Review

Fibrosis of two: Epithelial cell-fibroblast interactions in pulmonary fibrosis

Norihiko Sakai, a,b, Andrew M. Tager a,b,c,⁎

a Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA 02114, USA
b Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA 02114, USA
c Pulmonary and Critical Care Unit, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA 02114, USA

A R T I C L E  I N F O

Article history:
Received 12 December 2012
Received in revised form 3 March 2013
Accepted 4 March 2013
Available online 14 March 2013

Keywords:
Pulmonary fibrosis
Epithelial cells
Apoptosis
Fibroblasts
Myofibroblasts
Extracellular matrix

A B S T R A C T

Idiopathic pulmonary fibrosis (IPF) is characterized by the progressive and ultimately fatal accumulation of fibroblasts and extracellular matrix in the lung that distorts its architecture and compromises its function. IPF is now thought to result from wound-healing processes that, although initiated to protect the host from injurious environmental stimuli, lead to pathological fibrosis due to these processes becoming aberrant or over-exuberant. Although the environmental stimuli that trigger IPF remain to be identified, recent evidence suggests that they initially injure the alveolar epithelium. Repetitive cycles of epithelial injury and resultant alveolar epithelial cell death provoke the migration, proliferation, activation and myofibroblast differentiation of fibroblasts, causing the accumulation of these cells and the extracellular matrix that they synthesize. In turn, these activated fibroblasts induce further alveolar epithelial cell injury and death, thereby creating a vicious cycle of pro-fibrotic epithelial cell-fibroblast interactions. Though other cell types certainly make important contributions, we focus here on the “pas de deux” (steps of two), or perhaps more appropriately to IPF pathogenesis, the “folle à deux” (madness of two) of epithelial cells and fibroblasts that drives the progression of pulmonary fibrosis. We describe the signaling molecules that mediate the interactions of these cell types in their “fibrosis of two”, including transforming growth factor-β, connective tissue growth factor, sonic hedgehog, prostaglandin E2, angiotensin II and reactive oxygen species. This article is part of a Special Issue entitled: Fibrosis: Translation of basic research to human disease.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Fibrosis characterizes many chronic diseases that result in end-stage organ failure, and consequently is a major cause of morbidity and mortality. The pathogenesis of fibrosis in many of these diseases is thought to involve aberrant or over-exuberant wound-healing processes initiated to protect the host from injurious stimuli [1]. In response to noxious stimuli of many different types, aberrant repair processes can produce the common result of excessive deposition of extracellular matrix that disrupts normal tissue homeostasis. Repair processes involve multiple cell types, including epithelial cells, fibroblasts, endothelial cells, pericytes and leukocytes, all of which potentially interact with each other. Interactions between two cell types in particular, alveolar epithelial cells and fibroblasts, appear to be central to the pathogenesis of idiopathic pulmonary fibrosis (IPF) [2].

IPF is characterized by progressive fibrosis, with excessive matrix deposition leading to destruction of lung architecture and ultimately fatal impairment of lung function. IPF has a heterogenous clinical course, but the median survival after diagnosis is only 2.5–3.5 years [2]. Although much of the pathogenesis of IPF remains to be elucidated, fibroblasts and epithelial cells have emerged as principal players in this disease, in particular myofibroblasts and type II alveolar epithelial cells. Fibroblasts and myofibroblasts accumulate in IPF lungs in “fibroblastic foci” that, as the predominant sites of excess matrix production, can be thought of as the leading edge of active fibrosis [3]. Fibroblast activation and accumulation in IPF, however, appears to be fundamentally driven by recurrent and/or non-resolving injury to the alveolar epithelium, and therefore in another sense, the injured alveolar epithelium can be thought of as the leading edge of active fibrosis. With fibroblasts and alveolar epithelial cells being in close apposition in the lung, it is not surprising that the interactions between these two key cellular players contribute to the development of pulmonary fibrosis. Though other cell types certainly make important contributions, we will focus on

Abbreviations: IPF, idiopathic pulmonary fibrosis; AEC, alveolar epithelial cell; TGF-β, transforming growth factor; CTGF, connective tissue growth factor; Shh, sonic hedgehog; PGE2, prostaglandin E2; ROS, reactive oxygen species; H2O2, hydrogen peroxide

⁎ This article is part of a Special Issue entitled: Fibrosis: Translation of basic research to human disease.

⁎⁎ Corresponding author at: Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, 149 13th Street, Room 8301, Charlestown, MA 02129, USA. Tel.: +1 617 724 7368; fax: +1 617 726 5651.
E-mail address: antager@partners.org (A.M. Tager).

0925-4439/ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbadis.2013.03.001

Biochimica et Biophysica Acta 1832 (2013) 911–921

Contents lists available at SciVerse ScienceDirect
Biochimica et Biophysica Acta journal homepage: www.elsevier.com/locate/bbadis

Biochimica et Biophysica Acta 1832 (2013) 911–921

Contents lists available at SciVerse ScienceDirect
Biochimica et Biophysica Acta journal homepage: www.elsevier.com/locate/bbadis

Biochimica et Biophysica Acta 1832 (2013) 911–921

Contents lists available at SciVerse ScienceDirect
Biochimica et Biophysica Acta journal homepage: www.elsevier.com/locate/bbadis

Biochimica et Biophysica Acta 1832 (2013) 911–921

Contents lists available at SciVerse ScienceDirect
Biochimica et Biophysica Acta journal homepage: www.elsevier.com/locate/bbadis

Biochimica et Biophysica Acta 1832 (2013) 911–921

Contents lists available at SciVerse ScienceDirect
Biochimica et Biophysica Acta journal homepage: www.elsevier.com/locate/bbadis
the “pas de deux” (steps of two), or perhaps more appropriate to IPF pathogenesis, the “folie à deux” (madness of two) of epithelial cell-fibroblast interactions as critical drivers of pulmonary fibrosis. Whereas the source of the fibroblasts and myofibroblasts that accumulate in the lungs as fibrosis develops—whether these cells arise from resident fibroblasts, resident epithelial cells or circulating precursors—has been an area of controversy, the pro-fibrotic effects of the interactions of fibroblasts and myofibroblasts with resident lung epithelial cells has become increasingly clear. We describe the role of several important mediators in orchestrating the pro-fibrotic interactions of epithelial cells and fibroblasts in their “fibrosis of two”, including transforming growth factor-β, connective tissue growth factor, sonic hedgehog, prostaglandin E₂, angiotensin II and reactive oxygen species (Fig. 1).

2. Epithelial cells: targeted cells in IPF

Accumulating evidence points toward recurrent and/or non-resolving injury to the lung epithelium as the “prime mover” of pulmonary fibrosis. Although the cause of this injury in IPF remains enigmatic, the footprints of lung epithelial injury are manifest, both in the form of (1) increased epithelial cell death, and (2) phenotypic alterations of the surviving epithelial cells. Increased numbers of apoptotic and necrotic cells have been observed in both the alveolar and bronchial epithelia of IPF patients [4–6]. Surviving epithelial cells in IPF lungs demonstrate several altered phenotypes [7]. Cuboidal epithelial cells representing type II alveolar epithelial cell (AEC) hyperplasia and/or bronchiolar basal cell proliferation line thickened fibrotic alveolar septa; single layers of flattened epithelial cells suggestive of squamous metaplasia frequently are present overlying fibroblastic foci; and single-layered columnar or pseudostratified columnar epithelial cells often line the abnormally enlarged, restructured air spaces of honeycomb lung. These morphological changes are associated with modifications of epithelial cell expression of specific cytokeratins, suggesting that in addition to their morphology, the differentiation states and functions of epithelial cells are likely profoundly altered in IPF. Of note, although IPF has been traditionally viewed as affecting the parenchymal lung rather than the airways, a potentially central role for the bronchial epithelium in addition to the alveolar epithelium in IPF pathogenesis has been suggested by the recent association of a genetic variant in mucin 5B (MUC5B) with both familial and sporadic IPF [8].

Finally, in addition to noxious stimuli in the external environment, alterations in the internal environment of epithelial cells can also lead to their death and promote pulmonary fibrosis. For example, the mutations in the gene encoding surfactant protein C (SFTPC) that have been associated with familial pulmonary fibrosis (familial interstitial pneumonia) cause SFTPC misfolding, leading to protein accumulation and endoplasmic reticulum (ER) stress [14–17]. Unresolved or prolonged ER stress activates cellular apoptotic pathways, and the resulting epithelial cell death may cause the pulmonary fibrosis that affects these SFTPC mutation kindreds [18]. Thus epithelial cell injury and death, albeit due to a variety of causes and through a variety of mechanisms, appears to be a common initiating pathway to fibrosis in the lung.

3. Epithelial cell-to-fibroblast interactions: how injured epithelial cells activate fibroblasts

Areas of AEC apoptosis and foci of α-smooth muscle actin (αSMA)-positive myofibroblasts co-localize in the lungs of IPF patients [6], making it plausible for these two cell types to directly influence each other as fibrosis develops. The ability of injured epithelial cells to affect local fibroblast behavior in a paracrine fashion has been demonstrated by in vitro co-culture experiments. In these experiments, mechanical injury to epithelial cells induced the expression of α-SMA and type I and III collagen in co-cultured fibroblasts by stimulating the activation of TGF-β in the extracellular matrix [19]. In addition to TGF-β, a growing list of mediators has been found to contribute to the ability of injured epithelial cells to activate fibroblasts.

![Fig. 1. Epithelial-fibroblast interactions drive the progression of idiopathic pulmonary fibrosis. Environmental stimuli initially injure alveolar epithelial cells (AECs), inducing their apoptosis and their production and/or activation of pro-fibrotic mediators, including TGF-β, CTGF and Shh. These AEC-derived mediators direct fibroblast migration, proliferation, activation and myofibroblast differentiation, resulting in the accumulation of myofibroblasts and extracellular matrix in the lung. Myofibroblasts in turn secrete mediators that amplify AEC injury and apoptosis, including ANGII and reactive oxygen species such as H₂O₂, creating a vicious cycle of pro-fibrotic epithelial cell-fibroblast interactions that drives the progression of IPF. PGE₂ normally mediates anti-fibrotic interactions between epithelial cells and fibroblasts, but its production by AECs is reduced in IPF, as is fibroblast PGE₂--responsiveness. Green arrows indicate pro-fibrotic epithelial cell-fibroblast interactions; red arrows indicate anti-fibrotic interactions. TGF-β, transforming growth factor-β; CTGF, connective tissue growth factor; Shh, sonic hedgehog; ANG II, angiotensin II; H₂O₂, hydrogen peroxide; PGE₂, prostaglandin E₂.](image-url)
fibroblasts, including other cytokines/growth factors, such as connective tissue growth factor (CTGF); morphogens, such as sonic hedgehog (Shh), and lipid mediators, such as prostaglandin E2 (PGE2) [20–24]. Many of these mediators, such as TGF-β, CTGF and Shh promote fibroblast activation, whereas others such as PGE2 are suppressive (Fig. 1).

In the sections that follow, TGF-β, CTGF, Shh and PGE2 are discussed as important examples of mediators through which injured epithelial cells regulate fibroblast activation, but are certainly not a complete list of the molecules involved in epithelial cell-to-fibroblast interactions in fibrosis.

3.1. Transforming growth factor-beta (TGF-β)

TGF-β is a member of the TGF-β super-family, which in addition to TGF-β includes related cytokines such as bone morphogenic proteins, activins and inhibins [25]. Mammals have three different forms of TGF-β (TGF-β1, -β2, and -β3), each of which are widely expressed throughout the body [26]. All three isoforms initiate their cellular effects using the same high-affinity cell surface receptors (TGF-β type I and type II receptors) [27,28]. But despite their common receptor usage, the isoforms have differing biological functions, as indicated by the differing phenotypes of mice deficient for each. TGF-β1 null mice develop severe multi-focal inflammation and die within 3 weeks of birth [29,30], whereas TGF-β2 null mice die in the perinatal period due to cyanotic heart disease and pulmonary insufficiency [31], and TGF-β3 null mice die of craniofacial defects, most notably cleft palate [32]. Taken together, these phenotypes indicate that TGF-β1 signaling is important for tissue growth and morphogenesis during embryonic development, and for tissue homeostasis thereafter. When tissue homeostasis is perturbed by injury, however, TGF-β3 acts as a major pro-fibrotic cytokine, potently increasing fibroblast recruitment, proliferation, differentiation into myofibroblasts and production of extracellular matrix [33]. Delivery of this cytokine by itself to the rodent lung through intratracheal transfer of active TGF-β1 in an adenoviral vector is sufficient to induce pulmonary myofibroblast accumulation and fibrosis [34,35]. Conversely, inhibiting TGF-β1 with neutralizing antibodies or a type I receptor inhibitor suppresses experimental pulmonary fibrosis [36,37]. Importantly, increased endogenous expression of TGF-β1 is not by itself sufficient to increase TGF-β1 function, because all three TGF-β isoforms are generated and are present in tissues as inactive latent precursors. As discussed below, epithelial cells have been shown to mediate the activation of latent TGF-β1 in the lung, and consequently to play a critical role in the presentation of active TGF-β1 to fibroblasts in the pathogenesis of pulmonary fibrosis.

3.1.1. Activation of TGF-β1 by epithelial cell integrins

The three TGF-β isoforms are produced in the form of small latent complexes (SLCs), in which the bioactive TGF-β peptides form non-covalent associations with a latency-associated peptide (LAP) [26]. Further, SLCs are usually secreted in association with latent TGF-β binding proteins (LTBP) as large latent complexes (LLCs). LLCs are sequestered in the extracellular matrix through the covalent binding of LTBP to extracellular matrix proteins, such as fibrillin and fibronectin [26]. For TGF-β1 to exert biological effects, it must be activated from these latent complexes. The changes in TGF-β1’s interactions with its LAP required for TGF-β1 activity can be accomplished by either non-proteolytic or proteolytic mechanisms that result in conformational changes or cleavage of the LAP respectively [26,38,39]. The non-proteolytic activation of TGF-β1 by epithelial cells, and the presentation of the active TGF-β1 produced to fibroblasts, appears to be an epithelial cell-to-fibroblast interaction that is central to the development of pulmonary fibrosis.

Epithelial cells induce activating conformational changes in latent TGF-β1 complexes through their integrins. Integrins are cell adhesion molecules and transmembrane receptors that link the cytoskeleton to the extracellular matrix, and in addition to adhesion, regulate multiple fundamental cell processes including cell migration, proliferation and differentiation [40,41]. Integrins are composed of α and β subunits (18 α and 8 β subunits) that heterodimerize to form 24 αβ combinations [42]. Eight of these, including all five αα-containing integrins (α1β1, α1β3, α1β5, α1β6 and α1β8), are capable of binding ligands with an arginine-glycine-aspartate (RGD) motif. The LAPs of both TGF-β1 and TGF-β3 have RGD sequences, and these two TGF-β isoforms can be activated in vitro by at least four of the αα-containing integrins (α1β3, α1β5, α1β6, α1β8) [42].

Activation of latent TGF-β1 specifically by the α1β6 integrin appears to centrally important in the development of pulmonary fibrosis. Genetic deletion of the β6 subunit, or antibody blockade of α1β6, suppresses TGF-β1 signaling in the lung after injury, and protect mice from the development of pulmonary fibrosis induced by bleomycin or radiation [43–45]. Lung expression of α1β6 appears to be restricted to epithelial cells, underscoring the fundamental involvement of epithelial cell-to-fibroblast interactions in the pro-fibrotic behaviors induced in fibroblasts by TGF-β1 during the development of pulmonary fibrosis. The ability of epithelial cell α1β6 to mediate latent TGF-β1 activation is dependent on epithelial cell cytoskeletal function. The cytoplasmic tail of the β6 subunit binds to the actin cytoskeleton, and disruption of this binding by mutation of the β6 cytoplasmic domain, or inhibition of actin polymerization with cytochalasin D, abolish epithelial cell activation of latent TGF-β1 [43].

Consistent with the activation of this pathway in pulmonary fibrosis, markedly increased lung epithelial α1β6 expression is present in mouse lungs post-bleomycin challenge, and in human lungs with usual interstitial pneumonia (UIP) pattern pulmonary fibrosis [45,46]. This increased α1β6 expression may itself be driven by active TGF-β1. TGF-β1 induces the expression of the β6 subunit gene (tgfb6) in the transcription factor Ets1 [47,48], and this process is inhibited by neutralizing β6 antibody [49]. α1β6 integrin-mediated activation of latent TGF-β1 may therefore be amplified in pulmonary fibrosis by a feed-forward loop of increased TGF-β1 activation and increased α1β6 expression.

3.1.2. Pro-fibrotic effects of TGF-β on fibroblasts

Once freed from its latent complexes by epithelial cell integrins, active TGF-β1 interacts with its receptors expressed by fibroblasts to induce multiple pro-fibrotic behaviors. Active TGF-β1 first binds to TGF-β receptor type II (TBRII), which phosphorylates and heterodimerizes with TGF-β1 receptor type I (TBR1) to form an active ligand–receptor complex. This TGF-β1–TBR1/I complex initiates pro-fibrotic responses in fibroblasts through both canonical and non-canonical signaling pathways. In the canonical TGF-β1 signaling pathway, activated TBR1 phosphorylates effector Smad proteins (Smad2 and Smad3), which heterodimerize with Smad4 to form Smad2/4 or Smad3/4 complexes [50]. These complexes translocate to the nucleus, where they bind to Smad response elements located in the promoter regions of pro-fibrotic genes such as type I collagen, fibronectin and αSMA [51]. Consistent with canonical TGF-β1 signaling having pro-fibrotic effects, Smad3-deficient mice are protected from bleomycin-induced lung fibrosis [52].

Both the c-Ablelson tyrosine kinase (c-Abl) and mitogen-activated protein kinases (MAPKs) have been implicated in non-canonical TGF-β1 signaling. c-Abl is directly activated by TGF-β1 in fibroblasts, and signals through Egr-1 [53]. The small molecule imatinib potently inhibits c-Abl, as well as the platelet-derived growth factor receptor tyrosine kinase. Imatinib was demonstrated to prevent the development of bleomycin-induced lung fibrosis in mice [54], but a recently completed randomized placebo-controlled clinical trial of imatinib in IPF patients showed no benefit [55].

Mitogen-activated protein kinases (MAPKs) have also been shown to be involved in non-canonical TGF-β1 signaling. The MAPK family of serine-threonine protein kinases include extracellular signal–regulated
kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38, which mediate a wide variety of cellular responses, including proliferation, differentiation, and apoptosis [56,57]. In lung fibroblasts, TGF-β induction of αSMA and collagen expression has been shown to be dependent on ERK and p38 MAPK [56,57], and p38 MAPK activation by TGF-β has been shown to contribute to fibroblast resistance to apoptosis through the pro-survival phosphatidyl inositol 3-kinase (PI3K)/AKT pathway [58].

3.2. Connective tissue growth factor (CTGF/CCN2)

CTGF is a member of the CCN protein family, containing Cysteine rich protein 61 (cyr61/CCN1), CTGF/CCN2, and Nephroblastoma overexpressed gene (NOV/CCN3), as well as three WNT-1-inducible signaling proteins (WISP), WISP-1/CCN4, WISP-2/CCN5 and WISP-3/CCN6. These proteins are characterized by an extraordinarily high content of cysteine residues, with the position of 38 of these cysteines being conserved almost entirely across the six family members [59,60]. CCN family proteins including CTGF contain four structural modules: an insulin-like growth factor binding protein module (module I), a von Willebrand factor type C module (module II), a thrombospondin type I homology module (module III) and a carboxy-terminal cysteine knot motif, heparin-binding module (module IV). These modules each have specific binding partners, including insulin-like growth factor-like module I, TGF-β for module II, specific integrins (α4/β1, α5/β1, α6/β1 and α1/β3) and sulfated glycoconjugates for module III, and heparin sulfate-containing proteoglycans (HSPGs) such as syndecan 4 and perlecain for module IV [59–61].

3.2.1. Synthesis of CTGF by epithelial cells

CTGF is principally regulated at the level of transcription [62]. Its promoter region contains binding sites for multiple transcription factors, including Snads, AP-1, Sp1, Ets-1, hypoxia-inducible factor and serum response factor [62–66]. Stimuli able induce CTGF transcription, dependent on the cell type, include TGF-β, thrombin, lysophosphatidic acid, and mechanical stress [60,61]. Signaling pathways involved in the regulation of CTGF expression by these stimuli include MAPK, protein kinase C, the small GTPase RhoA, and PI3K [61,66–68]. Interestingly, most of the stimuli able to induce CTGF induction also have been implicated in the pathogenesis of pulmonary fibrosis [36,69–72]. CTGF was originally discovered as a ‘PDGF-related mitogen’ in the medium of cultured human umbilical vein endothelial cells [73], but has subsequently been shown to be expressed by other cell types, including alveolar epithelial cells [21,73]. In lung tissues obtained from IPF patients, CTGF mRNA and protein are both increased, localizing predominantly to AECs and activated fibroblasts. In contrast, CTGF-expressing AECs are sparse in the normal lung [21].

3.2.2. Pro-fibrotic effects of CTGF on fibroblasts

CTGF regulates multiple fibroblast behaviors that contribute to fibrosis, including fibroblast adhesion, migration, proliferation, differentiation, matrix production and apoptosis [74]. CTGF can induce these behaviors either by binding to fibroblast cell surface molecules directly, or by acting as an “adapter” molecule that brings other mediators into contact with their receptors on fibroblasts [74]. CTGF can directly affect fibroblast behaviors by binding to integrins, HSPGs and the low density lipoprotein receptor-related protein/β3-macroglobulin receptor (LRP) [60,75–77]. For example, CTGF binding to fibroblast HSPGs induces fibroblast adhesion and proliferation [77,78]. Specific CTGF binding to the insulin-like growth factor-II (IGF-II)/mannose 6-phosphate (M6P) receptor expressed on lung fibroblasts has also recently been shown to induce fibroblast proliferation [79]. CTGF has also been shown to directly stimulate lung fibroblast expression of multiple proteins known to be elevated in IPF, including type I collagen, the cytoskeletal proteins vinculin, moesin and ezrin, and IQ motif containing GTPase activating protein 1 (IQGAP1), a scaffold protein that regulates cell migration and is elevated in lung fibroblasts isolated from scleroderma patients with pulmonary fibrosis [80].

In its role as an adapter molecule [74], the cytokines and growth factors that CTGF can “present” to their specific receptors on fibroblasts include TGF-β [81], epidermal growth factor, and insulin-like growth factor-II [82]. This adapter function appears to be required for at least some of the pro-fibrotic effects of TGF-β on fibroblasts. Although CTGF-deficient fibroblasts show intact TGF-β-induced Smad signaling, the ability of TGF-β to induce the expression of multiple pro-fibrotic mRNAs and proteins, including αSMA and type I collagen, is impaired in these cells [83].

Pro-fibrotic effects of CTGF have also been demonstrated in vivo. Whereas BALB/c mice are resistant to bleomycin-induced pulmonary fibrosis [84], transient overexpression of CTGF in the lungs of these “fibrosis-resistant” mice by adenoviral gene transfer of CTGF enabled bleomycin to induce pulmonary myofibroblast accumulation and fibrosis similar in extent to that produced in “fibrosis-prone” C57Bl/6 mice [85]. The pro-fibrotic effects of CTGF that is specifically expressed by pulmonary epithelial cells were demonstrated in a transgenic mouse model with doxycycline-inducible overexpression of CTGF in respiratory epithelial cells directed by the clara cell secretory protein promoter. Overexpression of CTGF from postnatal days 1–14 resulted in increased αSMA expression, collagen deposition and dramatic thickening in the peribronchial/peribronchiolar and perivascular regions of the lungs [86]. Cooperation between the pro-fibrotic effects of TGF-β and CTGF has also been noted in vivo. Intraperitoneal co-administration of CTGF and TGF-β2 induced multilorgan fibrosis in the lungs, liver and kidneys, whereas administration of either cytokine alone failed to elicit a fibrotic response [87]. Conversely, anti-CTGF antibodies have been shown to mitigate the increases in αSMA and type I collagen protein expression, as well as total collagen content, induced in lung in the bleomycin model of pulmonary fibrosis [88,89].

3.3. Sonic hedgehog (Shh)

Some of the pathways that regulate embryological development but are subsequently quiescent appear to re-emerge during repair responses to tissue injury [90]. Consistent with re-activation of developmental pathways in response to injury, microarray analyses of IPF gene expression revealed enriched expression of genes associated with lung development, including Patched-1, a receptor in the hedgehog pathway [91]. The hedgehog (Hh) family in mammals consists of sonic hedgehog (Shh), desert hedgehog (Dhh), and indian hedgehog (Ihh), which play critical roles in embryonic development, tissue patterning, and organogenesis [92,93]. Of these three mammalian Hh homologues, Shh has been shown to be responsible for tissue patterning in the lung, by regulating branching morphogenesis [94–96]. During lung development, Shh is produced in the distal epithelium and stimulates mesenchymal cell proliferation. Shh overexpression in mice pre-natally results in lethally excessive accumulation of lung interstitial mesenchyme [96], emphasizing that the Shh pathway mediates important epithelial cell-to-mesenchymal cell interactions during embryological lung development.

3.3.1. Epithelial cell synthesis of Shh

In both lung development and pulmonary fibrosis, epithelial cells appear to be the major source of Shh. During embryological development, low levels of Shh mRNA are present throughout the epithelium, and high levels are present at the tips of the terminal buds that ultimately form alveoli [23,97]. Several studies of lung tissues obtained from IPF patients have demonstrated high Shh expression in reactive AECs, as well as in the epithelial cells that line honeycomb cysts, compared with little or no Shh expression in normal adult lung tissues [98–100]. In contrast, another recent study found that most of the components of the Shh system were expressed in normal adult alveolar epithelium, but that this pathway was activated specifically in IPF.
3.3. Pro-fibrotic effects of Shh on fibroblasts

Shh signaling is transduced by 2 transmembrane proteins, patched-1 (PTCH-1) and smoothened (SMO). PTCH-1 is an Shh-binding receptor, which in the absence of Shh constitutively blocks SMO activity. Shh binding to PTCH-1 activates SMO, and activated SMO directs the biochemical processing and subsequent nuclear translocation of the transcription factors glioma-associated oncogene homolog (GLI)1, GLI2, and GLI3 Target genes regulated by these transcription factors include PTCH-1 itself, as well as regulators of the cell cycle, enabling the Shh pathway to regulate cell proliferation [104–106]. One recent immunohistochemical analysis of Shh pathway components in IPF reported fibroblast expression of PTCH-1, SMO, and GLI1 in the lungs of IPF patients, whereas no staining was detected in normal lungs [100]. PTCH-1 mRNA was also upregulated in primary lung fibroblasts grown from IPF versus normal lung tissue, and in fibroblasts assessed by laser capture microdissection in IPF lung tissues versus lung tissues from patients with cryptogenic organizing pneumonia [100]. In vitro, recombinant Shh has recently been shown to increase fibroblast proliferation and migration, to increase fibroblast expression of collagen and fibronectin but not αSMA, and to protect fibroblasts from apoptosis [100]. Additionally, TGF-β1’s ability to drive and maintain myofibroblast differentiation was recently demonstrated to require SMO/GLI pathway activity [101].

3.4. Prostaglandin E2 (PGE2)

The paucity of fibroblasts in normal alveolar septae compared with the greater abundance of fibroblasts in the connective tissue surrounding pulmonary bronchi, arteries, and veins led some investigators to hypothesize that under homeostatic conditions, alveolar epithelial cells suppress fibroblast accumulation. Media conditioned by AEC-fibroblast co-culture was in fact demonstrated to inhibit fibroblast proliferation, and this inhibition in subsequent studies was determined to be attributable to PGE2 synthesized by AECs [24,107]. In contrast to the pro-fibrotic epithelial to-fibroblast interactions mediated by TGF-β, CTGF and Shh, PGE2 therefore carries anti-fibrotic signals from epithelial cells to fibroblasts.

3.4.1. Epithelial cell synthesis of PGE2

AECs have a large capacity for synthesizing PGE2, the most abundant arachidonic acid metabolite that these cells produce. Following the liberation of arachidonic acid from membrane phospholipids, the synthesis of prostanooids, including prostaglandins, thromboxane and prostacyclin, is initiated by two cyclooxygenase (COX) enzymes, COX-1 and COX-2. COX-1 is constitutively expressed in most cells and tissues, whereas COX-2 is expressed when induced by inflammatory or mitogenic stimuli [108]. The pulmonary epithelium represents an exception to this usual pattern, in that AECs constitutively express both COX isoforms [109]. Experiments using COX-2-deficient AECs demonstrated that the PGE2 synthetic capacity of these cells is predominantly COX-2-dependent [24].

PGE2 synthesis is reduced in IPF, limiting the anti-fibrotic epithelial cell-to-fibroblast interactions that are mediated by PGE2. PGE2 levels in the epithelial lining fluid of individuals with IPF were found to be 50% lower than those in normal subjects [110]. AEC production of PGE2 may be suppressed by increased levels of plasminogen activation inhibitor-1 (PAI-1) and CC chemokine ligand 2 (CCL2) in IPF lungs. Plasmin has recently been shown to upregulate AEC PGE2 production [111]. Plasmin upregulates AEC COX-2 expression, potentially through its ability to proteolytically activate and release hepatocyte growth factor (HGF) from these cells and/or the extracellular matrix. In bronchial epithelial cells, HGF has been shown to increase COX-2 gene expression through an Akt-, MAPK-, and β-catenin-dependent pathway [112]. PAI-1, which is markedly upregulated in fibrotic lungs [113], prevents the generation of plasmin by inhibiting urokinase-type plasminogen activator (uPA). By reducing the generation of plasmin, increased levels of PAI-1 could therefore reduce AEC PGE2 synthesis in IPF. In support of this hypothesis, PAI-1-deficient mice demonstrate increased lung production of PGE2, and are protected from bleomycin-induced pulmonary fibrosis [111]. This protection was abrogated by a selective inhibitor of the HGF receptor c-Met, which reduced lung COX-2 and PGE2 levels. In contrast to its upregulation by plasmin, AEC PGE2 synthesis is downregulated by CCL2. CCL2 is present in increased amounts in the bronchoalveolar lavage fluid of IPF patients [114], and may be induced in the AECs themselves by thrombin activation of the major thrombin receptor, protease-activated receptor-1 (PAR1) [115]. CCL2 and PAR1 are co-expressed and co-upregulated on the activated epithelium in fibrotic areas in IPF, and thrombin potently induces CCL2 expression in lung epithelial cells in vitro in a PAR1-dependent manner.

3.4.2. Anti-fibrotic effects of PGE2 on fibroblasts

PGE2 has been demonstrated to have multiple activities on fibroblasts that could suppress fibrosis, including inhibition of fibroblast migration [116], proliferation [117,118], collagen synthesis [119], and myofibroblast differentiation [120]. Of the 4 E prostanoid (EP) receptors, designated EP1, EP2, EP3, and EP4 [120], these inhibitory effects of PGE2 on fibroblasts are mediated by EP2 [120–122]. Lung fibroblast EP2 expression, however, is downregulated in pulmonary fibrosis. Diminished EP2 levels in fibroblasts isolated from mouse lungs following bleomycin challenge reduced the ability of PGE2 to inhibit their proliferation and collagen secretion [121]. Fibroblasts isolated from patients with IPF also exhibit decreased EP2 expression, and are similarly refractory to the anti-fibrotic effects of PGE2 [123]. The diminished EP2 expression levels in fibroblasts from fibrotic lungs are maintained by hypermethylation of the PGE receptor 2 gene (PTGER2) promoter [124]. Treatment of these fibroblasts with DNA methylation inhibitors or DNA methyltransferase-specific siRNA decreased PTGER2 methylation, increased EP2 mRNA and protein expression, and restored PGE2 responsiveness [124]. The anti-fibrotic epithelial cell-to-fibroblast interactions mediated by PGE2 therefore appear to be limited in IPF by decreased fibroblast EP2 expression as well as by decreased AEC PGE2 synthesis. Whereas epithelial-to-fibroblast interactions mediated by TGF-β, CTGF and Shh are attractive targets for new IPF therapies to inhibit, restoring PGE2-mediated interactions, by increasing AEC PGE2 production and fibroblast EP2 expression, represents an attractive therapeutic strategy for pulmonary fibrosis.

4. Fibroblast-to-epithelial cell interactions: how activated fibroblasts injure epithelial cells

As noted above, recurrent and/or non-resolving injury to the lung epithelium now appears to be the "prime mover" of pulmonary fibrosis. Increased numbers of apoptotic cells have been observed in the alveolar and bronchial epithelia of IPF patients [4,5], and the specific induction of epithelial injury and/or apoptosis has been shown to be sufficient to cause pulmonary fibrosis in several mouse models [9–12]. Although the initiating causes of epithelial injury in IPF remain enigmatic, activated fibroblasts appear to be able to amplify the epithelial apoptosis that results. As also noted above, the co-localization...
of αSMA-positive myofibroblast foci with areas of AEC apoptosis in IPF lungs [6] makes the paracrine interaction of these two cell types plausible in the development of fibrosis in vivo. The ability of activated fibroblasts to affect epithelial cells in a paracrine fashion has been demonstrated in vitro. In these experiments, media conditioned by fibroblasts isolated from the lungs of IPF patients markedly induced apoptosis of AECs in culture, whereas media conditioned by fibroblasts from control subjects did not [125]. Several mediators have been found to be responsible for this ability of activated fibroblasts to induce epithelial cell apoptosis, including angiotensin II and hydrogen peroxide (H$_2$O$_2$) (Fig. 1).

4.1. Angiotensin II

The renin–angiotensin system consists of renin, angiotensinogen (AGT), angiotensin I (ANG I), angiotensin converting enzyme (ACE) and angiotensin II (ANG II). The octapeptide ANG II is the primary effector molecule of this pathway, and is formed by enzymatic cleavage of AGT to ANG I by the aspartyl protease renin, followed by the conversion of ANG I to ANG II by ACE. Although best known for its role in blood pressure regulation due to its ability to mediate vasoconstriction, ANG II has been implicated in the pathogenesis of fibrotic diseases affecting multiple organs, including the lung [126].

4.1.1. Fibroblast synthesis of ANG II

ANG II was determined to be the soluble factor responsible for inducing AEC apoptosis in media conditioned by IPF lung fibroblasts [127]. Lung tissue from IPF patients and bleomycin-challenged mice demonstrate upregulation of AGT and ANG peptides specifically in myofibroblasts [128,129]. In vitro, human lung fibroblasts have been demonstrated to upregulate AGT expression in response to TGF-β, through a mechanism involving activation of AGT transcription by hypoxia-inducible factor-1α and Jun D [130]. Myofibroblasts, especially under hypoxic conditions, are consequently thought to be an important source of ANG II production in the fibrosing lung.

4.1.2. Pro-fibrotic effects of ANG II on alveolar epithelial cells

The biological effects of ANG II are mediated through its two specific G protein-coupled seven transmembrane domain receptors, ANG II type 1 receptor (AT1R) and ANG II type 2 receptor (AT2R) [126]. Although AECs express both AT1R and AT2R, experiments with AT1R- and AT2R-selective antagonists indicate that AT1R mediates ANG II-induced AEC apoptosis [131,132]. An AT1R-selective antagonist was able to prevent mouse AEC apoptosis and lung fibrosis in the bleomycin model, as was an antisense oligonucleotide targeting AGT mRNA and an ACE inhibitor [128,133,134]. AT1R-deficient mice similarly demonstrated reduced AEC apoptosis and collagen accumulation in the bleomycin model [134]. Further implicating the renin–angiotensin system in IPF, a single-nucleotide polymorphism (SNP) in the promoter region of the AGT gene that increases AGT transcription has been associated with more rapid disease progression [135].

4.2. Reactive oxygen species (ROS)

Experiments co-culturing small airway epithelial cells with TGF-β-stimulated fibroblasts isolated from IPF patients identified hydrogen peroxide (H$_2$O$_2$) as another diffusible paracrine signal produced by activated myofibroblasts that is able to induce epithelial cell death [136]. Production of ROS, such as H$_2$O$_2$, superoxide anions (O$_2^-$) and hydroxyl radicals (OH), in excess of the capacity of cells and tissues to detoxify or scavenge them is referred to as “oxidative stress”, and has been implicated in fibrotic diseases of multiple organs, including the lung [137]. Multiple lines of evidence indicate the presence of oxidative stress in IPF lungs. H$_2$O$_2$ concentrations are significantly higher in the exhaled breath condensates of IPF patients than control subjects, and correlate with disease severity [138]. Proteins in the bronchoalveolar lavage fluid of IPF patients demonstrate elevated levels of oxidative changes, such as oxidation of methionine residues to methionine sulfoxide, and introduction of carbonyl groups into other amino acid side-chains [139,140]. Additionally, epithelial cells in the lungs of IPF patients demonstrate the presence of ROS-induced DNA modifications [141].

4.2.1. Fibroblast synthesis of ROS

ROS are formed by the univalent reduction of oxygen, generally mediated by several ROS-producing enzymes, such as mitochondrial respiratory oxidases, xanthine oxidase, myeloperoxidase and NADPH oxidases [137,142]. NADPH oxidases comprise a seven member family, including NOX1, 2, 3, 4 and 5, Duox1 and 2. Both NOX1 and NOX2 generate superoxide anions, whereas NOX4 produces H$_2$O$_2$ [143]. Recent evidence has implicated NOX4 expressed by fibroblasts in the development of lung fibrosis. TGF-β induces expression of this NOX isoform in IPF lung fibroblasts in vitro. Further, the expression αSMA and extracellular matrix proteins induced in lung fibroblasts by TGF-β, as well as the proliferation of these cells induced by serum, require NOX4-dependent generation of H$_2$O$_2$ [144]. In IPF lung sections, H$_2$O$_2$ localizes specifically to αSMA-expressing myofibroblasts [136], and NOX4 similarly localizes to myofibroblastic foci [144]. A critically important role for fibroblast NOX4, and the H$_2$O$_2$ it produces, in the pathogenesis of pulmonary fibrosis was demonstrated in experiments in which siRNA-mediated knockdown of NOX4 expression suppressed fibrosis in vivo in both bleomycin- and FITC-induced mouse models of pulmonary fibrosis [144].

4.2.2. Pro-fibrotic effects of ROS on alveolar epithelial cells

In cultures of confluent primary distal lung epithelial cells, exogenous H$_2$O$_2$ inhibits monolayer closure after scratch wounding by inducing epithelial cell apoptosis [145]. As noted above, co-culture experiments demonstrated that endogenous generation of H$_2$O$_2$ by TGF-β-stimulated IPF lung fibroblasts induces apoptosis of small airway epithelial cells in a paracrine manner [136]. In the bleomycin mouse model of pulmonary fibrosis, alveolar epithelial apoptosis induced in vivo by bleomycin injury was demonstrated to be NOX4-dependent. The dramatic increase in apoptotic alveolar epithelial cells observed in wild-type mice at early time points post-bleomycin challenge was abrogated in NOX4-deficient mice, and the development of fibrosis in these mice was markedly reduced at later time points [146]. A pathogenic role for ROS in pulmonary fibrosis in vivo studies has been further underscored by the amelioration of bleomycin-induced fibrosis in mice administered the antioxidant superoxide dismutase, and the exacerbation of bleomycin-induced pulmonary fibrosis in mice genetically deficient for this enzyme [147,148].

5. Bidirectional epithelial cell-fibroblast interactions in pulmonary fibrosis

Many important mediators of pulmonary fibrosis may be produced by both epithelial cells and fibroblasts, and may exert important effects on both cell types as well. Several of the signaling molecules discussed above carry signals both from epithelial cells to fibroblasts, and from fibroblasts to epithelial cells, thus mediating bidirectional epithelial cell-fibroblast interactions in pulmonary fibrosis.

5.1. TGF-β

As described above, TGF-β is activated during the development of pulmonary fibrosis by epithelial cell integrins, and induces multiple pro-fibrotic activities in fibroblasts, including their recruitment, proliferation, myofibroblast differentiation and extracellular matrix production [33]. In addition to mediating these pro-fibrotic effects on fibroblasts, TGF-β delivers pro-fibrotic signals to the alveolar epithelium. In contrast to its induction of resistance to apoptosis in
lung fibroblasts, TGF-β promotes apoptosis of lung epithelial cells in vitro [149,150]. A pathogenic role for TGF-β-induced epithelial cell apoptosis is suggested by observations of mice with lung-specific overexpression of active TGF-β. In these mice, a transient wave of epithelial apoptosis precedes the development of lung fibrosis, and blocking the TGF-β-induced epithelial apoptosis by administration of a general or a 3/7-selective caspase inhibitor markedly ameliorated the subsequent fibrosis. A pathogenic role for TGF-β-induced epithelial cell apoptosis is further suggested by the protection from bleomycin-induced fibrosis observed in mice with epithelial cell-specific deletion of TGF-β type II receptor [151]. Lung fibroblasts produce TGF-β latent complexes, and have been shown to be able to activate these complexes themselves by the α5β1 integrins that they express [152]. TGF-β therefore could very plausibly mediate pro-fibrotic fibroblast-to-epithelial cells interactions, in which TGF-β produced and activated by lung fibroblasts mediates epithelial cell apoptosis. TGF-β thus could be an important mediator of bidirectional interactions between epithelial cells and fibroblasts in pulmonary fibrosis.

5.2. ANG II

As noted above, ANG II produced by myofibroblasts induces apoptosis in alveolar epithelial cells in a paracrine fashion. In addition to mediating this pro-fibrotic effect of fibroblasts on epithelial cells, ANG II may also carry pro-fibrotic signals from epithelial cells to fibroblasts. Apoptotic AECs have been shown to produce AGT and convert it to ANG II both in vitro and in the lung [129,153]. Lung fibroblasts express AT1R and AT2R, and ANG II-AT1R signaling induces multiple pro-fibrotic activities in cells, including their proliferation, migration, and extracellular matrix synthesis [129]. ANG II therefore could also mediate pro-fibrotic effects of epithelial cells on fibroblasts, in which case it would also be an important mediator of bidirectional interactions between these two cell types in pulmonary fibrosis.

6. Other mediators of epithelial cell-fibroblast interactions in pulmonary fibrosis

The signaling molecules discussed in this review are meant to serve as important examples, rather than as a complete list, of mediators that direct epithelial cell-fibroblast interactions in lung fibrosis. Multiple other pathways that may contribute to the pro-fibrotic interactions of these two cell types have been described. Additional examples of important such signaling molecules include members of the WNT pathway and of the found in inflammatory zone (FIZZ)/resistin-like molecule (RELM) family. The WNT pathway was recently demonstrated to deliver pro-fibrotic signals from epithelial cells to fibroblasts in the development of lung fibrosis [154]. Expression of canonical (β-catenin-dependent) WNT signaling components and the WNT pathway target molecule, WNT-1-inducible signaling protein-1 (WISP1, another member of the CCN family of matricellular proteins), are strongly upregulated in type II AECs in both mice challenged with bleomycin and humans with IPF [154]. Alveolar epithelial cell WISP1 can exert pro-fibrotic effects on lung fibroblasts: WISP1 stimulates fibroblast extracellular matrix synthesis and myofibroblast differentiation in vitro, and its neutralization suppresses bleomycin-induced lung fibrosis in mice in vivo [154]. FIZZ/RELM family members, including FIZZ1/RELM-α and FIZZ2/RELM-β, similarly have been shown to be expressed by pulmonary epithelial cells during the development of fibrosis, and to exert pro-fibrotic effects on fibroblasts; FIZZ1 is strongly induced in mouse AECs following bleomycin challenge [155], and induces fibroblast extracellular matrix production, myofibroblast differentiation, and resistance to apoptosis [155–157]. FIZZ2 can also be induced in lung epithelial cells, is highly expressed in the lungs of bleomycin-challenged mice and IPF patients, and stimulates fibroblast type I collagen synthesis and myofibroblast differentiation; genetic deletion of FIZZ2 significantly attenuates pulmonary fibrosis in the bleomycin mouse model [158]. We expect that the number of pathways found to contribute to the pro-fibrotic interactions between epithelial cells and fibroblasts will continue to grow as investigators continue to unravel the complex pathogenesis of pulmonary fibrosis.

7. Epithelial cell-fibroblast interaction as a common theme in the development of organ fibrosis

Epithelial cell-fibroblast (and mesothelial cell-fibroblast) interactions may importantly contribute to the pathogenesis of fibrotic diseases in multiple organs. These interactions may be particularly relevant in organs in which fibroblasts and epithelial cells (or mesothelial cells) normally reside in close proximity, such as the lung, kidney, liver and peritoneum.

Progressive and potentially lethal renal fibrosis occurs in diverse kidney diseases. Renal fibrosis most often involves the accumulation of fibroblasts and extracellular matrix in the tubular interstitium, and atrophy of the tubular epithelium. The degree of renal fibrosis correlates well with the prognosis of the renal diseases in which it is found, independent of their etiologies [159]. Accumulating evidence indicates that injured renal tubular epithelial cells activate and/or upregulate mediators such as TGF-β and CTGF that deliver pro-fibrotic signals to neighboring fibroblasts [160]. Apoptosis of renal tubular epithelial cells contributes to the progression of renal fibrosis [161], and both ANG II and ROS have been shown to induce apoptosis in these epithelial cells [162–164], as they do in alveolar epithelial cells in pulmonary fibrosis.

Epithelial cell–fibroblast interactions analogous to those occurring in IPF also appear to be important in the pathogenesis of hepatic and biliary fibrosis [165–167]. Hepatocyte injury and death leading to hepatic stellate cell activation in viral or toxin-induced hepatic fibrosis is analogous in many ways to AEIC injury activating myofibroblasts in pulmonary fibrosis. In the case of biliary fibrosis, it is injury to cholangiocytes that leads to the activation and/or upregulation of mediators such TGF-β and CTGF that promote the activation and recruitment of hepatic stellate cells and portal fibroblasts [165]. Moreover, ααβ6 integrin expression is upregulated by cholangiocytes in mouse models of biliary fibrosis, and contributes to the ability of these cells to activate latent TGF-β [168]. Biliary fibrosis in mice in vivo by bile duct ligation was significantly reduced in β6 integrin-deficient mice, and by administration of a blocking antibody to ααβ6 [169].

Peritoneal fibrosis is an important problem following acute peritoneal injury, as in the development of adhesions post-abdominal surgery, and following chronic peritoneal injury, as in the development of peritoneal fibrosis post-chronic peritoneal dialysis [170,171]. In the normal peritoneum, fibroblasts reside in a thin interstitial layer adjacent to the mesothelial cell monolayer. In the case of peritoneal dialysis-induced fibrosis, dialysis solutions that are hyperosmotic, hyperglycemic and/or acidic chronically injure the mesothelial cell layer, causing these cells to elaborate pro-fibrotic mediators including CTGF that drives peritoneal fibroblast proliferation and matrix deposition, resulting in progressive fibrotic expansion of the peritoneal interstitium [170,172,173].

8. Concluding remarks

IPF remains a devastating disease that as yet is without effective pharmacological therapy, despite intensive scientific and clinical investigation. Although the stimuli that initiate epithelial injury in IPF have yet to be identified, substantial evidence supports the hypothesis that progression of IPF is driven by a series of pro-fibrotic epithelial cell–fibroblast interactions. In a "pas de deux", or "folie à deux", of epithelial cells and fibroblasts, interactions between these two cell types create a vicious cycle in which repetitive cycles of AEC injury provoke the activation of fibroblasts, and these activated fibroblasts...
in turn induce further AEC injury. The mediators of these interactions represent attractive therapeutic targets for treatment of pulmonary fibrosis, as well as for fibrotic diseases of other organs such as the kidney and liver where epithelial cell-fibroblast interactions appear to be central to the pathogenesis of fibrosis. Several of the mediators of these interactions discussed in this review are already being targeted by drugs in various stages of evaluation for IPF and/or other fibrotic diseases: TGF-β signaling is the target of the anti-oxidant defense; and Ang II signaling is the target of the anti-oxidant defense; and Ang II signaling is the target of the anti-CTGF antibody pirfenidone (InterMune); CTGF is the target of the anti-CTGF antibody RO-5376; and these interactions discussed in this review are already being targeted by drugs in various stages of evaluation for IPF and/or other fibrotic diseases: TGF-β signaling is the target of the anti-oxidant defense; and Ang II signaling is the target of the anti-CTGF antibody pirfenidone (InterMune); CTGF is the target of the anti-CTGF antibody RO-5376; and Ang II signaling is the target of the anti-PTEN antibody TAK-003. A greater understanding of epithelial cell-myoﬁbroblast interactions appears to be central to the pathogenesis of pulmonary ﬁbrosis.

**Acknowledgements**

The authors gratefully acknowledge support from NIH ROI HL095732 and ROI HL012879, and Scleroderma Research Foundation, Coalition for Pulmonary Fibrosis, Pulmonary Fibrosis Foundation and Nierenberg Center for Advanced Lung Disease Grants to A.M.T., and from the Japan Society for the Promotion of Science (JSPS) Excellent Young Researcher Overseas Visits to N.S. The authors have no conﬂicting interests.

**References**


