Lost after translation: insights from pulmonary surfactant for understanding the role of alveolar epithelial dysfunction and cellular quality control in fibrotic lung disease

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Mulugeta S, Nureki S, Beers MF. Lost after translation: insights from pulmonary surfactant for understanding the role of alveolar epithelial dysfunction and cellular quality control in fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol 309: L507–L525, 2015. First published July 17, 2015; doi:10.1152/ajplung.00139.2015.—Dating back nearly 35 years ago to the Witschi hypothesis, epithelial cell dysfunction and abnormal wound healing have reemerged as central concepts in the pathophysiology of idiopathic pulmonary fibrosis (IPF) in adults and in interstitial lung disease in children. Alveolar type 2 (AT2) cells represent a metabolically active compartment in the distal air spaces responsible for pulmonary surfactant biosynthesis and function as a progenitor population required for maintenance of alveolar integrity. Rare mutations in surfactant system components have provided new clues to understanding broader questions regarding the role of AT2 cell dysfunction in the pathophysiology of fibrotic lung diseases. Drawing on data generated from a variety of model systems expressing disease-related surfactant component mutations [surfactant proteins A and C (SP-A and SP-C); the lipid transporter ABCA3], this review will examine the concept of epithelial dysfunction in fibrotic lung disease, provide an update on AT2 cell and surfactant biology, summarize cellular responses to mutant surfactant components [including endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and intrinsic apoptosis], and examine quality control pathways (unfolded protein response, the ubiquitin-proteasome system, macroautophagy) that can be utilized to restore AT2 homeostasis. This integrated response and its derangement will be placed in the context of cell stress and quality control signatures found in patients with familial or sporadic IPF as well as non-surfactant-related AT2 cell dysfunction syndromes associated with a fibrotic lung phenotype. Finally, the need for targeted therapeutic strategies for pulmonary fibrosis that address epithelial ER stress, its downstream signaling, and cell quality control are discussed.

alveolar type 2 cells; cell quality control; epithelial ER stress; fibrotic lung disease; surfactant proteins

BY THE VERY NATURE OF ITS JUXTAPOSITION WITH THE OUTSIDE WORLD, the epithelium of the distal lung, and specifically the alveolar type 2 (AT2) cell, in the course of performing the biosynthetic, metabolic, and repair/regenerative functions critical to maintenance of alveolar homeostasis, must also protect itself from significant stress imparted by its constant exposure to mechanical, metabolic, and environmental factors. To address this considerable threat to protein synthesis, macromolecular turnover, and maintenance of organelle mass and integrity, eukaryotic cells and higher organisms have evolved a complex network of cellular quality control pathways designed to improve, modify, compartmentalize, and/or remove abnormal molecular and organelar substrates that accumulate during the course of “daily life.” Disruption of this network inevitably results in the accumulation of misfolded proteins/aggregates, complex macromolecules, and/or dysfunctional organelles with elaboration of multiple signaling cascades that can drive disease initiation/progression through the triggering of inflammation, cell death, and organ dysfunction. Although cellular quality control and its derangement have been shown to play an important role in disease pathogenesis in other organ systems such as brain, liver, and pancreas, its contribution to pulmonary disease is only emerging.

Pulmonary fibrosis represents a heterogeneous group of postnatal interstitial lung disorders characterized by destruction of pulmonary architecture caused by an abnormal wound repair response that ultimately leads to scar formation, organ malfunction, disruption of gas exchange, and respiratory failure. In adults, idiopathic pulmonary fibrosis (IPF), the most common subtype of the larger family of idiopathic interstitial pneumonias, is a diffuse parenchymal lung disease of unknown etiology that affects over 5 million people worldwide and typically results in a need for lung transplantation or in death within 2–5 years of diagnosis (81). In the United States, the annual incidence of IPF appears to be rising and is estimated at 5–16/100,000 individuals with a prevalence of 13–20/100,000, is more common in men, and increases significantly with age. IPF is characterized histologically by a pattern of usual interstitial pneumonia (UIP), in which the peripheral subpleural parenchyma shows evidence of fibroelastic foci, traction bronchiectasis, and microscopic honeycombing lined by hyperplastic AT2 cells interspersed with areas of normal or nearly normal lung tissue. Familial forms of pulmonary fibrosis (FPF) can also present in children and are part of the larger spectrum of childhood interstitial lung disease (chILD) (38; 88). The incompletely defined pathogenesis of IPF (and chILD) has been a major obstacle in developing effective therapies capable of stabilizing or improving lung function for these disorders.

Although research into the details of pathways responsible for epithelial cell dysfunction in IPF is still in its infancy, the pulmonary surfactant system has provided an important platform and experimental substrates to study the role aberrant
quality control, cell stress, and cytotoxicity in these events. In this review, we describe developments in the understanding of the molecular mechanisms underlying childhood and adult interstitial lung diseases (ILDs) from the viewpoint of disease-related mutations in components of the surfactant system [surfactant protein C (SP-C); surfactant protein A (SP-A), ATP binding cassette class A3 (ABCA3) lipid transporter] and the cellular responses to their expression that could modulate disease pathogenesis. Data on the elicited quality control events and signaling pathways leveraged from a variety of in vitro and in vivo models expressing these mutant isoforms will be examined and placed in the context of translational data from patients showing similar molecular signatures in both familial and sporadic IPF and in preclinical and patient data from other non-surfactant-related AT2 cell dysfunction syndromes associated with a fibrotic lung phenotype.

Alveolar Epithelial Injury and Fibrotic Lung Remodeling: the Witschi Hypothesis Reborn

The health of the distal lung relies on the proper function of AT2 cells, which play a critical role in the production and maintenance of pulmonary surfactant, contribute to overall barrier function, perform xenobiotic metabolism, and participate in lung repair serving as a progenitor population following lung injury to replace damaged AT1 and AT2 cells (10, 16). The cuboidal-shaped AT2 cells comprise only 3–5% of the alveolar surface area while the remainder is occupied by juxtaposed AT1 cells that serve to facilitate gas exchange. Both AT1 and AT2 cells have important roles in ion transport in the distal lung (101). Nevertheless, AT2 cells constitute 60% of alveolar epithelial cells and 10–15% of all lung cells (36), rendering them a critical component in alveolar homeostasis.

Over 35 years ago, Haschek and Witschi published a theory on the pathogenesis of lung fibrosis that challenged the then contemporaneous view of lung fibrosis as an “inflammatory disease” (56, 165). Although chronic inflammation as a key to its pathogenesis had persisted in some circles, it is not a prominent feature in biopsies of IPF patients (144). Coupled with the low efficacy or even harmful effects of immunomodulatory therapies shown in many clinical trials involving these patients (62a, 67, 132, 133), a paradigm shift developed away from the so-called “alveolitis” theory of the 1970s and 1980s to again suggest a pivotal role for alveolar epithelial cells (AEC) and specifically AT2 cell dysfunction in the development of a fibrotic lung phenotype. Although not primary drivers of the fibrotic lesion, the exact roles of inflammation and the local immune milieu in IPF pathogenesis remain to be defined.

In this “epithelial injury/abnormal wound repair” model, rechampioned in 2001 (144), IPF development and progression were proposed to be initiated and sustained by microfoci of repeated cycles of AEC injury occur within a larger dysfunctional repair process reminiscent of aberrant wound healing described in other organs such as skin, kidney, and liver (18, 184). More recently, the “postmodern” link between intrinsic epithelial cell dysfunction and IPF/ILD postulated by Witschi has been firmly established based on multiple lines of experimental evidence and clinical correlations:

1) Monogenetic diseases such as Hermansky-Pudlak syndrome (HPS) are characterized by dysfunctional AT2 cells and ILD (114). Mutations in nine different HPS genes disrupt intracellular cargo protein trafficking leading to dysfunctional lysosome-related organelles (LROs) in various organs. Lamellar bodies (LBs), the LROs of AT2 cells, show marked increase in size due to overaccumulation of phospholipids. Two HPS genetic subgroups, HPS1 and HPS4, manifest a common lung phenotype characterized by the premature development of a UIP-like fibrotic pattern in the fourth decade of life (114). Some pathological features can be recapitulated in mouse models of HPS, where AT2 cells were shown to be important effector cells elaborating proinflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1) (5, 191, 192).

2) Depletion of AT2 cells induces spontaneous fibrosis in mice. In a transgenic mouse model using the SP-C gene promoter to express a diphtheria toxin (DT) receptor exclusively in AT2 cells, administration of DT in these mice induced AT2 cell death and increased lung collagen deposition.

3) AEC apoptosis is paramount in mouse models of lung fibrosis generated by single or repetitive exposures to bleomycin. Inhibition of apoptosis in these models by use of caspase inhibitors, Fas/FasL pathway blockade, or genetic deletion of proapoptotic Bcl2 proteins attenuates fibrosis (164).

4) It has been noted that aberrantly activated lung epithelial cells produce virtually all the mediators responsible for fibroblast migration, proliferation, and activation with the subsequent exaggerated extracellular matrix accumulation and destruction of the lung parenchyma (144, 187).

5) Some of the most compelling evidence for the role of AT2 cells in these processes is derived from studies linking patients with UIP or chILD histopathology with mutations in AT2 cell-restricted genes involved in the production of pulmonary surfactant. To date, more than 60 SP-C (SFTPC), and 150 ABCA3 mutations, as well as two mutations in the surfactant protein A2 gene (SFTPA2) have been identified in affected children and adults (25, 116, 175, 178). As will be detailed below, signatures of protein aggregation, endoplasmic reticulum (ER) stress, proinflammatory/profibrotic cytokine elaboration, altered macroautophagy, and apoptosis defined by model systems expressing SFTPC, SFTPA, and ABCA3 mutations are also present in the lungs and AT2 epithelia of sporadic and familial IPF patients (3, 23, 83, 91).

Thus substantial evidence exists that a vulnerable and/or dysfunctional AT2 epithelium is a pivotal player in aberrant injury/repair responses occurring in IPF and other forms of fibrotic lung remodeling.

AT2 CELLS AND THE BIOSYNTHETIC CHALLENGE OF SURFACTANT

The alveolar gas exchange surface is coated with a thin film of surfactant, representing a complex heterogeneous mixture of primarily lipids (90% by weight) and some protein that serves to promote alveolar stability by reducing surface tension at the air-liquid interface along the epithelial lining layer (129). In addition to lipids [mainly phosphatidylycerol (PC) with one (lyso-PC) or two (DPPC) palmitic acid side chains], biochemical analysis of surfactant systems expressing SFTPC, SFTPA, and ABCA3 mutations has identified four unique proteins designated surfactant proteins: SP-A, SP-B, SP-C, and SP-D (131). A large volume of literature has demonstrated that the surface tension-reducing function of surfactant stems from the interaction of phospholipids and the two low-molecular-weight hydrophobic proteins,
SP-B and SP-C (reviewed in Ref. 180). The relatively hydrophilic and more abundant oligomeric proteins, SP-A and SP-D, are members of the collectin family of C-type lectins that share distinct collagen-like and globular, carbohydrate-binding domains. Although SP-A and SP-D do not have a direct function in the surface tension activity of surfactant, they play an essential role in innate lung host defense (reviewed in Ref. 183). Numerous studies have shown that, under pathological conditions, SP-A and SP-D can each undergo a variety of posttranslational modifications (such as oxidation, nitration, S-nitrosylation) that can lead to loss of function (50, 53, 102, 196).

AT2 cells synthesize, secrete, and recycle all components of surfactant (Fig. 1). Compared with neuroendocrine and many secretory epithelia (exocrine and endocrine) in other tissues, both functionally and morphologically, the AT2 cell is somewhat unique. The diversity of cellular cargo that must be managed (phospholipids, hydrophobic proteins, multimeric hydrophilic proteins, cytokines) creates a significant burden on cell quality control and metabolic demands. Ultrastructurally, AT2 cells lack a dense secretory granule; but instead, transmission electron microscopy (EM) reveals the presence of cytoplasmic, osmiophilic, lamellated organelles (i.e., LBs) long recognized as the storage organelle from which surfactant is released into the alveolar lumen (9). Biochemically, LBs resemble other LROs in that they are acidic, express lysosomal markers (CD63; LAMP-1), but also contain surfactant lipids, SP-B, and SP-C as well as a fraction of intracellular SP-A.

![Fig. 1. Schematic representation of structure, biosynthetic processing, and life cycle of 3 surfactant system components associated with interstitial lung disease](http://ajplung.physiology.org/doi/abs/10.1152/ajplung.00139.2015)

**SP-A**
- **Gene**: SFTPA1
- **Function**: Essential for surfactant function.
- **Structure**: Consists of four domains: amino-terminal domain, collagenous domain, neck domain, and carbohydrate recognition domain (CRD).
- **Posttranslational Modifications**: Signal peptide cleavage, hydroxylation of proline residues, and N-linked glycosylation.

**SP-B**
- **Gene**: SFTPB
- **Function**: Essential for surfactant function.
- **Structure**: Consists of two domains: amino-terminal domain and COOH-terminal domain.
- **Posttranslational Modifications**: Signal peptide cleavage, hydroxylation of proline residues, and N-linked glycosylation.

**SP-C**
- **Gene**: SFTPC
- **Function**: Essential for surfactant function.
- **Structure**: Consists of a collagen-like domain and a globular carbohydrate-binding domain.
- **Posttranslational Modifications**: Signal peptide cleavage, hydroxylation of proline residues, and N-linked glycosylation.

**ABCA3**
- **Function**: Involved in lipid transport.
- **Structure**: Comprises 12 transmembrane helices.
- **Posttranslational Modifications**: Nedd4-2 binding "PY" targeting motif.

**Endoplasmic Reticulum (ER)**
- **Function**: Synthesis of surfactant components.
- **Posttranslational Modifications**:
  - Signal peptide cleavage
  - Hydroxylation of proline residues
  - N-linked glycosylation

**Golgi Apparatus**
- **Function**: Processing of surfactant components.
- **Posttranslational Modifications**:
  - Hydroxylation of proline residues
  - N-linked glycosylation

**Lamellar Bodies (LB)**
- **Function**: Storage of surfactant components.
- **Posttranslational Modifications**:
  - Signal peptide cleavage
  - Hydroxylation of proline residues
  - N-linked glycosylation

**Secretion**
- **Function**: Release of surfactant components into the alveolar lumen.
- **Posttranslational Modifications**:
  - Signal peptide cleavage
  - Hydroxylation of proline residues
  - N-linked glycosylation
C3.7) is a lipid-avid peptide composed of 33–35 highly con- 
form isolated and sequenced from lung lavage fractions (SP-
ments the mature SP-C domain forms a very stable and rigid 
propeptide (34).

4) Linker domain. As the name signifies, the proximal 
COOH-terminal propeptide (residues 59–93) is linearly posi-
tioned between the mature SP-C (TM) and BRICHOS domains 
and through formation of a hairpin structure thereby facilitates 
docking of the BRICHOS domain to the TM segment. Impor-
tantly, mutations in the linker domain do not induce cytosolic 
aggregation of proSP-C but instead result in its mistrafficking 
and accumulation in plasma membrane, early endosomes, and 
LE/MVB (15, 58) (discussed in detail later).

SP-C: a Unique Secretory Product

Of the four surfactant proteins, only SP-C is synthesized 
exclusively by the AT2 cell. The 2.4-kb gene encoding human 
SFTPC is located on chromosome 8p and is organized into six 
exons (I through V coding, VI untranslated) and five introns, 
which produce a 0.9-kb mRNA encoding either a 191- or 
197-amino acid 21-kDa proprotein (proSP-C21). The protein 
form isolated and sequenced from lung lavage fractions (SP-
proSP-C folding, the BRICHOS domain appears to have an 
intramolecular chaperone-like activity safeguarding the meta-
stable, β-sheet-prone, mature SP-C domain from amylod fibril 
formation (51, 65, 154, 182). Amyloid deposits composed of 
mature SP-C have also been observed in lung tissue samples 
from some ILD patients with mutations in the BRICHOS 
domain (181).

Surfactant Protein A: a Multimeric Glycoprotein AT2 
Product

SP-A, the most abundant surfactant protein, is a luminal, 
multifunctional sialoglycoprotein of Mr 28–36,000 that con-
tains a COOH-terminal C-type lectin motif, a triple collo-
dagen domain, and carbohydrate recognition domain (CRD) 
(183) (Fig. 1, inset). In humans, there are two genes (SFTPA1 
and SFTPA2), 4.5 kb each, located on chromosome 10. The 
amino acid differences that distinguish SP-A1 from SP-A2 
are in general conservative and located mainly in the colla-
gen-like domain with only one shown to affect protein 
structure. Like SP-D, SP-A is synthesized by airway cells, 
including nonciliated (club/Clara) cells. Although it has 
been localized to extrapulmonary sites, including salivary 
glands, lacrimal glands, and the female urogenital tract, the 
exact physiological relevance of its expression in these 
organs remains to be defined (reviewed in Ref. 78).

The biosynthesis of SP-A differs markedly from SP-C (and 
SP-B). In contrast to proteolytic processing of a larger pro-
protein, posttranslational processing of the SP-A primary transla-
tion product consists of NH2 signal peptide cleavage, N-linked 
glycosylation, and proline hydroxylation followed by trimeric 
association of monomers via coiled-coil bundling of a-helices 
in the neck thought to be composed of two SP-A1 and one 
SP-A2 monomer(s). The completed oligomeric structure of 
SP-A represents a higher order 18-mer assembled from six 
trimeric subunits linked by interchain disulfide bonding 
(Fig. 1, inset).
Metabolic labeling studies have demonstrated that the intracellular trafficking and secretion of nascent SP-A may involve both a LB-dependent (42) and a LB-independent (constitutive pathway (122). Since alveolar SP-A can also be taken up via endocytosis and incorporated into LBs it has been difficult to accurately gauge the contribution of each pathway. Beyond the presence of a signal peptide and N-linked glycosylation, structural motifs and signals regulating trafficking of SP-A are not defined.

ABCA3: a Transporter Critical to AT2 Cell Homeostasis

A key component in the formation of LBs is the ABCA3 glycoprotein, a member of the ABC superfamily TM transporter proteins that use ATP energy to drive various substrates, ranging from small ions (e.g., ABCC7 (CFTR) = Cl\(^-\)) to large molecules (e.g., ABCA1 = cholesterol) across the plasma and intracellular membranes. ABCA3 belongs to a subclass (class A) of ABC transporters that are involved in a variety of lipid translocation events in multiple cell types, including macrophages, epithelial cells, and neurons, with each localized in distinct subcellular compartments (71).

Human ABCA3 has been mapped to chromosome 16p13.3 and encodes a 1,704-amino acid protein (35). Although ABCA3 mRNA is detected in many tissues, the ABCA3 message is highly expressed in AT2 cells and ABCA3 protein localized in the LB-limiting membrane (110, 186). ABCA3 has been shown in vitro to transport phosphorylated cholamine, phosphatidylglycero1, sphingomyelin, and cholesterol into lysosomes of model cell line systems (8, 30, 31, 41, 110). Functionally homozygous null mutations of ABCA3 described in neonates with respiratory failure are associated with the presence of abnormal electron dense bodies on transmission EM, and other congenital malformations of model cell line systems (8, 171). Since each ABCA3 transporter has a unique subcellular destination and function, subsequent sorting of ABCA3 to LB requires additional unidentified signal(s). 2) N-linked glycosylation: site-directed mutagenesis of two asparagine residues within the first NH2-terminal luminal loop of the transporter impairs protein stability and disrupts its anterograde trafficking, resulting in proteasomal degradation and cell stress (14).

To summarize, AT2 cells represent a metabolically active cellular component of the distal lung responsible for the biosynthesis, assembly, and complex intracellular trafficking of a diverse and challenging set of cargo, resident proteins, and organelles that often mix in multiple subcellular compartments of the secretory, endocytic, and degradative (lysosomal) pathways.

CELLULAR QUALITY CONTROL

In 2001, Nogee and colleagues (117) first reported a mutation in the COOH-terminal encoding region of SFTP C that resulted in deletion of exon 4 and its 37 amino acids (SP-C\(^{\Delta exon4}\)) in both an infant and mother with ILD. Shortly after, a second report described a different SFTP C mutation resulting in substitution of glutamine for leucine at position 188 of the SP-C propeptide (SP-C\(^{\Delta 188Q}\)) in a large kindred of whom the biopsies of 11 adults showed IPF/UIP patterns and nonspecific interstitial pneumonia (NSIP) patterns were present in three children. Using a variety of model systems, we and others subsequently demonstrated that expression of SP-C\(^{\Delta exon4}\) or SP-C\(^{\Delta 188Q}\) in vitro resulted in intracellular SP-C protein aggregation and cell death by apoptosis. Importantly, all affected patients with SFTP C-related ILD reported to date have been heterozygous for the mutant allele, suggesting a toxic gain of function mechanism.

To preserve homeostasis in the face of a variety of exogenous and endogenous insults that challenge the integrity of their molecular and organelle components, eukaryotic cells have developed a quality control network integrating various combinations of the following processes: 1) sensing mechanisms designed to detect the presence of unwanted proteins, nucleic acids, macromolecules, and organelles; 2) effector pathways composed of chaperone/cochaperone/enzyme systems designed to improve macromolecular integrity (e.g., protein folding), facilitate normal trafficking, or repair damaged organelles and DNA-based structures (e.g., telomeres); and 3) degradative components such as the ubiquitin-proteasome system (UPS) and autophagosome-lysosomal-endosomal pathways (principally macroautophagy will be considered here) acting synergistically to restore protein/organelle integrity and remove defective or unwanted substrates. In addition, signaling pathways emanating from many of these component pieces serve to modulate the overall composition of the quality control network.

Quality Control Lessons for the Lung from Other Organs

At the NHLBI workshop “Malformed Protein Structure and Proteostasis in Lung Diseases,” the role that disorders of protein folding and degradation play in the pathobiology of lung disorders and their associated complications was highlighted (7). By extension, recent discoveries have also implicated organelle dysfunction and turnover, in particular in mitochondria or in the endosomal-lysosomal system, in the pathogenesis of some age-dependent (e.g., IPF), age-accelerated (e.g., HPS-induced fibrosis), and environmentally modified (e.g., tobacco) lung disorders, pointing to a critical role for the need to edit and remove damaged organelles to maintain lung health. A major conclusion from this workshop was that research into the mechanisms and pathways underlying protein (and organelle) quality control in the lung and the coupling of alterations in quality control to lung disease has been largely underdeveloped.

By contrast, much of the progress made in our understanding the role of quality control pathways in disease has been derived from studies of a seemingly diverse set of chronic, degenerative disorders observed in a variety of organ systems including the central nervous system (CNS), liver, and pancreas. Huntington’s and Alzheimer’s diseases represent progressive neurodegenerative disorders associated with expression or altered processing of a mutant TM protein (analogous to proSP-C) that undergoes a fatal conformational rearrangement that unmarks
an intrinsic ability to self-associate (aggregate), induce aberrant cell signaling/cytotoxicity, and/or be deposited in nonviable subcellular or tissue compartments. Similarly, liver disease from expression of the mutant Z allele of alpha-1-antitrypsin (Z-A1AT) is associated with ER retention and cytotoxic aggregation of Z-A1AT in hepatocytes leading to their eventual death. The sheer magnitude of biosynthetic capacity for insulin and other hormones in islet cells of the endocrine pancreas requires an equally robust quality control system, which appears to fail in models of Type 2 diabetes. Furthermore, disorders such as Parkinson’s disease have supplied the recognition that protein “conformational diseases” are often accompanied by mitochondrial dysfunction and, like all organelles, must be “recycled,” requiring an additional layer of cell quality control (130). Finally, lipid storage disorders such as Niemann-Pick C (NPC) disease that are marked by severe neurodegeneration and lung disease have been shown to acquire a defective cell quality control phenotype (primarily impaired macroautophagy) (140).

Together, from basic and translational studies of these diseases using these abnormal protein isoforms as substrates, the cellular responses to misfolded proteins and dysfunctional organelles have been dissected out in the context of cellular quality control.

Protein Quality Control (Proteostasis)

Mutant protein accumulation within the ER and cytosol is countered by a complex mechanism aimed at restoring protein homeostasis or “proteostasis” (6). Proteostasis refers to the control of concentration, formation, binding interactions, quaternary structure, and location of individual proteins making up the proteome by redapting the innate biology of the cell, often through transcriptional and translational changes. The proteostasis network comprises processes controlling protein synthesis, folding, trafficking, aggregation, and degradation and is regulated by the integration of inputs from numerous cell sensing and signaling pathways. Failure of proteostasis to cope with misfolding-prone proteins, aging, or metabolic/environmental stress can trigger or exacerbate conformational disease.

The major components of a prototypical quality control response to aberrant conformers include the following:

The unfolded protein response. As an initial response, highly specific signaling pathways, evolved to ensure that ER protein-folding capacity is not overwhelmed, are collectively termed the unfolded protein response (UPR) (reviewed in detail in Ref. 73). The UPR pathways are governed by three ER TM protein sensors: PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE-1) (illustrated in Fig. 2). Their activation by abnormally folded proteins initiate one or more of three signaling cascades with two objectives: 1) generation, translocation, and binding of select transcription factors (XBP1, ATF6p50, ATF4) to nuclear ER-stress-responsive elements to expand ER refolding capacity by upregulating a variety of chaperones (e.g., Bip, GRP94, calnexin, protein disulfide isomerase); and 2) through phosphorylated eIF2α to promote translation attenuation at the level of the ribosome. Importantly, molecular signatures generated by these events can be used to detect UPR activation in cells and in tissues: IRE1 activation is heralded by appearance of spliced XBP1 mRNA or XBP1 protein; PERK activation can be confirmed by detection of phosphor-eIF2α, CHOP/GADD153, and ATF4; ATF6 cleavage fragments confirm engagement of this pathway. The relative contributions of each UPR pathway to the proteostatic response are protein substrate and cell specific.

ER-associated protein degradation. If the UPR is unsuccessful at restoring normal protein conformation, misfolded or unassembled protein targets are removed from the ER by retrotranslocation and ultimately destroyed by the ubiquitin-proteasome system (UPS) depicted in Fig. 2 (107, 170). The proteasome is the main protein degradation machinery of the cell and degrades ~90% of all cellular proteins. Covalent attachment of one or more K48-linked polyubiquitin chain(s) to a lysine residue(s) represents the primary targeting signal for direction to 26S proteasomes and then degraded into small peptides. In addition to a role in protein quality control, protein degradation by the proteasome is essential for diverse cellular functions such as apoptosis, transcription, cell signaling, and immune responses (reviewed in Ref. 119, 141). The proteasome itself is composed of a cylinder-shaped catalytic core particle, the 20S proteasome, which can be capped at either end by several different 19S regulator complexes when ATP/Mg2+ is present (48). The 20S proteasome alone participates but is inefficient at protein degradation therefore activation mediated by these 19S regulatory particles is crucial and can contribute to differential proteasome responses to physiological or pathological conditions (reviewed in Ref. 106).

The 26S proteasome used for ER-associated protein degradation (ERAD) is subject to failure. In a variety of systems, overexpression of misfolded protein isomers and conformers can actually inhibit global UPS function, leading to the cytosolic accumulation of both the primary mutant as well as bystander proteins (17). In addition, environmental factors such as cigarette smoke can directly impair proteasome activity in lung cells (without affecting proteasome expression), resulting in accumulation of polyubiquitinated proteins (168).

Autophagy. The process of macroautophagy (hereafter referred to as autophagy) was originally described as a cellular response to fasting starvation. However, an important emerging concept is that autophagy also functions as a second major degradation pathway for the targeted removal of long-lived, aggregation-prone proteins (e.g., huntingtin, α-synuclein, and neuroserpins) (82, 89). An extensive discussion of the autophagic process is beyond the scope of this review; the reader is referred to several excellent recent reviews (32, 94, 113). In brief, as illustrated in Fig. 2, autophagy is a dynamic process controlled by at least 30 known autophagy (Atg) genes whose assembly and turnover enclose cytosolic materials and organelles by generation of isolation membranes (phagophores) that mature to form double membrane-bound vesicles (“autophagosomes”) that are readily identified by EM. Subsequently, the autophagosome containing captured cargo fuses with the lysosome for degradation of the contents. The entire process is highly regulated from inputs from a variety of sensing and signaling mechanisms including mammalian target of rapamycin (mTOR1), Class III phosphoinositide 3-kinases (PI3K), and several mTOR-independent pathways. The selectivity of autophagy for misfolded protein is dependent on the recognition of ubiquitinated aggregates by ubiquitin-binding receptors such as p62/sequestosome-1 (p62/SQSTM1) (125).

Various reports have confirmed the existence of a link between ERAD/UPS and autophagy in the clearance of mis-
folded proteins (28, 124), since perturbations in the flux through either pathway can affect the activity of the other system (85). Evidence also exists for cross talk between the UPR and autophagy mediated by several pathways including an eIF2α/H9251/ATF4 axis and JNK1 (105, 171).

Aggresomes, JUNQ, and IPOD. Current consensus holds that direct cellular toxicity from aberrant mutant protein conformers is associated with nonnative soluble protein oligomers and that the formation of large aggregates is instead cytoprotective. To limit toxicity from misfolded proteins accumulating in the cytosol or nucleus that escape degradation by either the UPS and/or autophagy, mammalian cells contain an additional cellular quality control-based defense that involves the recognition, segregation, and active compartmentalization of ubiquitylated protein oligomers/microaggregates in a microtubule-dependent manner to pericentriolar, membrane-free, vimentin-enwrapped perinuclear structures termed aggresomes (68). Work by several groups has shown that, in addition to protein aggregates, Atgs and lysosomes can also be directed to aggresomes in a process dependent on the microtubule deacetylase.
Fig. 3. Pathways to AT2 cell injury/dysfunction defined by using mutant proSP-C isoforms as prototype substrates. The expression of proSP-C mutants can produce 1 of 2 profiles of aberrant cellular responses. Misfolding BRICHOS proSP-C (Group A1): ER stress marks the state in which the UPR can no longer maintain ER homeostasis because of overwhelming expression of misfolded SP-C, leading to a cascade of cellular disruption and injury: 1) Dysfunction of ERAD due to an overloaded and defective UPS. This event can be further exacerbated by second hits such as cigarette smoke and viruses. 2) ER stress-dependent recruitment of TRAF2 by IRE1 to activate JNK that promotes upregulation and release of cytokines. 3) Induction of apoptotic pathways by ER stress-induced activation of either the PERK/EIF2α/ATF4 network to trigger CHOP activation, IRE1/TRAF2 activation of caspase 4/12, and/or cytochrome c release from dysfunctional mitochondria. All 3 intersect downstream at the activation of caspase 3. 4) A failure of aggresomal compartmentalization or autophagy also contributes to the eventual accumulation of toxic macroaggregates. Mistrafficked Linker (non-BRICHOS) proSP-C (Group B): mutations in the COOH-terminus outside BRICHOS domain of proSP-C reported to date appear to be initially mistargeted to the plasma membrane via a constitutive pathway. Because of proper processing failure, proSP-C in the plasma membrane can be reinternalized and trafficked through early endosomes to late endosomes (LE)/MVB. The enhanced presence of aberrantly trafficked and misprocessed proSP-C in these compartments leads to a functional disruption of normal endosome/amphisome/lysosome turnover within the autophagic machinery that ultimately results in a distal block in autophagy. Such a block leads to the accumulation of abnormally large autophagic vacuoles containing undegraded, organelar, and proteinaceous debris populated with autophagy markers including LC3 and P62, as well as parkin, a protein critical for autophagy-dependent removal of dysfunctional mitochondria (i.e., mitophagy). *Potential sites for organelle disruption by the expression of mistrafficked proSP-C.

Consequences of Failed Protein Quality Control: ER Stress, Aggregation, and Apoptosis

If the integrated proteostatic program (combined efforts of UPR, ERAD, and autophagy) fails to control mutant protein levels, a series of deleterious consequences for the cell can ensue. Illustrated in Fig. 3, these include the following events:

1) ER stress. In addition to its normal adaptive signaling described previously, prolonged or severe overload of the ER with client proteins can activate “alarm signals” that overlap with some of the same signaling transduction cascades associated with innate immunity (185, 194). Termined “ER stress,” UPR sensors (particularly IRE1) can then activate downstream MAP kinases such as JNK to promote the elaboration of proinflammatory cytokines and growth factors from affected cells (115, 166). The proof of principle that the balance of UPR/ER stress can determine cytoprotection/cytotoxicity has been demonstrated repeatedly whereby overexpression or knockdown of Grp78/Bip can
modulate both ER stress markers and susceptibility to cell death in vitro and in vivo (72, 179).

2) Intrinsinc apoptosis. In mammalian cells, ER stress can also induce “intrinsic apoptosis” via one or more pathways (80, 185). One involves the cleavage and activation of the ER-membrane-localized caspase-4/12 (60). In another, a complex generated by IRE1 and TNF-receptor-associated factor 2 (TRAF2) recruited to the ER membrane signals downstream effectors such as JNK (62). In some systems, PERK via an ATF4-mediated pathway and/or IRE1 via XBP-1 can upregulate C/EBP-homologous protein (CHOP), a transcriptional repressor of expression of prosurvival Bcl2 proteins. Additionally, ATF4-mediated pathway and/or IRE1 via XBP-1 can upregulate CHOP, a transcriptional repressor of expression of prosurvival Bcl2 proteins. Moreover, CHOP can also induce apoptosis via activation of caspases 4/12 (60). In another, a complex generated by IRE1 and TNF-receptor-associated factor 2 (TRAF2) recruited to the ER membrane signals downstream effectors such as JNK (62). In some systems, PERK via an ATF4-mediated pathway and/or IRE1 via XBP-1 can upregulate C/EBP-homologous protein (CHOP), a transcriptional repressor of expression of prosurvival Bcl2 proteins. Additionally, in a variety of model systems (52). Further downstream integrative death signaling can involve activated caspase cascades that vary among cell types, by pathway and by inducing substrate. As examples, numerous reports have linked ER stress-mediated apoptosis of pancreatic beta cells to a caspase 4/12-mediated activation of caspase 3 (reviewed in Ref. 123). Similarly, amyloid-beta (Aβ) also induces apoptosis via activation of caspases 4/12 and then caspase 3 in neuroblastoma cell lines (61); however, neurons expressing misfolded PolyQ72 huntingtin undergo caspase 8-mediated apoptotic events (138).

3) Protein aggregation. Best exemplified by chronic neurodegenerative disease, the intracellular or extracellular accumulation of an abnormal protein isoform (such as Aβ amyloid precursor protein, α-synuclein, or polyglutamine huntingtin-1) with inherent instability can lead to time-dependent neuronal cell toxicity and the histopathological appearance of inclusion bodies, amyloid fibrils, and protein macroaggregates in the cytosol or within the nucleus (29, 76, 135, 137). As will be discussed in detail subsequently, given the propensity of SP-C3,7 to form β-amyloid structures (51), it is not surprising that expression of some SFTPC mutations results in generation of SDS-insoluble proSP-C macroaggregates in vitro (111, 112) as well as intracellular amyloid formation in vitro and in vivo (182).

4) Mitochondrial dysfunction. ER stress can induce mitochondrial dysfunction through both physical and functional communications between mitochondria and the ER network, which can further exacerbate apoptotic events (reviewed in Ref. 169).

Quality Control for Dysfunctional Organelles: Mitophagy

In addition to the utilization of macroautophagy for selective removal of polyubiquitinated protein aggregates (aggrephagy), autophagy can target and remove specific subcellular components (selective autophagy) including invading pathogens (xenophagy), lipids (lipophagy), and dysfunctional cellular organelles such as mitochondria or ER. Degradation of dysfunctional mitochondria, termed mitophagy, is a remarkably specific process. When a single mitochondrion is uncoupled via photoreirradiation, this and only this mitochondrion will be degraded (79). Additionally, global disruption of mitochondrial function in cells treated by using an uncoupler such as carbonyl cyanide 3-chlorophenylhydrazone results in selective mitochondrial degradation, leaving other organelles intact.

The process of mitophagy requires the coordination of cytosolic factors and signals assembled at the outer mitochondrial membrane (reviewed in Refs. 189, 190). A key step in the initiation of the process involves PTEN-induced putative kinase 1 (PINK1), stabilized in the outer mitochondrial membrane in response to lowered TM potential (Δψ), which then recruits the E3 ligase parkin, resulting in K-63-linked polyubiquitin chains of a variety of mitochondrial protein substrates such as mitofusins (Mfn1; mfn2). The subsequent recognition and sequestration of the ubiquitin decorated mitochondrion utilizes much of the same autophagy machinery commissioned for misfolded protein removal. For example, the ubiquitin-binding adaptor p62/SQM1, which recruits ubiquitinated protein cargo into autophagosomes by binding to LC3, also accumulates on mitochondria following their parkin-mediated ubiquitination and facilitates their engulfment by the phagophore/autophagosome.

Aging, Cellular Quality Control, and Parenchymal Lung Disease

The effects of aging on cellular quality control capacity have been well documented, particularly in the CNS where perturbations to proteostasis and mitochondrial function are hallmarks of age-related neurodegenerative disorders (108). An age-dependent decrease in proteasome activity has also been observed in other tissues including skin, muscle, heart, liver, kidney, and eye, each with a variety of consequences (reviewed in Ref. 95). Aging has been shown to markedly downregulate autophagic activity (136). Given their key role in processing cellular constituents, either to the lysosome for degradation or to other cellular compartments for immune activation, autophagy and the UPS represent important cytoprotective strategies against aging, especially for postmitotic cells. As proof of concept, expansion of either proteasome activity or autophagy in the CNS extends lifespan and protects organisms from symptoms of proteotoxic stress (96).

Aging is a known risk factor for IPF (81). Many of the hallmarks of aging (e.g., genomic instability, telomere attrition, epigenetic alterations, and cellular senescence) have been proposed as essential mechanisms for the development of IPF; however, these disturbances are not restricted to IPF (reviewed in Ref. 145). Telomerase mutations have been described in IPF families but also in chronic obstructive pulmonary disease (4, 150). A recent report has described enhanced sensitivity of the alveolar stem cell function of AT2 cells from mice with short telomere syndromes subjected to exogenous “second hits” providing a potential mechanism for their role in aberrant injury/repair processes in IPF pathogenesis (2).

In addition to these pathways, several lines of evidence link age-dependent alterations in cell quality control, AT2 cell function, and susceptibility to fibrosis. Aged mice are susceptible to fibrosis from herpesvirus infection with increased expression of ER stress markers (161). In a preliminary report, lung tissue from aged mice displays decreased proteasomal activity and alterations in 26S proteasome assembly (27). Recently, Bueno et al. (23) have found that AT2 cells from the lungs of both IPF patients and aged wild-type mice exhibit marked accumulation of dysmorphic and dysfunctional mitochondria. This was associated with both an upregulation of ER stress markers and a downregulation of PINK1. Moreover, young PINK1-deficient mice had premature development of similarly dysmorphic, dysfunctional mitochondria in AT2 cells.
and were vulnerable to apoptosis and development of lung fibrosis in a viral challenge model (23). Interestingly, in a transgenic mouse model of atg5 deficiency, a 90% decrease in autophagy was well tolerated in young adult mice but resulted in alveolar septal thickening and altered lung mechanics in aged animals, consistent with accumulation of damage over time (54). Collectively, these data suggest that aging has marked effects on both the proteostasis network and autophagy in the lung epithelia and that IPF, like many neurodegenerative conditions, may reflect an acquired and potentially aged-related defect in one or more arms of cellular quality control.

SURFACTANT MUTATIONS, EPITHELIAL DYSFUNCTION, AND IPF PATHOGENESIS

The three components of the surfactant system associated with ILD opportunistically represent distinct substrates (a bitopic integral membrane protein, a soluble luminal protein, and a polytopic membrane transporter) with which to assess cellular responses and aberrant signaling present in the distal lung epithelia in IPF. As the first of these to be described, SFTPC represents a starting point to understand AT2 cell dysfunction in both sporadic IPF and FPF. Since the initial descriptions of the clinical proSP-C mutants, SP-C<sup>G100S</sup> and SP-C<sup>L188Q</sup>, nearly 15 years ago, over 60 additional SFTPC mutations have been reported in the literature in association with parenchymal lung disease (summarized in Table 1). These can be grouped on the basis of their functional behavior and domain location within the proSP-C sequence. The potential targets and various processes that have been revealed by their expression in cells, preclinical animal models, and patients are schematically illustrated in Fig. 3.

Table 1. Summary of reported phenotypic features for surfactant component mutations

<table>
<thead>
<tr>
<th>Mutation (Domain)</th>
<th>Clinical Diagnosis</th>
<th>Lung Phenotype (in vivo)</th>
<th>Subcellular Localization</th>
<th>Cellular Responses (in vitro)</th>
<th>References</th>
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<tr>
<td><strong>SFTP2</strong></td>
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<td>F198S (CRD)</td>
<td>Familial pulmonary fibrosis</td>
<td>Total BAL [SP-A] Normal</td>
<td>ER retention</td>
<td>(+) ER stress, cleared by ERAD (+)</td>
<td>99, 100, 175</td>
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<td></td>
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<td>Intrasacellar aggregation Not secreted</td>
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<td><strong>SFTPC</strong></td>
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<td>ΔExon4 (BRICHOS)</td>
<td></td>
<td></td>
<td>ER retention→ aggresomes Intrasacellar aggregates</td>
<td>(+) ER stress</td>
<td>21, 39, 97, 98, 100, 26, 49, 175</td>
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<td>L188Q (BRICHOS)</td>
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<td>ER stress (humans; mice)</td>
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<td></td>
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<td>Epithelial cytotoxicity</td>
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<td><strong>Group A1</strong></td>
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<td>G100S (BRICHOS)</td>
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<td>ER retention</td>
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<td>Intrasacellar aggresomes</td>
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<td>(+) ER stress, cleared by ERAD (+)</td>
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<td><strong>Group A2</strong></td>
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<td>L110R (BRICHOS)</td>
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<td>ER retention</td>
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<td>Intrasacellar aggregation</td>
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<td>(+) ER stress, cleared by ERAD (+)</td>
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<td><strong>Group B1</strong></td>
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<td>E66K (Linker)</td>
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<td>Intrasacellar aggregation</td>
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<td>(+) ER stress, cleared by ERAD (+)</td>
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<td><strong>Group B2</strong></td>
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<tr>
<td>∆91-93 (Non-BRICHOS)</td>
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<td>ER retention</td>
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<td>Intrasacellar aggregation</td>
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<td>(+) ER stress, cleared by ERAD (+)</td>
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<td><strong>Group C</strong></td>
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<td>P30L (NH2-terminal)</td>
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<td>ER retention</td>
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<td>Intrasacellar aggregation</td>
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<td>(+) ER stress, cleared by ERAD (+)</td>
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*Seen with homozygous or compound heterozygous ABCA3 expression; †found with heterozygous ABCA3 expression; ‡S. Mulugeta, unpublished observations. BAL, bronchoalveolar lavage; chILD, interstitial lung disease of childhood; CPI, chronic pneumonitis of infancy; CRD, carbohydrate recognition domain; EE, early endosomes; EEA1, early endosomal antigen-1; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; HSP, heat shock response; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; LBs, L<sub>ME</sub>/L<sub>MB</sub>, LRO, lysosome-related organelle; NSIP, nonspecific interstitial pneumonitis; PAP, pulmonary alveolar proteinosis; PAS, periodic acid Schiff; 4-PBA, 4-phenylbutyric acid; PC, phosphatidyl choline; PM, plasma membrane; RDS, respiratory distress syndrome of newborn; SP, surfactant protein; TM, UIP, usual interstitial pneumonitis.
Clues from SFTPC BRICHOS Mutations: Aggresomes, ER Stress, Cytokines, EMT, and Apoptosis

The Group A1 mutations prone to misfolding tend to cluster around the conserved cysteine residues (cys127/cys189) in the SP-C BRICHOS domain. When the prototypical SP-CΔ exon4 and SP-CΔ1,188Q BRICHOS mutants are expressed in a variety of in vitro model systems, they are initially retained in the ER, fail to undergo appropriate proteolytic processing, do not end up in LB, and have a high propensity to form intracellular aggregates. Alanine mutagenesis of either or both cysteines generates laboratory-mutant isoforms that indicate these aggregates are actually aggresomes whose formation can be blocked with microtubule inhibitors, exacerbated by proteasome inhibitors, and rescued by the chemical chaperone 4-phenylbutyric acid (4-PBA) (69, 153). Aggresomes are also observed with clinical BRICHOS mutants (173). Additional mutations in the more proximal BRICHOS domain termed Group A2 (A116D; L110R) are less well characterized but are also excluded from LRO/LB, partially localize in LAMP3/EEA1 (LE/MVB) compartments, induce a UPR, and form Congo red aggregates in vitro (160, 193). The sole Group C mutant reported to date (P30L) is also ER retained and polyubiquitinated (presumably for ERAD) (13, 160).

Using SP-CΔ exon4 and SP-CΔ1,188Q mutants as substrates, misfolded BRICHOS conformers are also capable of activating one or more of the three UPR sensors, IRE1/XBP1, ATF6, and PERK/eIF2α, in both cell lines and primary rodent AT2 cells (97, 98, 111, 112). The ultimate failure of the UPR to correct mutant protein load and alleviate ER stress appears to induce a series of downstream events that then contribute to AT2 cell dysfunction and promote aberrant injury/repair responses.

**Sound the alarm: inflammation.** Persistent UPR activation by these mutant isoforms appears to induce an ER-mediated “alarm” response. Transient expression of SP-C BRICHOS domain mutants promoted IL-8 release from cultured epithelial cells. The release of IL-8 was mechanistically linked to the activation of JNK signaling and was associated with activation of nuclear factor-kappa light-chain-enhancer of activated B cells (NF-κB) (97). Stably transfected cell lines expressing SP-CΔ exon4 appear to adapt to chronic ER stress via an NF-κB-dependent pathway and suggest this may be a prosurvival response, but one capable both of elaborating additional inflammatory mediators and of being exploited by second hits such as viral infections (22).

**Apoptosis.** Given the role of AT2 cells as progenitors (16), their depletion poses a threat to the overall capacity of the lung for repair/regeneration. ER stress induced by mutant proSP-C can induce intrinsic apoptotic death via induction of numerous pathways including caspase 4 activation, mitochondrial cytochrome c release, c-Jun NH2-terminal kinase (JNK) signaling, and caspase 3 activation (97, 98, 111, 112). Interestingly, apoptosis induced by pharmacological proteasome inhibition or overexpression of an SP-C BRICHOS mutant can be significantly inhibited by angiotensin receptor blockade (saralasin) or the angiotensin II counterregulatory peptide Ang1-7, showing that ER stress-induced intrinsic apoptosis in human AECs can involve autocrine pathways such as the ANGII/ANG1-7 system (163).

**Changes in cell phenotype.** Although somewhat controversial as to their contribution to total fibrotic burden, a variety of studies have suggested that epithelial cells in the lungs and other organs can undergo phenotypic changes with the adoption of mesenchymal phenotypes. Termed epithelial-mesenchymal transition (EMT), this phenomenon probably represents an adaptive response to cellular stress. In the alveolar epithelium and in cell lines, EMT driven by ER stress induced either pharmacologically or by using proSP-C mutants has been shown. In primary rodent AT2 cells, Zhong et al. (195) have shown that treatment with tunicamycin or thapsigargin induces α-smooth muscle actin expression and decreased E-cadherin that was accompanied by morphological changes consistent with a shift to a mesenchymal phenotype. Overexpression of SP-CΔ exon4 in this model largely recapitulated these effects. Tanjore et al. (157) reported similar findings following induction of ER stress by overexpression of the SP-CΔ,188Q mutant and identified SMAD2/3 signaling as a mediator of these events. Importantly, both studies demonstrated a role for SRC family kinases and suggest that therapies based on mitigating ER stress may have pluripotent benefits.

**Beyond SP-C: Aberrant Cellular Responses to Other Surfactant Component Substrates**

The cellular responses observed above are not idiosyncratic with proSP-C BRICHOS mutants, given that studies utilizing mutant SFTPA2 and ABCA3 substrates have both corroborated the pathways discussed above and extended the findings with several new observations.

**SFTPA** (SP-A). In 2009, Wang and colleagues (175) using genomewide linkage scanning, reported on two kindreds with pulmonary fibrosis and lung adenocarcinomas in which rare heterozygous missense mutations were identified in SFTPA2 alleles. Resulting from the substitution of valine for glycine at codon 231 (G231V) or serine for phenylalanine at codon 198 (F198S) in the CRD of the SP-A protein, when expressed in lung cell lines or primary murine AT2 cells, the resulting mutant SFTPA2 protein products were proximally retained, failed to be secreted, and activated the UPR (99). Subsequently the same group found elevated amounts of TGF-β1, a pleotropic profibrotic cytokine linked to both myofibroblast activation and EMT, in the bronchoalveolar lavage (BAL) of patients expressing the G231V mutation. Primary mouse AT2 cells expressing G231V in vitro also elaborated significant amounts of TGF-β as well as two TGF-β binding proteins (LTBP-1 and LTBP-4) (100).

**ABCA3.** Although not restricted to any particular domain, functional characterization of a subgroup of ABCA3 mutations (including L101P, L982P, L1553P, Q1591P, G1221S) by transient or stable expression results in their total or partial retention in the ER (30, 103). Designated as “Type I” (Table 1), this mutant subgroup can enhance expression of Bip, increase XBP1 splicing, and induce apoptosis in vitro (177). Recent work with both the Type I clinical ABCA3 mutants as well as laboratory-generated glycosylation-deficient constructs suggest that the misfolded isoforms are substrates for ERAD (14, 177). Taken in total these data are highly supportive of a common model of ER stress and failed proteostasis from misfolded surfactant components.
A Role for Induced AT2 ER Stress and Apoptosis in IPF Pathogenesis

Mechanistic relationships between ER stress-induced epithelial dysfunction and lung fibrosis identified from the in vitro studies described above have been further strengthened using mouse models. Bridges et al. (21) reported that transgenic mice constitutively expressing SP-CΔexon4 exhibit a gene dose-dependent arrest of lung morphogenesis, induction of Bip expression, and aberrant proSP-C processing. More recently, conditional expression of SP-CΔ118Q in AT2 cells of adult mice at induced levels of ~20% of the endogenous gene expression produced ER stress (90). Although lacking evidence of gross fibrosis at baseline, these mice exhibited a greater sensitivity to low-dose bleomycin with marked fibrotic changes and enhanced AT2 cell apoptosis. The direct role of ER stress in this process was further supported by exposure of wild-type mice to tunicamycin treatment to independently induce ER stress, which again enhanced the fibrotic response to bleomycin (90).

ER Stress Signatures Are Not Exclusive to Cells, Mice, or Even SFTPC IPF Families

The molecular signatures for ER stress and UPR pathways identified by the in vitro systems and mouse models have been subsequently detected in the lungs of IPF patients. Lawson and colleagues (91) demonstrated evidence of UPR activation markers (including Bip, EDEM, and XBP1) in IPF patients expressing SP-CΔ118Q mutations as well as in samples both from non-SFTPC IPF patients and in sporadic cases of IPF. Korfei et al. (83) extended these findings by reporting molecular signatures of multiple UPR pathways and apoptosis in AT2 cells including ATF6, ATF4, XBP1, CHOP, Bax, and caspase 3 exclusively in patients with sporadic IPF with pathological features of UIP. Thus chronic ER stress in the alveolar epithelium likely represents a broad mechanism for the pathogenesis of ILD (156).

AT2 Quality Control Responses

An important concept, especially in the context of quality control issues discussed above, is that the cellular pathology and molecular signatures of ER stress induced by BRICHOS proSP-C expression must invariably result from of an imbalance in or failure of the cellular quality control network to accommodate the offending protein conformers and dysfunctional organelles. Very little is currently known about the actual quality control repertoire of AT2 cells. Using microarray analyses, Dong et al. (39) identified genes specifically induced in response to expression of SP-CΔexon4 and SP-CΔ118Q and showed that two Bip co-chaperones, ER-localized DnaJ homologues ERdj4 and ERdj5, selectively bind to mutant but not wild-type proSP-C and were required for successful retrotranslocation and proteasomal degradation of BRICHOS mutant SP-C substrates. In a preliminary report, we have shown that although primary human AT2 cells exhibit a significant degree of both proteasomal activity and autophagy, these cells appear to rely primarily on ERAD for control either of misfolded proSP-C conformers or more generally of a variety of PolyQ huntingtin substrates (57). Similarly, Maitra and colleagues (99) have shown that primary mouse AT2 cells in vitro rely almost exclusively on MG132-sensitive proteasomal activity to clear misfolded SP-A2 mutants. Although not appearing to have a primary role in basal proteostasis, the function of autophagy either as a backup to ERAD and/or in the quality control of AT2 organelles such as mitochondria, LB, or nonprotein substrates remains an important area for further investigation.

Beyond ER Stress and Apoptosis: Dominant Negative or Loss-of-Function Effects

Patients heterozygous for the SP-CΔexon4 mutation do not express mature SP-C (117). Mechanistically, because proSP-C sorting involves homomeric association of proSP-C monomers mediated by the mature SP-C domain, through heterotypic interactions, SP-C BRICHOS mutants can act as dominant negatives to direct wild-type proSP-C away from normal routing and into aggregates (174). Because SP-B and SP-C are felt to be functionally redundant with respect to overall surfactant biophysical activity, the question of whether a lack of mature SP-C in surfactant would contribute to IPF pathogenesis is unanswered. As expected, SP-C null mice are viable at birth and grow normally without a lung phenotype (45) but exhibit enhanced sensitivity to bleomycin fibrosis (93) and respiratory syncytial virus infection (47). However, when the original strain of SP-C null mice are backcrossed onto a different genetic background (129/Sv), the absence of SP-C is associated with spontaneous inflammation and lung remodeling (46), suggesting that SP-C deficiency is a potential disease modifying factor.

In a related fashion, when expressed in primary rodent AT2 cells, the disease-causing human SPA2 mutants are not secreted (99). Furthermore, in epithelial cell lines simultaneously transfected with SP-A2 isoforms and wild-type SP-A1, coimmunoprecipitation resulted in recovery of wild-type SP-A2 from the medium but not SP-A2 mutants (G231V or F198S) (99). Furthermore, in epithelial cell lines simultaneously transfected with SP-A2 isoforms and wild-type SP-A1, coimmunoprecipitation resulted in recovery of wild-type SP-A2 from the medium but not SP-A2 mutants (G231V or F198S) (175). Although total SP-A levels do not appear to be altered by heterozygous expression of SP-A2 mutants in vivo, given that the SP-A trimer and 18-mer are from combinations of SP-A1 and SP-A2, the resultant consequences of an SP-A1 restricted SP-A on innate lung immunity and/or IPF pathogenesis remains to be clarified.

In contrast to posttranslationally acquired SP-C or SP-A deficiency, functionally impaired ABCA3 mutations (designated Type II mutations) have been identified, with homozygous expression producing severe loss of function phenotypes (30, 103, 104) (Table 1). Affected patients as well as ABCA3 null mice demonstrate complete absence of protein expression, deformed LBs, and die from respiratory distress shortly after birth (31, 43, 147, 172), indicating a critical role in production of pulmonary surfactant. However, heterozygous expression of ABCA3 mutations, with a resultant partial loss-of-function caused either by ER retention (Type I), improper lipid-pump function (Type II), or both (Type I/II compound heterozygotes) have each been reported and appear to act as genetic modifiers of lung diseases associated with SFTPC mutations in children (24). In one adult FPF kindred all carrying the same SFTPC mutation, a functional genetic variant in ABCA3 (E292V) also found in the previous pediatric study appeared to also affect disease penetrance, suggesting interplay between the two genotypes (37), not surprising given their overlapping biology within the AT2 cell.
SFTPC Trafficking Mutations: a New Phenotype of Acquired Quality Control Disruption

Beginning in 2004, a series of missense mutations clustering within a 40-amino-acid span encoded on exon 3 of human SFTPC was reported, heralding an emerging diversity of molecular and clinical phenotypes induced by SFTPC expression (19, 55, 152). The first of these detailed a de novo heterozygous missense mutation (g.1286T>C) resulting in substitution of threonine for isoleucine (I73T) in an infant with NSIP (19). Interestingly, the identical SP-C-I73T mutation (both inherited and sporadic) has reappeared in several other published reports of parenchymal lung disease in children and adults and is now felt to be the most common SFTPC mutation (26, 116).

Collectively the Group B mutations (E66K; I73T; Δ91–93) (55, 152) all reside within the linker domain of proSP-C (Fig. 1). Although presentation and course can vary (1, 128), overlapping features that emerge include interstitial lung remodeling with intra-alveolar accumulation of surfactant (lipids, SP-A, SP-B), the presence of aberrantly processed proSP-C, and abnormal AT2 cell cytoplasmic inclusions on EM, suggesting abnormalities in surfactant homeostasis (19, 44, 58, 152). Importantly since mature SP-C can be detected in BAL from these patients, the non-BRICHOS trafficking mutants do not appear to act as dominant negatives (19, 162).

The cellular phenotype induced by Group B SFTPC mutants is also distinct from that of Group A1 (Table 1). Functionally, Group B SFTPC mutants have been defined by in vitro expression studies (mainly using SP-C-I73T), demonstrating a unique and markedly aberrant intracellular trafficking pattern for proSP-C first directly to the plasma membrane with subsequent internalization and accumulation within early endosomes and LE/MVB (15, 153) (Fig. 3). This recapitulated the expression pattern seen on a biopsy of a patient with SFTPC-E66K showing proSP-C staining on the plasma membrane and in early endosomes of AT2 cells (19). Recently, two separate preliminary studies using knockin or transgenic expression strategies have each demonstrated that the distribution of mutant SP-C-I73T in AT2 cells in vivo is nearly identical with the pattern noted in cell lines and patients (118, 176).

The link between the mistrafficking behavior and cellular dysfunction, which does not involve ER stress (111), has recently been defined in vitro with cell lines expressing hSP-C-I73T. As illustrated in Fig. 3, SP-C-I73T induces a novel quality control phenotype punctuated by a striking disruption of macropathway characterization by the accumulation of dysmorphic, autophagic vacuoles containing organelar and proteinaceous debris (58). In the in vitro findings from hSP-C-I73T cell lines phenocopied ultrastructural changes seen in AT2 cells on EM of a lung biopsy from a SFTPC I73T patient (58). Biochemically, hSP-C-I73T cells exhibited increased expression of Atg6/LC3, SQST/p62, and RAB7, consistent with a late block in autophagic vacuole maturation that was confirmed by autophagy flux studies. The observed disrupted autophagy resulted in a diminished functional capacity of hSP-C-I73T cells to degrade model protein aggregate substrates and impairment in mitophagy, producing an accumulation of dysfunctional mitochondria. The disruption of autophagy-dependent proteostasis and mitophagy induced by expression of SP-C-I73T is reminiscent of cellular quality control phenotypes observed in organellar storage disorders such as Niemann-Pick disease (140, 146), which has been associated with a premature development of fibrotic lung disease.

In translational studies of IPF from two separate groups, lung tissue from IPF patients demonstrates evidence of decreased autophagy as defined by increases in LC3, p62, and polyubiquitinated protein expression and decreased numbers of autophagosomes (3, 126). These findings, when combined with the aforementioned work on aging (23), emphasize the potential important role of impaired autophagy, mitophagy, and mitochondrial dysfunction in lung epithelia to IPF.

Impact of Environmental Factors on Lung Quality Control

Substantial clinical variability in the age of onset and disease severity in both surfactant mutation-associated (SP-C, ABCA3, SP-A2) ILD and sporadic IPF suggests a possible role for environmental factors and/or for modifier genes. In addition to the aging lung and its effects on quality control discussed above, given the direct exposure of the lung epithelium to the “outside world,” environmental insults may participate in a two-hit model of AT2 dysfunction to promote fibrosis. In support of this, IPF is found more frequently in cigarette smokers (11, 142), and cigarette smoking is one of the strongest associated risk factors for the development of diffuse parenchymal lung disease (DPLD) (151). Cigarette smoke exposure induces ER stress and proteasomal dysfunction in alveolar epithelial cell lines in vitro (149, 168) and signatures of UPR activation have been detected in the smoke-exposed human lung in vivo (77).

Similarly, emerging evidence also implicates human herpes-viruses (including herpesvirus-8; Epstein-Barr virus; cytomegalovirus) in IPF, and one or more of these viruses have been detected in the lungs of up to 97% of tested patients with IPF (40, 86, 91, 155, 188). In a mouse model of aging, infection with the murine herpesvirus lead to upregulation of multiple components of the UPR/ER stress pathway and accompanied by the development of lung fibrosis (161). In human IPF, cytomegalovirus antigens and the UPR transcription factor XBP-1 were colocalized in alveolar epithelial cells from an individual with UIP (91). More recently, sampling of asymptomatic relatives of patients with familial IPF showed evidence of herpesvirus infection and ER stress (87).

In a recent clinical study, IPF exacerbations were significantly associated with antecedent 6-wk increases in air pollution exposures of ozone and nitrogen dioxide, suggesting that air pollution may contribute to the development of this clinically meaningful event (64, 91). Although the mechanism was not assessed, given the oxidative-nitrative stress applied by these agents, it seems likely that additional proteotoxic and mitochondrial stress to the quality control network in the distal lung from the enhanced generation of “challenged substrates” by these exposures may be contributing factors.

CONCLUSIONS, UNANSWERED QUESTIONS, AND OPPORTUNITIES

Over the past 15 years much progress has been made in understanding the pathogenesis of IPF. There is now substantial evidence to show that the AT2 epithelium is altered in the fibroblastic foci of IPF lungs and that the AT2 cell is a pivotal regulator of lung injury, inflammation, and repair. Although
SFTPC, SFTPA, and ABCA3 mutations are rare (2–20% in most ILD cohorts) (92, 167), understanding both the spectrum of clinical phenotypes and the molecular pathogenesis of the AT2 cell dysfunction caused by these mutations adds value to understanding the broader mechanisms that underlie the pathogenesis of sporadic IPF.

The data summarized here generated by using surfactant protein mutations as a platform has revealed the role for both ER stress and alterations in cellular quality control pathways in epithelial cells in IPF pathogenesis (Fig. 3). The fact that characteristic cellular phenotypes are induced by distinct classes of surfactant component mutations and that these converge at the epithelial cell should spur further focus on efforts to understand AT2 biology in health and disease. Coupled with the emergence of confounding factors in IPF pathogenesis such as age, environmental exposures, and infection, the findings summarized in this review strongly support a two-hit (or multiple-hit) model whereby the repetitively injured AT2 cell population serves as a driver of disordered repair and injury. Although not discussed here, the well-known associations of telomerase mutations with familial IPF (4), which can also be thought of as a “quality control disorder” (for telomeres), also converge on this epithelial-centric model of IPF and highlight the importance for aging-associated processes and senescence that were not previously linked to IPF. In this context, the role of the recently described Muc5B promoter polymorphism in IPF remains to be clarified (127, 143).

Given the above model, it should come as no surprise that the therapies to date have done little to stabilize or reverse the fibrotic process (18). Analogous to cancer, it is likely that future therapeutic options for IPF should involve directed therapies aimed at engaging multiple targets including the epithelial cell dysfunction, fibroblast activation, and matrix deposition. This approach is probably not amenable to single drug therapy. Furthermore, experience with the surfactant protein component mutations reveals that the final common histopathological pathway seen as UIP or chILD likely reflects distinct combinations of several different upstream molecular events. Identifying the specific pathogenic pathway signatures in patients will be critical to identify personalized therapies based on pathogenesis not histology.

Finally, given the protracted period for drug development and the overlap of many of the pathways discussed above (ER stress, misfolding, aggregation, mistrafficking, autophagy, apoptosis, and MAP kinase signaling) with those seen in other chronic degenerative diseases (Alzheimer’s disease, Huntington’s disease, cystic fibrosis, and α1-antitrypsin deficiency), it may be worth considering a cross-fertilization of IPF research/treatment strategies with these disease areas by adopting experimental paradigms and reagents, coupled with readily available compound libraries, and approved drugs that already target some of these signatures in an attempt to open new therapeutic avenues for treatment of this devastating disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.M., S.-I.N., and M.F.B. conception and design of research; S.M. and S.-I.N. performed experiments; S.M. prepared figures; S.M. and M.F.B. edited and revised manuscript; S.M., S.-I.N., and M.F.B. approved final version of manuscript.

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