. Healthy epithelial regeneration is highly dependent on AEC viability and proliferative ability. However, increased AEC2 apoptosis and senescence are large contributors in IPF, due to the loss of progenitor function and consequently failure of re-epithelization. Downregulation of miR-29c (a transcriptional target of TGF-β) is observed in IPF patients when comparing to healthy patients, corresponding with a reported increase in AEC apoptosis and decreased ability of epithelial recovery (30,32). Whereas, *in vivo* overexpression increased proliferation and

cellular viability, resulting in diminished fibrosis and increased recovery. This increased cellular viability can be explained by the fact that Foxo3a, a cell cycle and apoptotic regulator, is directly suppressed by miR-29c (33). Next to this, increased miR-34a expression resulted in higher p53 acetylation and is suspected to regulate cellular senescence of AEC2s in IPF (30). By inhibition of miR-34a in injured AECs, apoptosis was reduced, AEC2 senescence decreased (only in aged mice) and development of IPF prevented (30,34). Therefore, implying miR-34a to be involved in programmed cell death, cellular senescence and cell cycle arrest. Other evidence showed miR-30a to be downregulated in AECs of murine bleomycin-induced injury models and in IPF patients (30). If upregulated, AEC apoptosis suppression was limited via lowered Drp-1 production (30). Besides this effect on the epithelium, suppression of myofibroblast accumulation and a reduction in fibrotic lesions by supplemented miR-30a administration, has been reported in murine bleomycin-induced fibrotic models (6). Merged, these results show a multi-potent governing function of miR-30a, which possesses the ability to attenuate or suppress the progression of IPF in an anti-fibrotic and re-epithelializing manner. When combining all miRNA studies, their influence on epithelial viability and regeneration can be considered as comparable, although miR-30 may be the most promising strategy based on the synergistic anti-fibrotic effect. Because only a limited number of miRNAs regarding epithelial repair are known today, further research must be performed in order to establish more repressing possibilities and to completely map the multiple miRNA-mRNA interactions. Only then their full regulatory function can be established, and adverse effects ruled out.

Based on the pleiotropic repressing ability, where one miRNA can modulate multiple transcripts, implementation of miRNAs as therapeutic agents for complex diseases could be a promising strategy. However in contrast, therapeutic use of miRNA entails adverse effects as well. Synthetically derived miRNA can transfect the pulmonary cells in naked form but is

negatively charged, therefore preferably aided with non-viral vectors, ionizable lipid nanoparticles or liposomes in order to be efficiently delivered into the cytosol (6). Once present, it induces a short-lasting transient effect only. In order to extend this duration or even create a permanent effect, pDNA encoding for primary miRNA (pri-miRNA) can be introduced into the DNA(6). Subsequently, pri-miRNA is transcribed which is post-transcriptionally processed into miRNA by Drosha and Dicer enzymes and exported into the cytosol where the initial miRNA machinery is activated **(Figure 6-B)**. Nevertheless, caution must be taken with pri-miRNA because transcriptional regulators, which implement the pDNA, may become saturated, ultimately causing altered miRNA transcription. Other challenges may arise from targeting specific cell-types or drug delivery through the dense and strong interconnected fibroblast foci. Based on current information known on their exerting effect, future miRNA therapeutics could provide a pro-regenerative environment so AECs can proliferate and prevail over the excessive fibrosis. However, these singular effects could be insufficient and therefore may require additional combination therapies with anti-fibrotic agents in order to attain a curing effect for IPF. Because cellular viability is not solely limited by miRNA interference but deteriorates with age, other therapy strategies based on markers of age and cellular proliferation or senescence may become increasingly important in the future as IPF is a highly age-related disease.

## TERT

Throughout life, tissues gradually lose their proliferative ability due to telomere shortening as a result of insufficient telomerase activity (35). Telomerase repetitively adds TTAGGG repeats and is coupled with a six-protein shelterin complex **(Figure 7)**(36). After DNA damage, critically-short telomeres will cause senescence or apoptosis. To prevent this, telomere lengths are constantly extended by telomerase which consists of out of two subunits, an RNA subunit

(Terc) and the enzyme telomerase reverse transcriptase (TERT). Abnormal telomere shortening can be influenced genetically (e.g. mutations in TERT, Terc) or environmentally (e.g. cigarette smoke) during life (36). As IPF is highly age related, telomere dysfunction is commonly observed, giving reason to suspect a link between age-related dysfunctional telomere extension and loss of AEC renewal in IPF (36).

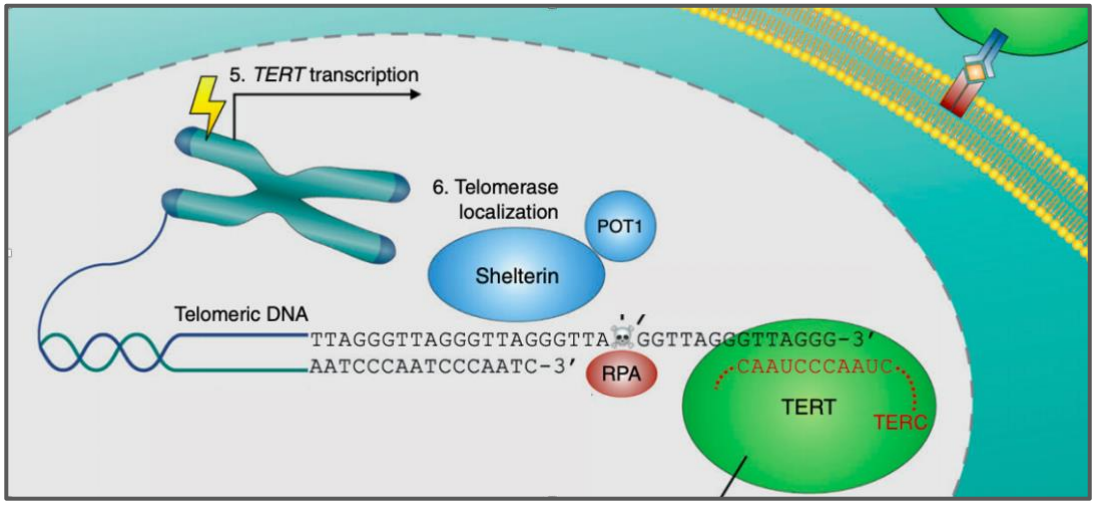


*Figure 7: TERT; The role of TERT in the telomerase dependent elongation of telomeric DNA with repetitive sequential repeats*(37).

To confirm a possible link, Liu et al. (2019) and Povedano et al. (2018) studied telomerase deficiency in mice AEC2s. From this study, no spontaneous IPF developments were reported. However, the minimal dose to induce fibrotic injury with BLM was significantly reduced and interestingly led to enhanced fibrotic symptoms (34,36). Next to this, AEC2 proliferation decreased, and apoptosis and senescence increased in telomerase knockout mice. To substantiate this, a combined murine model with shortened telomeres and induced low-dose bleomycin injury was used to establish the effect of transient TERT delivery via adeno-associated vectors (AAV9-TERT), which specifically target AEC2s and locally activate telomerase **(Figure: 8)**(36). In weeks 1-3 after induced injury, reduced inflammation and fibrosis was observed, confirmed by transcriptome analysis. Interestingly, in week 8 fibrosis disappeared or was highly reduced. As predicted, increased AEC2 proliferation and lengthening

of telomeres was observed in isolated AEC2s, with diminished DNA damage, senescence and apoptosis as result. From these results, TERT treatment can be reported as highly potent regarding epithelial regeneration and viability. However, as with miRNA, the targeted use of vectors and their therapeutic outcome is still under investigation and requires further research in order to establish a cell specific effect in hard-to-reach fibrotic lesions. For instance, ensuring AEC2 specific vector delivery or preventing reduced fibroblast apoptosis and senescence. Whether TERT also exerts extra-telomeric effects remains unclear and should also be investigated in future studies. Despite these challenges, direct intervention on a genetic level, by circumventing highly interconnected homeostatic signaling routes (e.g. TGF-β, FGF), could provide more effective or possibly curing therapies for IPF. As every strategy shows individual strengths and weaknesses, the following section will address the challenges and recommendations that must be further explored in follow-up studies.

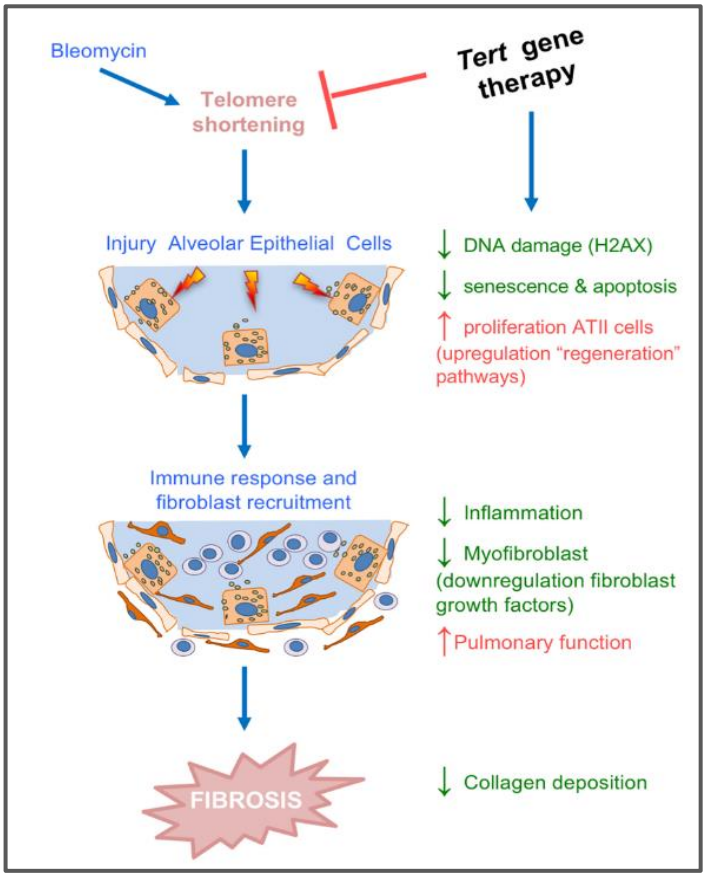


*Figure 8: A proposed animal model with short telomeres, a condition addressed as a molecular implication in IPF, was treated with TERT gene therapy delivered with AAV9 vectors. This resulted in anti-fibrotic and pro-regenerative responses (green and red text), ultimately reducing fibrosis and ameliorating pulmonary function*(36).

## Challenges and recommendations

In the past years, IPF has been studied extensively and knowledge has significantly increased. As a result, the vision of it being solely a fibrotic and inflammatory disease has shifted towards a more prudent approach with the epithelium playing a key role. As discussed in this literature study, multiple therapeutic strategies or interventions have shown to protect, attenuate or even reverse disease progression, providing promising treatment strategies regarding epithelial preservation and regeneration. However, multiple challenges have been identified that must be solved first in order to safely progress to clinical trials. Improving model design which better replicate IPF pathology is such a challenge. Furthermore, treatment effectiveness must be increased by local and targeted drug delivery, as this decreases adverse effects (6). Because all four discussed treatment strategies show different molecular entities (protein, miRNA, small-molecule drug) and target separate pathways, pulmonary drug delivery techniques cannot be generalized. Moreover, the state of IPF tissue could impede site-specific delivery as thick and highly connected fibrotic foci could prevent therapeutics from reaching myofibroblasts, covered behind collagen fibers. Instead, therapeutics may end up in the ECM where interaction with present fibroblasts could have counteracting effects. Yet, alveolar epithelium is well accessible as they present the first alveolar cell line. If in the end site-specific delivery does succeed, adverse effects are limited, efficiency is increased, and costs are decreased. This emphasizes its clinical importance in IPF patients. In the following sections, implications on clinical translation, drug delivery and model design that are currently faced will be discussed and provided with future recommendations.

## TGF- β/FGF

Though multiple efforts have been made to target TGF-β activation, no significant clinical benefits have yet been reported (12). As TGF-β is strongly involved in numerous homeostatic events (e.g. immunosuppression, wound healing, epithelial hyperplasia), clinical interventions often resulted in adverse effects (12). Comparably, TGF-β induced disease models also showed altered cellular homeostasis, therefore decreasing their clinical reliability (4). As addressed in this paper, several reports studied TGF-β signaling by depleting its receptor(s). In this way, other essential signaling, which are not related to IPF, can be dysregulated and exert unwanted effects. FGF faces similar problems as TGF-β, based on its heterogenic physiologic involvement. Where one ligand-receptor interaction induces favorable effects, different cell type targets or alternative signaling routes may exert negative effects. This ultimately increases the risk of developmental or metabolic diseases (21). Treatment strategies which distinctively target cell types (e.g. AEC) or, more specifically, downstream signaling pathways (e.g. Smad) are for this reason highly desired. Site-specific delivery may require vectors with, for instance, desirable fibroblast or epithelial affinity. However, high affinity for collagen must be prevented, as this may sequester administered therapeutics. Although vectors propose good targeting potential, these systems also have some disadvantages like immunogenic responses. Moreover, where IPF involves numerous fibrinogenic factors, a single mediator approach with one growth factor could be insufficient for disease attenuation. Though, this must be investigated in further detail. Altogether, future use of TGF-β/FGF treatment strategies seem likely but signaling mechanisms must be investigated thoroughly in order to ensure no counteractive homeostatic pathways are initiated.

## miRNA

Three different miRNAs have been discussed in this paper, all positively influencing AEC proliferation, senescence or apoptosis, consequently attenuating IPF. From these, miR-30 was addressed as most potent therapeutic agent based on its anti-fibrotic and AEC regenerative effects. In general, their pleiotropic suppressive function can be seen as clinical advantage, but it may bring safety risks, as multiple effects of a single miRNA may counteract or mask each other, possibly causing adverse effects. For instance, other targets of miR-30 include B-cell lymphoma 6 proteins (contributors of diffuse large B-cell lymphoma), runt-related transcription factor 2 (essential for osteoblast differentiation) and p53 (apoptotic regulator)(30). To prevent this interaction, transcriptome analysis must be used to completely map miRNA signaling pathways. Moreover, their negative charge, large size and nuclease susceptibility also present some challenges, as cytosolic delivery is only possible with vectors (6). Vector delivery must be local and ionizable lipid nanoparticles could be used to enable efficient pulmonary deposition, cytosolic delivery and retain miRNA stability (6,38). Use of dry powder inhalers (DPI) is preferred when delivering genetic material and could be proposed as suitable system for IPF (6). Unfortunately, it is currently unclear whether DPIs can be used in IPF patients, as the highly disturbed lung architecture might disturb powder flow and delivery. Considering all findings, miRNA shows great treatment potential but presented challenges must be solved first before miRNA therapy can proceed to clinical trials.

## TERT

TERT gene knockout resulted in aggravation of the fibrotic response and alveolar regenerative inability. In a later study which reported vector-mediated TERT gene therapy, fibrotic lesions were significantly reduced or even disappeared. Combined, the role of TERT in alveolar homeostasis appears crucial and interfering strategies may give rise to highly effective fibrotic

reduction or alveolar regenerative induction. Yet, interference with TERT also poses multiple challenges. Because apoptosis and senescence were decreased and AEC2 proliferation increased, cells could enter a state of excessive growth and induce carcinogenic effects. However, this has not yet been reported (36). Integration of the genetic material could pose another challenge, as active host loci and DNA repair factors showed to interfere with vector integration. Incorrect genetic integration results in mutagenic alterations which can have detrimental consequences (36). In order to deliver TERT site specifically, AEC2 targeting vectors can be used (36). These vectors could initiate an immunogenic response, possibly interfering with the fibrotic environment. Nevertheless, reported immunological effects of AAV9 were classified as moderate. To circumvent immunogenic responses, chimpanzee adenoviral vectors could be used as alternative, as such vectors reportedly showed low seroprevalence and high loading capacity of foreign genes in humans (39). Viral vectors can be administered intravenously which limits concentrations ending up in the lungs, due to distribution via the blood through the whole body (36). Formulating gene therapy in a way which enables pulmonary inhalation (e.g. ionizable nanoparticles with DPIs) could give rise to higher local concentrations, increased efficiency and reduced costs. If delivery is not site- or cell-specific, counteracting effects may arise. Taking these challenges into account, further research must be performed on mutagenicity, carcinogenicity or possible extra-telomeric roles of TERT, as these are not reported in the discussed papers. In addition, vector specificity should be increased further so administered concentrations are limited, ultimately lowering the risk of these side effects occurring.

## Model design

All presented studies of potential treatment strategies are performed on IPF resembling pre-clinical models. However, several differences between these models and the actual IPF pathogenesis exist, possibly displaying a distorted image of reported effects (4). The desired

phenotype consists of a progressive fibrotic model including AEC hyperplasia, increased EMT and limited inflammation (7). Initiation of fibrosis can easily be performed in many ways but often lacks progressive characteristics (4). Furthermore, these initiations mostly lead to inflammatory responses, which are undesirable due to broad homeostatic functions of regulatory mediators (e.g. cytokines). Improved model design which fully recapitulates IPF phenotype is therefore of great importance in order to ensure clinical effectiveness of future treatments. In the following sections, currently used and new model techniques accompanied with their strengths and weaknesses will be discussed. Eventually, illustrating current *in vitro/ex vivo/in vivo* model progression and their caveats in IPF research.

### *Cell culturing*

*In vitro* culturing of different cell types (e.g. AECs, fibroblasts) remains a relevant method in clinical research. As IPF pathology includes multiple involved cell types, culturing of specific primary cell types under controlled conditions, obtained from IPF patient tissues, could provide better understanding of specific independent effects when excluded from external cellular signaling (4). Use of these models will always remain, mainly because of the practical ease regarding isolation and culturing. However, the majority of current used *in vitro* systems are characterized by their 2D structure, therefore lacking cellular interaction and mechanical similarities associated with 3D composed cell cultures (4). For this reason, progress on 3D culture structures must be made to establish models more comparable to human lung tissues.

Innovative multicellular systems *in vitro* models, called lung organoids, replicate pulmonary organ models more accurately compared to 2D models (4). Organoids can be developed for multiple organ types and are characterized by their self-assembling formation out of organ specific progenitors obtained from human or animal tissues (40). Interestingly, applied genetic mutations to some stem cells remain in formed organoids, thus enabling development of fibrotic

disease models by targeting genes which increase IPF susceptibility (4). Despite this, organoids also pose some disadvantages including culturing challenges (e.g. development, maintenance) and absence of air perfusion and vascularization (4). These limiting factors combined, reduce clinical resemblance. Overall, organoids show better clinical translation compared to 2D cell cultures and remain the preferred *in vitro* model, based on their basic 3D composition which better replicates lung architecture stiffness, ECM protein composition and inter-cellular communication of multiple cell types (4). However, a more advanced approach is needed to translate *in vitro* findings to *in vivo* or even clinical practice.

### *Precision-cut lung slices*

With this in mind, interest is increasingly directed towards three-dimensional organ models which validate therapeutic evidence based on original cellular architecture, presence of multiple cell types and their interactive role in pulmonary tissue (41). Such 3D-models are precision-cut lung slices (PCLS), which originate from human/animal lung resections or transplants and preserve original pulmonary architecture, ECM composition and viable cell populations (4). Therefore, enabling productive and more sustainable investigation of pathological conditions or direct drug effects in structural cells. PCLS strongly reduce research cost regarding animal housing, as multiple slices can be harvested from one mice lung (4). Yet, this technique is not eminent, as limited cellular viability and model standardization pose challenges (4). Nevertheless, PCLS pave the way for more sustainable and IPF resembling models from which more reliable data can be collected.

### *Animal models*

Due to relative low costs, practical convenience and phenotypic equities compared to humans, animal models (e.g. rodents) are still recommended and most used in pre-clinical research. Creating an IPF animal model which recapitulates most phenotypic aspects remains

challenging, as no perfect system seems to exist. Though multiple models have been developed to this day, the fibrotic model, in which fibrosis is initiated by the chemotherapeutic antibiotic bleomycin (BLM), is most widely used in clinical practice (7). By breaking DNA strands, ultimately resulting in free radical production, the cytostatic adverse effects of BLM accurately present an IPF mimicking phenotype including acute lung injury and fibrosis (4,12). After intratracheal administration, multiple alveolar cells are damaged, consequently leading to disruption of alveolar architecture and inflammation, followed by excessive fibrosis. However, because IPF patients show little inflammation and fibrosis is highly progressive, research on anti-inflammatory or anti-fibrotic therapeutics must consider the standard inflammatory presence and standard absence of progressive fibrosis in BLM-induced models, in order to conclude for therapeutic effectiveness (4,6,42). Furthermore, timing of intervention after bleomycin injury is of great importance, because the majority of inflammation must have subsided, and excessive fibrosis fully started (4,6).

Besides inducing fibrosis therapeutically, creating models by making them more genetically susceptible for IPF might provide more replicating phenotypes, as reports show a clear link between IPF and several genetic alterations including MUC5B or telomerase related genes (4). Trf1 (a TERT component) deletion in mice AEC2s resulted in spontaneous pulmonary fibrosis after 9 months and MUC5B overexpression led to increased susceptibility towards bleomycin-induced injury, although not spontaneously forming pulmonary fibrosis (43). Though, caution is advised, as gene knockouts may lead to compensatory genetic responses, possibly affecting the expected outcome of proposed models (4). Additionally, aged mice might be more suitable models, as they showed epigenetic reprogramming and reduced regenerative ability after induced injury. Such effects are also observed in IPF patients (4,44). In the end, the quest for a fully resembling *in vivo* model for IPF remains. However, combining discussed techniques may

create less inflammatory and increased progressive models which provide better clinical translation of future research. For instance, by lowering the required inductive therapeutic dose as a result of genetically increased susceptibility in aged mice.

### *Further prospects*

When combining all discussed models which are currently used to study pathological implications and interventions of IPF, no complete model system exists. Despite bleomycin being used most frequently and addressed as best model, all models present unique features which all could contribute to research progression in their own way. However, the need for one overarching IPF resembling model remains, and systems must be carefully designed or selected in order to establish maximal clinical translation and treatment efficiency. Lately, promising model developments have been made regarding fibrotic persistence, genetically modified induced IPF models, aged mice models and 3D *ex vivo* models. The future lies at limiting *in vivo* studies by combining or replacing current methods and generating models which maximally resemble the complex IPF pathology in a sustainable way.

## Conclusion

In recent years, significant progression regarding targeting of epithelial repair has been made. In this review we discussed several potent strategies (TGF-β, FGF, miRNA, TERT) which show promising results regarding new treatment or curing developments for IPF. Even though only TERT gene therapy managed to reverse disease progression, there is not one single strategy preferred over the other, as all therapeutic strategies have shown to protect, attenuate or even reverse disease progression by increasing epithelial preservation and/or regeneration. However, developments in this area are still in an early stage and accompanied with several challenges regarding mediator pleiotropism, model design, targeted drug delivery and dosage forms. In order to ensure better therapeutic outcomes in IPF patients, counteracting effects of

treatment strategies regarding fibrotic reduction and increasing epithelial regeneration must be further investigated before proceeding to clinical trials. Similarly, knowledge on efficiency and safety of different pulmonary vector systems remains limited and therefore requires further exploration. To improve clinical translation, follow-up studies on treatment strategies should be performed in models which better resemble IPF phenotypes. Taking all addressed remarks into account, better or even curing treatment option could arise for IPF patients in the near future, as there are still very limited treatment options available for this severe and pathologically complex disease.

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