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Oxidative Stress and Pulmonary Fibrosis

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Abstract

Oxidative stress is implicated as an important molecular mechanism underlying fibrosis in a variety of organs, including the lungs. However, the causal role of reactive oxygen species (ROS) released from environmental exposures and inflammatory / interstitial cells in mediating fibrosis as well as how best to target an imbalance in ROS production in patients with fibrosis are not firmly established. We focus on the role of ROS in pulmonary fibrosis and, where possible, highlight overlapping molecular pathways in other organs. The key origins of oxidative stress in pulmonary fibrosis (e.g. environmental toxins, mitochondria / NADPH oxidase of inflammatory and lung target cells, and depletion of antioxidant defenses) are reviewed. The role of alveolar epithelial cell (AEC) apoptosis by mitochondria- and p53-regulated death pathways are examined. We emphasize an emerging role for the endoplasmic reticulum (ER) in pulmonary fibrosis. After briefly summarizing how ROS trigger a DNA damage response, we concentrate on recent studies implicating a role for mitochondrial DNA (mtDNA) damage and repair mechanisms focusing on 8-oxoguanine DNA glycosylase (Ogg1) as well as crosstalk between ROS production, mtDNA damage, p53, Ogg1, and mitochondrial aconitase (ACO2). Finally, the association between ROS and TGF- β 1-induced fibrosis is discussed. Novel insights into the molecular basis of ROS-induced pulmonary diseases and, in particular, lung epithelial cell death may promote the development of unique therapeutic targets for managing pulmonary fibrosis as well as fibrosis in other organs and tumors, and in aging; diseases for which effective management is lacking.

Keywords

Reactive oxygen species; Epithelium; Mitochondria; NADPH Oxidase; Apoptosis

1. Introduction

Fibrosis is characterized by exuberant extracellular matrix (ECM) protein deposition in the basement membrane and interstitial tissue in the setting of an injured overlying epithelium and expansion of activated mesenchymal cells (myofibroblasts). Fibrosis is pathologically evident in numerous diseases involving nearly every organ. Accumulating evidence over the past several decades have identified many of the important pathogenic mechanisms that promote fibrosis, yet the precise molecular mechanisms involved and the crosstalk between implicated pathways are not fully understood. Oxidative stress is one important molecular mechanism underlying fibrosis in a variety of organs, including the lungs. However, the

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causal role of reactive oxygen species (ROS) released from environmental exposures and inflammatory / interstitial cells in mediating fibrosis as well as how best to target an imbalance in ROS production in patients with fibrosis are not firmly established.

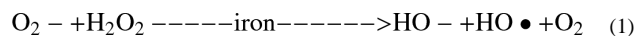
The term “oxidative stress” encompasses all the molecular, cellular and tissue abnormalities resulting from excess ROS production and/or depleted antioxidant defenses [1–3]. As compared to other organs, the lungs are particularly vulnerable to oxidative stress because they are exposed to the highest levels of oxygen; oxygen pressure of inhaled air is 150 mmHg and that of alveolar air is 100 mmHg while venous blood oxygen pressures returning from various other organs ranges from a high of ~45 mm Hg to a low of ~1 mmHg [2]. As emphasized by others, the traditional concept of fibrosis resulting from an imbalance in ROS production and antioxidant defenses is overly simplistic given the diverse pathways that are independently regulated and the ineffectual results from numerous antioxidant trials [1–3].

The purpose of this review is to highlight our current understanding of the causal role of oxidative stress in promoting fibrosis. Given the enormity of the topic and space constraints, we have chosen to restrict our focus to the role of ROS in mediating pulmonary fibrosis. In particular, we emphasize the role of ROS in the pathobiology of asbestosis, which is pulmonary fibrosis resulting from asbestos exposure. Asbestosis shares radiographic and pathologic features with idiopathic pulmonary fibrosis (IPF), a much more common disease that has a worse prognosis, and lacks effective treatment and ideal animal models. We underscore some of the important overlapping oxidative stress-induced molecular pathways resulting in fibrosis in other organs (e.g. liver, heart, etc.) that have recently been reviewed in detail elsewhere [4–6]. We first describe the principal origins of oxidative stress that can lead to pulmonary fibrosis including environmental toxins (e.g. tobacco, occupational exposure such as asbestos or silica, radiation, and drugs, such as bleomycin), mitochondria and NADPH oxidase (NOX), especially NADPH oxidase-4 (NOX4) in inflammatory and lung target cells. Oxidative stress also results from inadequate or deficient antioxidant defenses. Reactive nitrogen species, which are beyond the scope of this review, also contribute to the free radical burden of fibrotic lungs (see for review: [7]). We explore the evidence for alveolar epithelial cell (AEC) apoptosis being important in promoting asbestosis and IPF with an emphasis on ROS derived from the mitochondria- and p53-regulated death pathways as well as recent evidence for an activated endoplasmic reticulum (ER) stress response in patients with IPF. After briefly summarizing how ROS trigger a DNA damage response, we discuss the role for mitochondrial DNA (mtDNA) damage and repair mechanisms focusing on 8-oxoguanine DNA glycosylase (Ogg1) as well as crosstalk between ROS production, mtDNA damage, p53, Ogg1, and mitochondrial aconitase (ACO2), which is a mitochondrial redox-sensor molecule involved in mtDNA maintenance. Finally, the association between oxidative stress and transforming growth factor β (TGF- β)-induced fibrosis is discussed. We examine recent investigations highlighting important crosstalk between ROS, apoptosis, and inflammation. A general hypothetical model depicting the major ROS-driven pathways described in this review is illustrated in Figure 1. Collectively, these studies are revealing new insights into the molecular basis of ROS-induced pulmonary fibrosis and that may prove useful in the development of novel anti-fibrotic treatment strategies.

2. Origins of Oxidative Stress in Pulmonary Fibrosis

Biological systems are continually exposed to both extrinsic sources of reactive oxidants (e.g. tobacco, asbestos/silica, radiation, bleomycin and other drugs, etc.) and those that arise endogenously in inflammatory cells as well as epithelial, mesenchymal and endothelial cells within tissues [8]. Several enzymatic systems contribute to ROS production including NOXs, xanthine oxidase, nitric oxide synthase (NOS), and the mitochondrial electron

transport chain. [8, 9]. They are also produced from the metabolism of a wide spectrum of drugs and xenobiotics [8]. ROS, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), play a central role in host defense by killing microbes in phagocytic cells. Although excess amounts of ROS are toxic and can promote fibrosis, lower levels of ROS are 'physiologic' by functioning as signaling molecules that mediate various cellular responses including proliferation, migration, differentiation, and gene expression [7–9]. The highly reactive $HO\bullet$ can be generated from O_2^- and H_2O_2 in the presence of small amounts of redox-activate ferrous iron complexed with toxins such as asbestos fiber from outside of cell, via the Fenton-catalyzed Haber-Weiss reaction shown in equation 1 [9, 10]. Alkoxy radicals are also formed by iron catalysis of organic hydroperoxides as shown in equation 2.



Redox cycling is a common mechanism by which quinones and related species are reduced by a flavoenzyme (e.g. cytochrome P450 reductase) to a free radical that then reacts with oxygen to generate O_2^- [9]. Some fibrogenic agents implicated in redox cycling include the herbicide paraquat, the anticancer drug doxorubicin and the diabetogenic compound alloxan [8]. Free radicals also arise through the oxidation of phenols, aromatic amines and hydrazines by heme proteins, and some compounds undergo autoxidation catalyzed by transition metal ions [9, 11]. Because these ROS-producing reactions are associated with fibrosis, they have been the focus of investigative interest [10]. Finally, as reviewed in this section, oxidative stress can also be caused by depletion of antioxidant defense mechanisms, resulting in increased levels of ROS. In general, low levels of ROS production can result in cell proliferation and activation of antioxidant defenses, but higher ROS levels trigger DNA damage, p53 activation, cell cycle blockade, and cell death via apoptosis and/or necrosis; all of these may be important in a culminating fibrotic response.

At least 4 lines of evidence convincingly show that ROS generated internally or induced by environmental exposure play a vital role in the development of pulmonary fibrotic diseases as reviewed elsewhere [1, 7, 12–14]: (1) Oxidized proteins and lipid products like 8-isoprostane and carbonylated proteins have been identified in exhaled air, bronchoalveolar lavage fluid, and lung tissue from patients with fibrotic lung diseases. (2) Bleomycin-induced pulmonary fibrosis, the most commonly utilized experimental model, is associated with marked increases in the levels of ROS, oxidized proteins, DNA and lipids. (3) Increased oxidative DNA damage is detected in patients with IPF as well as workers with silicosis and asbestosis and animal models with silica or asbestos induced lung fibrosis. (4) Antioxidants and iron chelators can attenuate bleomycin- and asbestos-induced pulmonary fibrosis in rodent models.

2.1. Mitochondria-derived ROS

ROS generated from the mitochondria of key target cells appear important in mediating pulmonary fibrosis. Mitochondrial dysfunction results in the generation of ROS (e.g. H_2O_2 and O_2^-) as the electron transport chain is uncoupled from proton pumping and ROS are released into the cytosol [15]. Carter and associates [16–19] have established a key role for H_2O_2 production by the mitochondria of alveolar macrophages (AM) in causing asbestosis. They reported that AM exposed to asbestos fibers produce H_2O_2 , which is blocked by catalase or mitigation of AM mitochondrial stress. Further, Rac1, a Rho GTP binding protein family member, increases AM mitochondrial H_2O_2 production while asbestos-induced AM H_2O_2 production is reduced by knockdown of the iron-sulfur protein of

complex III in the mitochondrial electron transport chain, a major site of ROS production. Increased levels of Rac1 are localized in the mitochondria of AM of asbestosis patients, and asbestos exposed mice with a conditional deletion of Rac1 have less oxidative stress and pulmonary fibrosis [16–19] (Figure 2). Taken together, these studies demonstrate that asbestos triggers AM H₂O₂ production by transferring electrons from complex III to Rac1, which then drive down-stream signaling pathways, inflammation and cellular injury that result in asbestosis in mice. Carter et.al have proposed that Rac1 is a novel biomarker for pulmonary fibrosis. An important role for H₂O₂ mediating pulmonary fibrosis is also supported by the protective effects of catalase in a rodent model of asbestosis [20]. As reviewed elsewhere, exogenous toxins, such as asbestos fibers can activate mitochondrial ROS production in other important lung target cells implicated in pulmonary fibrosis, including epithelial cells [14, 21]. However, additional studies are warranted to better understand the relevance of these findings in pulmonary fibrosis and to determine which cell should be primarily targeted.

2.2. NAD(P)H Oxidase

NOX family of oxidoreductases catalyze one or two electron reductions to form O₂⁻ and H₂O₂, which contribute to cellular signaling, killing of invading microbes, and damage of surrounding host tissues when released primarily by activated inflammatory cells but also by non-inflammatory cells (See for reviews: [22, 23]). Recent studies have established the central role of NOX, especially isoforms NOX1, NOX2 and NOX4, in the pathogenesis of pulmonary fibrosis [22, 23]. NOX4 is induced by TGF-β1, which subsequently promotes key events in the development of fibrotic lung disease such as myofibroblast differentiation and impaired re-epithelialization discussed later (see Section 5). Thannikal and colleagues [24] demonstrated that NOX4-dependent generation of H₂O₂ is required for TGF-β1-induced myofibroblast differentiation and ECM production, and that genetic knockdown or pharmacologic inhibition of NOX4 prevents bleomycin-induced fibrosis in mice. Moreover, NOX-4 is upregulated in lungs of mice treated with bleomycin and humans with IPF [24]. TGF-β1 mediated myofibroblast differentiation is associated with the enzymatic production of extracellular H₂O₂ that can promote AEC cell death, a key event initiating and perpetuating pulmonary fibrosis as reviewed in section 3.1 [25, 26]. NOX4 is strongly expressed in the hyperplastic alveolar epithelium of IPF patients, particularly in alveolar type 2 (AT2) cells [25]. Interestingly, NOX4-deficient mice are completely protected against bleomycin-induced AT2 cell apoptosis and pulmonary fibrosis, but not against bleomycin-induced pulmonary inflammation [25]. NOX-4 deficient epithelial cells and/or AEC treated with NOX-4 chemical inhibitors (fluvone-5- inhibits NOX-4/2- and GK13601-inhibits NOX-1/4) decrease TGF-β1-mediated ROS generation and AEC apoptosis [25]. Collectively, these findings firmly implicate that ROS produced by NOX4 are crucial for mediating AEC apoptosis during the development of pulmonary fibrosis. In an experimental model of diabetic lung fibrosis, NOX activation in fibroblasts by angiotensin II also appears important [27]. A role of NOX2 in pulmonary fibrosis is supported by the finding that mice genetically deficient in NOX2 are protected from bleomycin- or carbon nanotube-induced lung fibrosis [22, 28].

NOXs are also important in mediating fibrosis in other organs. For example, a mutated SOD, which enhances O₂⁻ production, increases NOX1 in hepatic stellate cells during liver fibrosis [29]. Hepatocyte apoptosis and liver fibrosis are attenuated by GKT137831, an inhibitor of NOX4/NOX1, *in vivo* [29, 30]. In the heart, NOX4 is also implicated in promoting myofibroblastic differentiation of cardiac fibroblasts and is a major source of mitochondrial oxidative stress in cardiac myocytes in the failing heart [31, 32]. Taken together, these data firmly support a causal role of NOXs in tissue fibrogenesis and indicates that targeting NOXs could afford a novel approach to treatment of fibrogenic diseases.

Future studies are necessary to further characterize the primary source of NOXs and role of NOX family members in the setting of ROS-induced organ fibrosis. It will also be of interest to determine the translational significance of targeting NOXs in humans with various forms of fibrosis.

Oxidative stress by NOXs and mitochondria-derived ROS can also trigger inflammatory signaling via the Nalp3 inflammasome sensing that may be important in driving silicosis, asbestosis and perhaps other forms of fibrosis [33–36]. The inflammasome, which is primarily expressed within macrophages and neutrophils, is a protein complex that serves as a host defense cellular danger sensor by activating IL-1 β -driven innate pathway. Studies using pharmacologic ROS inhibitors, iron chelated asbestos, and targeted murine knockouts, demonstrate that ROS generated by NOXs during phagocytosis and/or by mitochondrial oxidative phosphorylation, are essential for activation of Nalp3 inflammasomes [33–36]. Thus, environmental toxins such as silica and asbestos, induce oxidative stress from NOXs and/or the mitochondria of inflammatory cells thereby activating multiple signaling pathways, including Nalp3, which may represent another novel therapeutic target for the remediation of pulmonary fibrosis.

2.3. Depletion of Antioxidant Defense Mechanisms

All organs, including the lungs, express a wide variety of antioxidants to protect against oxidative stress. Depletion of antioxidant defenses can result in oxidative stress that augments fibrosis. Herein, we focus on some of the primary lung antioxidant defenses implicated in animal models of pulmonary fibrosis and humans with IPF, including catalase, glutathione (GSH), and superoxide dismutase (SOD) as well as nuclear factor erythroid 2-related factor 2 (Nrf2), the primary regulator of antioxidant enzymes.

2.3.1 Catalase—a scavenger of H₂O₂ expressed in lung AEC and inflammatory cells, can inhibit H₂O₂-mediated activation of fibroblasts in IPF lungs [7]. Continuous intravenous infusion of polyethylene glycol catalase blocks asbestosis in a rat model [20]. More recently, intratracheal catalase administration to asbestos-treated mice was shown to prevent pulmonary fibrosis by inhibiting H₂O₂ production through Rac 1-activated inflammatory cells [18].

2.3.2. Glutathione—a low molecular weight antioxidant found in normal lungs, is reduced in the epithelial lining fluid and fibrotic foci in IPF lungs [7, 37]. The anti-fibrotic effects of N-acetyl cysteine (NAC), a GSH precursor, are observed in rodent models of fibrosis in which NAC increases lung GSH levels and attenuates bleomycin-induced fibrosis [38, 39]. Administration of aerosolized NAC to patients with IPF augments their antioxidant/oxidant balance [40]. One phase III clinical trial suggested a possible benefit of NAC therapy [41]. However, the results of an ongoing NIH-sponsored, multi-center, Phase III trial (PANTHER) should definitively address whether NAC is efficacious in IPF.

2.3.3. Superoxide dismutase—SOD decomposes superoxide radicals to H₂O₂. All three isoforms of mammalian SOD (intracellular copper-zinc SOD; mitochondrial manganese SOD; and extracellular SOD [EC-SOD]) exist in lung cells but cell-specific localization and expression varies (see for review: [2, 7]). EC-SOD is highly expressed in the lung and is implicated in the pathogenesis of ROS-induced pulmonary diseases, including pulmonary fibrosis [2, 7]. EC-SOD is protective in several murine models of pulmonary fibrosis (e.g. bleomycin, asbestos and radiation) whereas EC-SOD knockout mice have significantly more fibrosis following exposure to environmental toxins [2, 7]. Although beyond the scope of this review, accumulating evidence demonstrates that EC-SOD exerts its anti-fibrotic effects, in part by preventing oxidative degradation of the ECM and release of ECM

degradation products that can augment fibrosis by effects on lung epithelial, mesenchymal and inflammatory cells [2, 7]. The role of the other two SOD isoforms in the pathogenesis of pulmonary fibrosis is less well understood. Mitochondrial Cu, Zn, SOD^{-/-} mice have less oxidative stress and do not develop asbestosis [19].

2.3.4. Nuclear factor-erythroid 2-related factor 2—Nrf2 is a transcription factor considered the “master regulator” of the antioxidant response (see for review: [42]). When Nrf2 is released from its negative regulator Kelch-like erythroid cell-derived protein CNC homology – associated protein 1 in the cytosol, Nrf2 traffics to the nucleus and activates the antioxidant response element (ARE) that controls hundreds of genes, including NAD(P)H quinone-oxidoreductase 1, antioxidant-related genes involved in glutathione biosynthesis, Phase II detoxifying “stress response” genes, and genes limiting the inflammatory and fibrotic responses. [42] Several recent studies underscore the importance of Nrf2 in regulating pulmonary fibrosis. First, Nrf2 knockout mice are more sensitive to bleomycin- and paraquat-induced pulmonary fibrosis as compared to wild-type animals [43, 44]. Second, in a study of primary lung fibroblasts cultured from healthy or IPF patients, decreased Nrf2 expression was associated with a myofibroblast phenotype (increased α -smooth muscle actin [α -SMA], and type 1 collagen expression) while Nrf2 activation increased antioxidant defenses and myofibroblastic dedifferentiation in IPF fibroblasts [45]. Moreover, a Nrf2 activator, sulfaphane, increased Nrf2 expression in cultured fibroblasts from normal and IPF patients; effects that were associated with increased expression of antioxidants, decreased ROS levels, and myofibroblastic dedifferentiation. Sulfaphane inhibited TGF- β 1- mediated profibrotic effects in both normal and IPF fibroblasts but not in Nrf-2 knockdown fibroblasts [45]. Resveratrol, a phytoalexin polyphenol produced by several plants, attenuates bleomycin- and paraquat-induced pulmonary fibrosis in part by augmenting Nrf2 levels and downstream antioxidant enzymes [44]. Collectively, these results in animal models suggest that Nrf2 may be a novel target for IPF therapy as well as other oxidative stress-induced fibrogenic diseases

3. The Role of ROS and AEC Apoptosis in Pulmonary Fibrosis

3.1. AEC apoptosis is important to the pathophysiology of pulmonary fibrosis

Cells undergo apoptosis by two mechanisms, the extrinsic (death receptor) and intrinsic (mitochondria-regulated) pathways. Several lines of evidence convincingly show that lung epithelial cell death / apoptosis is one of the critical pathophysiologic events limiting normal lung repair and thereby facilitating pulmonary fibrosis. These studies have been extensively reviewed in detail elsewhere and will only be briefly summarized here (see for review: [1, 14, 21, 46, 47]). First, patients with IPF and animal models of pulmonary fibrosis show significant lung epithelial cell injury and apoptosis. In contrast to catastrophic lytic cell death that can trigger an inflammatory response, apoptosis is a regulated, ATP-dependent process of cell death that results in the elimination of cells with extensive DNA damage without eliciting an inflammatory response. Second, fibrogenic environmental toxins, including silica and asbestos, can induce both lytic and apoptotic AEC death in part by generating ROS derived from the mitochondria or NOXs. Third, DNA strand break formation, a potent trigger of apoptosis, occurs in the AEC of human patients with IPF. p53, a critical DNA damage response molecule reviewed below (see Section 4), is activated in patients with IPF and rodent models of asbestosis. Fourth, several murine models have established that AEC apoptosis is sufficient for inducing pulmonary fibrosis and that blocking AEC-targeted apoptosis prevents fibrosis [48, 49]. Janssen-Heininger and colleagues [47] have established that redox-based alterations in the FAS death receptor by protein S-glutathionylation are required for mediating extrinsic apoptosis in lung epithelial cells and pulmonary fibrosis. Fas is S-glutathionylated independently of NOX-induced ROS

after caspase-8 degradation of glutathione reductase 1, thereby increasing caspase-3 activation and apoptosis. Glutathione reductase 1 overexpression attenuates S-glutathionylation and attenuates Fas ligand induced apoptosis and pulmonary fibrosis. In the ER of lung epithelial cells the Fas ligand induces Fas glutathionylation with ERp57 (protein disulfide isomerase 3) and glutathione S-transferase π -1, which subsequently increases caspase-3 and -8 activity, AEC apoptosis, and bleomycin-induced lung fibrosis [50]. Notably, RNAi mediated knockdown of ERp57 and glutathione S-transferase π -1 attenuates the development of bleomycin-induced pulmonary fibrosis. Finally, preventing α v β 6 integrin release from damaged lung epithelial cells, blocks activation of latent TGF- β and pulmonary fibrosis following radiation or bleomycin exposure [51]. Thus, these studies establish a crucial role of AEC apoptosis in the pathophysiology of pulmonary fibrosis following exposure to oxidative stress. Future work is required to further characterize the precise molecular mechanisms involved and to determine the translational significance of identified targets in animals exposed to fibrogenic agents and humans with IPF.

3.2. Genetic predisposition to pulmonary fibrosis – Role of oxidative stress and the lung epithelium

IPF appears to have a genetic predisposition in nearly 5% of patients (see for review: [46]). Numerous mutations are associated with the development of pulmonary fibrosis, some expressed only in genes of epithelial cells, and others more ubiquitously expressed. Mutations in surfactant protein C and A2 genes, which are expressed only in the alveolar epithelium, are present in unique cohorts of familial pulmonary fibrosis patients [46]. Mutated surfactant proteins can induce an ER stress in AEC and this is important in mediating pulmonary fibrosis (discussed below). The most common gene mutations identified in familial pulmonary fibrosis are in the telomerase genes (TERT and TERC), and unlike surfactant proteins, are expressed outside the lung, especially in stem cells and progenitor cells (see for review: [52]). Notably, AEC from 60 of 62 patients with IPF had shortened telomeres that were < 50% of predicted [53]. Since shortened telomeres are directly associated with aging-related diseases in part due to oxidative stress, shortened AEC telomeres in IPF may in part account for their susceptibility to fibrosis following injury [3, 52, 53]. There is also some evidence that mitochondrial ROS production is a crucial determinant of telomerase-dependent senescence [54]. However, additional genetic and/or environmental factors appear important since AEC telomerase shortening alone does not promote bleomycin-induced fibrosis in mice [55]. Polymorphisms in the MUC5B gene promoter are evident in 38% of patients with sporadic IPF and 34% of patients with familial pulmonary fibrosis [56]. Further studies are necessary to determine how alterations in MUC5B, which is expressed in the bronchial epithelium but not in AT2 cells, augments pulmonary fibrosis. It will also be of interest determining how oxidative stress adversely impacts these various mutant genes and their protein products in the development of pulmonary fibrosis in animal models as well as in humans.

3.3. Mitochondria-regulated AEC death pathways in pulmonary fibrosis

Diverse fibrotic stimuli (e.g. ROS, DNA damage, asbestos, etc.) activate the intrinsic death pathway by increasing the permeability of the outer mitochondrial membrane, reducing the mitochondrial membrane potential ($\Delta\psi_m$), and releasing numerous apoptotic proteins, including cytochrome c (see for reviews: [14, 21, 46, 57–59]). The Bcl-2 family of BH3 domain proteins are grouped into a four-class hierarchy dependent on structure and function (either pro- or anti-apoptotic). Pro-apoptotic proteins, such as Bax and Bak, can induce outer mitochondrial membrane permeabilization, which is the point of no return in the intrinsic apoptotic pathway causing the release of apoptogenic molecules from the mitochondria and caspase activation. Proapoptotic Bid, which is activated by the death receptor pathway and triggers intrinsic apoptosis by blocking anti-apoptotic molecules enabling Bax/Bak-mediated

apoptosis, is necessary for mediating bleomycin-induced fibrosis in mice [49]. It will be interesting to know whether AEC or other cell types are primarily responsible for mediating the effects of Bcl-2 family members in pulmonary fibrosis.

Oxidative stress resulting from exposure to asbestos fibers activates the mitochondrial death pathway in AEC that may be important in the development of pulmonary fibrosis. Since this area has been extensively reviewed elsewhere (see for reviews: [1, 14, 21, 46, 58, 59]), herein we highlight the evidence with asbestos. Asbestos fibers cause mitochondrial dysfunction as evidenced by decreased $\Delta\psi_m$, the release of cytochrome c from the mitochondria into the cytosol, and caspase-9 and 3 (but not caspase-8) activation [60]. These effects are blocked by phytic acid (an iron chelator), benzoic acid (a free-radical scavenger), and overexpression of Bcl-XL [60]. A crucial role for mitochondrial ROS in mediating AEC apoptosis is suggested by two lines of evidence: (1) asbestos-induced AEC intrinsic apoptosis and p53 activation are blocked in cells lacking mitochondrial DNA and mitochondrial ROS production (2) asbestos induces mitochondrial ROS production using a highly sensitive rho-GFP probe targeted to the mitochondria [60–63]. An important role for mitochondrial ROS and p53 in mediating intrinsic AEC following asbestos exposure is suggested by the protective effects of pifithrin- α , an inhibitor of p53-dependent transcription [63]. Activated protein kinase delta (PKC δ) migrates to the mitochondria of lung epithelial cells *in vitro* and *in vivo* following asbestos exposure and is necessary for inducing asbestos-induced intrinsic apoptosis and fibrosis via Bim activation [64, 65]. Taken together, mitochondrial ROS production and PKC δ activation following asbestos exposure appear important for inducing p53 activation and intrinsic lung epithelial cell apoptosis. However, the translational significance of these findings for pulmonary fibrosis awaits further study.

3.4. AEC ER Stress in fibrotic lung injury

Several groups have documented that the alveolar epithelium in patients with IPF have evidence of ER stress response in cells undergoing apoptosis [66, 67]. However, the pathophysiologic significance of these findings and the role of oxidative stress are uncertain. The ER primarily regulates protein folding and transport as well as Ca²⁺ homeostasis (see for reviews: [58, 68]). The ER can modulate intrinsic apoptosis, but the detailed mechanism underlying crosstalk between the ER and mitochondria and how this regulates mitochondrial metabolism, mitochondrial Ca²⁺ levels, and cell survival/death signaling are not fully understood. Close tethering of the ER to the mitochondria appears crucial for transferring ER-derived Ca²⁺ that is important for triggering intrinsic apoptosis [58, 69]. Mitofusin 2, one of the several proteins localized at mitochondrial associated membranes, physically attaches the mitochondria to the ER and is crucial for mitochondrial Ca²⁺ uptake from the ER [58, 69]. Exogenous and endogenous stressors, including mitochondrial ROS production, augment unfolded protein accumulation in the ER, leading to ER stress and activation of three unfolded protein response (UPR) signaling pathways including: (1) inositol requiring protein 1 (IRE1- α)/ X box binding protein 1 (Xbp-1), (2) activated transcription factor 6, and (3) protein kinase RNA-like endoplasmic reticulum kinase/eukaryotic initiation factor-2a. (see for review: [58, 68]). The UPR is vitally important for coordinating numerous adaptive cellular responses that enhance cell survival in the setting of oxidative stress and other noxious stimuli, but apoptosis / autophagy (cell death with extensive cytoplasmic vacuolization) occur in the setting of a sustained ER stress response [57, 58, 68].

The Bcl-2 family members function at both the mitochondria and the ER, although the precise molecular mechanisms regulating crosstalk between these two organelles and the role of oxidative stress are not fully established [58, 59, 68, 69]. Transient ER Ca²⁺ release favors pro-survival signaling within cells while sustained ER Ca²⁺ release generally results

in intrinsic apoptosis [58, 59, 69]. Sarcoplasmic ER Ca^{2+} ATPase (SERCA) overexpression in Bax/Bak double knockout cells, which are resistant to mitochondria-regulated apoptosis, restores ER Ca^{2+} levels and intrinsic apoptotic response following exposure to oxidative stress [69]. The antiapoptotic effects of Bcl-2 are linked to Ca^{2+} mobilization from the ER to the mitochondrion and blocks homodimerization of pro-apoptotic Bax that displaces SERCA from the mitochondrial associated membranes [58, 59, 69]. Oxidative stress-induced NF- κ B transcriptionally regulates the expression of sigma-1 receptors located in the mitochondrial associated membranes, and downstream of Bcl-2 expression [70]. ER Ca^{2+} release to the mitochondria is necessary, but not sufficient for induction of intrinsic apoptosis by coordination of ER stress survival signals through IRE1- α / TNF associated factor-2, resulting in increased apoptosis signal regulating kinase 1 and JNK [71].

Accumulating evidence implicate an important role for AEC ER stress response in patients with IPF and possibly in mediating asbestos pulmonary toxicity. First, as noted above, AEC in patients with IPF that are undergoing apoptosis co-localize with markers of ER-UPR stress [66–68]. Second, ER stress augments epithelial-mesenchymal transition (EMT) and the formation of myofibroblasts, one of the primary effector cells in IPF [72]. Third, AEC ER stress and EMT can result from over-expression of a mutant, and accumulation of misfolded forms of surfactant protein C in the ER and are associated with familial pulmonary fibrosis [72, 73]. Notably, transgenic mice conditionally expressing mutated SP-C^{L188Q} in their AT2 cells and them exposed to ER stress (e.g. intratracheal tunicamycin) do not develop pulmonary fibrosis unless a profibrotic stimuli (e.g. bleomycin) is added [74]. Thus, AEC ER stress appears to render the cells susceptible to oxidative stress-induced apoptosis following exposure to environmental toxins or drugs, and thereby promotes pulmonary fibrosis. As reviewed elsewhere [14], preliminary studies from our group show that asbestos-exposed AEC cells release AEC ER Ca^{2+} and activate the UPR signaling proteins (IRE1- α and Xbp-1). Further, the ER chemical chaperone 4-phenyl butyric acid (4-PBA) abolishes asbestos-induced increases in IRE1- α and Xbp-1, but does not prevent ER Ca^{2+} release or apoptosis. A recent study demonstrates that ROS are responsible for mediating TGF- β -induced UPR and that 4-PBA suppresses both TGF- β -induced UPR and myofibroblast differentiation [75]. Aging, which is a major cofactor in the development of IPF, is associated with increased oxidative stress, a decline in ER protein folding, reduced ability of the UPR to maintain cellular homeostasis during ER stress, and increased AEC apoptosis following injury (see for review: [1, 3, 57, 68]). Unlike young mice, older mice challenged with murine gamma herpesvirus 68 (MHV68) develop lung fibrosis that is associated with increased AER ER stress and apoptosis [76]. Taken together, these data show that AEC ER stress occurs in IPF as well as following exposure to various toxins that can promote fibrotic remodeling. Figure 3 illustrates a hypothetical model by which crosstalk between the ER and the mitochondria prevent AEC apoptosis that can lead to pulmonary fibrosis. It will be of considerable interest to better understand the molecular mechanisms by which oxidative stress following exposure to environmental toxins, causes an ER-UPR and apoptosis and also to determine whether modulation of this pathway affords a unique therapeutic target in the management of pulmonary fibrosis as well as other fibrotic diseases.

4. Oxidative stress and DNA damage response in Pulmonary Fibrosis

4.1. Oxidative stress induces a p53-dependent DNA damage response

Oxidative stress activates p53, which is considered the genome gatekeeper because p53 integrates key signals regulating cellular responses such as cell-cycle arrest, differentiation, apoptosis, senescence, and antiangiogenesis (see for review: [21, 77–81]). p53 is a transcriptional activator modulating downstream target gene expression involved in DNA-damage responses and tumor suppression that are important in preventing the accumulation

of gene mutations [82]. As such, p53-dependent DNA damage response following oxidative stress serves as a barrier to tissue injury/fibrosis and tumor progression by augmenting DNA repair and/or promoting cell death of cells with DNA damage that overwhelms the repair mechanisms [78]. The key role of p53 is highlighted by the fact that over half of all human cancers have p53 mutations and also by the increased cancers seen in p53 null mice [83]. p53 is also redox sensitive and its transcriptional function is integrally linked to oxidative stress by orchestrating downstream cellular effects including the induction of apoptotic cell death [21, 81]. Stabilization of p53 can also promote further ROS generation via effects on the mitochondria [21, 81]. By regulating thousands of genes, either directly or indirectly, p53 modulates numerous vital cellular roles, including mtDNA maintenance (discussed below) [84, 85].

Accumulating evidence implicate p53 in the pathophysiology of pulmonary fibrosis, including that caused by asbestos [21, 63, 86–89]. Asbestos increases p53 and p21 expression in lung epithelial and mesothelial cells that result in cell-cycle arrest and apoptosis [63, 90–92]. p53 levels are elevated in lung cancers of patients with asbestosis [93], and p53 point mutations are present in the lung epithelium of smokers and asbestos-exposed patients [94]. Crocidolite asbestos augments *p53* gene mutations in BALB/c-3 T3 cells [95]. In lung-specific dominant-negative p53 mice, chrysotile asbestos induces TGF- β and other proinflammatory/fibrotic signaling and increases adenocarcinoma formation [96]. Finally, gene-expression microarray studies in lung epithelial and mesothelial cells demonstrate that p53 activation has a very prominent role along with nearly 2,500 other genes of over 54,000 genes profiled [97, 98]. Collectively, ROS-induced p53 is crucial in regulating the lung cellular DNA-damage response, as occurs with fibrogenic agents such as asbestos and tobacco smoke.

ROS-induced DNA damage can trigger p53-dependent apoptosis that may be important in driving a fibrogenic response. Further, p53 can promote additional ROS generation, often via effects on the mitochondria [77, 85]. p53-regulated apoptosis can occur via activating the intrinsic and extrinsic death pathways either directly or indirectly via crosstalk with other ROS-activated signaling, such as MAPK [63, 99, 100]. Prompt repair of DNA lesions in most instances will restore the DNA. However, abnormal DNA repair may occur resulting in gene mutations, chromosomal aberrations and ultimately cell transformation. Damaged DNA can trigger apoptosis and contribute to the fibrogenic and malignant potential of oxidative stress [21, 101, 102]. A variety of DNA lesions, such as single or double strand breaks, intra- and inter-strand cross-linking and base damage produced by oxidant-induced ROS induce a DNA damage response [77, 103]. Under normal conditions, p53 expression is maintained at low levels through constitutive degradation by mouse double-minute 2 protein (MDM2), an E3 ubiquitin ligase, whereas upon DNA damage, p53 is released from MDM2-mediated degradation and is rapidly stabilized [104–106]. Further, deubiquitinating enzymes including herpesvirus-associated ubiquitin-specific protease (HAUSP) and ubiquitin specific peptidase (Usp) 10 have been shown to stabilize p53 protein by antagonizing MDM2-dependent ubiquitination in response to DNA damage [104, 107]. Transcriptional activity of p53 is also regulated by interacting partners such as transcriptional coactivators or corepressors [108]. The functional consequences of DNA damage-dependent p53 stabilization and transcriptional activation are induction of downstream target genes, such as p21, Bax, Noxa and BBC3 (encoding PUMA), that are involved in mediating p53's cellular functions noted above [98, 105]. p53 also modulates apoptosis, at least partially, via transcription-independent mechanisms through associations with several members of the Bcl2 family of proteins in the cytoplasm or at the mitochondria [80, 101]. As illustrated in Figure 4, p53-Bcl2 and/or p53-BclXL complexes can promote Bax- and/or Bak-mediated intrinsic apoptosis; supporting a dual role of p53 in gene transactivation as well as in mitochondria-regulated apoptosis [80, 109]. To maintain the appropriate cellular redox state,

ROS levels are tightly controlled; a task which is performed by two p53-regulated interconnected systems: thioredoxin and glutathione [79, 110]. The anti-apoptotic function of p53 is attributed to the effects on genes regulating the thioredoxin and glutathione systems including glutathione peroxidase, manganese SOD, aldehyde dehydrogenase 4, p53-induced glycolysis, apoptosis regulator, PA26 and Hi95 [79].

4.2. The role of p53 during DNA repair triggered by oxidative stress

Damaged DNA following oxidative stress and other genotoxic exposures can be corrected by p53-related repair systems; a clearly protective role for p53 [79]. The observation that p53 translocation to the mitochondria does not necessarily lead to apoptosis and may enhance mtDNA repair also suggests an important role for p53 in maintaining normal cellular homeostasis in the setting of oxidative stress [84, 85, 111]. Further, there appear to be distinct genes activated by p53 for mediating the DNA damage response and tumor suppression following exposure to genotoxic stress as occurs with ROS [82]. p53 transactivation-dependent and -independent functions act as coordinators of the DNA repair process and involve most of the cellular DNA repair systems, such as mismatch repair, non-homologous end-joining, homologous recombination, nucleotide excision repair, and base excision repair (BER) [112]. Deficiency of components of the BER induced by oxidative stress, such as apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE/Ref-1) or DNA polymerase β , cause a p53-dependent pre- and postnatal lethality, respectively, and lead to hypersensitivity toward certain types of DNA-damaging agents [79]. APE/Ref-1 or 8-oxoguanine-DNA glycosylase (OGG1), another important BER enzyme, are up-regulated in various cancers and promote apoptosis resistance [112, 113]. OGG1 has glycosylase activity that removes abnormal bases in DNA, especially 8-hydroxyguanine (8-oxoG), and generates an apurinic or apyrimidinic (AP) site recognized by the multifunctional enzyme APE/Ref-1 [112]. Oxidative stress causes multiple types of DNA base damage, however 8-oxoG has been most widely studied since 8-oxoG pairs with adenine rather than cytosine during DNA replication potentially resulting in a G:C to T:A transversion that is a pre-mutagenic lesion implicated in cancer and aging [21, 114]. Notably, p53 activation by oxidative stress *in vitro* enhances the excision of 8-oxoG nucleotides from the DNA by enhancing the sequential activities of OGG1 and APE/Ref-1 [81]. The transactivation-independent functions of p53 during DNA repair and recombination are also involved during p53-induced apoptosis [113]. Although much has been uncovered recently in how p53 regulates DNA repair in the setting of oxidative stress, the precise molecular pathways and their *in vivo* relevance in the setting of pulmonary fibrosis are not firmly established.

Mitochondrial ROS production following oxidative stress causes mtDNA damage that must be efficiently repaired or else mtDNA damage will trigger cell death or possibly mutagenic abnormalities contributing to the fibrogenic and malignant potential of ROS [21, 114, 115]. The mtDNA is particularly vulnerable to oxidative attack given its location at the inner mitochondrial membrane in close proximity to the mitochondrial electron transport chain and given that ~1–5% of total molecular oxygen used by the mitochondria for energy production results in the generation of ROS [21, 114–117]. Malignant transformation involves periods of increased mitochondrial ROS production when select cells survive oxidative stress-induced cell death [115, 116, 118]. As recently reviewed [21, 114, 115, 119], several lines of evidence suggest that mtDNA oxidative injury triggers apoptosis that is important in driving ROS-induced fibrogenesis and, perhaps, inflammation-associated cancers including: (1) cell death is better associated with mtDNA oxidative lesions than with nuclear DNA damage, (2) mtDNA damage results in ATP depletion and mitochondrial dysfunction, (3) augmenting mtDNA repair can block apoptosis, and (4) defective mtDNA repair promotes apoptosis. Because mtDNA oxidative damage is primarily repaired by BER, defects in mitochondrial BER enzymes, all of which are encoded in the nucleus and

imported into the mitochondria, can promote mtDNA damage and instability [114, 120]. We focus on mitochondrial Ogg1 (mt-Ogg1) since it is the best-characterized mitochondrial BER enzyme involved in preventing the deleterious effects of oxidative stress in a wide variety of cells.

4.3. The role of hOgg1 during mitochondrial DNA repair caused by oxidative stress

The first step of the BER involves recognition and removal of the aberrant 8-oxoG base pair catalyzed by DNA glycosylases, such as Ogg1. DNA glycosylases recognize the modified or inappropriate base and cleave the N-glycosidic bond, creating an abasic site [114]. Notably, *in vitro* fluorometric techniques document that oxidative stress preferentially activates mt-Ogg1, rather than nuclear Ogg1 [121]. Further, Ogg1-deficient mice have increased 8-oxoG levels in mtDNA and nuclear DNA suggesting the importance of Ogg1 for most of the BER activity following exposure to oxidative stress [122]. An emerging role for mt-Ogg1 in regulating apoptosis is suggested by the findings that mt-OGG1 overexpression reduces mtDNA damage and intrinsic apoptosis caused by ROS-exposed vascular endothelial and asbestos-exposed cells [61, 123–129]. Crocidolite asbestos fibers, which are among the most potent amphiboles for inducing fibrosis and malignancies, cause preferential mtDNA damage, rather than nuclear DNA damage, in lung mesothelial cells that results in apoptosis [129]. The human *OGG1* gene produces two major-alternatively spliced *OGG1* mRNAs in humans: α -Ogg1, and β -Ogg1 [130]. α -Ogg1 is mainly located in the nucleus and present to a lesser extent in the mitochondria whereas β -Ogg1 is localized exclusively in mitochondria [130, 131]. A curious unexplained observation is that β -Ogg1 levels in the mitochondria are 20-fold higher than α -Ogg1 levels, yet only the α -Ogg1 isoform has 8-oxoG DNA repair glycosylase activity [132]. We reasoned that there must be a role for Ogg1 that is independent of DNA repair [61]. Support for this possibility was our observation that overexpression of mitochondrial α -Ogg1 mutants lacking 8-oxoG DNA repair activity were as effective as wild type mt-Ogg1 in preventing oxidant (asbestos and H₂O₂)-induced caspase-9 activation and intrinsic apoptosis in AEC cells yet did not affect the levels of mitochondrial ROS production [61]. It is unclear whether β -Ogg1 acts similarly in preventing oxidant-induced mitochondrial dysfunction and apoptosis. Interestingly, overexpression of mitochondrial α -Ogg1 preserved mitochondrial aconitase (ACO2) protein levels and activity in the setting of oxidative stress suggesting a novel role for Ogg1 that is considered in more detail below [61].

ACO2, which is an essential mitochondrial enzyme for energy metabolism and involved in the interconversion of citrate to isocitrate in the tricarboxylic acid (TCA) cycle, contains four iron–sulphur moieties which render ACO2 as a source for ROS and iron *in vitro* and *in vivo* [133–136]. ACO2 is uniquely sensitive to O₂^{•-}-mediated oxidative inactivation because of the presence of a single unligated iron atom, such that oxidation of the [4Fe-4S]²⁺ cluster renders it unstable and promotes removal of the labile iron atom, consequently forming H₂O₂ by the reduction of O₂^{•-} [135]. Inactivation of ACO2 reduces mitochondrial manganese SOD levels [137], decreases the life span in *Drosophila* [134], and may have a pathophysiologic role in neurodegenerative diseases such as progressive supranuclear palsy [138], Friedreich's ataxia [139], and Huntington's disease [140]. In yeast, ACO2 preserves mtDNA independently of ACO2's catalytic activity, which was the first suggestion of a novel dual function for ACO2 in mtDNA maintenance as well as a mitochondrial TCA enzyme [141]. We reported a novel role for mt-hOgg1 in chaperoning ACO2 in the setting of oxidative stress that prevents AEC apoptosis [61]. Notably, several groups have reported that suppression of Ogg1 expression increases oxidant-induced apoptosis [142–144]. Collectively, these findings suggest that intrinsic AEC apoptosis following exposure to oxidative stress is regulated by a novel interaction between mt-Ogg1

and ACO2. However, the role of Ogg1 and ACO2 in mediating pulmonary fibrosis following AEC apoptosis requires further study.

The precise molecular mechanisms by which mt-hOgg1 and ACO2 interact to prevent oxidant-induced AEC apoptosis are unclear, but there are at least two possibilities, which are not mutually exclusive. First, mt-hOgg1 may chaperone ACO2 and thereby block ACO2 oxidative modification that results in its degradation by mitochondrial Lon protease [145]. An example of this mechanism was uncovered with Friedreich's Ataxia in which ACO2 coprecipitates with a mutant frataxin incapable of preventing oxidative inactivation of ACO2 [146]. Frataxin, which is an iron chaperone protein that blocks ACO2 oxidative inactivation and/or augments ACO2 reactivation, may function similarly to mt-hOgg1. Second, overexpression of mt-hOgg1 or ACO2 may preserve the mtDNA levels necessary to prevent activation of p53 and intrinsic apoptosis. Overexpression of mt-Ogg1 or ACO2 may attenuate the accumulation of mtDNA lesions in the setting of oxidative stress that can result in intrinsic apoptosis via p53-dependent DNA damage signaling. Support for this latter possibility includes accumulating evidence showing a potentially crucial association between p53, Ogg1 and ACO2. p53 regulates the transcription of the *OGG1* gene in colon and renal epithelial cells [142]. Further, p53 deficient cells have reduced expression and activity of OGG1 due partly because of reduced p53 binding to the putative *cis* elements within the *OGG1* promoter; a defect that can be overcome by p53 overexpression. p53 may also modulate ACO2 since thymoquinone, a p53-dependent antineoplastic drug, decreases ACO2 activity in liver mitochondria [147]. Both endogenous p53 induction by camptothecin and exogenous transient p53 overexpression decrease *ACO2* gene expression in prostate carcinoma cells [148]. Camptothecin does not affect ACO2 reporter activity in p53-null PC-3 cells suggesting that the decrease in *ACO2* gene expression by camptothecin occurs via p53 activation. These findings may account for the observation that Ogg1 decreases oxidative stress-induced fibroblast apoptosis through p53-mediated signaling [142]. Additional investigations are warranted to further explore these various possibilities and to determine the molecular mechanism by which mt-hOgg1 interacts with aconitase to affect p53 signaling. The *in vivo* relevance of these findings to oxidative stress-induced fibrosis also requires study.

5. Oxidative Stress and TGF- β -induced Fibrosis

The interactive effects between oxidative stress and TGF- β are important for promoting fibrosis (see for review: [12]). TGF- β , which is the most potent profibrogenic cytokine evident in nearly all fibrotic diseases, is a multi-function protein that exists in different isoforms (TGF- β 1, 2, and 3) and plays a vital role in ECM deposition by regulating crucial cellular activities such as cell proliferation, differentiation, adhesion, migration, apoptosis, and EMT. TGF- β stimulates ROS production leading to oxidative stress while oxidative stress can activate latent TGF- β setting up a vicious profibrogenic circle. Considerable evidence firmly implicate that TGF- β is required for the development of fibrosis including: (1) There is an increase in TGF- β mRNA and/or protein expression in fibrotic diseases of almost all organ systems in humans and in experimental animal models of fibrosis. (2) Transgenic mice that overexpress activated TGF- β globally have fibrosis in multiple organs and tissue. (3) Increased constitutively active TGF- β in lungs and peritoneum leads to lung and peritoneal fibrosis, respectively. (4) Fibrosis is attenuated in experimental animal models by blocking activated TGF- β in various organ systems by administration of either TGF- β binding proteins, anti-TGF- β antibody, or TGF- β type 1 receptor, as well as by the overexpression of dominant-negative TGF- β type 2 receptor [12]. Herein, we focus on some of the recently identified important molecular pathways by which TGF- β and oxidative stress collude to drive pulmonary fibrosis.

5.1 ROS activate latent TGF- β and induces TGF- β gene expression

All three isoforms of TGF- β are secreted in an inactive, latent form attached to a latency association protein (LAP). ROS increase TGF- β -induced fibrosis in part by activating latent TGF- β by two proposed mechanisms: (1) directly through oxidation of the LAP thereby releasing TGF- β and (2) indirectly by ROS-induced activation of matrix metalloproteinases (MMPs), which then cleave LAP and release active TGF- β [12]. ROS generated by asbestos fibers can directly activate latent TGF- β [149]. Using a site mutagenesis approach, a redox switch located on methionine 252 of the LAP has been identified as a possible extracellular ROS-sensor, since mutant LAP were incapable of ROS-induced TGF- β activation [150].

In addition to activating latent TGF- β , ROS can augment gene expression and secretion of TGF- β in many cell types including AEC and macrophages (see for review: [12]). Notably, free radical scavenging agents, iron chelators, as well as NOX2 null mice all substantially reduce ROS-induced TGF- β gene expression in several fibrogenic model systems [12]. Also, H₂O₂ promotes EMT in lung epithelial cells via a TGF- β 1-dependent mechanism [151]. The fibrotic response caused by adenoviral vectors encoding constitutively activated TGF- β 1 are blocked by concomitant treatment with adenoviruses encoding EC-SOD [152]. Collectively, these studies show the closely linked interactive effects between ROS and TGF- β activation in promoting pulmonary fibrosis.

5.2 TGF- β increases ROS production

Although ROS promote activation of TGF- β gene expression and release from LAP, TGF- β can also increase ROS production. TGF- β -induced generation of oxidative stress occurs in part by decreasing antioxidant defenses as well as by increasing ROS production. TGF- β decreases the concentration of important antioxidants in the lungs and hepatocytes, such as catalase, GSH and SOD, all of which have the capacity to inhibit fibrogenic responses due to oxidative stress as discussed in Section 2.3 [12, 153]. Intra-nasal instillation of adenoviral vectors encoding for constitutively activated TGF- β 1 in murine lungs suppresses enzymes involved in GSH synthesis before the development of pulmonary fibrosis and that this occurs by inducing Activating Transcription Factor 3 (ATF3), a transcriptional repressor involved in regulation of glutamate-cysteine ligase [13]. These changes in GSH synthesis result in oxidative stress, lung epithelial cell apoptosis and pulmonary fibrosis [13].

There are several mechanisms by which TGF- β can augment the production of ROS that include the following: (1) activating oxidases in the cell membrane that promote H₂O₂ release to the extracellular space in human lung fibroblasts and bovine pulmonary artery endothelial cells. (2) inducing mitochondrial ROS production in part by decreasing complex IV activity in lung epithelial cells and hepatocytes. (3) activating Nox4 to generate ROS in various types of cells *in vitro* and *in vivo* [12, 154–156]. Taken together, these studies establish that TGF- β can augment oxidative stress, important in the pathogenesis of pulmonary fibrosis.

5.3 Possible mechanisms by which ROS mediates TGF- β -induced fibrogenesis

The detailed molecular mechanisms by which TGF- β promotes fibrosis is beyond the scope of this report but has recently been reviewed [12]. Herein we highlight some of the newer findings centered on the four possible mechanisms by which ROS mediate TGF- β -induced fibrogenesis including: (1) activation of fibroblast proliferation and collagen production, (2) induction of EMT, (3) promotion of epithelial cell apoptosis, and (4) activation of ECM gene expression and suppression of ECM protein degradation. Using primary human cardiac fibroblasts, TGF- β increases NOX4 and α -SMA, a myofibroblast marker, while knockdown of NOX4 by siRNA reduces production of ROS and expression of α -SMA mRNA suggesting that ROS mediate cardiac fibroblast differentiation to myofibroblasts [31].

Further, these investigators showed that ROS activate Smad2/3 and that activated Smad2/3 alone can cause cardiac fibroblast differentiation to myofibroblasts. As noted above (Section 2.2), TGF- β induced NOX4 expression is crucial for mediating ROS production that drives myofibroblast activation in the lungs of patients with IPF and mice with bleomycin-induced fibrosis as well as increase in AEC apoptosis [33, 157]. TGF- β -induced NOX4 expression and oxidative stress also appear important in mediating hepatocyte apoptosis and liver fibrosis [158, 159]. ROS can induce EMT; a process that results in a loss of epithelial markers and acquisition of mesenchymal phenotype as seen in many fibrotic diseases, including IPF [160–162]. TGF- β is a key mediator of EMT *in vitro* [163, 164] and *in vivo* [165, 166]. A role for ROS is suggested by the observation that antioxidants block TGF- β -induced EMT in lung and renal epithelial cells [151, 167]. A recent study demonstrated that TGF- β coordinately regulates EMT by interactions with β -catenin through its interaction with cAMP-response element-binding protein (CREB)-binding protein (CBP) in AECs [168]. Using the bleomycin mouse model of pulmonary fibrosis, these investigators demonstrate that ICG-001, a small molecule specific inhibitor of β -catenin /CBP interaction, attenuates pulmonary fibrosis and α -SMA and collagen induction in AEC. Given the robust β -catenin signaling in pulmonary fibrosis as well as emerging evidence of crosstalk between ROS levels and β -catenin signaling pathway, this suggests a novel mechanistic link that may be relevant to our understanding of pulmonary fibrosis [57, 168–170].

6. Conclusions

Table 1 summarizes some of the important molecular mechanisms underlying ROS-induced pulmonary fibrosis that we reviewed as well as some of the crucial areas that require further investigation. Patients with pulmonary fibrosis have increased oxidative stress as measured by various techniques as well as reductions in crucial anti-oxidant defenses. With the possible exception of NAC, none of the strategies aimed at limiting oxidative stress to date have proven efficacious in the management of pulmonary fibrosis. A simple imbalance between ROS levels and anti-oxidant defenses is unlikely to fully account for the multiple, independently regulated pathways involving various cells and organelles [1–3]. Targeted anti-oxants to the mitochondria or limiting NOX4 activation warrant further study. Emerging evidence demonstrates that mitochondria- and p53-regulated death pathways as well as ER stress mediate AEC apoptosis, which is an important early event in patients with IPF and asbestosis, all of which are modulated by the levels of ROS. There may also be an important interactive effect among mtOgg1, ACO2, and p53 in mtDNA repair and oxidant-induced intrinsic apoptosis. It seems likely that many of the interactive effects among mtOgg1, ACO2, p53, and intrinsic apoptosis described herein with asbestos fibers will have important broader implications regarding pulmonary fibrosis and, perhaps, mutagenesis. Future investigations exploring the role of these various interactive effects in transgenic animal models and in humans are warranted. Furthermore, the role of mt-Ogg1 and ACO2 in preventing oxidant-induced mtDNA damage, p53 activation, and intrinsic apoptosis requires additional study. ROS activate latent TGF- β , TGF- β gene expression and fibroblast collagen production by a RhoA-dependent mechanism. Finally, and perhaps most importantly, the ROS / pulmonary fibrosis paradigm is shedding light into the molecular basis underlying fibrosis in other organs involving the liver, heart and kidney as well as our understanding of tumorigenesis, for which effective treatment regimens are urgently required.

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Acronyms

AEC	alveolar epithelial cell
AT2	alveolar epithelial type II
AM	alveolar macrophage
ARE	antioxidant response element
BALF	bronchoalveolar lavage fluid
JNK	c-Jun N-terminal kinase
EGFR	epidermal growth factor receptor
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
ERK	extracellular-signal-regulated kinase
H₂O₂	hydrogen peroxide
IPF	idiopathic pulmonary fibrosis
IRE1α	inositol requiring kinase 1 alpha
IL-1β	interleukin-1 beta
ACO2	mitochondrial aconitase
mtDNA	mitochondrial DNA
mt-hOgg1	mitochondrial human 8-oxoguanine-DNA glycosylase 1
$\Delta\psi_m$	mitochondrial membrane potential
MDM2	mouse double-minute 2 protein
NAC	n-acetyl cysteine
NOX	NADPH oxidase
NOX4	DPH oxidase-4
NOS	nitric oxide synthase
Nrf2	nuclear factor erythroid 2-related factor 2
PKCδ	protein kinase C delta
ROS	reactive oxygen species
SERCA	sarcoplasmic ER Ca ²⁺ ATPase
αSMA	α smooth muscle actin
O₂⁻	superoxide
SOD	superoxide dismutase
SP-C	surfactant protein C
TGF-β	transforming growth factor beta
TNFα	tumor necrosis factor-alpha
UPR	unfolded protein response
XBP1	x-box binding protein 1

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Highlights

- Oxidative stress is an important mechanism underlying fibrosis
- ROS derived from the mitochondria and NOX4 can promote fibrosis
- AEC apoptosis in lung fibrosis is coupled with mitochondria, ER, and p53 activation
- ROS-induced AEC apoptosis is blocked in cells overexpressing Ogg1 or ACO2
- The interactive effects between oxidative stress and TGF- β augment fibrosis

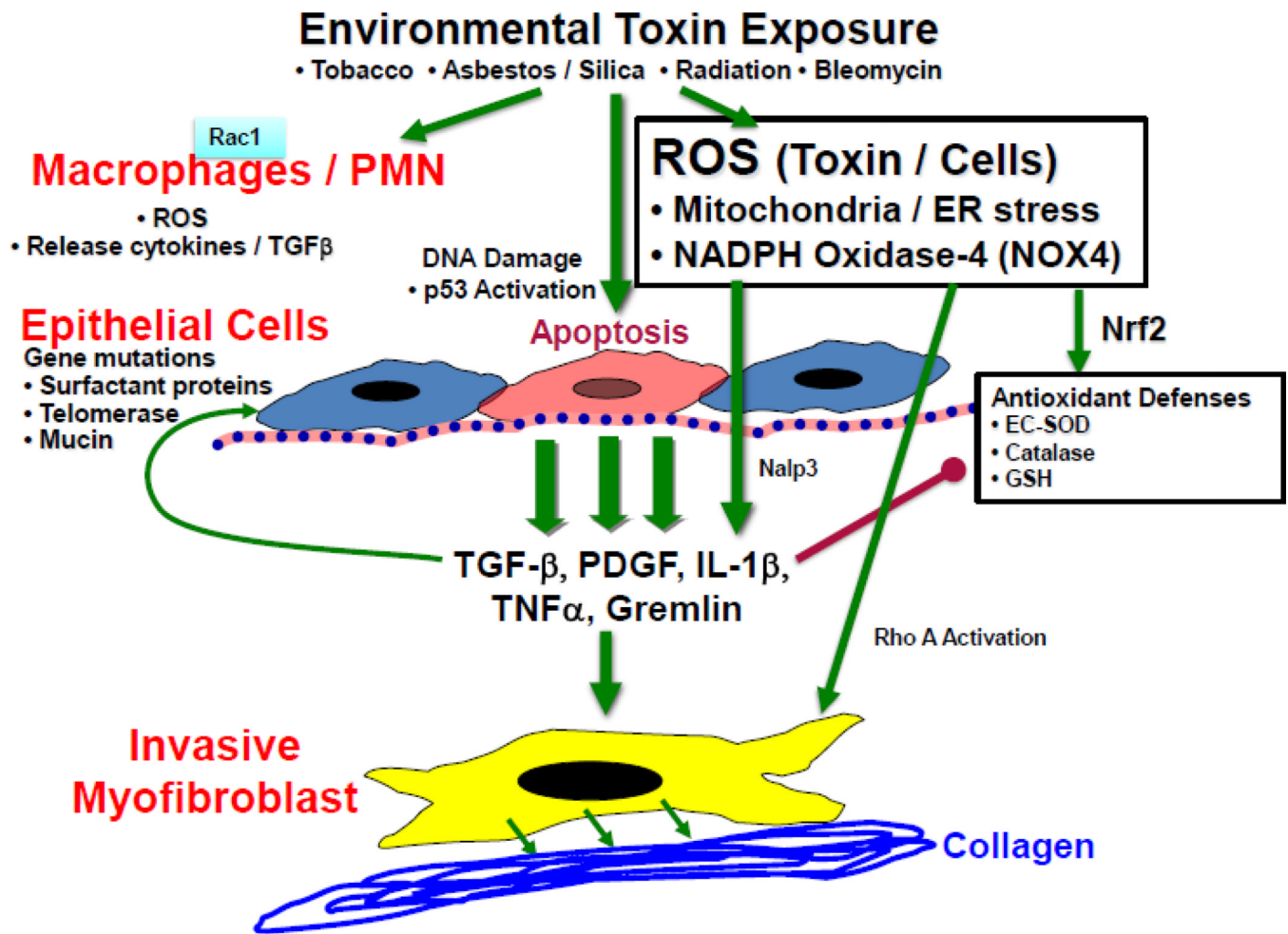


Figure 1. Hypothetical model of environmental stress/oxidant induced pulmonary fibrosis
Environmental toxins/oxidants may induce the generation of ROS via induction of ER stress, NOX4 and uncoupling of mitochondrial electron transport or Rac 1 mediated activation of macrophages and/or PMNs. In conjunction with DNA damage and p53 activation, ROS promote apoptosis of airway epithelial cells, and elicit the production of cytokines and growth factors that may be important for invasive myofibroblastic differentiation and collagen deposition and may alter antioxidant defenses. The susceptibility of epithelial cells to apoptosis may be affected by mutations in surfactant, telomerase and mucin genes.

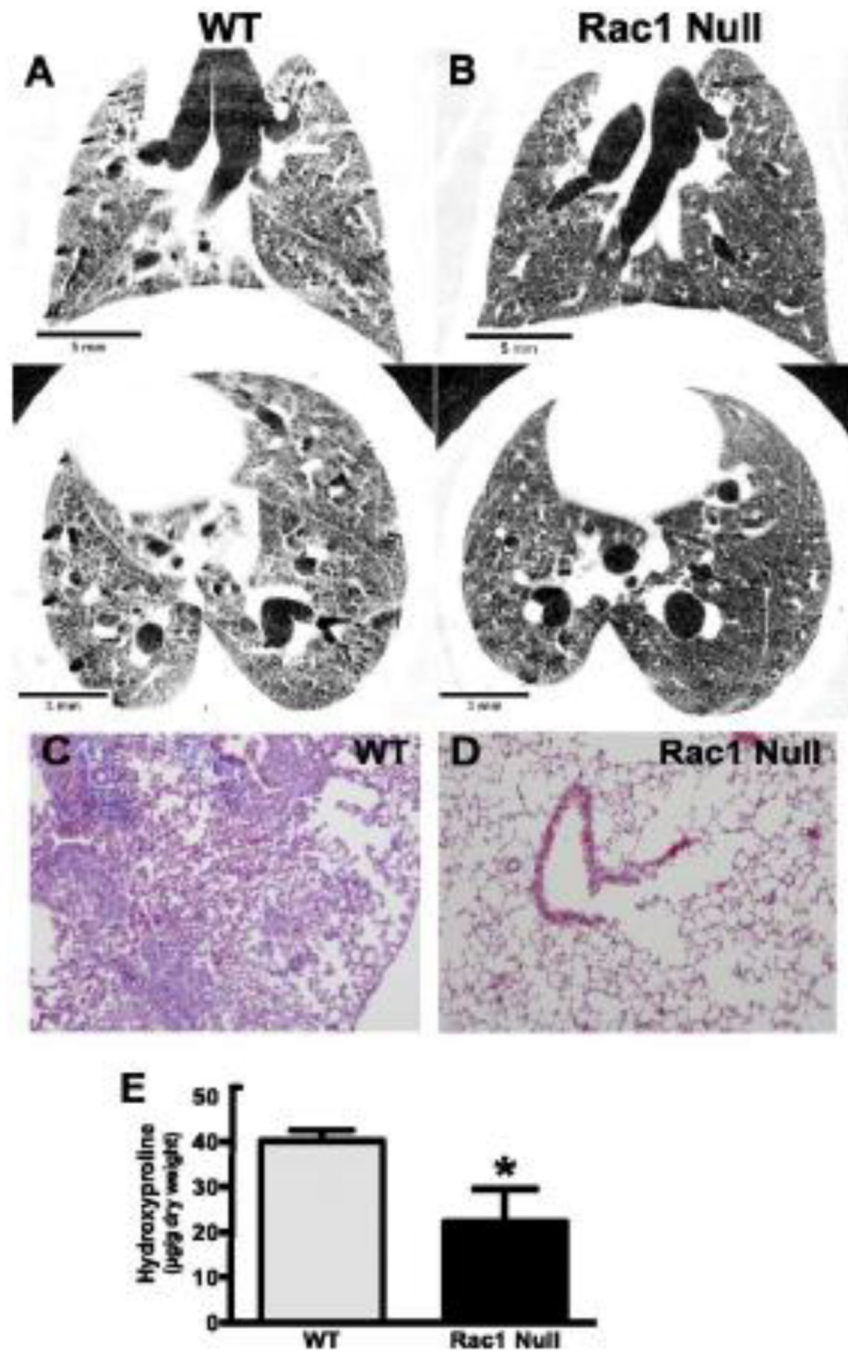


Figure 2. Asbestos-induced pulmonary fibrosis is blocked in Rac-1 null mice
 Wild-type (WT) and Rac1 null mice were intratracheally exposed to chrysotile asbestos (100 µg). Micro-CT scan images were obtained on live mice (*in vivo*) 21 days after exposure. Images are representative of three WT (A) and three Rac1 null (B) mice. Twenty one days after exposure, the mice were euthanized and the lungs were removed and processed for collagen deposition using Masson's trichrome staining. Micrographs are representative of 10 WT (C) and 10 Rac1 null (D) mice. (E), mice were euthanized 21 days after exposure, and lungs were removed and homogenized for hydroxyproline assay. WT ($n = 4$) and Rac1 null

($n = 4$); * $p < 0.0208$. *Experimental data from Osborn-Heaford et.al (reference 16) was used with permission.*

Environmental Toxin Exposure

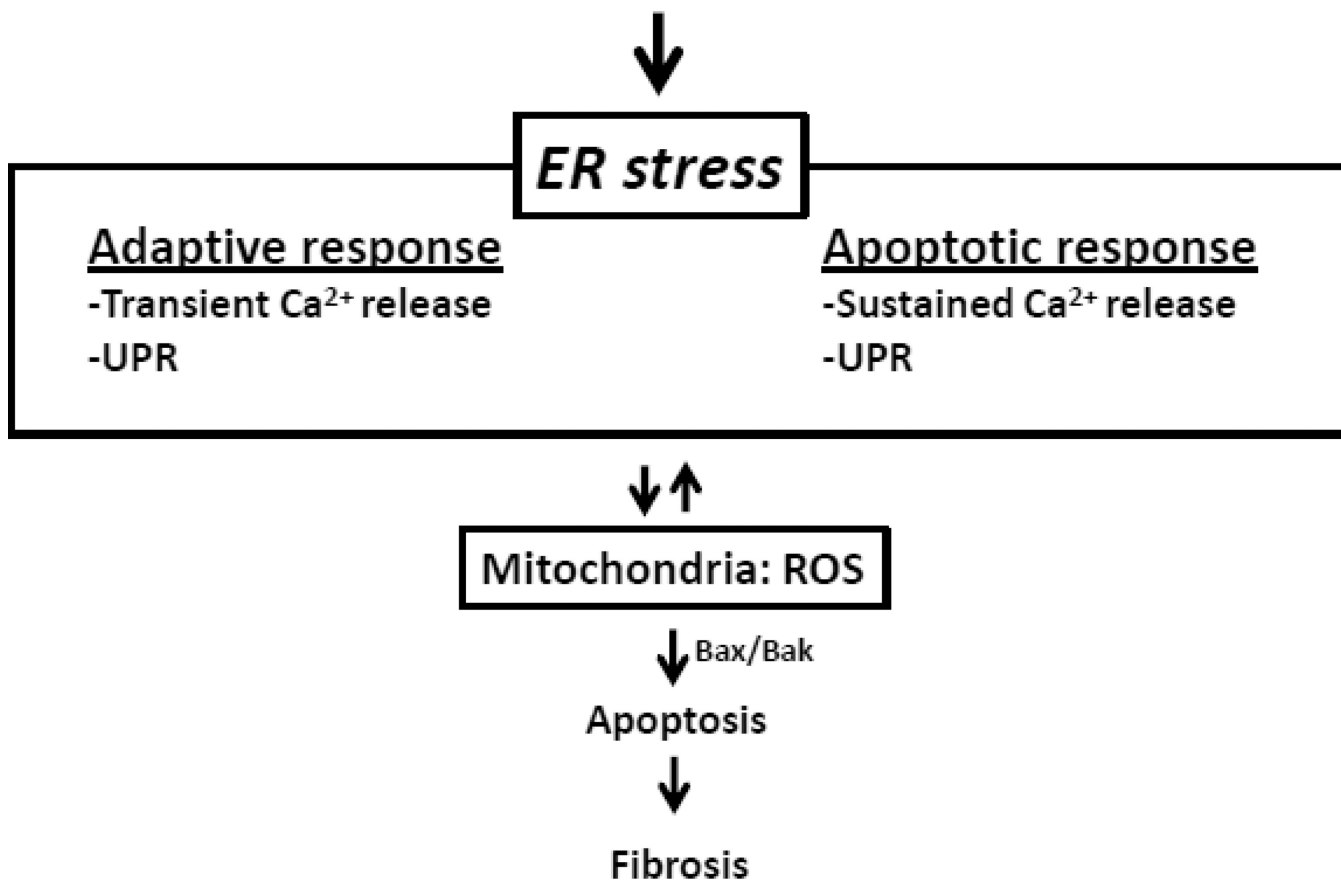


Figure 3. Hypothetical model of AEC ER-mitochondria crosstalk-induced apoptosis and fibrosis
Exposure of AEC cells to environmental toxins induces ER that can lead to the release of ROS from the mitochondria that promotes apoptosis and pulmonary fibrosis (see text for details).

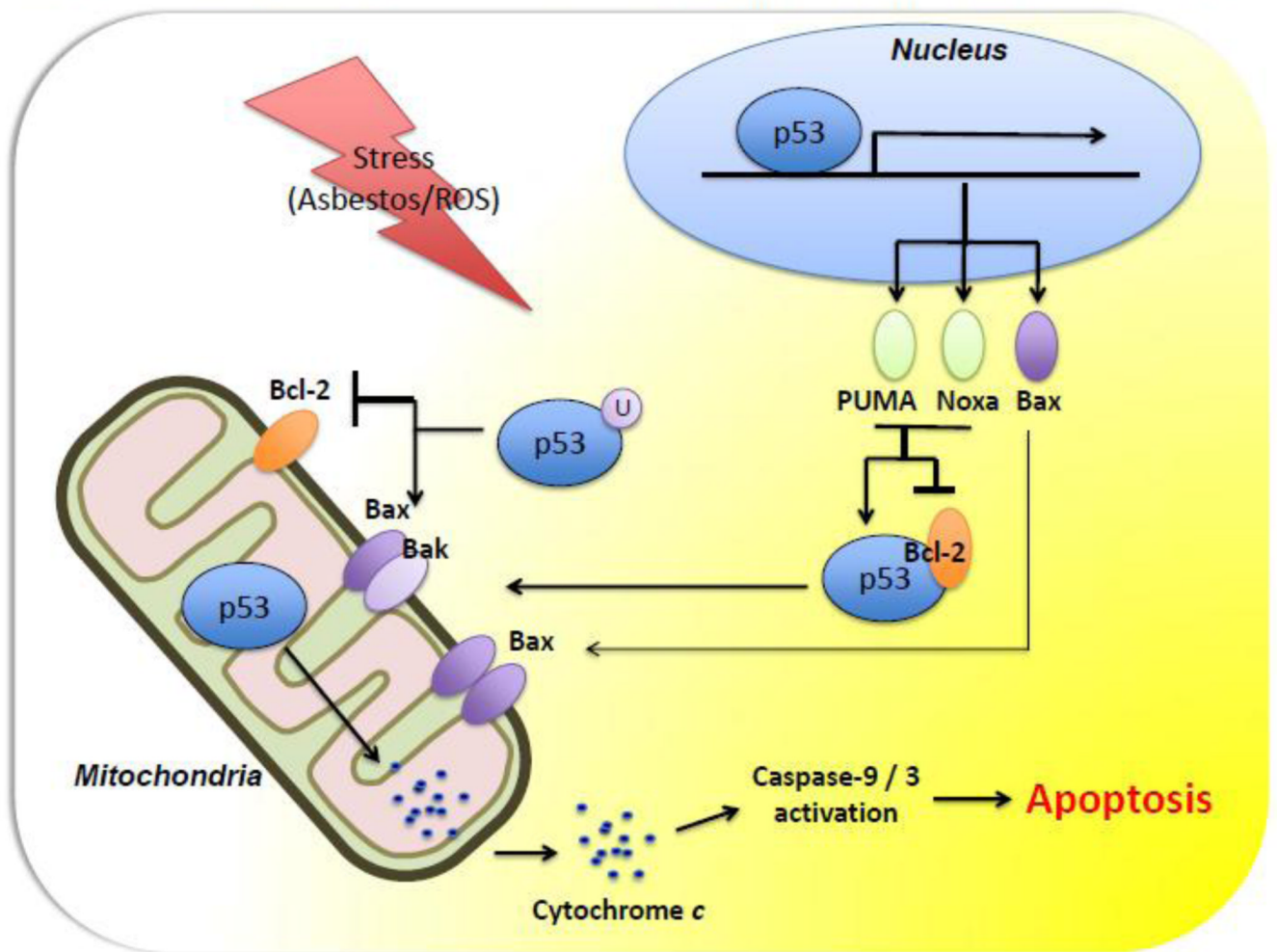


Figure 4. Hypothetical model of p53-dependent cytosolic and mitochondrial apoptotic pathways
 In response to stressors such as oxidative stress, nuclear-localized p53 induces the transcription of target genes that mediate p53's function (see text for details).

Table 1

Some of the important molecular mechanisms underlying ROS-induced pulmonary fibrosis and areas requiring further investigation

What we know	What we need to know
There is increased oxidative stress in the lungs of patients with pulmonary fibrosis.	<ol style="list-style-type: none"> 1 What is the most reproducible technique to quantitate ROS in tissue and BALF? 2 What is the <i>in vivo</i> relevance of these findings in the context of treating pulmonary fibrosis and which cell(s) should be targeted?
There is decreased antioxidant defenses in the lungs of patients with pulmonary fibrosis.	<ol style="list-style-type: none"> 1 What is the best way to quantitate anti-oxidant defenses in tissue and BALF? 2 What is the <i>in vivo</i> relevance of these findings in the context of treating pulmonary fibrosis and which cell(s) should be targeted?
Apoptosis of AECs occurs in patients with IPF and asbestosis.	Is AEC apoptosis essential for mediating pulmonary fibrosis?
Pulmonary fibrosis is associated with activation of an ER stress response that may be due partly to ROS production.	What is the causal role of ROS in activating the ER stress response in AEC as well as the <i>in vivo</i> relevance of ER stress in the context of treating pulmonary fibrosis?
ROS cause AEC apoptosis <i>in vitro</i> while AEC apoptosis occurs in patients with IPF and asbestosis.	Is AEC apoptosis essential for mediating pulmonary fibrosis?
Asbestos induces AM Rac1-dependent mitochondrial ROS production that is important in murine asbestosis.	What role does AM Rac1 play in humans with asbestosis, and is it a useful biomarker of pulmonary fibrosis?
p53-dependent transcription mediates ROS/asbestos-induced intrinsic AEC apoptosis <i>in vitro</i> and is evident in gene-profiling studies of normal and malignant cells.	Is p53-dependent transcription necessary for pulmonary fibrosis following ROS exposure in animal models or humans?
Mitochondrial hOgg1 and ACO2 prevent oxidant-induced AEC mitochondrial dysfunction and intrinsic apoptosis <i>in vitro</i> .	What is the <i>in vivo</i> relevance of these findings in the context of asbestosis and other forms of pulmonary fibrosis?
ROS activate latent TGF- β , induce TGF- β gene expression, and activate fibroblast collagen production	What is the <i>in vivo</i> relevance of these findings in the context of treating pulmonary fibrosis and which cell(s) should be targeted?
ROS activates Nalp3 inflammasomes.	Is Nalp3 activation induced by ROS necessary and sufficient for inducing pulmonary fibrosis, and if so, which cell is responsible?

Abbreviations: ACO2, mitochondrial aconitase; AEC, alveolar epithelial cell; AM, alveolar macrophage; BALF, bronchoalveolar lavage fluid; ER, endoplasmic reticulum; hOgg1, human 8-oxoguanine-DNA glycosylase 1; IPF, idiopathic pulmonary fibrosis; ROS, reactive oxygen