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The Self-fulfilling Prophecy of Pulmonary Fibrosis: A Selective Inspection of Pathologic Signaling Loops

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Introduction

"He jests at scars that never felt a wound" – Shakespeare

Acute and chronic lung diseases represent a tremendous financial, personnel, and resource burdens on worldwide health systems. In this review we will focus on the pathophysiology of a chronic lung disease that is characterized by scarring of the lung tissue. Idiopathic pulmonary fibrosis (IPF) is a complex disease with only theorized potential contributory etiologies (genetic susceptibility, environmental exposure, and lifestyle habits). In a similar manner, there is tremendous variability in the natural disease course and response to therapy. This unpredictability makes IPF difficult to diagnosis, manage, and study. As such, there remains an unmet need for new therapeutic targets. This review attempts to highlight the complex interplay of seemingly disparate signaling pathways to help readers identify potential new targets and consider novel approaches when designing future clinical trials.

A scar develops as part of a normal response to tissue injury. Tissue or organ fibrosis is pathological scarring which occurs when the wound healing pathway is dysregulated. In other words, the appropriate healing response does not abate and thus becomes pathologic. Wound healing involves the recruitment of fibroblasts that contribute to structural repair, homing of inflammatory cells and the release of chemokines and cytokines that induce extracellular matrix (ECM) production. When this process becomes pathologic, it causes the accumulation of fibroblasts and myofibroblasts, destruction of normal tissue structure, the development of fibroblastic foci and loss of organ function. The abnormal architecture observed in pulmonary fibrosis results in restrictive lung physiology and impaired gas exchange. There are many known causes of pulmonary fibrosis including environmental exposures, auto-immune diseases, radiation exposure, medications and respiratory infections. However, some forms of pulmonary fibrosis lack an identifiable etiology, the most notable being idiopathic pulmonary fibrosis (IPF).

The FDA has approved only two drugs for the treatment of IPF, pirfenidone (precise mechanism of action uncertain, but likely decreases production and activity of Transforming Growth Factor-Beta 1 (TGF-β1) and nintedanib (a triple tyrosine kinase receptor inhibitor (Platelet Derived Growth Factor (PDGF)), Fibroblast Growth Factor (FGF), and Vascular Endothelial Growth Factor (VEGF)). These medications slow the progression of the disease but do not stop or reverse the scarring and do not make patients feel less breathless (1, 2). Currently, the only cure for IPF is lung transplantation, but this is only suitable for a small subset of patients, donated organs are a limited resource, and which comes with significant potential co-morbidities. Many other therapeutic targets have been studied but have failed to demonstrate clinical efficacy. We propose that the lack of efficacy may be related to the considerable redundancy in pro-fibrotic pathways involved in the complex pathogenesis of IPF.

Lung scarring develops and progresses through multiple mechanisms that involve the activation of pro-fibrotic pathways which in turn promote their own ongoing activation (3-5). This leads to persistent up-regulation of mediators that drive fibrosis and a concurrent downregulation of negative mediators of fibrosis. Therefore, targeting any one single fibrotic pathway may be inadequate due to the simultaneous activation of multiple pro-fibrotic, converging and cross-amplifying, pathological pro-fibrotic loops. In this review we highlight the relationships between these complex wound healing signals and explore how dysregulation of these pathways may result in pathophysiology as well as how they may be exploited for therapeutic benefit. We conceptualize these pathologic loops as a parallel induction of multiple pro-fibrotic stimuli, coalescing in the synergistic and perpetual propagation of cyclical patterns of pro-fibrotic signaling. These convergent signals then work in concert to drive pathologic fibrosis.

In addition, anti-fibrotic or homeostatic pathways are down-regulated in pulmonary fibrosis, functionally limiting the natural “brake” on pro-fibrotic pathways. Although there are several examples of self-sustaining pro-fibrotic signaling loops, in this review we focus on: (1) epithelial injury and senescence and (2) the (re)-activation of developmental pathways (Figure 1). We also address how the down-regulation of anti-fibrotic mediators contributes to fibrosis promotion.

Epithelial Cell Damage, ROS and Senescence Generate a Pathologic Signaling Loop

The best supported hypothesis for the inciting event(s) in IPF is repeated injury to the alveolar epithelium (6, 7). Sustained injury of the epithelium is hypothesized to produce an abnormal wound healing response, resulting in the activation and differentiation of fibroblasts. This hypothesis is supported by the finding that in the alveolar epithelium of fibrotic lung tissue there are greater numbers of apoptotic type I and II cells, particularly around areas of increased myofibroblast activity (8, 9). Interestingly, myofibroblasts have demonstrated resistance to apoptosis and senescence (10, 11). This pattern of adjacent, phenotypically different, cells exhibiting divergent behavior highlights another aspect of the complex heterogeneity observed

in IPF. Numerous *in vivo* studies have highlighted the importance of alveolar epithelial cell injury in the promotion of pulmonary fibrosis (12-15).

Many rodent models of pulmonary fibrosis involve the use of agents which damage the lung epithelium resulting in epithelial cell death and subsequent development of fibrosis. These agents include bleomycin, diphtheria toxin, fluorescein isothiocyanate (FITC), silica, and ionizing radiation (16). Alveolar damage has been shown to activate fibroblasts, which leads to additional epithelial injury, resulting in a self-propagating pro-fibrotic signaling loop (Figure 2).

Although the specific cause of epithelial cell death remains unknown in IPF, there are genetic factors which may increase the risk of epithelial injury. Mutations in genes such as surfactant protein A1 (SFTPA1) (17), A2 (SFTPA2) (18), and C (SFTPC) (19, 20), genes associated with telomere-related biology such as telomerase encoding reverse transcriptase (TERT), telomerase encoding RNA component (TERC) (21), regulator of telomere elongation helicase 1 (RTEL1), poly(A)-specific ribonuclease (PARN) (22), and dyskerin (DKC1) (23) and polymorphisms in the Mucin 5B (MUC5B) (24-26) promoter are all associated with the development of familial pulmonary fibrosis (FPF) and/or sporadic IPF. In fact, up to one third of patients with IPF have at least one common genetic variant (27).

The mechanisms involved in how an injured epithelium induces fibrosis remain incompletely understood, but it is hypothesized that in a healthy lung, normal alveolar epithelial cells help maintain homeostasis in neighboring fibroblasts. However, when epithelial cells are injured, they release Damage (or danger) Associated Molecular Patterns (DAMPs) and reactive oxygen species. This disrupts the epithelial barrier through epithelial apoptosis and subsequent stimulation of local fibroblasts. For more specific information on the role of alveolar injury and the development of pulmonary fibrosis, we refer the readers to an excellent review article (28).

ROS and Mitochondrial dysfunction

As organisms age there is an increase in reactive oxygen species (ROS) production. ROS has long been thought to play a role in pulmonary fibrosis. It is well established that damage to the epithelium results in increased ROS, which then impacts neighboring epithelial cells and fibroblasts. The capacity to regulate ROS production declines with age and this altered capacity may be one of the mechanisms through which senescent myofibroblasts and alveolar epithelial cells generate excess ROS (29, 30). The largest endogenous source of ROS is the mitochondria, and the increased mitochondrial dysfunction that occurs with aging is thought to be responsible for this increased production of ROS. In pulmonary fibrosis, there is increased ROS and mitochondrial dysfunction. For example, alveolar epithelial cells in patients with pulmonary fibrosis have mitochondria with morphological abnormalities and impaired enzymatic activity of the electron transport chain (31). Furthermore, the oxidative stress generated from mitochondrial dysfunction can damage mitochondrial DNA in nearby lung epithelial cells, perpetuating a cascade of mitochondrial dysfunction and apoptosis ultimately resulting in a pathologic fibrotic response (32).

Mitochondria play a critical role in the development of lung scarring as evidenced by the spontaneous formation of alveolar scarring and fibroblast proliferation in homozygous knockout mice of Translocase on the Outer Mitochondrial Membrane 5 (TOMM5) (33). Increased mitochondrial ROS activates TGF-β1 and PDGFR (34); PDGFR expression is increased in IPF (35) and is one target of nintedanib (36). In IPF fibroblasts NADPH Oxidase 4 (Nox-4), a marker of mitochondrial ROS, is elevated (37). Genetic knockdown and small molecule inhibitors of Nox-4 in human lung fibroblasts inhibit TGF-β1-induced expression of myofibroblast markers and cell contractility (37, 38). Furthermore, inhibition of Nox-4 reduces the expression of a senescence markers and increases cellular susceptibility to apoptosis (39). Both chemical inhibition of Nox-4/Nox-1 and Nox-4 RNA knock down attenuate the development of lung fibrosis in rodents (38, 40). TGF-β1 induces Nox-4 activity and expression, which leads to increased production of ROS (37, 39, 40). The paracrine effect of ROS propagates an abnormal wound healing response, which includes cell death and aberrant premature aging (41). Fibrosis and inflammation promote the subsequent generation of ROS and TGF-β1 negatively regulates the anti-oxidant pathway (42)

Stress to mitochondria, including oxidant imbalance, causes the release of mitochondrial DNA which acts as a DAMP recognized by the immune system (43). Patients with IPF have increased levels of mtDNA in bronchoalveolar lavage (BAL) fluid and serum compared to healthy subjects (44). Patients who respond to pirfenidone, defined as a stable forced vital capacity (FVC) for 1 year with treatment, experience decreased levels of circulating mtDNA in serum (44). This suggests that treatment strategies that preserve proper mitochondrial function, reduce ROS production, or decrease the levels of circulating mtDNA, may serve as valuable therapeutics.

Multiple *in vivo* studies have shown that antioxidants, such as N-acetylcysteine (NAC), inhibit bleomycin-induced pulmonary fibrosis (45). However, a clinical trial found that NAC did not prevent the progression of fibrosis in patients with mild-to-moderate IPF (46). In fact, the trial was stopped prematurely because the “triple therapy arm” (prednisone, NAC, and azathioprine) demonstrated a higher discontinuation rate, adverse reactions, and increased mortality. Yet, subsequent subgroup analysis demonstrated that a group of patients with a specific genotype of Toll Interacting Protein (TOLLIP), polymorphism TT, were found to have improved or stable lung function after treatment with NAC, but those with the CC genotype had disease progression (47). This provides supportive evidence that personalized treatment strategies could be applied clinically based on the genetic background of the patient. Indeed, a clinical trial entitled PRECISIONS has recently received NIH funding to determine if NAC is a viable treatment for IPF patients with the TOLLIP TT genotype based on this data (NCT04300920). This is one of the first studies to provide personalized medicine within the context of IPF and represents an exciting step forward in clinical trial development.

Senescence and Senescence-Associated Secretory Phenotype

Oxidative stress accelerates aging and propagates signals that induce a senescent phenotype throughout the interstitium. Cellular senescence is a marker of normal aging, but it is

also more abundant in chronic lung disease (known as premature aging). The process of senescence was initially thought to be protective from insults such as malignancy and infection because instead of inducing apoptosis or other detrimental paracrine signals, an injured cell could “hibernate” and not affect surrounding cells. However, accumulation of senescent cells has been shown to result in tissue dysfunction, age-related diseases, and shortened lifespans (48). Senescence has also been linked to telomere attrition, defective autophagy, failure of repair mechanisms, stem cell depletion and mitochondrial dysfunction (49). Both epithelial cells and fibroblasts in the fibrotic lung experience senescence, as characterized by “irreversible growth arrest” of cells. Phenotypically identified by an increase in cell size, metabolic activity, the production of inflammatory mediators, and apoptosis-resistance, senescence is a component of several chronic lung diseases and is particularly associated with aging. This process has been implicated in the pathogenesis of lung scarring (30, 50, 51), particularly because IPF is a disease of aged individuals.

Markers of senescence including p16, p21, and senescence-associated β-galactosidase (SA β-gal) and are all increased in fibroblastic foci as well as in alveolar epithelial cells (29, 50). Human lung epithelial cells treated with TGF-β1 develop a senescent phenotype. Senescent alveolar epithelial cells demonstrate a distorted morphology, have decreased barrier function and secrete an increased abundance of ROS. Importantly, senescent alveolar epithelial cells themselves are capable of inducing neighboring fibroblasts to differentiate into myofibroblasts (50). Similarly, fibroblasts isolated from active areas of lung fibrosis also exhibit *in vitro* signs of enhanced cellular senescence including abnormal cell morphology and resistance to apoptosis (30). They also exhibit accelerated replicative senescence, are more resistant to ROS signaling and exhibit enhanced expression of myofibroblast markers (29, 30). This creates a microenvironment in which epithelial injury, resulting in senescence, induces senescence in fibroblasts, which then develop resistance to apoptosis, continue to deposit matrix and release pro-senescent signals to perpetuate this cycle throughout the lung. Thus, senescent myofibroblasts and alveolar epithelial cells both contribute to a pro-fibrotic microenvironment, which communicates cellular stress signals and propagates a pathologic wound healing response in the lung (Figure 3).

Senescent cells are also metabolically active and release inflammatory signals into the microenvironment, termed the senescence-associated secretory phenotype (SASP)(52). This signaling cascade is activated by $p21^{CIP1}$, which then activates p38 mitogen-activated kinase (p38 MAPK) and Janus-activated kinase (JAK). Activation of JAK causes secretion of pro-inflammatory cytokines (for example Vascular Endothelial Growth Factor (VEGF), Tumor Necrosis Factor-alpha (TNF-α) and TGF-β1), chemokines (CXCL1 and CXCL8), and MMPs (MMP 2 and 9). While the inflammatory consequences of SASP are established in COPD and other chronic lung diseases, there is increasing evidence that support the role of JAK activation in IPF. For example, JAK inhibitors have been shown to attenuate bleomycin-induced pulmonary fibrosis (53). In addition, JAK expression and activity was increased in lung tissue from patients with IPF compared to healthy controls (54). As demonstrated in Figure 3, the SASP is a downstream product of TGF-β, PI3K/mTOR, and p38 MAPK signaling pathways. This phenotype then produces a cyclical, pro-fibrotic signaling loop that activates p38 MAPK

and JAK that induces more cellular senescence, VEGF signaling and telomere attrition, leading to more fibrosis.

Loss of Negative Regulation: PTEN and mTOR

The upregulation of tissue remodeling pathways cause pathologic fibrosis, but the loss of anti-fibrotic signaling modalities, which serve as a homeostatic brake in the wound healing process, also contributes to fibrosis. We present PTEN as a critical negative regulator of the wound healing process and explore the consequences of the loss of such a “brake” on the wound healing process. In this section we will first discuss how the loss of PTEN induces cyclical pathological signaling pathways and, second, how this loss causes the up-regulation and dysregulation of the mammalian target of rapamycin (mTOR) pathway, which also drives fibrosis (Figure 3).

Often examined in the context of cancer as a tumor suppressor protein, the presence of Phosphatase and Tensin Homolog (PTEN) attenuates tissue fibrosis. PTEN downregulation occurs following epithelial injury (Figure 3). Lung fibroblasts isolated from pulmonary fibrosis patients also display reduced protein levels and enzymatic activity of PTEN (55, 56). Furthermore, fibroblastic foci in lung tissue from patients with pulmonary fibrosis have decreased expression of PTEN (56, 57). Supporting the idea that a loss of PTEN is pro-fibrotic, chemical inhibition or genetic knockout of PTEN in fibroblasts is necessary and sufficient to drive myofibroblast differentiation (56, 57). *In vivo*, bronchoalveolar epithelial or myeloid-specific deletion of PTEN display more histological features of pulmonary fibrosis and collagen content in the lung in response to bleomycin (58, 59). These deleterious effects are mitigated by restoration of PTEN. On the other hand, overexpression of PTEN inhibits the development of a TGF-β1-induced fibrotic phenotype in fibroblasts (56, 57). This underscores the importance of homeostatic PTEN signaling in the wound healing process.

PTEN serves as a critical signaling node, limiting several pro-fibrotic pathways. At baseline levels, PTEN inhibits the phosphorylation of FAK, which plays a role in fibrosis through the regulation of cell migration and α-smooth muscle actin expression in fibroblasts (57). The loss of PTEN thus allows for increased FAK phosphorylation and myofibroblast differentiation. In addition, loss of PTEN directly increases the production of TGF-β1, which then promotes the further downregulation of PTEN via transcriptional inhibition (60). This cyclical signaling loop thus amplifies pathologic signaling associated with fibrosis.

In the epithelium, PTEN expression is reduced in response to injury, including in models of fibrosis. In rodent models utilizing bleomycin, there is observed PTEN depletion in epithelial cells (61). Immunohistochemistry of lung tissue isolated from patients with IPF show decreased PTEN expression in the alveolar epithelium as well as increased markers of senescence, p21 and β-gal (62). *In vitro*, the loss of PTEN in epithelial cells induces senescence (61) and overexpression of PTEN reverses this effect (62). There are multiple mechanisms associated with PTEN-mediated senescence in epithelial cells, including activation of NFkB, phosphorylation of Akt and regulation of mitophagy (31, 61, 62). This evidence suggests that

PTEN regulates the response to injury of the epithelium as well as the adoption of a senescent phenotype.

An emerging area of research is interrogating how the loss of PTEN leads to the increase in other pro-fibrotic pathways such as mTOR, a developmental pathway that is dysregulated in IPF (63). mTOR regulates a variety of essential events in embryogenesis and fetal development, from the fertilization of oocytes (64) to the formation of complex structures in soft organ development (65). In the lung, mTOR regulates the growth and development of branching airways (66) by coordinating expression of HIF-1α and VEGF to facilitate angiogenesis and nutrient exchange in distal regions of the lung (67). In developed organs, mTOR regulates cellular metabolism, differentiation, apoptosis and senescence (65, 68, 69).

The reactivation of the mTOR pathway in fibrosis contributes to fibroblast proliferation, encourages cellular senescence, decreases sensitivity of fibroblasts to pro-apoptotic signals and leads to dysregulated autophagy. mTOR protein expression, as well as its downstream effector, p-S6, are both increased in fibroblastic foci (70, 71). Furthermore, TGF-β1 activates mTOR signaling in human lung fibroblasts *in vitro* (70, 72), which in turn promotes the activation of Akt and its downstream effects including an increase in cell proliferation, resistance to apoptosis and fibroblast migration (72). Endogenous inhibition of mTOR is accomplished by 5'-AMP activated kinase (AMPK). This kinase is activated by low intracellular ATP concentrations, caloric restriction, and metformin. Interestingly, metformin inhibits and reverses bleomycin-induced pulmonary fibrosis in mice (73). Pharmacologic inhibition of mTOR signaling by rapamycin or MLN0128 attenuates myofibroblast differentiation *in vitro* and bleomycin- and radiation-induced lung fibrosis *in vivo* (72, 74). However, inhibition of mTOR may also increase expression of other pro-fibrotic cytokines such as CTGF, thus raising concern about the clinical utility of inhibiting mTOR (75, 76).

Mechanistically, PTEN and mTOR are connected via disinhibition of PI3K. The loss of PTEN leads to the sustained activation of phosphatidylinositol-3 kinase (PI3K) and/or the focal adhesion kinase (FAK)/Src kinase (57, 59). Activated PI3K also generates phosphatidylinositol-3,4,5-triphosphate, which promotes the phosphorylation of Akt. mTOR, specifically mTOR complex 2 (mTORC2) stabilizes the catalytic site of Akt which allows for maximal activation of Akt (77). Activation of Akt induces cellular proliferation, increased glycolytic metabolism, and apoptotic resistance (72, 78) all of which support pathologic lung scarring. Once activated, Akt causes the activation of mTOR signaling via multiple substrates (79). This causes a cyclical activation of Akt and mTOR, which occurs because of PTEN downregulation and subsequent PI3K activation.

Recent exciting work by the Chambers lab and others has explored how dual inhibition of PI3K and mTOR may be effective in attenuating lung fibrosis. GSK2126458 is a dual PI3K/mTOR inhibitor originally developed for the treatment of cancer (80). *In vitro*, treatment with GSK2126458 inhibits TGF-β1 induced collagen secretion in myofibroblasts. In ex-vivo lung tissue from patients with IPF, GSK2126458 also attenuated collagen formation, as assessed by decreased P1NP levels and Akt phosphorylation (81). Dual targeting therapies such as these

pave the way for more in depth studies of how multiple, interrelated pathways drive fibrosis, and exemplify why single molecule targeting may not be efficacious in clinical practice.

Activation of mTOR signaling has also been linked with a decrease in autophagic activity. Autophagy is the process of recycling damaged organelles and cellular components resulting in the homeostatic turnover of intracellular bodies. Markers of autophagy including p62 and beclin 1 are decreased in the lung tissues of patients with pulmonary fibrosis, notably in fibroblasts and within active areas of fibrosis. Impaired autophagy likely promotes an aging phenotype in epithelial cells via up-regulation of senescence and mitochondrial dysfunction (82). This increase in mTOR is correlated with increased mitochondrial biogenesis and increased ROS production, both of which can also encourage the senescent phenotype and may contribute to epithelial cell dysfunction or exhaustion in IPF.

Although mTOR may play a theoretical role in the development of pulmonary fibrosis, clinical trials studying mTOR inhibitors have been disappointing. A clinical trial utilizing Everolimus, a derivative of rapamycin, worsened fibrosis (83). The reason behind this outcome is not clear but may have been related to the high dose of the drug causing unanticipated detrimental off-target effects, or the fact that mTOR inhibition may increase some pro-fibrotic cytokines while inhibiting others (83). This outcome underscores yet again the complexity of the pathogenesis of IPF and the need for a more complete understanding of the ways in which multiple intersecting pro-fibrotic pathways influence one another. Blocking a single, aberrant developmental signaling pathway may not effectively treat fibrosis due to the overwhelming activation of various competing fibrosis inducing signaling pathways. However, the introduction of dual targeting therapeutics, such as GSK2126458 targeting both PI3K and mTOR show exciting efficacy and promise in targeting signaling nodes to target multiple pro-fibrotic pathways.

Fibrotic Pathogenic Cascades from the Activation of Developmental and Aging Pathways

It is estimated that 20% of genes dysregulated in IPF are related to early developmental pathways (84). This includes signaling molecules related to cellular growth, migration, morphogenesis, and differentiation, such as Wingless/Integrase-1 (Wnt)/β-catenin, bone morphogenic protein (BMP), TGF-β1 and mTOR. Additionally, age related DNA alterations of the lung have demonstrated roles in the pathogenesis of IPF. This includes telomere shortening, telomerase associated mutations, mitochondrial dysfunction and generation of reactive oxygen species and cellular senescence (21, 29, 69, 85).

When considering pathways associated with development, it is important to acknowledge that the timing of activation is critical to the outcome in that cell type or tissue. The research community is only beginning to understand the importance of temporal regulation of pro-fibrotic cytokines in the normal wound response of the lung. A recent single cell sequencing study by Riemondy et al. demonstrates the importance of TGF-β1 temporal activation and

subsequent downregulation in the epithelium following LPS-injury (86). This highlights the need for more such research to understand the processes of resolution to develop novel therapeutics to attenuate fibrosis.

Wnt and TGF-β1, control critical developmental signaling pathways that act cooperatively to create a self-perpetuating, cyclical pathologic signaling loop within the context of pulmonary fibrosis (reviewed in more detail in (87, 88), respectively). These pathways are activated by repeated and/or unresolved epithelial injury, resulting in the sustained activation of pro-fibrotic signals, which is then propagated and amplified throughout the mesenchyme resulting in persistent pathologic fibrosis. These master regulators of fibrosis cross-activate, or actively suppress the negative regulators, of other tissue remodeling pathways. This exacerbates the tissue remodeling response by simultaneously pressing the gas and removing the brakes on the wound healing process resulting pathologic fibrosis.

Wnt and Fibrosis

Wingless/Integrated (Wnt)/β-catenin signaling plays an important role in the developing lung through the regulation of cell fate, proliferation, and determination of cellular polarity (89). Early in development, Wnt signaling regulates airway formation and epithelial branching (90). In the post-natal lung Wnt is a key mediator of homeostasis by participating in alveologenesis and progenitor cell regulation, including the maintenance and differentiation of lung stem cells (91, 92) as well as the response to injury (93, 94). In the adult, increased Wnt activity has been demonstrated in several types of disease, including chronic lung diseases (95, 96) and it may serve to regulate the regenerative potential of the lung (97, 98). However, aberrant reactivation of the Wnt pathway later in life could lead to exhaustion of stem and progenitor cell populations, leading to a premature aging phenotype and reducing the capacity of the lung to respond appropriately to injurious stimuli (96, 99). This highlights the duality of signaling proteins like TGF-β1 and Wnt; some activity is necessary for homeostasis, but prolonged activity, or improperly timed signaling leads to pathology.

The Wnt family consists of 19 secreted glycoproteins which bind to transmembrane receptors including lipoprotein receptor related proteins (LRPs) and Frizzled 1-10 (FZD 1-10). Canonical Wnt signaling is initiated when Wnt binds to Frizzled and a co-receptor (LRP5/6) which allows nuclear translocation of β-catenin (100). Once in the nucleus, β-catenin activates several transcription factors including the main transcriptional effector, the T cell factor/lymphoid enhancing binding factor (TCF/LEF) family. When Wnt signaling is inactive, β-catenin is phosphorylated and subsequently degraded by the ubiquitin-proteasome pathway. Detection of β-catenin protein is frequently used as an indicator of active, canonical Wnt signaling. Initiation of this transcriptional program results in the broad activation of fibrosis-associated pathways including extracellular matrix deposition, cell cycle progression, growth factor secretion and cellular regeneration (101). Wnt signaling also occurs independent of β-catenin, also known as the non-canonical Wnt pathway. In this framework, a Wnt ligand binds a Frizzled receptor, which leads to the potential activation of several intracellular messengers including protein kinase A (PKA), calcium/calmodulin, paxillin and c-Jun N-terminal kinase (JNK) (102).

Researchers have identified increased canonical and non-canonical Wnt signaling in lung tissue of patients with IPF, indicated either by the presence of Wnt ligands, receptors and/or nuclear β-catenin. Specific examples of Wnt members that are increased in IPF include the ligands Wnt1, Wnt3a, Wnt7b; the receptors Fzd1-4; β-catenin and its downstream targets, including frizzled-related protein (FRZB), cyclin D1, and WNT1-inducible-signaling pathway protein-1 (WISP-1) (103-105). Furthermore, Wnt/β-catenin signaling is elevated in mouse alveolar epithelial type II cells after bleomycin challenge (51, 106-109) (Figure 2).

Molecules up-regulated by Wnt/ β-catenin signaling, such as WISP-1, fibronectin, and plasminogen activator inhibitor-1 (PAI-1) induce both epithelial and myofibroblast proliferation and differentiation resulting in the production of pro-fibrotic mediators (91, 110). In patients with pulmonary fibrosis, the increase in Wnt ligands and downstream mediators is observed in circulating peripheral blood mononuclear cells (PBMCs) (111) as well as in the fibrotic lung in bronchiolar lesions, damaged alveoli, fibroblastic foci (90) and type II airway epithelial cells (AECIIs) (112). AECIIs serve as one of the major progenitor cells in the lung, and prolonged Wnt expression in these cells may deplete the regenerative capacity of the lung and impair the ability of the lung to respond to injury.

The canonical Wnt/β-catenin pathway is well studied in pulmonary fibrosis, with most research focusing on the activation of β-catenin. However it is important to note β-catenin can be activated via Wnt-independent mechanisms (113). Within the context of pulmonary fibrosis, the upregulation of Wnt ligands, receptors and downstream mediators (112, 114, 115) indicates that the upregulation in β-catenin is at least partially dependent upon Wnt signaling. β-catenin is known to induce epithelial-mesenchymal transition (EMT), myofibroblast differentiation, increases fibroblast migration (116-118) and potentially prevents mesenchymal apoptosis (119). In the bleomycin model of pulmonary fibrosis, β-catenin induces macrophage differentiation, which antagonizes the resolution of the wound healing process (120, 121). In rodent models of fibrosis, β-catenin inhibitors suppress myofibroblast differentiation and induce epithelial differentiation resulting in barrier preservation and a reduction in collagen deposition (122-125).

An endogenous family of negative regulator of active Wnt signaling are the Dickkopf 1-4 proteins (DKK 1-4). The best studied is Dkk1, which is thought to antagonize Wnt signaling by inducing receptor internalization of the Wnt co-receptors LRP5/6 or by preventing the assembly of a receptor ternary complex involving LRP5/6 and FRZ receptors (126). The DKK proteins remain relatively understudied in pulmonary fibrosis, although Dkk1 is downregulated in whole lung samples from patients with pulmonary fibrosis as well as in skin from patients with systemic sclerosis (126, 127). However, other researchers have demonstrated DKK1 is increased in whole lung tissue, with the most abundant DKK1 staining present in basal bronchial epithelial cells. *In vitro*, DKK1 induced dose-dependent epithelial proliferation, indicating a regulatory role for the DKK proteins in epithelial maintenance and wound repair. The concentration of DKK protein was also increased in the BAL of patients with pulmonary fibrosis, compared to patients without lung disease. The upregulation of DKK proteins may be a protective mechanism and could be a response to wound healing that becomes overwhelmed by other pro-fibrotic signals. Additionally, DKK proteins could have different roles in the epithelium versus the mesenchyme, which may explain these conflicting results.

TGF-β1 and Fibrosis

Transforming Growth Factor-beta 1 serves an essential role in development and is required for embryonic stem cell fate commitment, proliferation and tumor suppression (128). In lung development specifically, TGF-β1 is integral to lung branching and alveolar formation (129). In the adult lung, low levels of TGF-β1 are required to maintain homeostasis, with this expression being localized to type 1 and 2 epithelial cells, mesenchymal cells, macrophages and endothelial cells (130). TGF-β1 is one of the classic cytokines responsible for the initiation of the inflammatory response and wound healing (94). However, when present in excess, it also is critical to the development of pulmonary fibrosis. The activation of TGF-β1 in the extracellular space induces multiple downstream pro-fibrotic mediators. It is also unique in that it is able to induce its own production and subsequent activation. The self-induced activation of TGF-β1 likely plays an important physiologic role in normal wound healing, however perpetual activation may sustain a pathological environment that promotes fibrosis.

TGF-β1 signals by binding to the TGF-β receptor 1 and 2 (TGFβR1 and TGFβR2) which causes TGFβR1 to phosphorylate TGFβR2. In the canonical TGF-β1 pathway, TGFβR1 then phosphorylates Smads 2 and 3. This allows for the association of Smad4 with Smads 2/3 and this complex translocates to the nucleus to induce downstream gene transcription. There are other Smads such as Smad7 which inhibit the association of Smad 2 and 3, as well as having several other mechanisms of TGF-β1 antagonism (129).

TGF-β1 is one of many cytokines that is significantly elevated in lung tissue and bronchoalveolar lavage fluid (BALF) of patients with IPF (131-134). Type I and type II alveolar epithelial cells along with fibroblasts, monocytes and macrophages (131, 135, 136), produce and secrete TGF-β1 into the extracellular space where it is maintained in an inactive form by a latent TGF-β1 complex. *In vitro*, TGF-β1 induces myofibroblast differentiation, activating production of extracellular matrix (ECM) proteins (137). TGF-β1 is unique in that active TGF can induce additional TGF-β1 activity (138). *In vivo*, over-expression of active TGF-β1 in rodent lung tissue induces progressive and irreversible fibrosis (139, 140). Conversely, inhibition of TGF-β1 signaling with neutralizing antibodies, receptor inhibitors, or via genetic silencing attenuates the development bleomycin-induced pulmonary fibrosis *in vivo* (141-146). TGF-β1 is therefore thought to be a key regulator of pulmonary fibrosis (Figure 2).

Wnt and TGF-β1 Cross-Propagation

In development, TGF-β1 and Wnt pathways share many physiologic roles including organ formation, organization and cellular differentiation (147). During development these processes are tightly regulated spatially and temporally. After development, both pathways are then downregulated, and remain relatively pedestrian in adult organisms. However, both Wnt and TGF-β1 signaling is induced in response to injury (94) to encourage tissue remodeling. In pathologic fibrosis, where wound healing has become dysregulated, both Wnt and TGF-β1 signaling pathways are perpetually activated. Unfortunately, the mechanisms that govern the

homeostatic activation and eventual down-regulation of Wnt and TGF-β1 during normal wound healing are not well understood.

TGF-β1 is known to directly induce the expression of several Wnt ligands in bone marrow stromal cells including Wnt2, Wnt4, Wnt5A, Wnt7A, Wnt10A and the co-receptor LRP5 (148). In primary human lung fibroblasts, TGFβ induces Wnt5A and activates β-catenin (149) Dermal fibroblasts isolated from patients with systemic sclerosis display hyper-responsiveness to Wnt ligands, compared to fibroblasts isolated from patients without disease. Utilizing a transgenic mouse with constitutive TGF-β1 activity in fibroblasts, researchers demonstrated reduced Axin-2 expression, a key negative regulator of the Wnt pathway. Reducing the levels of Axin-2 inhibits the β-catenin destruction complex from forming, and this allows β-catenin to translocate into the nucleus and activate Wnt transcriptional programs when it would otherwise be degraded (150). This indicates chronically high levels of TGF-β1 may prime fibroblasts to be more sensitive to Wnt ligands and to have higher basal Wnt activation, even in the absence of a ligand. This would drive activation of both pathways contributing to a pathologic, pro-fibrotic signaling loop on the receptor/ligand level. At the ligand level, TGF-β1 is able to increase Wnt ligand secretion directly and prolonged exposure to TGF-β1 sensitizes fibroblasts to Wnt ligands, increases basal Wnt activation even in the absence of stimulation (Figure 2).

TGF-β1 is able to both increase Wnt pathway activation while also inhibiting the negative regulators of the Wnt pathway. In a study of myocardial fibrosis by Blyszczuk et al., the activation of TGF-β1 leads to abundant Wnt ligand secretion, which is required for both the initiation and propagation of pathologic fibrosis in the heart. However, activation of the Wnt pathway while the TGF-β1 pathway was blocked was not sufficient to induce myofibroblast differentiation (151). This suggests that some of the pro-fibrotic effects of TGF-β1 signaling may be dependent on Wnt, demonstrating the cross-talk and potential for synergism of these pathways in fibrotic disease. The pleiotropic actions of pro-fibrotic cytokines such as TGF-β1 support the concept that multiple pathologic signaling loops are not only initiated in pulmonary fibrosis but also capable of perpetuating their own induction and activity across signaling cascades.

TGF-β1 suppresses DKK1 (149, 152) which is an important negative regulator of the Wnt pathway. Suppressing this negative regulator enhances Wnt/β-catenin signaling by a loss of brakes on this system. In dermal fibroblasts *in vitro* and an *in vivo* model of skin fibrosis using a TGF-β1 adenovirus, there was a TGF-β1 mediated decrease in DKK1, showing that with increasing TGF-β1 there is decreasing DKK1 protein. This imbalance increased Wnt signaling, as measured by nuclear translocation of β-catenin and activation of TCF/LEF elements (127). Interestingly, active DKK1 attenuates active TGF-β1 secretion, indicating inhibition of Wnt signaling also suppresses TGF-β1 signaling (153). The redundant regulation of both pathways indicates that a loss of DKK1 may further allow both pathways to remain active without proper negative regulation (Figure 2).

Shared downstream mediators of TGF-β1 and Wnt, such as Smads 2/3 and β-catenin bind to common transcriptional elements, including Smad binding elements (SBE) and TCF/LEF regions resulting in dual activation of TGF-β1 and Wnt signaling at the transcriptional level. In

addition, promoter regions of many Wnt and/or TGF-β1 responsive genes contain both SBEs and LEFs, allowing for co-transcriptional control of both signaling pathways and amplification of target genes, such as αSMA (154). Once these genes are induced, they further signal to perpetuate Wnt/TGF-β1 signaling to cause a positive, cyclical signaling loop of fibrosis (Figure 2). In addition, there is convergence of the Wnt and TGF-β1 pathways at the promoter level, where signal transducers of both pathways, such as Smads and β-catenin, form complexes, which bind promoter elements to induce transcriptional activation (155, 156). Thus, the activation of Wnt can promote sustained canonical TGF-β1 signaling and vice versa. Active TGF-β1 then suppresses the expression of negative regulators of the Wnt/β-catenin pathway, allowing for further activation of Wnt.

As an example of the delicate balance that is achieved with these interacting pathways, TGF-β1 signaling also leads to Wnt suppression. Upon TGF-β1 induction, the mediator Smad3 can form a complex with Axin and GSK-3β, which then induces phosphorylation of the complex, ultimately resulting in propagation of Wnt signaling without negative regulation (157). Smad3 can also support canonical Wnt signaling by shuttling β-catenin into the nucleus (158, 159). It is thought that, in the context of pulmonary fibrosis, this balance ultimately favors perpetuation of both signaling pathways (Figure 2). However, there is likely to be some degree of temporal, spatial, and cell-specific nuance to the influence that Wnt and TGF-β1 have on one another.

There are several pro-fibrotic signaling pathways that center around developmentally activated gene programs, specifically Wnt activation. This indicates Wnt may be a critical control point in the development of a sustained pathological fibrotic response. The pathways and mechanisms associated with aberrant Wnt activation within the context of fibrosis include the promotion of canonical TGF-β1 signaling. The convergence of the TGF-β1 and Wnt pathways is well-established and essential for proper development early in life. However, reactivation of the Wnt pathway later in life, in combination with aberrant TGF-β1 signaling is associated with several diseases that include cancer and fibrosis. We propose that recurrent epithelial injury chronically activates both TGF-β1 and Wnt pathways leading to cross-perpetuation of both pathways as each pathway causes a loss of negative regulators in the other (Figure 2).

Future Directions

Several exciting frontiers of research provide hope to patients, caregivers, physicians, and scientists. For example, the recently funded PRECISIONS trial represents the power of collaboration between philanthropic organizations (Three Lakes Partners), a national registry and biorepository (the Pulmonary Fibrosis Foundation), and physician-scientists through the National Heart, Blood, and Lung Institute at the National Institutes of Health. Precision medicine, as defined by the US National Library of Medicine, is “an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person”. The PRECISIONS study, as the first precision medicine trial in IPF, will establish the groundwork for future trials. We also argue that innovative trial designs and data collection will lead to more ethically responsible studies with

fewer participants and a more efficient arrival at clinical outcomes. For example, adaptive trials are more flexible are a novel method to study the efficacy of drug(s) and allow flexibility down to the individual participant level. As this is a relatively new approach, collaboration among biostatisticians, physicians, and scientists is needed to overcome the challenging statistical methods required to assess outcomes in an adaptive trial. Harboring the power of other non-traditional data collection methods, such as daily in home spirometry(160), provides the ability to have a rich data set and reduce the number of participants required to reach pre-determined outcomes. Clinical trials can also evolve by incorporating clinically measured outcomes (i.e. forced vital capacity) patient-centered ones (e.g. efficiency and effectiveness). From a research perspective, there are several potential therapeutic targets on the horizon (e.g. JAK 2 inhibitors, pan-FGF inhibitors, etc.). The use of big data combined with biorepositories will promote a more comprehensive understanding of individual variability and genetic differences to identify new therapeutic targets and, potentially, to discover clinically relevant biomarkers. With the explosion in publically available RNA sequence data, basic scientists should confirm these findings at the protein and cell signaling/activity levels. Finally, using novel approaches such as organoids, co-cell culture (i.e. epithelial and fibroblasts), and creating new non-inflammatory small animal models of pulmonary fibrosis will further our understanding of the mechanisms involved in the natural progression of disease. Specifically determining what is involved in the normal damping down of the healing process and harnessing this information to reverse fibrosis. There is evidence to suggest that IPF is a subclinical disease before patients develop symptoms (161). Therefore dissecting the differences between wound healing initiation, propagation and termination would add invaluable insight into pathologic signaling that could be exploited to therapeutic advantage.

Conclusion

"The knot loops in upon itself; I cannot find the end" – J.M. Coetzee

This review outlines several cyclical pathologic signaling pathways of pulmonary fibrosis that stimulate the development of scar tissue, the pathologic consequences of repeated and/or self-propagated epithelial damage, and premature cellular aging phenomena. In addition to the increased activation of pro-fibrotic signaling cascades in pulmonary fibrosis, there is a loss of the inhibitors of pro-fibrotic signaling. Impaired regulators of fibrotic pathways lead to uncontrolled ECM production and scarring. Additionally, pro-fibrotic cyclical signaling pathways remain unabated in the absence of anti-fibrotic mediators, enhancing the progression of lung fibrosis.

Fibrotic signaling loops involving the loss of negative regulators of pro-fibrotic cascades contribute to the development of pulmonary fibrosis and make discovery of single target therapeutics for lung scarring challenging. At present there are few, if any, truly effective treatments for pulmonary fibrosis. Probably due to the significant redundancy of these pathways, many clinical trials of single-targeted therapies have failed to improve patient outcomes. Lung fibrosis is difficult to treat due to the activation of multiple fibrotic pathways as

well as the loss of several negative regulators of fibrogenesis that convert a normal physiologic response into an irreversible pathologic one.

The combination of increased pro-fibrotic and loss of anti-fibrotic proteins induces an unchecked self-fulfilling prophecy that enhances pathologic lung scarring. Therefore, we propose employing the use of multi-modal therapies, in combination with precision medicine techniques and novel methodology, in future clinical trials in an attempt to capture the complexity of simultaneous gain and loss of function changes that occur in idiopathic pulmonary fibrosis. Strengthening the partnerships between academia, the pharmaceutical industry, philanthropic organizations and patient advocacy organizations within the ILD community will serve as model as we move into the era of precision medicine.

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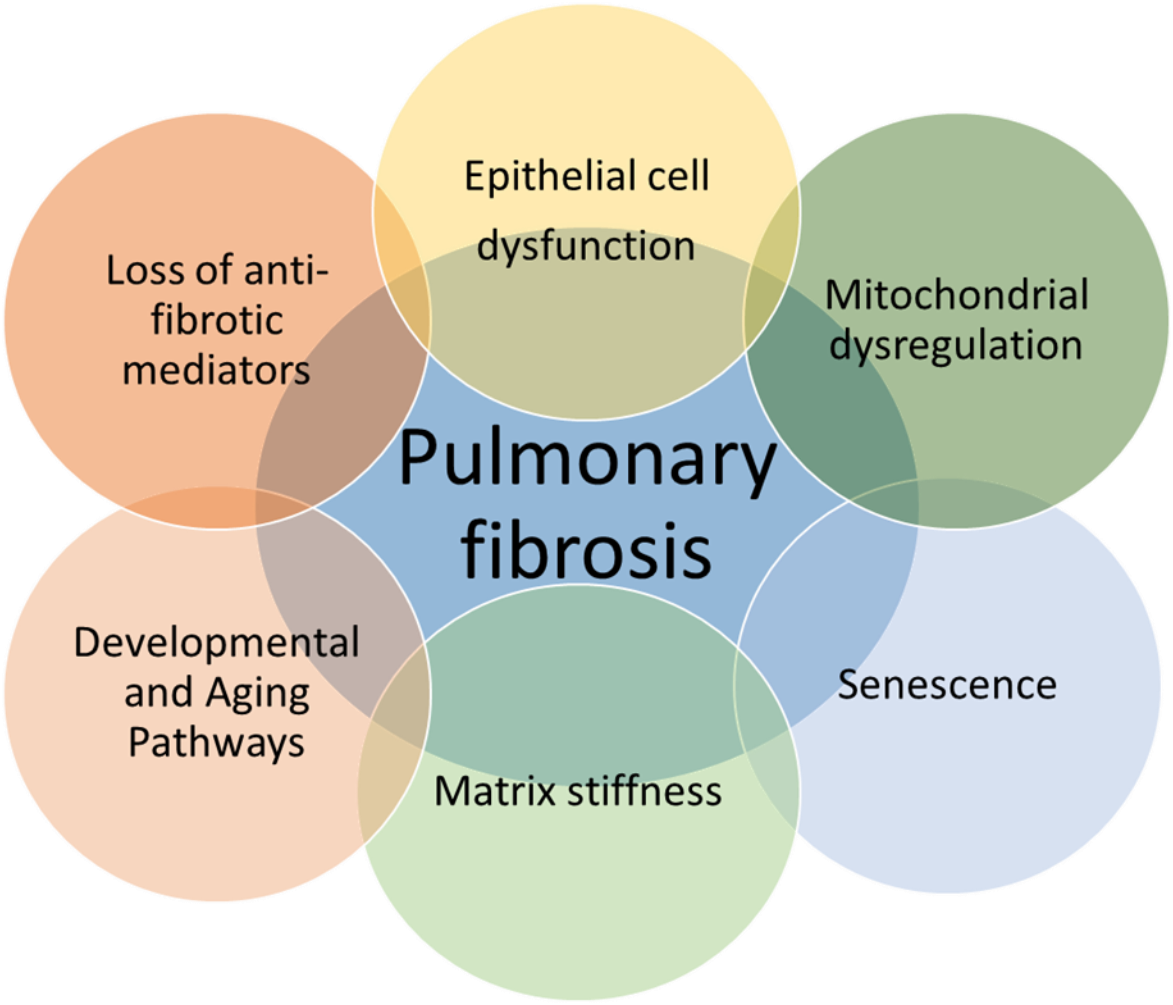

Figure 1. Overview of the interaction among various feed-forward loops involved in pulmonary fibrosis whereby all pathways contribute to the development of the disease.

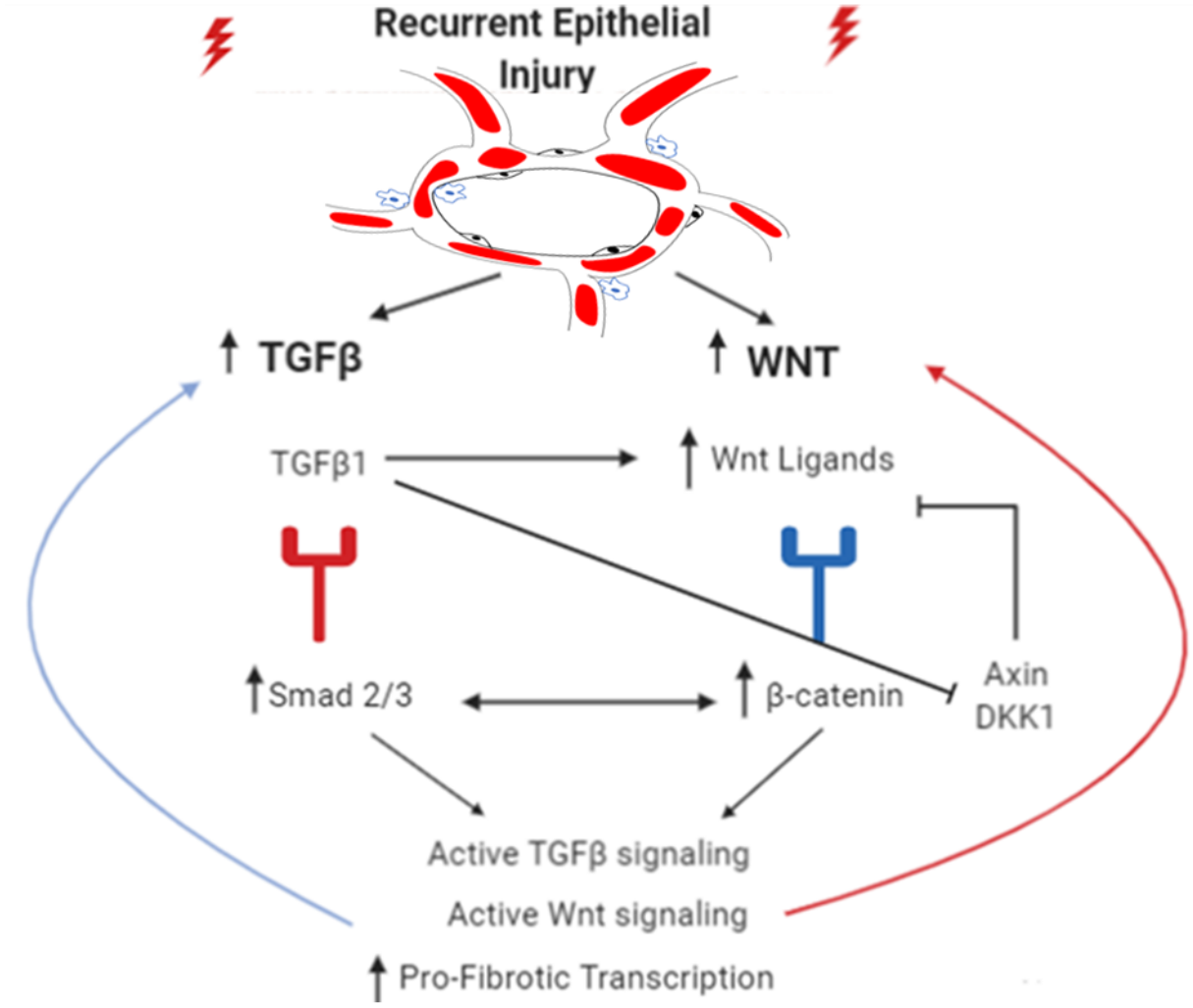

Figure 2. Recurrent epithelial injury leads to chronic activation of TGF-β1 and Wnt signaling cascades. The activation of TGF-β1 can suppress negative regulators of the Wnt pathway and ultimately activate Wnt signaling. Activation of Wnt signaling also propagates TGF-β1 signaling. This, coupled with chronic epithelial damage drives further drives this cycle to result in pathologic, unresolving wound healing.

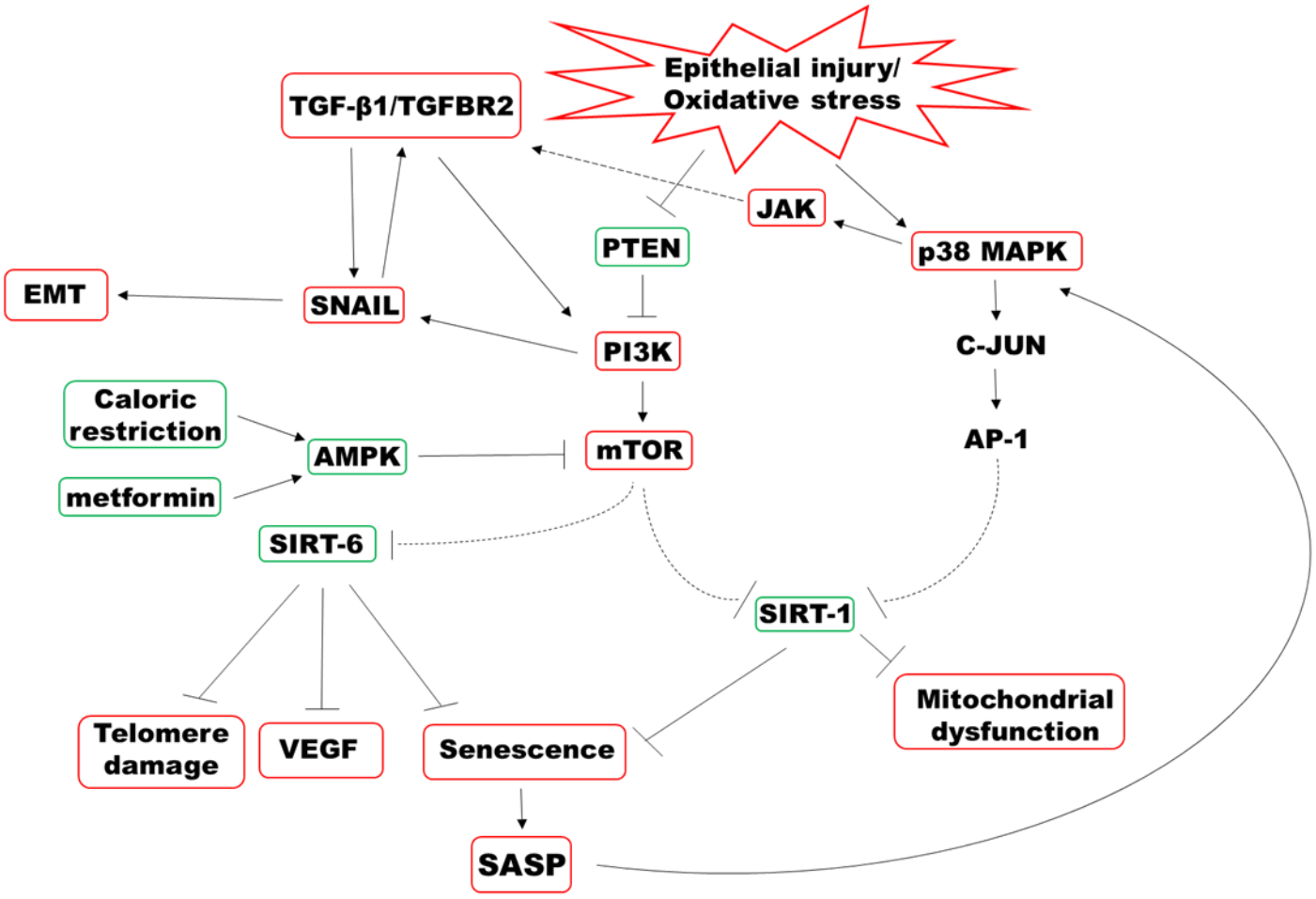

Figure 3. Signaling cartoon of the interaction among TGF-β1, epithelial to mesenchymal transition (EMT), epithelial injury, oxidative stress, telomere attrition and senescence. Messengers with red boxes depict pro-fibrotic pathways and green boxes indicate anti-fibrotic ones.